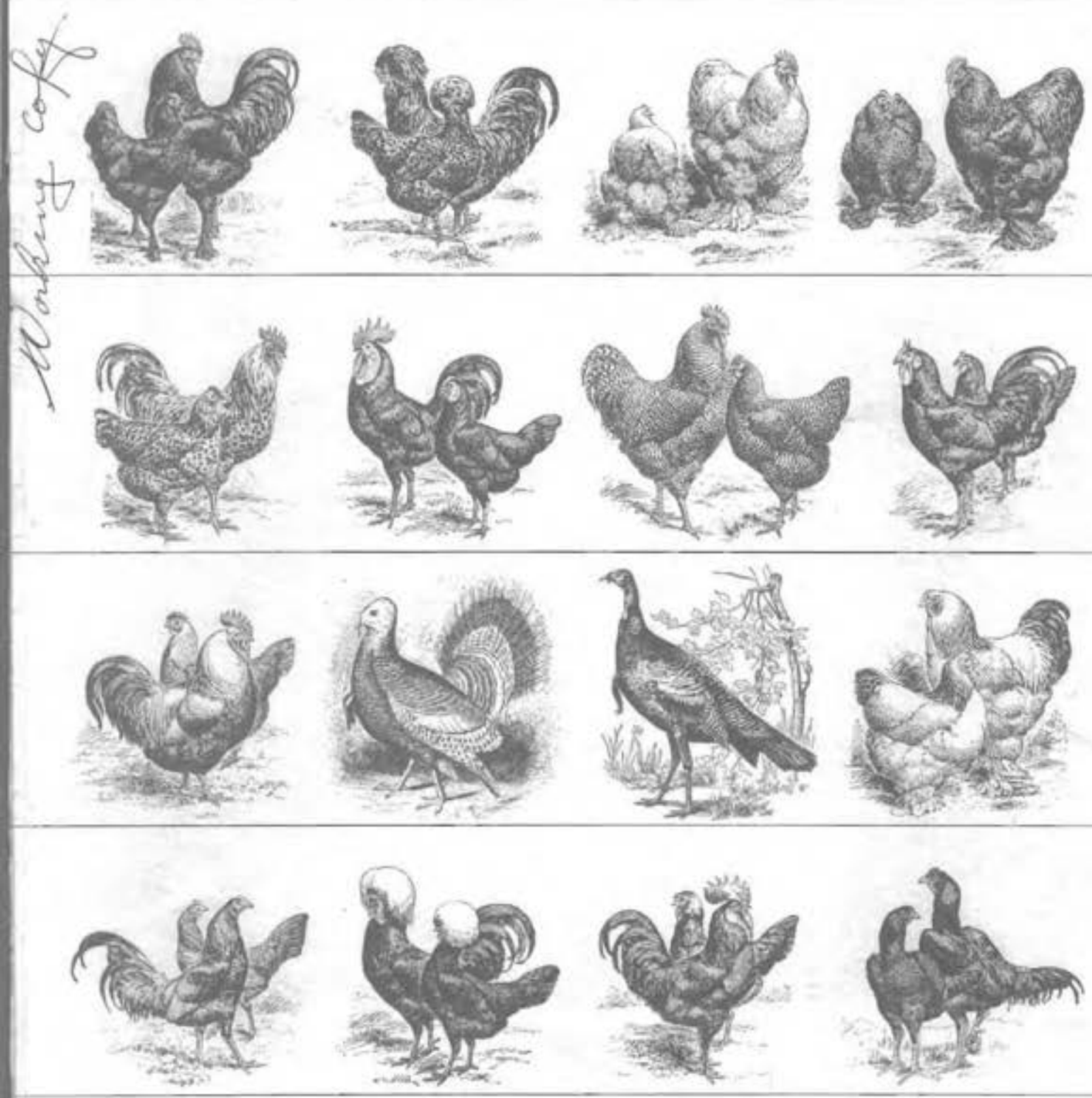
PROCEEDINGS OF THE THIRTY-EIGHTH WESTERN POULTRY DISEASE CONFERENCE

March 6–9, 1989 · Tempe, Arizona

AND PROCEEDINGS OF THE MONOCLONAL ANTIBODY WORKSHOP · *March 6, 1989*



SPECIAL ACKNOWLEDGMENT

The Western Poultry Disease Conference (WPDC) is pleased to acknowledge substantial contributions to its Speaker's Fund. These contributions provide support for outstanding scientists whose participation might otherwise not be possible. Over forty organizations and companies have given substantial financial support, and many companies and organizations, including some contributing to the Speaker's Fund, send speakers at no expense to the conference. We thank all these.

We especially thank the American Association of Avian Pathologists for its generous travel grant to bring speakers from other regions or overseas.

Our distinguished sustaining and donor members are listed on the following pages. We are most grateful for their generosity. We especially thank the donors, whose contributions—each $500 or more—are particularly noteworthy.

Many have provided special services that contribute to the continued success of this conference. We express special thanks to the following: Dr. Richard Chalquest, who has been in charge of local arrangements at Tempe; members of the translation crew, who make a major contribution in providing the excellent English/Spanish translation and other bilingual assistance; the program chairman; and all others who contribute to the program and host committees.

We express our gratitude to all authors who submitted manuscripts. We give thanks and acknowledgment to Brigham Young University, office of Scholarly Publications, and senior editor Howard Christy, who handled the publication of the proceedings. We especially thank Louise Williams, assistant editor of Scholarly Publications, who coordinated design, copy editing, and computer desk-top publishing production of the manuscripts. We also thank Bruce Patrick (Graphic Communications, Brigham Young University) for the cover design and interior layout.

38TH WESTERN POULTRY DISEASE CONFERENCE OFFICERS 1989

The proceedings of the 38th Western Poultry Disease Conference, including the proceedings of the Monoclonal Antibody Workshop, are presented as a service and a source of information to those attending the conference and to others who wish to gain some insight as to the discussion and information presented. As has been stated in the past, these proceedings are not considered or regarded as an official "publication" but simply as a source of information.

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MINUTES OF THE 37TH WPDC MEETING

President Marcus Jensen called the meeting to order at 4:00 P.M. on March 1, 1988 in the Faculty Club at the University of California, Davis. The first order of business was to review the minutes of last year's meeting. Unanimous approval of the minutes was obtained by voice vote and as printed in the proceedings of the 37th meeting of WPDC.

The next order of business was the treasurer's report. Dr. Rosenwald reported a balance of $9,041.00 in the conference account, and approximately $10,000.00 received from registration at the current conference. From the $19,041 total, several expenses of the current conference still must be paid but a balance of approximately $6,000.00 is expected after all the current expenses are paid. Dr. Rosenwald acknowledged the continued support of the American Association of Avian Pathologists (AAAP), the Distinguished Donor Members, the Sustaining Members, and the Friends of the Conference, all of which are vital to the successful mission of the conference.

President Jensen reminded all members that the site of the next conference was approved at the last meeting to be Arizona State University with the dates now established as March 6–9, 1989. A call for invitations for a meeting site for 1990 was issued and after some discussion it was decided that the exact site could not be identified at the moment but that a site in the general vicinity of central California will be selected by the officers of the conference. Possible sites are Sacramento, Davis, and San Francisco.

Dr. Art Bickford then presented a report on the activities of the ad hoc committee that has been working for the last few years to suggest a long-range plan of management of the WPDC. A final report was included in the announcement of the meeting and was reviewed with the membership. A new organization of eight officers was recommended as follows:

1. Executive secretary and conference coordinator
2. Treasurer and fund raiser
3. President
4. Program chairperson
5. Program chairperson-elect
6. Local arrangements chairperson
7. Local arrangements chairperson-elect
8. Proceedings editor

The executive secretary, treasurer, and proceedings editor are considered to be semi-permanent positions, with all other officers serving one-year terms. The chairperson-elect will automatically move up to become chairperson at the end of the one-year term. All officers will be subject to approval by the membership each year at the annual business meeting.

Dr. Zander complimented the committee for devising a plan to assist the organization but requested a nominating committee be added to select officers in the future. Following discussion of that proposal, Dr. Zander moved that the current officers of the conference each year serve as a nominating committee for future officers. The motion was seconded and was passed unanimously by all members present.

The final report of the ad hoc committee was reviewed and a listing of the current officers was proposed as follows:

1. Executive Secretary	Dr. R. Chalquest
2. Treasurer	Dr. A. Rosenwald
3. President	Dr. B. Kelly
4. Program Chairperson	Dr. M. Matsumoto
5. Program Chairperson-elect	Dr. Jeanne M. Smith
6. Local Arrangements	Dr. R. Chalquest
7. Local Arrangements-elect	To be selected
8. Proceedings Editor	Dr. M. Jensen

The president requested additional nominations from the floor, whereupon Dr. Zander moved that the nominations be closed and that the final report of the ad hoc committee with the listed slate of officers be approved. The motion was seconded by Dr. Mathey and was unanimously approved by all of the members. The president then dissolved the ad hoc committee, thanking them for their work.

One additional act requested by the ad hoc report was the establishment of a support committee with a representative of each of the following districts of the conference:

1. Northwest and Canada
2. California

3. Mexico
4. Other Western States

It was decided that each of these districts should choose a representative by the next meeting of the conference in 1989.

The president then asked the members for any new business. No new business was forthcoming, and Dr. Jensen turned the gavel of the conference over to Dr. Barry Kelly. Past-president Duncan McMartin presented a plaque to outgoing President Marcus Jensen in appreciation of his service.

The meeting was then adjourned.

PROCEEDINGS EDITOR NATIONALLY RECOGNIZED

Louise E. Williams, of Scholarly Publications at Brigham Young University and editor of the *Proceedings of the Thirty-Seventh Western Poultry Disease Conference*, was a Third Prize winner in the *Ventura Publisher* "Design for Excellence" contest sponsored by XEROX. Her accomplishment has been widely publicized. Her photograph has prominently appeared nationally (with a bird on her shoulder, of course), along with a photograph of the cover of the *Proceedings*, on which publication Mrs. Williams was judged. Congratulations, Mrs. Williams! And thank you for so significantly enhancing the prestige of the WPDC.

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Note: Papers of both the proceedings of the 38th WPDC and proceedings of the Monoclonal Antibody Workshop are listed together in alphabetical arrangement by author. Authors and titles of all papers scheduled for presentation at the conference are listed. However, the texts of several papers are not included in this proceedings owing to the fact that they were not received in time.

PROCEEDINGS OF THE THIRTY-EIGHTH WESTERN POULTRY DISEASE CONFERENCE

EMERGING NEW DISEASES OF POULTRY

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Introduction

Emerging and new may convey similar meanings, but for purposes of this presentation, new is defined as not previously reported while emerging includes diseases in which the cause(s) and/or features are often well known. In the case of emerging diseases, variations in agent or clinical syndrome may be recognized. Economic significance or public opinion may be changed, thus causing an "old" disease to emerge. Use of emerging and new is illustrated here by having chicken anemia agent (CAA) included with salmonellosis.

Emerging diseases was the topic of a recent review.[63] The present paper updates that review and includes only diseases of chickens and turkeys. Diseases are grouped into categories to facilitate organization and presentation.

New Diseases

Egg drop syndrome (EDS76) is no longer a "new" disease, but is an excellent example of a disease that emerged to cause economic loss. Recognition that a new syndrome existed, documentation of the clinical and epidemiologic features, isolation of the etiologic agent, reproduction of the disease, and prevention through vaccine development and application are demonstrated in this example.[1,1,54,80,96,98,120,143]

CAA is perhaps the new disease of greatest interest currently in the USA. Isolated by Japanese investigators while studying a "vaccine" accident, CAA caused anemia and mortality in chicks inoculated at day-of-age. CAA caused no lesions in inoculated embryos nor did the virus produce lesions in cell culture systems. CAA was identified as a parvo or parvo-like virus.[87,155,184,197,168] Enhanced pathogenicity was demonstrated when CAA was combined with other agents such as Marek's disease virus (MDV) or adenovirus.[143,144,145,146,147] Antibodies against CAA are detected using an indirect immunofluorescent procedure in which virus is grown in a lymphoblastoid cell line (MDCC–MSB1).[54,156] Yuasa recently reviewed research finding on CAA.[184] Antibody to CAA is widespread in broiler breeders, commercial layers, and parent layer stock. Antibody was also detected in a number of SPF flocks in several countries, including the USA.[180] Rosenberger (personal communication) isolated CAA from sentinel birds in the Delmarva region of the USA. CAA is the topic of several presentations which follow later in this conference, thus further indicating current interest in this disease. The practical significance of CAA as a disease of poultry is being investigated. Box et al. provided evidence suggesting that CAA may be responsible for poor antibody response to killed NDV vaccine.[14]

Avian nephritis virus (ANV) is a picornavirus isolated from the rectal content of a clinically normal broiler chicken.[161] Inoculation of day-old chicks causes lowered body weight, degeneration of proximal convoluted tubules of the kidney and presence of viral antigen in the kidney.[71,81] Antibody to ANV is widespread in poultry flocks in Japan and N. Ireland.[69] Nicholas et al.[109] recently tested 400 sera from 35 chicken or turkey flocks in England. They found ANV antibodies in 14 of 25 chicken and 4 of 10 turkey flocks. Several (2 of 3) SPF flocks had ANV antibodies. No ANV was found in any of the vaccines tested by these workers. The significance of ANV in clinical disease or economic loss is not known at this time.

Turkey rhinotracheitis (TRT) is an acute, rapidly spreading respiratory disease of turkeys,[6,7,15,50,73,74,76,87,150] TRT must be differentiated from rhinotracheitis or turkey coryza caused by *Bordetella avium* and sometimes referred to as ART because of the early identification of the organism as *Alcaligenes fecalis.* TRT is considered to be caused by a virus. Current evidence indicates that this virus is a pneumovirus.[100] A possible role for the TRT virus in causing swollen head syndrome in chickens was suggested.[100,105,141] *B. avium* is the accepted cause of rhinotracheitis of turkeys in the USA. Neither antibody against TRT nor TRT virus has been found in turkeys in the USA.

Other diseases of complex etiology and/or poorly understood pathogenesis include runting-stunting syndrome,[24,44,45,46,47,50,52,84,103,110,116,120,126,140] poult enteritis,[99,101,110,117,153,152,154] ascites,[32,63,65,66,77,78,83,94,95] sudden death syndrome,[65,116] and hypertensive angiopathy of turkeys.[66]

Variants

This category includes variations of recognized diseases as well as diseases due to antigenic

variants. Infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV) are among the best examples for this category.

Use of sentinel birds led to the identification of variant IBDV viruses.[121,123,130,134] Monoclonal antibodies were used for rapid diagnosis of IBDV[94] and for detection of variants using an antigen capture ELISA system.[126,130,137] At least 3 variant IBDV serotype I viruses are recognized using the monoclonal antibody approach (Snyder, D. B., personal communication). The presence of IBDV variants which cause bursal atrophy without the classic lesions of necrosis and inflammation appear to be complicating factors in design of appropriate control measures by vaccination.[73]

Antigenic differences among IBV have long been recognized as causing significant problems in immunization. Davelaar et al. reviewed studies on the isolation and characterization of Dutch isolates of IBV.[28] In spite of the use of technics including limited proteolysis of glycoproteins and monoclonal antibodies for characterization, rapid field diagnosis of IBV and the specific strain responsible is not available. Problems in interpretation of serological data are recognized.[11,22,23,24,33,35,36,43,65,75,79,80,81,104,106,112,120,130] The role of IBV as a cause or contributing cause in urolithiasis is currently of interest.[27,30,31]

Very virulent pathotypes of MDV represent another example in this category.[113] The complicating role of CAA in causing enhanced pathogenicity of MDV must be considered. Future studies on these pathotypes of MDV will require elimination of CAA as a possible complicating factor.

Variations in Clinical Syndrome

Agents which are well-characterized may now present a newly recognized or appreciated syndrome. *Cryptosporidium* sp. were described in poultry by Tyzzer.[105] Within the past 10 years, this organism has been found with increasing frequency.[13,42,54,135] Sinusitis in turkeys was caused by *Cryptosporidium* sp.[51] Cryptosporidia were found in small intestine of chickens,[42,55] respiratory system of turkeys,[114] lungs, air sacs, and ureters of layers,[107] in addition to reports of the organism parasitizing the cloacal bursa.[88] Dual infections of *C. baileyi* and reovirus seemed to promote the shedding of both organisms. Combined infections caused a significant reduction in weight gain.[59,88] Methods for the rapid diagnosis of *Cryptosporidium* sp. in chickens used readily available stains applied to imprints of trachea.[87] When *Cryptosporidium* sp. isolated from commercial turkeys were used to inoculate other turkeys, no differences in body weight, no mortality, and no clinical signs resulted. Organisms were shed from the experimentally inoculated turkeys by 3 days post-inoculation and organisms were found on epithelial cells in the ileum, cecum and cloacal bursa.[13]

Other examples of clinical syndrome variation include biliary hyperplasia in which *Clostridium perfringens* was suspected.[118,138] *Pasteurella anatipestifer* caused a clinical syndrome resembling chlamydiosis in turkeys.[120] *Mycoplasma iowae* is reported to cause synovitis, arthritis, and rupture of the digital flexor tendons in turkeys. *M. iowae* was less effective than other mycoplasma in stimulating circulating antibody production, thus raising the possibility of finding ruptured tendons in birds with no serologic evidence of *M. iowae* infection.[15,16,138] Chlamydiosis occurred in Minnesota turkeys with atypical signs and lesions. The clinical picture was complicated by the isolation of *Erysipelas* sp. from these turkeys. Chlamydiosis was confirmed later after processing plant workers developed flu-like disease.[108]

Diseases with Renewed Interest

Diseases in this category have a long recognized etiology. Interest is renewed because of the economic impact of the disease and/or the public health implications. Pigeon paramyxovirus and spread to commercial poultry provide a classic example of the application of monoclonal antibody technology to epidemiology.[1,2,3,4] The 1983 outbreak of avian influenza (AI) provides another example of the economic impact which such disease have in our industry.[10,16,61] The application of molecular biology technics to the assessment of the pathogenicity of AI viruses illustrates the extension of our efforts to understand and control these diseases.[4,140] Laryngotracheitis virus (LT) caused outbreaks in several regions of the USA during the past 3–4 years.[39,70]

An increasing concern and public awareness of salmonellosis is occurring. The television show "60 Minutes" and articles in popular magazines like Newsweek served to inform or misinform the public. Increased incidence of food poisoning associated with *Salmonella enteritidis* (SE) contamination in eggs caused redirection of research efforts in several areas. A recent (12/17/88) newspaper article (The Atlanta Journal and Constitution, Saturday, December 17, 1988) described the resignation of a high ranking British government official because of comments she made about salmonella contamination of poultry in Great Britain. Egg sales fell 60% overnight as a result of this officials statement. Diseases which have little or no clinical effect on the health of birds may have devastating effect when public

opinion about food safety is influenced. SE caused by phage type 4 caused clinical disease in chickens in England.[87,88] In the USA, SE infection is not usually associated with clinical disease in birds. Other bacteria of interest because of their role in human disease are *Campylobacter* sp.[121] and *Listeria* sp.[43,80]

Other Conditions

Mycotoxins acting alone or in combination with infectious agents pose significant threats to poultry health.[13,20,83,92,126]

Musculoskeletal disorders, frequently referred to as "leg weakness," are problems of importance to turkey and broiler breeder producers.[22,27,56,58,65,118]

Adjuvant vaccine-induced granulomas in breast muscle caused significant economic loss to some producers and represent a classic example of a "man-made" condition. Avian encephalomyelitis (AE) was associated with certain lots of vaccines.[53] Salpingitis and osteomyelitis are two conditions seen with increased frequency by poultry inspectors (Hollingsworth, J., personal communication).

Conjunctivitis and blepharitis resulted from a mixed bacterial infection of *Staphylococcus hyicus, Escherichia coli,* and *Streptococcus* sp.[53]

In Australia, a syndrome associated with drops in egg production, enlarged liver and spleen is referred to as big liver spleen (BLS) disease.[81]

Conclusion

Emerging or new diseases serve as models to help diagnostic and research people improve our service to the poultry industry. Each disease is important on its on merit, but certain concepts seem common to these examples. Several of these concepts are listed below:

1. The need for keen observation and description of clinical disease is critical.
2. A protocol for disease investigation and data collection is essential.
3. Routine procedures for culture and identification, while important, are not adequate. Failure of routine embryo inoculation and cell culture methods to demonstrate CAA is an example.
4. Applications of molecular biology technics have identified additional types (strains) of agents and improved our appreciation of the complexity of current diseases. This statement is illustrated by the application of monoclonal antibody methods for the demonstration of antigenic variants of IBDV. Use of monoclonal antibodies as an epidemiologic tool is illustrated by the pigeon paramyxovirus story.
5. Many current disease problems are of complex etiology. Runting-stunting syndrome is a good example of such a syndrome for which the precise cause(s) and pathogenesis are not known. In fact, this particular syndrome may not be a single disease, but a reflection of multiple possible causes resulting in similar clinical signs. There is an ever increasing awareness of the interactions resulting in new or emerging diseases. Interactions between IBDV, CAA, mycotoxins, coccidia, stress, and other viruses and bacteria to cause disease are receiving increased attention. More research work is now being done in attempting to demonstrate these interactions.
6. Disease of limited importance to the health of birds may be of enormous importance for public health and the economic impact for the industry.
7. A global awareness of new or emerging diseases is essential. Sharing of information and diagnostic reagents is of major importance. This need exists at a time when major poultry disease resources for research are being reduced or closed. The need for central reference laboratory support is increasing. Support for this activity will likely come increasingly from the industry itself rather than from government.

It was the intent of this presentation to serve as an introduction to the topics covered in more depth by others in this conference.

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CURRENT STATUS OF CHICKEN ANAEMIA AGENT

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Recent work in Belfast on chicken anaemia agent (CAA) is summarized in this paper.

Using the indirect immunofluorescence test, it was shown that antibody to CAA is widespread in the UK in commercial chicken flocks, both in layer and heavy breeds. Longitudinal studies showed that after losing maternally derived antibody, breeder flocks seroconverted fairly rapidly around 8 to 9 weeks of age, and at 18 to 24 weeks of age 80% to 90% birds had developed antibody.[1] It is not known whether birds without detectable antibody are susceptible to infection, and whether such birds are likely to transmit the virus vertically to a proportion of the progeny following challenge. Nor is it known whether CAA can induce latent infections, resulting in reactivation and re-excretion at intervals throughout the lifetime of the bird. No antibody to CAA was found in a small survey of turkey and duck sera. Day old turkey poults inoculated intramuscularly with high doses of CAA did not develop anaemia or seroconvert. This indicates that turkeys are not susceptible to infection with CAA.

A serosurvey of SPF chicken flocks from France, Holland, West Germany, UK[2], Australia, and USA showed that many SPF flocks have also been infected with CAA. These included flocks of organizations selling SPF eggs commercially for vaccine manufacture. Because CAA is known to be vertically transmitted, infection in SPF flocks raises the possibility that avian vaccines could be contaminated with CAA. We have shown that low doses of CAA do not produce anaemia in intramuscularly inoculated, susceptible chicks. Therefore if vaccines are tested for CAA contamination by chick inoculation, inoculated birds must be examined for evidence of seroconversion to CAA as well as for anaemia and characteristic histopathology.

We have made 2 isolates of CAA for broilers in the UK with runting stunting syndrome and one isolate from broilers with blue wing in the USA. These isolates were obtained by inoculation of susceptible chicks with field material, followed by isolation of CAA from livers of thymuses of inoculated birds in MDCC-MSB1 cells. Isolation of CAA in MSB1 cells required 9 to 10 subcultures of passages. Isolates were classified as CAA on the basis of growth in MSB1 cells, antigenic relationship by cross-immunofluorescence with the Cux-1 isolate of CAA, chloroform and heat resistance, and ability to produce anaemia and characteristic histopathological changes in experimentally inoculated chicks. Al isolates produced anaemia in almost all susceptible chicks inoculated intramuscularly at one day old, but not in chicks in contact with inoculated chicks. In 7-day old chicks, the UK and the USA isolates produced anaemia in 15% to 20% and 50% respectively of inoculated chicks. Similar results were obtained by Yuasa and Imai[3] using a collection of 11 Japanese isolates of CAA. Using cross-neutralization tests, the UK and USA isolates, the Cux-1 isolate from West Germany and the Gifu-1 isolate from Japan were shown to belong to the same serotype. As the 11 Japanese isolates also belonged to the same serotype as Gifu-1,[3] available evidence indicates that only one serotype of CAA has so far been recognized worldwide. No naturally occurring apathogenic isolates of CAA have yet been reported. These would have obvious potential for use as live virus vaccines. It is important to make and characterize more isolates of CAA worldwide to establish the degree of diversity in terms of both pathogenicity and antigenicity.

Following development of a purification procedure for CAA which yielded preparation of CAA capable of eliciting a serological response in mice, it was possible to produce monoclonal antibodies to the Cux-1 isolate of CAA.

Screening of hybridomas was carried out by indirect immunofluorescence. Over 30 hybridomas secreting antibodies which reacted with CAA-infected MSB1 cells were identified. A number of these were cloned and ascites produced in mice. Three patterns of staining were recognized following immunofluorescent or immunoperoxidase staining of CAA-infected MSB1 cells with monoclonal antibodies:

1. Fine, granular nuclear staining
2. Large nuclear inclusions and
3. Diffuse unclear staining

Monoclonals with patter 1 could be subdivided into those which were Cux-1 specific and those

which reacted with all isolates tested. Monoclonals with pattern 3 reacted with more cells than those with patterns 1 or 2. Only monoclonals with pattern 2 reacted with material from infected MSB1 cells in Western blots. The monoclonals have been used to:

1. identify structures in infected MSB1 cells by immunogold labelling
2. develop antigen detecting ELISAs which have facilitated the development of improved purification procedures for CAA
3. develop an ELISA for detecting antibodies to CAA in chicken sera

Following the demonstration of antibodies to CAA in SPF chickens, and recognizing the desirability of maintaining SPF flocks free from infection, it is obvious that widespread serological monitoring of SPF flocks for CAA antibodies will be necessary. Indirect immunofluorescence is unsuitable for this purpose, as it is unsuitable for examining large numbers of sera and requires the availability of specialized equipment of considerable expertise. For these reasons, we attempted to develop an ELISA to detect antibodies to CAA. In initial experiments using plates coated directly with partially purified CAA, we were unable to detect any differences in reaction between sera found positive and negative by indirect immunofluorescence. However, when a monoclonal antibody was used to coat the plates and capture MSB1 cell derived CAA antigen, such sera were readily distinguished. In the current ELISA procedure, sera are reacted with captured antigen at a dilution of 1 in 1,000 and bound chick immunoglobulins are detected with anti-chick globulin linked to horseradish peroxidase. Field and SPF sera from the UK, USA, West Germany, and Australia have been examined for antibodies to CAA by indirect immunofluorescence and ELISA and the results compared. Similar results were obtained by both tests.

Nucleic acid extracted from partially purified preparations of CAA was sensitive to DNase I and S1 nuclease, but not RNase A, indicating that CAA contains a single-stranded DNA. Following electrophoresis on agarose gels under denaturing conditions, the size of the DNA was estimated to be 2.3 kb. A double stranded DNA of the same size (2.3 kbp) as the viral DNA was recognized in CAA infected MSB1 cells. This double stranded DNA was successfully cloned into the pGEM-1 transcription plasmid. Following excision from the plasmid and ligation, the cloned DNA was able to transect MSB1 cells. This evidence indicates that the size of CAA DNA is considerably smaller than that of parvoviruses. The taxonomic position of CAA has therefore not yet been established.

DNA probes made from cloned DNA have been used to demonstrate the presence of CAA in tissues from experimentally infected chicks. The availability of such probes means that it is now possible to learn more about the epidemiology of CAA infection in commercial flocks than was previously possible using virus detection by isolation in MSB1 cells or susceptible chicks. Virus isolation is too cumbersome to allow large scale epidemiological studies to be undertaken.

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CAA: REVIEW AND RECENT PROBLEMS

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Chicken anemia agent (CAA) is a viral agent which was first isolated from field chickens by Yuasa et al.[20] in Japan in 1979. Since then, CAA has been isolated in several countries throughout the world.[3,6,11,20] Yuasa et al. named CAA from its nature to produce aplastic anemia experimentally in chickens. CAA is presumed to be a virus because of its filterable and transmissible nature; however, it has not yet been classified into any virus group since its nucleic acid type has not yet been determined. CAA fails to grow in standard cell cultures made from chicken tissues. It propagates, however, in some lymphoblastoid cell lines established from Marek's disease (MD) and lymphoid leukosis (LL) lymphomas.[3,23] CAA is prevalent extensively in chicken flocks and most breeder chickens possess antibody against CAA.[19,27] Pathogenicity of CAA is enhanced by concurrent viral infections.[4,8,21,22,41] Recently, CAA is attracting attention for its involvement in several poultry diseases in the field[8,13,28,39] and its immunosuppressive effect.[1,21,35,41] Studies on CAA are reviewed mainly from its pathological and epidemiological aspects.

Physical and Chemical Nature

The physical and chemical nature of CAA is presented in Table 1. CAA was resistant to treatment with organic solvents and passed through a membrane filter of 50 nm in pore size. After filtration through a filter of 25 nm, the infectivity was retained but was reduced significantly. The infectivity remained unchanged by heating at 60°C for an hour, and treatment with several detergents, enzymes, and commercial disinfectants. These results indicate that CAA is a small, unenveloped, and stable agent.

Goryo et al.[12] identified viral particles with a diameter of 19nm as CAA particles. The particles observed resembled a virus belonging to the parvovirus group. The buoyant density of CAA at the peak of infectivity in cesium chloride was 1.35 to 1.36/cm^3 by Goryo et al. The generally accepted buoyant density of parvovirus group is about 1.40/cm^3. The density of CAA in his report was different from that of general parvoviruses. In brief, CAA is a different kind of agent from any known avian viruses. However, the taxonomic position of CAA as a virus can not be determined from the results obtained up to now.

Growth of CAA In Vitro

CAA produced no cytopathic effect (CPE) and failed to grow in any cultured monolayer cells which were made from chicken tissues and chicken embryos.[20,33] It grows, however, in some lymphoblastoid cell lines established from (MD) and LL lymphomas such as MSB1 and 1104B1. CPE and specific immunofluorescent (IF) positive antigens are observed in the cells infected with CAA.[3,23,27] These cell lines can be used to assay CAA for isolation, titration and the detection of antibody by the neutralization and IF tests.

Bülow and Fuchs[3] reported that the pathogenicity of CAA was decreased by passage through MSB1 cells. Boryo et al.[12] asserted, however, that CAA did not decrease in pathogenicity even after 40 passages. We also found no marked decrease in the pathogenicity of CAA after cell passages.[22] The reason for this difference in results is unknown.

Multiplication of CAA in the Chicken

When CAA is inoculated into day-old chicks, infection accompanied by viremia is seen in the chicks 24 hours after inoculation. CAA is recovered from tissues throughout the body, including the brain. A peak of infective titer is reached in all tissues at 7 days after inoculation. The infective titer begins to decrease gradually 3 weeks after inoculation. CAA is recovered from the brain, liver and feces up to 5 weeks after inoculation. It is also recovered from organs throughout the body when inoculated into 4- or 6-weeks-old chickens.[22]

CAA multiplies in chicken embryos,[3] and since it generally does not kill embryos, the embryos can hatch, but they died of anemia after hatching.[20]

Pathogenicity of CAA for Chickens

CAA affected the bone marrow and lymphoid tissues to induce aplastic anemia. The incidence and severity of anemia by CAA infection depend on many factors such as the age of infected birds, presence or absence of maternal antibody,

pathogenicity among strains of CAA, genetic difference in the susceptibility of chickens, and dose and route of infection. Especially, the pathogenicity of CAA is influenced greatly by the age at which birds are exposed to infection. With the advance in age, birds rapidly acquire a resistance to CAA. The pathological changes mentioned below are of those in day old chicks inoculated intramuscularly with Gifu-1 strain of CAA.[36,37,50]

Symptoms

Depression was observed in some of the inoculated chicks about 10 days after inoculation with CAA. Death occurred 12 days after inoculation at the earliest. In general, the utmost onset of symptoms and death appeared 15 to 17 days after inoculation. Chicks exhibited anorexia, lethargy, drooping of the neck, and anemia, which made the whole body pale in color. The incidence of anemia was 100%, and the rate of mortality varied with the experiments, generally being less than 50%. Insufficient growth was clear in inoculated chicks.

Changes in Peripheral Blood

The hematocrit values starts to decline from 8 days and reach a minimum about 16 days after inoculation. Cell numbers in all kinds of cell series such as red and white blood cell and thrombocyte decrease at the same time. Anemia in CAA infection is pancytopenia. Chicks died at this state. In the surviving chicks, these peripheral blood changes returned to almost the same levels as those in normal chicks.

Macroscopic Changes

Discoloration of the bone marrow to yellow and atrophy of the thymus were characteristic changes always found in chicks affected with CAA. Discoloration of the bone marrow began to be noticed in some chicks 4 days after inoculation, and the bone marrow changed to light pink or yellowish white in all the chicks 10 to 18 days after inoculation. Atrophy of the thymus lobes was noticed in chicks 4 to 8 days after inoculation, and it became so conspicuous in all the chicks that the lobes were almost rudimentary by 12 to 18 days after inoculation. Atrophic changes of bursa of Fabricius were also observed. Hemorrhages appeared in the skin, skeletal muscle, and proventricular mucus membrane.

Histological Changes

In chicks inoculated with CAA, histopathological changes appeared in accordance with macroscopical changes at the same time, frequency, and severity. In the bone marrow, all the cells known as the erythrocytic, thrombocytic, and granulocytic series were reduced remarkably in number and disappeared and were displaced by adipose cells. Eventually, 10 to 16 days after inoculation all the chicks showed an aplastic picture of hematopoietic foci in the whole bone marrow. In the thymus, marked disappearance of lymphocytes throughout the cortex and medulla was observed 10 to 18 days after inoculation and later. Thymic lobules were distinctly atrophic. Lymphocytes decreased in number and disappeared in the spleen, the bursa of Fabricius and the other lymphoid tissues throughout the body.

In addition to the changes in lymphoid tissues, histological changes were also observed in other tissues throughout the body when anemia prevailed. These changes were characterized by degeneration, necrosis, and necrobiosis, which seemed to be caused by circulatory disturbance or secondary bacterial infection. Cell filtration were scarce in these lesions.

In chicks which survived infection, these pathological changes disappeared gradually and the chicks were restored to almost the same condition as normal chickens 5 weeks after inoculation or later.

Factors Exerting an Influence on the Pathogenicity

Differences in Pathogenicity Among Virus Strains

The properties among CAA strains isolated by different investigators have not been compared directly. We compared pathogenicity and antigenicity among the 11 strains of CAA isolated in Japan. There was some difference in pathogenicity among the strains for chicks depending on the difference in the age of chicks at the time of inoculation. This difference, however, was not significant. There was no difference in antigenicity among the 11 strains when they were compared by the cross neutralization and IF methods.[38]

Difference in Susceptibility Among Ages in Chicks

The susceptibility of chicks to CAA decreased with the advance in age.[37,38] When chicks were inoculated with CAA within 4 days of age, almost all of them developed anemia. The later the infection with CAA, the lower the incidence of anemia. Chicks inoculated with CAA at 2 weeks-old produced no anemia, although CAA multiplied throughout the body. Such age susceptibility of chicks is related with the development of immunological competence in chicks. It is presumed that CAA produces anemia more readily

in day-old chicks, because the antibody is produced later than in older chickens.

Influence of Maternal Antibody

Since it is common for breeder flocks in the field to possess antibody against CAA, commercial chickens are generally refractory to CAA infection by maternal antibody derived from dams.[21] It is unknown, however, what titer of antibody inhibits the infection with CAA. The age susceptibility of chicks, and the presence and absence of maternal antibody are important factors in discussing infection of CAA in the field.

Pathogenicity of CAA by Concurrent Infection with Other Viruses

It has been reported that the pathogenicity of CAA is enhanced by concurrent infection with viruses such as infectious bursal disease virus (IBDV), MDV, herpesvirus of turkeys (HVT), and reticuloendotheliosis virus (REV).[4,23,25] Chicks inoculated with CAA alone at 2 weeks of age or later showed no anemia. On the other hand, chicks inoculated with IBDV at hatching and also with CAA 2, 3, or 4 weeks later developed anemia. Whereas uninoculated chicks raised with chicks inoculated with CAA alone revealed no anemia. However, when chicks inoculated with IBDV were placed in the same house, anemia was noticed among the uninoculated chicks.[23]

The enhanced pathogenicity of CAA by concurrent infection may be caused by the immunosuppressive effect of viruses such as IBDV, MDV, or REV and by further intensified affection in the lymphoid tissues by coinfection with these viruses.

Since the pathogenicity of CAA is also enhanced in bursectomized chickens, it is considered that humoral antibody may be closely related with the production of anemia by CAA.[42] In bursectomized chicks, CAA persists in the body for a long time.

Route of Infection

CAA infects chickens by various routes such as nasal, ocular, oral, muscular or cerebral. Therefore, the nasal and respiratory routes may be routes of natural infection. The incidence of anemia by the natural routes is lower than that by the intramuscular or intraperitoneal route. Chickens exhibiting anemia by contact infection are rarely observed even if infection took place. However, when a small number of chicks raised among a relatively large number of affected chicks in the same house, chicks sometimes exhibit clinical signs by contact infection. A horizontal infection may occur. However, some factors such as concurrent viral infections may play an important role to produce clinical infection with CAA.

Egg transmission of CAA was proved experimentally.[29] It is supposed that the vertical transmission may be an important route of infection to produce anemia in chicks in the field.

Infective Dose

Morbidity and mortality in chicks inoculated with CAA are related with the dose of CAA used for inoculation; that is, the larger the dose, the higher the severity of anemia and mortality. Larger doses always produce anemia in chicks, but smaller doses do not always produce anemia even if infection occurs. In fact, the doses of CAA which can cause anemia is about 100 times as large as the tissue culture infective doses.

Susceptibility Among Genetically Different Strains of Chickens

There are differences in the rate of appearance of anemia and the severity of viremia among genetically different strains of chickens; however, these differences are not very large (Yuasa, unpublished data).

Epizootiology

Mode of Infection in the Field

The field broiler chicken flocks were chronologically examined for CAA infection.[43] All the day-old chicks had the maternal antibody. The antibody-positive rate began to decrease at about 3 weeks of age, but it began to increase again at about 5 to 7 weeks of age, and finally reached 100%. Although the time of infection may vary among flocks, CAA infection generally appears at 3 to 6 weeks of age, when maternal antibody is decreased. As a result, antibody is supposed to become positive. In fact, CAA was isolated from chickens at 4 to 7 weeks of age. The experimental chicken flocks showed no signs of anemia during the observation period, indicating that CAA may be present commonly in chicken flocks and that chickens may readily become infected with it. However, most of the infected chickens manifested no clinical symptoms, remaining in a state of inapparent infection.

Distribution of Antibody

Antibody to CAA in 40 commercial breeder flocks in Japan was examined.[37] Of 381 serum samples, 375 (93.7%) gave a positive result. By flock, all the chickens were positive in 36 flocks. All the chickens tested were negative in one flock. In the remaining 3 flocks, some chickens were negative and others positive. The antibody-

positive rate was very high among commercially bred chickens. However, negative flocks were also found. Chicks derived from such antibody-negative flocks may possess the possibility to be infected with CAA and show clinical diseases. CAA was found to exist in several countries, Germany, Australia, New Zealand, the United States, Sweden, England and Malaysia.

Most of the breeder flocks raised as SPF chicks were negative for antibody, although a limited number of such flocks were positive. The positive rate was distinctly lower in SPF flocks than in commercial ones, which indicated that raising chickens in an isolated condition is an effective method to prevent of CAA infection. It seems that it is inevitable to assay for CAA in the SPF flocks from which eggs were obtained to produce the biological products.

CAA and Anemic Diseases in the Field

CAA was isolated routinely from chickens suffering from anemic diseases in the field.[22,24] The isolation rate was higher than that of adenoviruses, which is regarded as a main cause of anemic diseases.[24] It was assumed that CAA may be closely related with the occurrence of anemic diseases in the field. However, since CAA is widely spread among commercial chicken flocks, even when CAA is isolated from diseased chickens it cannot be deduced simply that CAA caused the diseases. Yuasa et al.[39] isolated CAA at a high rate from chickens suffering from aplastic anemia, which broke out among 16- to 26-days-old chickens. In this case, it was suggested that some of the breeder chickens, from which the chicks involved in this outbreak had been derived, might be negative for antibody against CAA. The condition of this outbreak was similar to the experimental CAA infection. It is highly possible that this outbreak might have been caused by CAA.

Outbreak of a disease accompanied with aplastic anemia in the field is usually seen among 3- to 15-weeks old chickens, and most frequently among 5- to 9-weeks old chickens.[23] Judging from the results of our experiments, it is hardly possible for chickens exceeding 5 weeks of age to show clinical signs by infection with CAA alone. When complicated by other infective or non-infective factors, CAA may be considered as a cause of diseases in the field, which is a generally accepted assumption.

Bülow et al. reproduced inclusion body hepatitis (IBH) experimentally by coinfection with CAA and adenovirus. The produced disease is corresponded with syndrome of IBH and aplastic anemia seen in the field.[7] Engstrom reported that the lesions similar to those found in field case of blue wing disease (BWD) were produced experimentally in chicks by dual infection with CAA and reovirus.[8] The concern of CAA with complicated diseases in the field is very interesting.

Immunosuppression by CAA Infection

Since CAA damages hematopoietic and lymphoid tissues, it is presumed that CAA might have induced immunosuppression in the chickens. Depression of vaccinal immunity against MD and Newcastle disease vaccines have been reported.[1,21,28,41] We observed experimentally the significant depression of the efficacy of HVT vaccine by CAA infection.[41] In two experiments, the protective index against MD in chickens inoculated with HVT at 1 day and CAA at 4 days, and challenged with MDV at 8 days of age was 41% in experiment 1 and 46% in experiment 2. On the other hand, the protective index in chickens not inoculated with CAA was 93% and 88%, respectively. However, when chickens were challenged with MDV at 18 days of age, HVT protected well against MD. Though CAA infection depressed the vaccinal immunity, the vaccine was still effective in vaccinated groups compared with unvaccinated ones. CAA may be responsible for some of the problems that occur with MD vaccines, however, the incidence of such probably occurs under limited conditions in the field. Otaki et al. reported an impairment of T cell-mediated immunity in CAA affected chickens.[31]

Discussion

This paper summarizes the studies on CAA obtained up to now. Pancytopenia (Aplastic anemia) is a blood dyscrasia caused by disturbance in the hematopoietic function of bone marrow. Some of the diseases of chickens characterized by aplastic anemia, hemorrhages in the muscles and internal organs and atrophic changes of the lymphoid organs are hemorrhagic syndrome,[14] hemorrhagic anemia syndrome,[17] aplastic anemia,[15] hemorrhagic aplastic anemia syndrome,[24] IBH,[18] and infectious anemia.[25] These anemic diseases are called as such from clinical and pathological diagnosis. Each disease is considered to be induced by multiple factors. American investigators suggest that a viral etiology (adenoviruses) plays a major role in these diseases.[18,23,28] However, the results of anemic diseases, including IBH, produced experimentally with adenoviruses have been generally much less severe than those of field diseases.[18] CAA produces clinical and post mortem manifestations similar to those of the anemic diseases mentioned above. It is highly possible that CAA participate in the out-

break of such anemic diseases in the field.[28] At present, it is considered that the incidence of the anemic disease themselves is not very high throughout the world, therefore, they are not regarded as a major economic threat to the poultry industry. However, the presence of CAA should not be neglected as one of the causes of so-called complicated infections nor as a factor aggravating the efficacy of vaccines. SPF flocks from which the biological products are produced should be checked for CAA infection. Much information on experimental CAA infection is available; however, the role of CAA in the field requires further clarification. Especially, more information is needed on the involvement of CAA in complicated infections, immunosuppression, and on the taxonomy of CAA.

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TABLE 1
Physico-chemical characteristics of CAA propagated in cell culture

Treatment		Infectivity (log $TCID_{50}$/0.1 ml) Pre	Post
Ethyl ether	10%[a], 2 hours (h)	5.5	5.5
Chloroform	10%, 2h	5.5	5.5
Filtration (Millipore)	50 nm	5.0	5.5
	25 nm	3.0	0.5
Heat	60°C, 1h	5.0	5.5
	70°C, 1h[b]	4.5	4.0
	80°C, 0.5h	4.5	–[c]
UV irradiation			
10W, 20 cm distance,	1 minute	7.0	2.5
	2 minutes	7.0	1.0
	3 minutes	7.0	–
Enzymes			
DNase	1 mg/ml, 3a°C, 2h	5.5	5.0
RNase	1 mg/ml, 37°C, 2h	5.5	5.0
Trypsin	1 mg/ml, 37°C, 2h	5.5	5.0
Proteinase K	1 mg/ml, 37°C, 2h	5.5	5.0
Commercial disinfectants			
Quternary ammonium compound	5%, 37°C, 2h	5.5	5.5
Amphoteric soap	5%, 37°C, 2h	5.5	5.0
Orthodichloro-benzene	5%, 37°C, 2h	5.5	4.5
Hypochlorite	5%, 37°C, 2h	5.5	–
Iodophor	5%, 37°C, 2h	5.5	1.5≥
Formalin	5%, 37°C, 2h	5.5	1.5
Detergents			
Sodium dodecylsulfate	10%, 37°C, 1h	4.5	5.0
Nonidet P–40	10%, 37°C, 1h	4.5	5.0

[a]Final concentration in culture fluid containing CAA
[b]Results of heating at 70°C are fluctuating
[c]Negative at undiluted material

ISOLATION AND IDENTIFICATION OF A PARVOVIRUS-LIKE VIRUS (THE SO-CALLED CHICK ANEMIA AGENT [CAA]) THAT CAUSES INFECTIOUS ANEMIA IN CHICKS

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Introduction

Commonly called the "chick anemia agent [CAA]," parvovirus-like viruses (PVLVs) currently are globally distributed and cause infectious anemia[4] and immunosuppression[12] in chickens. Disease caused by PVLV is worse when chicks are immunosuppressed by other viruses.[2,21] When simultaneous or secondary infections occur, chick morbidity and mortality usually is marked.[2,4,12,21]

Although serum samples from the United States of America (USA) have contained specific antibody against PVLVs,[11] isolation of PVLV from USA chickens has not been reported.

In this paper we describe a spontaneous outbreak of PVLV-associated disease in Georgia chickens, and the production of anemia in GA–1 PVLV-inoculated specific-antibody-negative embryos and chicks.

Materials and Methods

Clinical History and Case Features

Chicks owned by a major broiler-producing company had a history of opportunistic infections that seemingly followed immunosuppression. Epidemiologic data suggested an infectious etiology.[8]

Chicks on one farm suddenly experienced an increase in mortality at 18 days of age. When necropsied, ventral abdominal and wing tip cutaneous and subcutaneous edema, hemorrhage, and necrosis were present. Disseminated coalescing <1 cm slightly raised pale areas with fine red verminous streaks were seen in livers. Bursas of Fabricius (BFs) and thymuses were small.

Case Work-up

Histology. Organ portions were collected and routinely-processed. Fresh portions of spleens, thymuses, and BFs were homogenized, centrifuged, stained and examined for virus particles with a transmission electron microscope (TEM).

Microbiology. Portions of skin and livers were cultured for aerobic and anaerobic organisms.

Virology. Portions of spleens, BFs, and thymuses were frozen, shipped overnight, received, and kept frozen at–70°C. After thawing, specimens were homogenized, and processed through MDCC–MSB1 culture passages.[8]

Cell preparation and indirect immunofluorescent antibody testing (IIFAT). Briefly, Cuxhaven–1 PVLV– (antigen control supplied by Dr. J. Pearson) or GA–1 PVLV-infected MDCC–MSB1 cells were incubated, harvested, then suspended in phosphate buffered saline (PBS).[8] Air-dried and fixed cell smears on clean glass microscope slides were stored at –20°C. The protocol for staining also has been described.[8]

Briefly, primary antibody (antibody control; specific immune serum containing antibodies against the Cuxhaven–1 isolate of PVLV; supplied by Dr. M. S. McNulty) was applied to thawed Cuxhaven 1–PVLV– and GA–1 PVLV-infected cell smears. After incubation the slides quickly were rinsed with PBS, then washed twice with PBS. Cells then were covered with secondary antibody (fluorescein isothiocyanate—labeled rabbit-anti-chicken antibody) and were incubated as above. After rinsing, washing, and coverslipping, cell preparations were examined for fluorescence.

Experimental Procedures

Seventh-passage GA–1 PVPV-infected MDCC–MSB1 culture supernatant was heat-treated, chloroform ($ChCl^3$) treated, or examined (TEM) for virus particles.[8] Six-day-old specific-antibody-negative (SAN) chick embryos, one-day-old, and three-day-old chicks from parents without antibody against PVLV (negative IIFAT) were identi-

fied individually, arbitrarily divided among groups, and housed in an isolated biocontainment facility. The embryos were yolk-sac inoculated with treated GA–1 PVLV or culture medium. The one-day-old and the three-day-old chicks were intraperitoneally inoculated with treated GA–1 PVLV or culture medium.

At appropriate intervals, blood samples were collected in heparinized micro-hematocrit tubes, centrifuged, and the packed cell volumes (PCVs) were determined and recorded.

Biostatistics

For each group, the numbers of chicks with anemia were compared (2x3 chi-square test). The calculated chi-square value then was appropriately partitioned to locate differences. For each time period, the percentage of chicks with anemia was regressed using an analysis of variance for curvilinearity.

Results

Case Findings

Septic necrotizing dermatitis, hepatitis, cellulitis, and myositis, non-fibrinopurulent necrotizing bursitis, and thymic and splenic lymphoid hypocellularity (maturation arrest, hypoplasia, or atrophy) were diagnosed in spontaneously ill chicks.[5] Nuclear or cytoplasmic inclusion bodies or foreign materials were not seen. Virus particles were not seen in homogenized and processed TEM-examined organ portions.

Clostridium perfringens was isolated from portions of skin and livers.

Virus infection was suspected on the third passage of homogenate-inoculated MDCC–MSB-1 cultures. TEM examination confirmed 18–22 µm diameter (19.2 +/– 0.2 µm) PVLV particles.

Specific cytoplasmic fluorescence was seen in GA–1-infected MDCC–MSB1 cultures.

Experimental Findings

There was an association between anemia and chick and embryo inoculation with GA–1 PVLV (Tables 1, 2, and 3).[5] There were no significant differences in anemia when the GA–1 PVLV-inoculum was either heat- or $ChCl^3$-treated.

There was no effect of age on the development of anemia following inoculation with GA–1 PVLV (Table 4).

Discussion

Clinically-ill Georgia chickens were found to be infected with a virus (GA–1 PVLV) that has physicochemical and morphologic characteristics of members of the Family Parvoviridae.[5,9] Isolation of GA–1 PVLV from Georgia broilers confirms the suspicion that PVLVs are present in the American commercial poultry population.[11]

GA–1 PVLV is significantly associated with the induction of anemia in chicks. Our findings are similar to those of others who have found that PVLVs resist heat- and $ChCl_3$-treatments and produce anemia in chicks.[5,9,10,20]

In our case, clinical signs, and gross lesions in GA–1 PVLV-infected chicks resembled signs and lesions in chicks reported by others.[4,6,7,17,18,20] Opportunistic bacterial and coccidial infections were seen during histological examination of the organs of these clinically-ill chicks. PVLVs reportedly induce immunosuppression.[5,12,20] If this is true, then it is likely that GA–1 PVLV is immunosuppressive. Hypoplasia or atrophy of lymphoid organs in GA–1 infected chicks was similar to the appearance of organs in cases of PVLV infection described by others.[6,7,10,15,18,19,20]

Immunofluorescence-testing indicates that antigenic similarity exists among the Cuxhaven–1-PVLV and GA–1 PVLV isolates.[5] It is obvious that the various PVLVs should be compared for similarities and differences.

Because several infectious and not-infectious etiologies are known causes of anemia in chickens,[1,13,14] the nomenclature for avian anemias caused by infectious etiologies (e.g., anemia induced by avian retrovirus, etc.) should be standardized. Imprecise acronyms such as chick anemia agent (CAA) and avian infectious anemia (AIA) be avoided. Instead, where sufficient evidence confirms a precise etiology, the name or identity of that specific etiologic agent should be used. We suggest "PVLV-associated transient anemia" be used whenever appropriate experimental designs and findings of anemia have been made. Because PVLV infection causes hematologic abnormalities other than anemia,[18] an even more appropriate name for PVLV-induced disease should eventually be coined. We believe that "PVLV-induced transient pancytopenia" is the most appropriate clinical pathological descriptor of this disease in chickens.

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TABLE 1[f]

The association[a] of anemia[b] and parvovirus-like virus in specific-antibody-negative embryos

Treatment[c]	Anemia[d] no	Anemia[d] yes	% yes
a = cell culture	20	0	0
b = chloroform-treated PVLV	22	4	15.3
c = heat-treated PVLV	19	5	20.8
$x^2 = 4.46$, df = 2, not significant			
Partitioning the chi-square	df	x^2	Significance level
i a vs b + c	1	4.13	$P < 0.05$
ii b vs c	1	0.33	n.s.[e]

[a]2x3 chi-square test, appropriately partitioned
[b]Anemia is defined as a packed cell volume of ≤ 26
[c]Cell culture acted as the control because it did not have parvovirus-like virus.
[d] Number of observations
[e]Not significant
[f]From *Avian Diseases* with permission

TABLE 2[f]

The association[a] of anemia[b] and parvovirus-like virus in specific-antibody-negative one-day-old chicks

Treatment[c]	Anemia[d] no	Anemia[d] yes	% yes
a = cell culture	32	0	0
b = chloroform-treated PVLV	46	7	13.2
c = heat-treated PVLV	37	6	14.0
$x^2 = 4.48$, df = 2, not significant			
Partitioning the chi-square	df	x^2	Significance level
i a vs b + c	1	4.82	$P < 0.05$
ii b vs c	1	0.02	n.s.[e]

[a,b,c,d,e] See Table 1
[f]From *Avian Diseases* with permission

TABLE 3[c]

The association[a] of anemia[b] and parvovirus-like virus in specific-antibody-negative three-day-old chicks

Treatment[a]	Anemia[d] no	yes	% yes
a = cell culture	103	0	0.0
b = chloroform-treated PVLV	95	30	24.0
c = heat-treated PVLV	86	23	21.0
$x^2 = 28.05$, df = 2, P< 0.001			
Partitioning the chi-square	df	x^2	Significance level
i a vs b + c	1	27.68	P< 0.001
ii b vs c	1	0.37	n.s.[e]

[a,b,c,d] See Table 1

[c]From *Avian Diseases* with permission

TABLE 4[c]

The effect[a] of age of chicks on the development of anemia[b] following inoculation with a parvovirus-like virus

Age of inoculation	Anemia[d] no	yes	% yes
In ovo	41	9	18.0
1 day	83	13	13.5
3 day	181	53	22.6
	305	75	

$x^2 = 3.67$, df = 2, not significant

[a]2x3 chi-square test

[b]see legend Table 1

[d]Number of observations

[c]From *Avian Diseases* with permission

ENHANCED PATHOGENICITY OF CHICKEN ANEMIA AGENT BY INFECTIOUS BURSAL DISEASE VIRUS RELATING TO THE OCCURRENCE OF MAREK'S DISEASE VACCINATION BREAKS

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Introduction

Marek's disease (MD) generally has been controlled by vaccinating one-day-old chicks with turkey herpesvirus (HVT) or attenuated serotype 1 MD virus (MDV). Nevertheless, excessive losses due to MD occasionally occur in properly vaccinated chicken flocks. Recently, we[3,4] reported the following findings: A virulent MDV and a chicken anemia agent (CAA), first described by Yuasa et al.,[8] were isolated from commercial chicken flocks that had excessive losses from MD, despite proper vaccination with HVT. Subsequently, dual inoculation of young chicks with both agents in the laboratory induced an early mortality syndrome, similar to that caused by very virulent MDV strains, by breaking vaccinal immunity produced by HVT.[7] Apparently CAA infections at early stages of life depressed protective vaccinal immunity of HVT. These findings suggest that CAA may be associated with MD vaccination breaks occurring in commercial chicken flocks.

It has been reported that the pathogenicity of CAA was enhanced by infectious bursal disease virus (IBDV).[1,2] In these studies, however, effects of dual infection of CAA and IBDV have been examined only in chicks inoculated with IBDV at one day of age. The present study was undertaken to examine lesions of chicks inoculated simultaneously with CAA and IBDV at various ages and to evaluate effects of such infections on immunity induced by HVT.

Methods

Two experiments were conducted. In the experiment 1, chicks were inoculated with CAA and/or IBDV at 14, 21, or 28 days of age. Chicks were reared up to postinoculation (PI-day 14, and their hematocrit values and relative weights (organ-weight divided by body-weight times 100) of the thymus and bursa of Fabricius were determined. The thymus, bursa, and bone marrow were collected at necropsy and processed for histological examinations. In the experiment 2 day-old chicks were vaccinated with HVT. The vaccinated and unvaccinated control chicks were challenged with virulent MDV, JM strain, at 7 days of age. The vaccinated and MDV-challenged chicks were divided into groups and inoculated simultaneously with both CAA and IBDV at 14, 21, or 28 days of age, or left uninoculated. Chicks were reared up to 76 days of age and then examined grossly.

Results and Discussion

The results obtained in the present work are summarized as follows: Atrophy of the thymus and aplasia of the bone marrow were more severe in chicks inoculated dually with CAA and IBDV at 14, 21, or 28 days of age than in chicks inoculated with CAA alone. Marked atrophy of the bursa of Fabricius was observed in chicks inoculated with IBDV alone or in chicks inoculated dually with CAA and IBDV. The extent of the bursal damage was similar between the two groups. An early mortality syndrome characterized by severe atrophy of the lymphoid organs as well as anemic bone marrow occurred in HVT-vaccinated and MDV-challenged chicks when both CAA and IBDV were inoculated at 14 or 21 days of age (20% in 14-day and 16% in 21-day inoculated groups). In HVT-vaccinated and MDV-challenged chicks, gross MD lymphomas were developed at a relatively high rate when chicks were dually inoculated with CAA and IBDV at 14 to 28 days of age (25% in 14-day, 36% in 21-day and 11% in 28-day inoculated groups). In contrast, no gross MD lymphomas were detected in HVT-vaccinated, MDV-challenged, and CAA- and IBDV-uninoculated chicks. The incidence of gross MD lymphomas was significantly ($P<0.05$, Fisher's exact test) higher in chicks inoculated with both CAA and IBDV at 14 or 21 days of age than in chicks that received neither CAA nor IBDV.

Our previous work[4] demonstrated that in chicks vaccinated with HVT at one day of age followed by MDV-challenge eight days later, the early mortality syndrome characterized by marked atrophy of the lymphoid organs and aplasia of the bone marrow occurred when CAA was inoculated at one to eight days of age, and

that in chicks treated in the identical manner, protective vaccinal immunity of HVT was significantly depressed if CAA was inoculated at one to 14 days of age. It was presumed that this may be due partly to transient but severe impairment of immune functions resulting from enhanced pathogenicity of CAA in HVT-vaccinated and MDV-challenged chicks and due partly to enhanced pathogenicity of MDV by CAA infections.[3,4,5] The present study indicates that in HVT-vaccinated and MDV-challenged chicks, the early mortality syndrome occurred when both CAA and IBDV were inoculated at 14 or 21 days of age, and that simultaneous inoculation with CAA and IBDV significantly depressed HVT vaccinal immunity even if chicks were inoculated at 21 days of age. These results suggest that pathogenicity of CAA may be enhanced by IBDV infection. On the other hand, Sharma[6] has demonstrated that IBDV infection at very early stages after hatching depressed vaccinal immunity of HVT. It is, therefore, possible that enhanced pathogenicity of IBDV by CAA may amplify the depression of HVT vaccinal immunity. However, such a possibility may be negligible, if any, because there was no evidence suggesting that CAA enhanced the pathogenicity of IBDV in any of the chicks examined in the present.

CAA and IBDV are widespread in commercial chicken flocks in Japan.[8,10] Therefore, there is a possibility that chicks become infected with both CAA and IBDV around three weeks of age when maternal antibodies are faded, and this may lead to the occurrence of MD vaccination breaks.

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ENHANCING EFFECTS OF CHICK ANEMIA AGENT (CAA) ON MAREK'S DISEASE PATHOGENESIS

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Infections with chick anemia agent (CAA), presumably a parvovirus, are widely prevalent in commercial poultry. CAA replicates preferentially in fast-dividing cells, therefore symptoms of disease appear only in young birds with a developing immune response. Clinical symptoms are transient anemia, retardation of growth, and hypoplasia of bursa of Fabricius, thymus, and bone marrow.

CAA infection is reported to occur concurrently with various other virus infections in young chickens and is considered to be responsible for the failure of vaccinations against Marek's disease. Recently, we recovered CAA during severe outbreaks of Marek's disease in the USA, Israel, and The Netherlands. The foreign flocks had been vaccinated with bivalent vaccines. The MDV isolates were of "normal" virulence. In the present study, we developed a microtitre serum neutralization test specific for anti-CAA antibody.

The isolated CAA evoked in laboratory experiments the complete clinical picture including depletion of both B- and T-cell populations. Combined CAA and MDV infections enhanced the spread of MDV via feather follicles. Without CAA less neoplastic MD lesions were observed. The immunopathological effects of CAA are further investigated by immunohistochemical staining techniques using MAbs specific for subsets of lymphoid and non-lymphoid chicken cell populations.

CAA: EXPERIENCES WITH AN EXPERIMENTAL VACCINE

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Introduction

Anemia dermatitis caused high losses in German broiler flocks in the beginning of the 1980s. In Southern Germany alone the losses reached millions of German marks.

We have proven that the disease is spread from the breeder to the progeny, and this mainly in the beginning of the hatching egg production.[6] Always and whenever parent birds were kept under highly hygienic conditions they became infected at point of lay, they then transmitted the infectious agent to the broiler progeny.

By virological examinations of the diseased birds we were at first able to isolate Adeno- and Reoviruses, but a reproduction of the disease with these agents was not possible. It was also not possible to reproduce the disease with bacterial isolates derived from the infected birds.

In 1984/85, we could demonstrate that "chicken anemia agent" (CAA) must be regarded as causative. This was first described by Yuasa et al. in 1979.[6] We called the disease "anemia/dermatitis" or "avian infectious anemia."[6]

By intensive observations on the farms, the epidemiology of this new disease could be elucidated: clinical non-apparent infections of the breeder flocks during hatching egg production, with connatal infections of the chickens via the hatching egg with losses due to anemia in the first weeks of life. These chickens are immunocompromised and they are concurrently infected with various bacteria, which will lead to gangrenous dermatitis in the 3rd to 5th week of life.

Bülow et al. reported an aggravation of anemia by other immunosuppressants, such as Marek, IBV, and Reticuloendotheliosis-virus.[1,2,3,4]

For the prevention of the disease in chickens it is essential to have CAA immune breeders before the start of hatching egg production. Then an infection during laying won't lead to an infection of hatching eggs and the young chickens will be protected by maternal antibodies. The following is a report on our trials for the development of a vaccine.

Material and Methods

Birds

For laboratory trials we had at our disposal SPF-chickens (Valo) which were partially free of CAA antibodies. Vaccination trials were carried out in the field in broiler breeder flocks from different German hatcheries.

Vaccine

As vaccine-virus we used an anemia agent which was isolated from field cases (isolate Cux-1); the agent was passed and propagated in CAA-susceptible SPF-embryos.[5]

Virus-titration

For the determination of virus content in the vaccine, MSB-cell cultures were used by a method described by v. Bülow et al.[5] The endpoint was calculated by the method of Read and Münch, and the virus dose was expressed as tissue culture infective dose 50% ($TCID_{50}$).

Serological Tests

SPF-birds and vaccinates before and after vaccination were tested by immunofluorescence- and serum neutralization-tests, as described by v. Bülow et al.[5]

Housing

Birds were kept in isolation units or modified Horsefall units in our installations. Breeders were kept in farms at various places in Germany, deep-litter management.

Results

Test for Innocuity

Absence of foreign agents. Twenty SPF-chicks, free of CAA antibodies, were inoculated at an age of 5 weeks. Ten birds each received the virus dose of $10^{4.5}$ $TCID_{50}$ by the intramuscular and oral route respectively.

Before vaccination and 42 days after injection the chicks were tested serologically for antibodies against the major chicken diseases, according to the monitoring list of SPF-flocks. Test birds were free of those antibodies. At present there are 15 agents in the monitoring list, besides reo- and adenoviruses there are also leukosisvirus and mycoplasma of birds (Table 1).

Test for pathogenicity. For this trial 20 nine-week-old pullets CAA antibody free were used. They were injected with $10^{4.5}$ $TCID_{50}$ CAA intramuscularly. On day 7, 14, 21, and 28 PI, 5

birds each were killed and examined. They were free of gross lesions. Thymus, bursa, bone marrow, liver, spleen and caecal tonsils were histologically examined. There were no abnormalities in these tissues. Only 1 bird showed, on day 14 PI minor lymphopenia in the thymus, which was considered to be a non-specific reaction. All birds were free of clinical diseases during the observation period (Table 2).

Lack of immunosuppressive effects of the experimental vaccine. This test was carried out in 5-week-old CAA antibody free SPF-birds. Chickens were infected with $10^{4.5}TCID_{50}$ per bird intramuscularly. Four weeks after the CAA infection these chickens and 5 non CAA infected birds were vaccinated with live virus ND-vaccine intranasally. Three weeks after the ND vaccination, HI antibodies were determined. The results showed no differences between the CAA vaccinates and the non CAA infected birds.

Birds were also challenged with a virulent NDV. CAA infected and ND vaccinated were as protected against Newcastle as non CAA infected ND vaccinated chickens. All nonvaccinated SPF-birds died within one week after ND challenge.

An immunosuppressive effect could not be demonstrated (Table 3).

Potency Tests

Determination of infectivity of the experimental vaccine in day old chicks. Ten-fold dilutions of the experimental vaccine were injected intramuscularly into 5 SPF birds, each free of CAA antibodies. Fourteen days after infection, hematocrit values and gross pathological lesions were recorded.

By this means an infectivity up to 1:100 was demonstrated which equals $10^{3.5}TCID_{50}$. Two tenths ml of the undiluted vaccine contains therefore approximately 100 chicken infective dose 50% (CID_{50}) (Table 4).

Evaluation of the minimum-effective dose. Ten-week-old birds were infected with decreasing virus doses by oral application. On day 21, 28, and 35 after infection, blood samples were drawn for the detection of CAA antibodies. Vaccinates which received $10^{2.5}TCID_{50}$ or more of the vaccine virus were antibody-positive 21 days after infection. The minimum effective dose in 10-week-old birds was determined to be $10^{2.5}$ $TCID_{50}$, a value which almost equals the ID_{50} in day old SPF chicks (Table 5).

We recommend for a protective application of the vaccine a 5-fold higher vaccine virus dose, i.e., $10^{3}TCID_{50}$.

Field trials with the experimental CAA vaccine in broiler breeders. After finishing laboratory trials, in 1986 we started vaccination trials in broiler breeder flocks. These flocks were all vaccinated against the following diseases: ND, IB, AE (live), Reo, and IBD (live and inactivated).

During the year 1986, we vaccinated 830,000 birds in 28 farms in different areas of Germany. CAA vaccine was given via drinking water in a dose of approximately $10^{4}TCID_{50}$/bird, at an age of 13 to 16 weeks.

Six weeks after vaccination blood samples were drawn to determine the CAA antibody status. In all vaccinated flocks we detected CAA antibodies. New cases of anemia dermatitis in the progeny of vaccinated broiler breeders were no longer found (Table 6).

More and more it became a practice to test breeder birds before vaccination for CAA antibodies; only those which had been negative were vaccinated.

By this means we found in that 50% of the flocks in Northern Germany were already antibody-positive before vaccination; in Southern Germany the percentage was even higher (70–75%).

Table 7 shows the number of birds and farms vaccinated in the last 2 years. Almost 2.5 million broiler breeders in 69 farms were vaccinated with the experimental vaccine. They all seroconverted within 4–6 weeks post vaccination. No adverse effects could be seen. Offspring remained free of anemia.

To prove CAA immunity of breeders experimentally, broiler progeny was challenged on the first day of life. We used the progeny of parent stock which was vaccinated at 13 weeks of age via drinking water. Hatching eggs were taken from these breeders at the age of 26 weeks, i.e., at the beginning of production.

SPF-birds, free of CAA antibodies, were used as controls. A CAA field isolate was given on day 1 intramuscularly in a dose of $10^{4.5}TCID_{50}$. It is known that infectious bursal disease virus (IBDV) infection aggravates the anemia, therefore, half of the experimental chickens were challenged simultaneously with the virulent IBDV strain 52/70, $10^{3}EID_{50}$/bird, by conjunctival application. On day 14 thymus and bone marrow lesions were recorded. While SPF-birds showed thymus lesions and bone marrow aplasia to a high degree, the offspring of vaccinated broiler breeders showed those symptoms only to a minor degree.

Discussion

CAA infections are widely spread. They are causing a severe anemia and immune deficiency

in young chickens by vertical infection via hatching egg from shedding breeder flocks. Chickens with maternal CAA antibodies are not showing any lesions after infection.[5]

In pullets and older birds, CAA infections are not causing any symptoms or lesions; birds develop immunity and transfer antibodies via yolk to the offspring. In general the epidemiology of infectious anemia caused by CAA infections is similar to that of avian encephalomyelitis.

For the prevention of the syndrome it is therefore essential to immunize breeders in their rearing period. Our vaccine has proven in laboratory and extensive field trials to be potent for breeder vaccination. The vaccine was shown to be pure and free of contaminants, and safe in birds of 12 to 16 weeks of age.

Breeders developed a solid immunity which was protective for the progeny. Immunity lasted for the whole production cycle of the breeder flocks; broiler progeny of CAA antibody positive breeders remained free of anemia.

In our field trials, breeder flocks were also vaccinated against IBD, another immunosuppressive virus infection, providing maternal protection to the offspring. We consider this fact as important to avoid detrimental effects caused by IBD infection, which could enhance susceptibility to CAA.

In a two year trial period, it became evident that about 50 to 75% of breeder flocks were naturally infected with CAA. Vaccination is therefore only necessary on those farms where natural CAA infections are not occurring.

Regular serological monitoring for CAA antibodies in breeder flocks is recommended. More specific and sensitive test procedures which are easier to perform have not yet been developed.

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TABLE 1
Innocuity of a CAA vaccine; absence of foreign agents

Time of Sampling	Presence of antibodies against diseases on monitoring list of SPF flocks, including Leukosis
Before Vaccination	None
6 Wk after Vaccination	None

TABLE 2
Innocuity of a CAA vaccine; test for pathogenicity

Days P I	Histological Lesions: Thymus	Bursa	Bone marrow	Liver/Spleen	Caecal tonsils
7	Neg	Neg	Neg	Neg	Neg
14	Neg*	Neg	Neg	Neg	Neg
21	Neg	Neg	Neg	Neg	Neg
28	Neg	Neg	Neg	Neg	Neg

*Minor Lesions (lymphopenia)

TABLE 3
Innocuity of a CAA vaccine; test for immunosuppression. Chicks were vaccinated with ND-La Sota vaccine, 10^6EID$_{50}$/bird I.N., 4 weeks after CAA vaccination. NDV HI antibodies were determined and all birds were challenged with virulent NDV 3 weeks after ND vaccination

Group	NDV HI mean titer Log_2	Mortality after NDV challenge
CAA vaccinated	6.0	0/8
Not CAA vaccinated	6.2	0/5
Non vaccinated controls	0	5/5

TABLE 4
Efficacy of a CAA vaccine* in chickens

Dilution of vaccine	Dose/bird ($log_{10}TCID_{50}$)	Pathology on day 14 P I: Thymus	Bone marrow	Hematocrit mean
10^0	5.8	5/5	4/5	18.3
10^{-1}	4.8	4/5	3/5	25.5
10^{-2}	3.8	3/5	2/5	27.2
10^{-3}	2.8	0/5	1/5	30.1
10^{-4}	1.8	0/5	0/5	30.3
Controls		0/5	0/5	30.7

* 0.2 ml i.m. per day old bird

TABLE 5
Efficacy of CAA vaccine; evaluation of minimum effective dose by oral route[a]

Group (5 birds each)	Doses $TCID_{50}$/bird	CAA Antibodies, days P I		
		21	28	35
1	$10^{4.5}$	+[b]	+	+
2	$10^{3.5}$	+	+	+
3	$10^{2.5}$	–	–	–
4	$10^{1.5}$	–	–	–
5	None	–	–	–

[a]Into beak, 0.1 ml/bird
[b]Detectable antibodies

TABLE 6
Efficacy of a CAA vaccine. Number of farms and birds in 1986 field trials in 13–15 week old broiler breeders vaccinated via drinking water

Location of farms	No. of farms[a]	No. of birds ($x10^3$)
Lower Saxony	68	169
Schleswig Holstein	3	66
Oldenburg	16	462
Bavaria	3	132
Total	28	829

[a]All flocks seroconverted and offspring remained free of anemia

TABLE 7
CAA vaccinations of broiler breeders 1987/88

Location of farms	No. of farms	No. of birds ($x10^3$)
Schleswig Holstein (including Cuxhaven)	37	852
Lower Saxony	12	498
Bavaria	20	1,138
Total	69	2,488

TABLE 8
Potency test in day old offspring of vaccinated broiler breeders when challenged only with a field isolate of CAA and simultaneously with a field isolate of CAA plus 2 virulent IBVD

Group	Challenge infection	Pathology on day 14 P I	
		Thymus	Bone marrow
Broiler	CAA	0.7*	0.4
	CAA + IBDV	1.1	0.8
SPF controls	CAA	2.2	2.2
	CAA + IBDV	3.0	3.0

*Lesions scores, mean of 10 birds. 0 = negative, 1 = low, minor, 2 = medium, 3 = severe

IDENTIFICATION OF THE CHICKEN ANEMIA AGENT, REPRODUCTION OF THE DISEASE, AND SEROLOGICAL SURVEY IN THE UNITED STATES

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Introduction

Chicken infectious anemia (CIA) is caused by an unclassified virus named chicken anemia agent (CAA). CAA was first isolated during the investigation of a Marek's disease vaccine contaminated with reticuloendotheliosis virus.[11] CAA is resistant to chloroform and to heating at 80°C for 15 minutes, passes through 25 nm filter membranes, and is distinct from other known viruses of chickens.[1,4,8,13]

Susceptible chickens inoculated with CAA at one day of age develop severe anemia by 14 days of age. The main findings are associated with destruction of erythrocytes of the bone marrow, as well as lymphocytes of the thymus and of the bursa of Fabricius.[1,4,8,13]

CAA has been isolated in Japan,[4,8,13] Germany,[1] and the United States[10] (R. Wellenstein, personal communication) and there is serological evidence of its presence in England, Northern Ireland,[7] and Australia.[13]

This paper reports the isolation of an agent, named CIA–1 with antigenic, physicochemical and pathological characteristics consistent with those of CAA.[1,4,8,13] It reports also on the geographical distribution of flocks with antibodies against the agent in the United States.

Materials and Methods

Chickens

Chickens free of CAA antibodies (Bülow, V. v., McNulty, M. S., and Yusaa, N., personal communications) from our Departmental specific-pathogen-free flocks were used. They were housed in Horsfall-type cages under filtered-air, positive-pressure for the entire experimental period.

Virus Strains

Cux–1 strain of CAA[1] was obtained from Mr. R. Wellenstein (SPAFAS, Inc. RFD #2, 67 Baxter Rd. Storrs, CN. 06268) under an USDA import permit.

The original source of CIA–1 was liver and bone marrow from broilers, with signs and lesions suggestive of CIA kindly provided by Dr. T. Holder (Pitman–Moore. Manager, Technical Services. Pitman–Moore, Inc. P.O. Box 537. Millsboro, DE 19966). The tissues were ground, and clarified by centrifugation. The supernatant fluid was then mixed with an equal volume of chloroform for 5 minutes in an ice bath, and clarified again by centrifugation. The final supernatant fluid was filtered in succession through 800, 450, 220, and 50 nm pore membranes (Millipore Products Division. Bedford, MA 01730), and frozen at –70°C. After the chloroform-treated supernatant fluid was passed through the 50 nm pore membrane, the integrity of the membrane was tested with a suspension of chicken embryo fibroblast (CEF) adapted IBDV vaccine assayed before and after filtration. Loss of infectivity of the IBDV suspension after filtration was considered evidence of filter integrity.

Primary isolation of CIA–1 was done by injecting 0.1 ml of inoculum intramuscularly (IM) in each leg and each side of the breast muscle of one-day-old embryonally bursectomized (EBx) chickens.[5] At 18 days postinoculation (dpi), the chicks were weighed, bled for hematocrit determination, euthanized, and examined for lesions. Liver and bone marrow were harvested, processed and frozen as above for further passage in intact chickens. The isolate obtained from the EBx chickens is hereby called CIA–1/1ChP.

Two additional passages were made using 10-fold dilutions of inoculum. Liver and bone marrow were harvested from chickens given the highest dilution to cause signs and lesions of CIA. Material from the second of these "terminal dilutions," designated CIA–1/3ChP, was used for the virus-neutralization test, for the pathology study and antibody production.

Chicken Anemia Agent Antibodies

Antiserum against CAA (CAA +ve) and serum free of antibodies against CAA (CAA –ve) were kindly provided by R. Wellenstein.

Antiserum against CIA–1 was produced by intramuscular (IM) inoculation of 1.0 ml of

CIA–1/3ChP into 4-week-old chickens. This antiserum was tested for antibodies against Cux–1 CAA by IFA test, and against avian encephalomyelitis virus, infectious bursal disease virus, reovirus, and adenovirus by indirect ELISA using commercially available plates (SPAFAS).

Field Sera and Yolk Samples

Field sera were kindly provided by Mark Martin (ISA–Babcock. P.O. Box 280. Ithaca, N.Y. 14850). Yolk samples were obtained from breeder hen eggs, diluted 1:4 in PBS, and kept frozen at –20°C. Included were 29 flocks, great-grandparent, and grandparent stock for both egg- and meat-type, and parent flocks and commercial flocks of egg-type chickens from 10 states of the continental United States, from 6 to 78 weeks of age.

Histopathology

Liver, heart, spleen, thymus, lung, intestine, kidney and bursa of Fabricius were fixed in 10% formalin for histologic examination.

Virus-neutralization Test

Equal volumes of a 1:500 dilution of antiserum CAA positive or CAA negative serum were mixed with ten-fold dilutions of CIA–1/3ChP, and incubated 1 hr at 37°C. One tenth of 1 ml of each virus or virus-antisera dilution were injected IM into the leg of seven one-day-old intact chickens. Chickens with thymus atrophy, pale bone marrow, and/or an hematocrit value lower than 30% at 18 dpi, were considered positive for infection. Titers were determined by the Kärber method.[8]

Indirect Fluorescent Antibody (IFA) Test

The test was carried on MSB–1 cells infected 48 hours earlier with Cux–1 CAA following the protocol described by McNulty et al.[7] Ten µl of serum or yolk samples diluted 1:500 in PBS were placed on CAA-infected and uninfected MSB–1 cells, CAA positive and negative sera were used as controls on each slide. Samples were read as positive only when clear granular fluorescence was observed in the nuclei of CAA-infected MSB–1 cells, and fluorescence was not observed in the uninfected cells. When fluorescence was observed in the uninfected smear the result is reported as uninterpretable.

Results

Virus Isolation

Only one of the four EBx chickens inoculated with the original field inoculum had a low (22%) hematocrit value at 18 dpi, two others could be considered suspicious (29%). Pale bone marrow, thymic and bursal atrophy, and enlarged liver were observed in all four inoculated chickens. Uninoculated controls had hematocrit values of 38% to 40%, and no alterations of the thymus or bone marrow.

Histological changes in infected chickens were characterized by mild to severe depletion of both erythrocytes and lymphoid elements in the bone marrow. There was also a moderate to severe depletion of lymphoid elements in both the medulla and the cortex of the thymus. In severe cases the depletion was such that the distinction between the cortex and the medulla of the thymus was lost. Inflammatory changes were present in the liver, heart and kidney.

When examined at 18 days post-inoculation intact chickens inoculated with the second chicken passage of CIA–1 had low hematocrit values and mild to moderate depletion of lymphoid cells in the medulla of the bursa of Fabricius, in addition to the already described lesions.

Antiserum Against CIA–1/3ChP

Bright granules of fluorescence were present in the nuclei of 10 to 15% of the Cux–1 CAA-infected MSB–1 cells treated with dilutions up to 1:100 of antisera against the CIA–1/3ChP. Fluorescence was not observed in smears of similarly treated uninfected MSB–1 cells. The antisera were negative for antibodies against AEV, IBDV, reovirus, and adenovirus by indirect ELISA.

Virus-neutralization Test

Data in Table 1 show the titers of mixtures of CIA–1/3ChP and CAA –ve or CAA +ve sera inoculated into intact chickens. Neutralization was marginal when low hematocrit values or thymus atrophy were the criteria of infection. However, using pale bone marrow as the criterion revealed at least a 100-fold neutralization of CIA–1/3ChP.

Field Samples

Of the 30 flocks tested for antibodies against the Cux–1 CAA, 23 were found positive in the IFA test. Flocks positive for CAA were from Arkansas, Connecticut, Georgia, Kansas, Maine, Michigan, New York, and Pennsylvania. It should be pointed out that serum samples from two flocks, one in Maine and the other in Michigan, could not be interpreted because there was strong fluorescence against the MSB–1 uninfected cells, possibly because Marek's disease antibodies, masked a CAA-specific reaction. Flocks negative for antibodies against Cux–1 CAA were found Florida, Minnesota, Texas, and West Virginia. Antibodies against Cux–1 CAA were found in

egg-type great-grandparents, grandparents, parents, and commercial chickens. Three flocks of broiler great-grandparents were found negative, and the 5 flocks of broiler grandparents tested were found positive. Antibodies against Cux-1 CAA were found in flocks from 10 to 78 weeks of age.

Discussion

The three most important findings of this work are: 1) the isolation of a CAA, CIA-1, 2) the reproduction of the disease by its inoculation in susceptible chickens, and 3) the detection of CAA antibodies in a high proportion of chicken flocks in the United States.

Several facts strongly support the possibility that CIA-1 is indeed a CAA isolate: 1) CIA-1 is resistant to chloroform treatment and passed through 50 nm membranes like CAA isolates. 2) CIA-1 inoculated in EBX and/or intact chickens produced signs and lesions reported by other authors as characteristic of chicken infectious anemia: low hematocrit values, pale bone marrow, thymic and bursal atrophy, and enlarged liver. The histological changes were also compatible with CAA: mild to severe depletion of erythrocytes and lymphoid cells in the bone marrow, moderate to severe depletion of lymphoid cells of the medulla and the cortex of the thymus and inflammatory changes in the liver, heart, and kidney.[3,4,8,10] 3) CIA-1 induced antibodies against the Cux-1 CAA isolate. 4) CIA-1 is free of other infectious agents which have been associated with anemia in chickens: infectious bursal disease virus and adenovirus, and it is also free from avian encephalomyelitis virus and reovirus, as evidenced by the absence of antibodies against these agents in the sera of chickens inoculated with CIA-1.

The results of the IFA test suggest a wide spread of CAA in chicken flocks in the USA, consistent with studies in other countries[7,10] and of Yuasa et al.[12] in the USA. CAA antibodies were found in flocks from eight out of the twelve states tested. The negative results obtained in four states may be due to the fact that three of these states are not very densely populated with poultry and only one flock was sampled from each. The three flocks from Texas were great-grandparent stock; these would be expected to be well isolated and therefore less likely to be exposed. It is likely that CAA may be more prevalent than this study suggests.

Acknowledgments

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TABLE 1
Neutralization of CIA-1/3ChP by CAA +ve antiserum, assayed in chickens

Inoculum	Log$_2$/ml titer based on:		
	Low hematocrit value (<29%)	Thymus atrophy	Pale bone marrow
CIA–1/3ChP + 1:500 –ve antiserum	1.2	4.0	≥4.5
CIA–1/3ChP + 1:500 CAA +ve antiserum	0.6	3.1	2.6

ANTICOCCIDIAL EFFICACY OF DICLAZURIL IN CHICKENS UNDER SIMULATED USE CONDITIONS

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Introduction

Diclazuril is a novel anticoccidial agent with broad-spectrum activity against 6 species of Coccidia in chickens and 3 important species in turkeys. A series of critical laboratory studies showed that the optimal use level of diclazuril was 1.0 ppm. In the present series of studies two floor pen experiments were done to confirm the results of laboratory studies and to study the response of chickens to diclazuril under simulated use conditions.

Materials and Methods

Animals

Day-old commercial broiler chicks (Peterson x Arbor Acres) were obtained from a local hatchery. Vaccinations were given at the hatchery for Marek's disease and Newcastle disease. Chicks were sexed at the farm and distributed to floor pens, with equal numbers of each sex in each pen.

Facilities

Thirty-six pens in a floor pen house, 5 x 10 feet, were used. Each pen was equipped with an automatic watering fountain and two hanging tube-type feeders. Clean pine shavings were used for litter. Brooding heat was provided by electric lamps and gas fired brooders.

Experimental Feeds and Treatments

A standard commercial type broiler ration was used to mix feeds containing diclazuril at 0, 0.5, 1.0, or 1.5 ppm, or with monensin at 100 g/ton, salinomycin at 60 g/ton or halofuginone at 3 ppm. Feeds were given *ad libitum* until 42 days of age, then unmedicated feed was given to all pens until 49 days of age.

Coccidiosis Exposure System

Recent field isolates of 6 important species of coccidia from broiler farms in the USA were propagated in young chickens and tested for virulence prior to use. Measured quantities of oocysts in aqueous suspension were mixed into the feed in each pen when the birds were 17 days old. The intestinal lesion score system of Johnson and Reid[1] was used for evaluation of lesions when 4 birds per pen were killed for necropsy 7 and 14 days later.

Date Collected

Birds in each pen were weighed at 24, 42, and 49 days of age. The feed issued was weighed in, and weighed back at 24, 42, and 49 days of age. Birds were removed from each pen for lesion score at 24 and 31 days of age. All dead birds were necropsied and their weights recorded.

Results

Experiment 1

The results of this study are summarized in Table 1. The unmedicated controls suffered mortality of 13.2% from coccidiosis, while medication with diclazuril allowed no mortality. Intestinal lesion scores were reduced from a total (upper, mid, lower, and cecal) of 8.08 in unmedicated controls, to 0.29 or less in treatments receiving diclazuril, at 24 days of age. At 31 days of age the scores totaled 4.25 in controls, compared with 0.42, 0.54, or 0.00 in treatments receiving diclazuril at 0.5, 1.0, or 1.5 ppm, respectively. Average live weight at 24 days of age was 0.9 Kg for unmedicated controls, compared with 0.954 or higher for birds receiving diclazuril. Final weights averaged 2.143 Kg for unmedicated controls, compared with 2.218–2.231 Kg for birds receiving diclazuril. Feed conversion averaged 2.046 in unmedicated controls, compared with 1.990–1.955 in birds receiving diclazuril. Birds receiving monensin or salinomycin also had improved lesion scores, lower mortality and improved weight gain and feed conversion relative to the unmedicated controls.

Experiment 2

The results are summarized in Table 2. The mortality from coccidiosis in unmedicated controls averaged 15.7%. There was no mortality from coccidiosis in birds receiving diclazuril at 0.5, 1.0,

or 1.5 ppm. Unmedicated controls had average live weight of 0.760 Kg at 24 days of age, compared with 0.844–0.854 Kg for birds receiving diclazuril at 0.5–1.5 ppm. Intestinal lesion scores averaged 8.79 in unmedicated controls, compared with 1.29, 0.96, or 0.96 for birds receiving diclazuril at 0.5, 1.0, or 1.5 ppm, respectively. Feed conversion averaged 2.257 for unmedicated controls, compared with 1.985, 1.983, or 1.977 for birds receiving diclazuril at 0.5, 1.0, or 1.5 ppm, respectively. Treatment with monensin or halofuginone also reduced mortality from coccidiosis, lowered intestinal lesion scores, improved gain and lowered feed conversion, in comparison with unmedicated controls.

Discussion

These results confirm the previous findings in laboratory studies that diclazuril possesses potent, broad spectrum activity against coccidia commonly found in broiler chickens. The optimum use level of diclazuril demonstrated in these studies, when all factors were considered, was 1.0 ppm. The product was well tolerated by the birds, producing excellent growth and feed conversion, in addition to the control of coccidiosis.

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TABLE 1
Efficacy of diclazuril against coccidiosis in broilers under simulated conditions of natural exposure in floor pens (CST-153)

Treatment	Average wt (Kg)		Feed conversion		Lesion score		% Cocc.
	Day 24	Day 49	Day 24	Day 49	Day 24	Day 31	Mortality
1 Unmedicated Control	.900	2.143	1.711	2.046	8.08	4.25	13.2
2 Diclazuril 0.5 ppm	.953	2.218	1.619	1.990	0.29	0.42	0.0
3 Diclazuril 1.0 ppm	.964	2.227	1.604	1.959	0.08	0.54	0.0
4 Diclazuril 1.5 ppm	.954	1.121	1.611	1.955	0.20	0.00	0.0
5 Monensin 100 g/ton	.916	2.207	1.632	2.035	4.50	1.88	0.5
6 Salinomycin 60 g/ton	.947	2.215	1.624	1.988	2.79	1.25	1.6

TABLE 2
Efficacy of diclazuril against coccidiosis in broilers under simulated conditions of natural exposure in floor pens (CST-152)

Treatment	Average wt (Kg)		Feed conversion		Lesion score		% Cocc.
	Day 24	Day 49	Day 24	Day 49	Day 24	Day 31	Mortality
1 Unmedicated Control	.760	2.005	1.752	2.257	8.79	5.75	15.7
2 Diclazuril 0.5 ppm	.844	2.149	1.524	1.985	1.29	0.46	0.0
3 Diclazuril 1.0 ppm	.849	2.160	1.502	1.983	0.96	0.46	0.0
4 Diclazuril 1.5 ppm	.854	2.193	1.512	1.977	0.96	0.17	0.0
5 Monensin 100 g/ton	.724	2.040	1.649	2.026	6.50	2.38	8.9
6 Halofuginone 3 ppm	.850	2.155	1.530	1.991	1.17	0.46	0.0

BATTERY EVALUATION OF CYGRO MADURAMICIN AGAINST RECENT FIELD ISOLATES OF COCCIDIA IN BROILERS

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Introduction

CYGRO maduramicin ammonium is a fermentation product produced by Actinomadura yumaensis. It is a monoglycoside polyether ionophore anticoccidial. Its anticoccidial activity has been reported in battery studies[1] and floor pen trials.[2-4] Excellent efficacy against 60 field isolates of coccidia from South America was also reported by McDougald et al. in 1987. The present paper represents an extensive evaluation of maduramicin ammonium against recent isolates of chicken coccidia from North America. Forty-one studies involving 73 strains of mixed Eimeria species were conducted in batteries to determine the optimal level of maduramicin and to compare its anticoccidial activity with the major ionophore products on the U.S. market—monensin and salinomycin.

Materials and Methods

The following experimental design (Table 1) was used for each study. Treatments consisted of noninfected, nonmedicated control (NNC); infected, nonmedicated control (INC); infected, medicated with maduramicin at 2, 4, 5, 6, or 7 ppm; infected, medicated with monensin at 99 or 121 ppm; and infected, medicated with salinomycin at 60 ppm.

The treatments were replicated 3 times with 5 Hubbard x Hubbard cockerels in each study. Pretitrated inocula containing sporulated oocysts of a single or mixed Eimeria spp. were inoculated by gavage into 10-day-old chicks. CYGRO 1% premix was used for preparation of various levels of maduramicin in the starter diet and commercial premixes were purchased for monensin and salinomycin medication. The medicated feeds were given from 2 days before coccidial inoculation to 7 days postinoculation (PI). Feeds and water were provided ad libitum.

The parameters (Slide 2) used for evaluation of efficacy were weight gains during 3 to 7 days postinoculation, lesion scores at day 7 PI, and mortality due to coccidiosis.

Modified Johnson and Reid scoring system[5] was used for lesion scores "0" or "1" were considered to have no and light lesions, respectively; those with "2" or "3" were considered to have moderate to severe coccidiosis.

Weight gain and lesion score were analyzed statistically by analysis of variance and their treatment means were compared using Fisher's protected LSD procedures. The Linear Plateau Model analysis was used for determining optimal levels of maduramicin.

Results and Discussion

Maduramicin at 4–7 ppm completely protected experimental chicks from the pathogenic effects of *E. tenella* which caused an average of 26% mortality in the infected, nonmedicated control (Table 3). A significant percentage of chick mortality due to coccidiosis was observed in the monensin medicated groups and some coccidial mortality also occurred in the salinomycin group. Despite the severe challenge during days 3–7 PI, weight gains for the maduramicin 4–7 ppm groups and the salinomycin group were comparable to that of the noninfected, nonmedicated controls. Maduramicin even at a dose as low as 2 ppm significantly improved weight gain over the infected control group.

Birds receiving 5–7 ppm maduramicin gained significantly better than those fed monensin at either 99 or 121 ppm. A similar trend was observed with lesion scores. As the concentration of maduramicin increased from 2 to 7 ppm, the mean lesion scores were reduced from 2.1 to 0.5. Maduramicin at 6 to 7 ppm provided the best moderation of lesions and lesion scores were significantly lower than the monensin or salinomycin groups.

E. acervulina complex included *E. acervulina*, *E. mivati*, and *E. mitis* and are prevalent species in North America. A total of 32 field isolates were tested (Table 4). Maduramicin at 5–7 ppm and salinomycin at 60 ppm provided weight gains comparable to the noninfected, nonmedicated control and significantly better than the infected, nonmedicated control and the monensin-medicated groups. All three ionophores allowed development of intestinal lesions, with the maduramicin 7 ppm group and the salinomycin group having the lowest lesion scores.

Against 17 strains of E. maxima (Table 5), maduramicin at 5–7 ppm and two other ionophores had statistically comparable weight gains

and lesion scores; however, birds fed maduramicin at 5–7 ppm and salinomycin at 60 ppm had the best numerical weight gains during the critical period of 3–7 days PI. All medicated groups, even the suboptimal level of 2 ppm maduramicin, had significant improvements in weight over the infected, nonmedicated group.

E. necatrix was relatively rare in North America and only 3 isolates were tested (Table 6). Weight gains for birds receiving maduramicin at 4, 6, or 7 ppm, monensin at 121 ppm, and salinomycin at 60 ppm were statistically comparable to the noninfected, nonmedicated group. Weight gains and lesion scores were comparable among the CYGRO 4–7 ppm groups, the monensin 99–121 groups, and the salinomycin group, and were statistically better than the infected, nonmedicated control group.

E. brunetti was also a less prevalent species. Only three field isolates were tested (Table 7). Except for the maduramicin 2 ppm group, all other medicated birds maintained weight gains comparable to the noninfected, nonmedicated group and significantly better gains than the infected, nonmedicated group. For lesion moderation, maduramicin at 6 ppm was most effective, followed by maduramicin at 5 and 7 ppm.

Salinomycin and monensin were less effective. For overall assessment, maduramicin at 6 ppm provided the best protection against the *E. brunetti* challenge in terms of weight gains and lesion moderation.

Based on the Linear Plateau Model Analysis, the optimal maduramicin level is 4 ppm for maximizing day 3–7 weight gains and 6 ppm for lesion control of economically important species. A dose range of 5–6 ppm will be recommended for maduramicin for prevention of coccidiosis in broiler chickens.

In conclusion, all three ionophore anticoccidials at recommended dose levels were effective in various degrees against all pathogenic species of coccidia. Maduramicin particularly is more effective than salinomycin and monensin against *E. tenella* and *E. brunetti* and comparable to salinomycin against other species. Monensin was consistently less effective in controlling the majority of field isolates of chicken coccidia from North America.

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TABLE 1
Experimental design

Coccidial Inoculation	Medication[a] Drug	Replicate[b] Level (PPM)	/Group
No	No	0	3
Yes	No	0	3
Yes	CYGRO	2	3
Yes	CYGRO	4	3
Yes	CYGRO	5	3
Yes	CYGRO	6	3
Yes	CYGRO	7	3
Yes	MONENSIN	99	3
Yes	MONENSIN	121	3
Yes	SALINOMYCIN	60	3

[a]From day –2 to day 7 postinoculation
[b]Five 10-day-old cockerels/replicate

TABLE 2
Evaluation parameters

- **Weight gains** from day 3 to 7 pi
- Lesion scores at day 7 pi
- Mortality

TABLE 3
Comparative efficacy of CYGRO, monensin, and salinomycin against *E. Tenella*

Treatment (PPM)	% Coccidial Mortality	Wt. Gain (G) Day 3–7	Lesion Score at day 7
N. N. Control	0[d]	125[a]	–
I. N. Control	26[a]	41[f]	2.9[a]
CYGRO (2)	1.4[bc]	83[e]	2.1[b]
CYGRO (4)	0[d]	116[ab]	1.3[cd]
CYGRO (5)	0[d]	122[a]	1.0[def]
CYGRO (6)	0[d]	122[a]	0.7[ef]
CYGRO (7)	0[d]	120[a]	0.5[f]
MONENSIN (99)	1.2[bc]	95[e]	1.6[c]
MONENSIN (121)	1.6[b]	99[de]	1.2[cd]
SALINOMYCIN (60)	0.1[cd]	118[ab]	1.1[cde]

[a] Means of 18 field isolates

TABLE 4
Comparative efficacy of cygro, monensin, and salinomycin against *E. Acervulina* complex*

Treatment (PPM)	Weight Gain (G) Day 3–7	Lesion Score at day 7
N. N. Control	128[a]	–
I. N. Control	63[f]	2.8[a]
CYGRO (2)	91[e]	2.5[b]
CYGRO (4)	115[bcd]	1.9[e]
CYGRO (5)	119[abc]	1.5[d]
CYGRO (6)	121[ab]	1.2[e]
CYGRO (7)	120[ab]	1.0[ef]
MONENSIN (99)	108[cd]	1.3[de]
MONENSIN (121)	107[d]	1.2[e]
SALINOMYCIN (60)	122[ab]	0.8[f]

*Means of 32 field isolates

TABLE 5
Comparative efficacy of cygro, monensin, and salinomycin against *E. Maxima**

Treatment (PPM)	Wt Gain (G) Day 3–7	Lesion Score at day 7
N. N. Control	133[a]	–
I. N. Control	59[d]	2.5[a]
CYGRO (2)	94[c]	2.0[b]
CYGRO (4)	110[b]	1.5[c]
CYGRO (5)	117[b]	1.3[cd]
CYGRO (6)	117[b]	1.0[d]
CYGRO (7)	116[b]	1.0[d]
MONENSIN (99)	110[b]	1.3[cd]
MONENSIN (121)	109[b]	1.1[d]
SALINOMYCIN (60)	118[b]	1.1[d]

*Means of 17 field isolates

TABLE 6
Comparative efficacy of cygro, monensin, and salinomycin against *E. Necatrix*

Treatment (PPM)	Wt Gain (G) Day 3–7	Lesion score at day 7
N. N. Control	129[a]	–
I. N. Control	38[d]	2.8[a]
CYGRO (2)	72[c]	1.8[b]
CYGRO (4)	107[ab]	1.6[b]
CYGRO (5)	100[b]	1.4[bc]
CYGRO (6)	113[ab]	1.0[bc]
CYGRO (7)	107[ab]	1.3[bc]
MONENSIN (99)	99[bc]	1.5[bc]
MONENSIN (121)	102[ab]	1.2[bc]
SALINOMYCIN (60)	118[ab]	0.8[c]

*Means of 3 field isolates

TABLE 7
Comparative efficacy of cygro, monensin, and salinomycin against *E. Brunetti*

Treatment (PPM)	Wt Gain (G) Day 3–7	Lesion score at day 7
N. N. Control	128[a]	–
I. N. Control	20[f]	3.1[a]
CYGRO (2)	62[de]	2.6[ab]
CYGRO (4)	95[ab]	1.4[cde]
CYGRO (5)	96[ab]	1.2[de]
CYGRO (6)	108[a]	0.9[e]
CYGRO (7)	104[ab]	1.2[de]
MONENSIN (99)	90[ab]	2.1[bc]
MONENSIN (121)	93[ab]	2.0[bc]
SALINOMYCIN (60)	104[ab]	1.6[cd]

[1]Means of 3 field isolates

A REVIEW OF ENTERIC PROTOZOA IN TURKEYS

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Case Report

Enteric protozoa are often seen in wet smears of intestinal scrapings taken at necropsy. Cytologic characteristics can provide rapid identification of these organisms and determine their relevance in relation to clinical signs.

Protozoa that might be identified histologically in a static, colorful, two dimensional tissue section, can be difficult to recognize cytologically. This report (Table 1) will compare size, shape, membrane and nuclear structure, locomotion and other identifying cytologic characteristics of enteric protozoa as they appear in wet mounts. The organisms that will be discussed include, *Trichomonas gallinae*, the cecal trichomonads, *Histomonas meleagridis*, *Spironucleus meleagridis*, *Chilomastix gallinarum*, Cochlosoma sp., *Entamoeba gallinarum*, cryptosporidia, and coccidia. Where available, photomicrographs of these organisms in wet mounts of mucosal scrapings and tissue sections will be projected.

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TABLE 1
Enteric protozoa of turkeys

	Location	Shape	Structure	Nucleus	Locomotion	Cyst	Pathogenicity/Prevalence
FLAGELLATES							
Trichomonads							
–*Trichomonas gallinae*	Proximal to ventriculus	ellipsoid 5–19 x 2–9μm	short undulating membrane	anterior oval	rapid erratic; 4 anterior flagella; no trailing flagellum	No	disease of young; variable pathogenicity/ prevalence; caseous masses
–Cecal Trichamonads							
Tritrichomonas eberthi	ceca	piriform 7–15 x 3–9μm	prominent easily seen undulating membrane extends full body length	anterior oval	rapid spiral oscillation; 3–5 anterior flagella depending on species; trailing flagellum	No	cecal trichomonads are very common and are not thought to be pathogenic
Tetratrichomonas gallinarum							
Pentatrichomonas sp							
Histomonas meleagridis	(Most commonly identified by tissue lesions in ceca and liver)						
Tissue Stages	Usually extracellular	pleomorphic	variable	eccentric oval	no discernable flagella	No	pathogenic; 3–12 week old birds; prevalence depends on management
Invasive Stage	peripheral in early lesions	8–17μm	basophilic	oval	no discernable flagella		
Vegetative Stage	central in chronic lesion cecum liver	pleomorphic 12–21μm	in packets basophilic or clear	oval	no discernable flagella		
Resistant Stage	in chronic lesions, ceca liver	4–11μm	dense membrane; acidophilic in packets and in giant cells	oval	no discernable flagella		
Flagellated Stage	cecal lumen	ameboid 5–30μm		vesicular with dense karyosome	jerky oscillation; no undulating membrane; single short flagellum	No	difficult to find
Parahistomonas wenrichi	(Generally larger than *H. meleagridis* but difficult to differentiate)						
	ceca, no tissue stage	ovoid 9–27μm	cytoplasm clear to granular	vesicular < 4μm	4 flagella; locomotion by pseudopods; sluggish	No	nonpathogenic

Spironucleus meleagridis (Hexamita meleagridis)	duodenum in young; ceca and bursa of older bird	ovoid symmetrical 6–12 x 2–5μm	no undulating membrane or ventral sucker	two prominent anterior nuclei each with large round endosome	fast darting; no spiraling; 8 symmetrical flagella	No	no longer common; infectious catarrhal enteritis in young birds poor management
Chilomastix gallinarum	ceca	pear-shape; asymmetrical 11–20 x 5–12μm	prominent figure "S" cytostomal cleft; no undulating membrane	anterior oval	3 anterior flagella	Rare	common—probably nonpathogenic
Cochlosoma sp.	lower intestine	tapered oval	large ventral sucker	central	6 flagella; erratic spiral motion	No	nonpathogenic
AMEBAE							
Entamoeba gallinarum	ceca	ameboid; 9–25μm; cyst 12–15μm	granular vacuolated cytoplasm	large central nucleus; prominent eccentric endosome	sluggish pseudopods	Yes	probably nonpathogenic
Endolimax gregariniformis	ceca	oval 9 x 5μm; cyst 10 x 7μm; bean shape	granular vacuolated cytoplasm	large irregular endosome	sluggish pseudopods	Yes	nonpathogenic
APICOMPLEXA (Eimeria oocysts are difficult to differentiate.) Less pathogenic coccidia: *E. dispersa, E. innocua, E. meleagridis, E. subrotunda*							
Eimeria meleagrimitis	duodenum jejunum	cyst 19 x 16μm	colorless double wall; ovoid	single	none	Yes	common—pathogenic in young birds
Eimeria adenoides	ileum; cecum rectum-deep in glands and crypts	cyst 26 x 17μm	colorless double wall; ellipsoid	single	none	Yes	common—pathogenic in young birds
Eimeria gallipavonis	ileum; rectum tip of villi	cyst 27 x 17μm	colorless double wall—ellipsoid	single	none	Yes	uncommon—pathogenic in young birds
Cryptosporida meleagridis	intracytoplasmic extracellular distal ileum	gametes, meronts oval 1.5–5μm cyst—5μm	cellular organelles are indistinct by light microscopy		none	Yes	thought to be nonpathogenic or mildly pathogenic

INTESTINAL PARASITES OBSERVED IN BIRDS AT THE CALIFORNIA VETERINARY DIAGNOSTIC LABORATORY SYSTEM—TURLOCK BRANCH

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The Turlock Branch of the California Veterinary Diagnostic Laboratory System (CVDLS) provides diagnostic services to large commercial poultry operations, game bird operations, backyard flocks, and pet bird owners. The following case reports illustrate the diversity of specimens submitted and intestinal parasites identified. Parasites found in the avian digestive tract can be categorized into two basic groups; the Helminth group consists of Nematodes (roundworms), Trematodes (flukes), and Cestodes (tapeworms) and the Protozoa group includes such genera as Histomonas, Trichomonas, Hexamita, and the subclass Coccidia.

A four year old Blue Streak Lori was submitted to the Turlock Diagnostic Laboratory for necropsy to rule out the possibility of psittacosis. This female bird was one of a breeding pair which had died suddenly. The bird was in fair nutritional condition but presented with slight distention of the abdominal wall. No lesions were observed in the heart, lungs, spleen, or kidney. The proventriculus was enlarged, had a markedly thickened wall, and was distended with a mucoid exudate. Scrapings of the proventricular mucosa revealed numerous roundworms approximately 8–10 mm in length. Direct microscopic examination of these round worms revealed wavy cuticular ridges (or cordons) originating from the anterior portion of the body or lips. The roundworm was identified as *Dispharynx* sp. based on its morphologic appearance and location in the proventriculus.[1,2] Histopathology of the proventriculus showed extensive reticular cell hyperplasia, edema, and numerous nematodes embedded in the mucosa. *Dispharynx* has an indirect life cycle involving beetles as intermediate hosts and infected birds pass embryonated eggs in their feces.

The second case involved two Old English Standard game hens and was investigated by Dr. George Cooper of the Turlock Branch of CVDLS. A review of the case history included an upper respiratory problem consisting of nasal exudate and puffy eyes which was unresponsive to sulfa treatment. Both birds were infested with stick-tight fleas *(Echidnophaga gallinacea)* and the proventricular wall was thickened with red globular worms visible through the serosa. This globular worm is characteristic of *Tetrameres* sp.[3] One bird was extremely thin, had firm cystic-like nodules covering the serosal surface of the entire intestines and numerous tapeworms present in the intestinal lumen. This nodular appearance is characteristic of *Raillietina echinobothrida* or "nodular tapeworm."[4] The second bird had thick foul-smelling nasal exudate and sinusitis from which Mycoplasma gallisepticum was isolated. *Tetrameres* also have an indirect life cycle involving insects (grasshoppers and cockroach) as the intermediate hosts and sheds embryonated eggs. Only the female *Tetrameres* attains the globular appearance, the male has a similar appearance to other round worms and is found on the mucosal surface. *Raillietina* uses the ant as the intermediate host and the intestinal nodules form at the attachment site of the scolex.

The final case involved a large turkey ranch with 42,000 birds eleven weeks of age. Six birds were submitted with a history of increased mortality, gasping, and coughing. The hens had been treated with Nitrofurazone, the toms with bluestone and iodine, and all birds had been vaccinated for cholera two weeks previously. Five of the six birds had mucoid tracheitis from which *E. coli* was isolated. The sixth bird showed moderate congestion of the proximal intestinal tract with whitish fibrinous plaques on the mucosa and moderate numbers of roundworms in the lumen. Histopathology of the intestine showed numerous larvae embedded in the mucosa, hypertrophy of mucous cells, numerous monocytic cell accumulation, and moderate heterophil accumulation of the lamina propria. Although positive identification of the larvae was not performed, *Ascaridia sp.* is highly suspected. *Ascaridia dissimilis* is the only ascarid found in

turkeys and it is not known if any pathology occurs.[2] Although, severe ascarid infestation is seldom a problem in modern management practices, decreased weight gain, synergistic interaction with coccidia and transmission of reoviruses has been observed with *A. galli* in chickens.[3]

Intestinal helminthic diseases are not a frequently encountered problem in today's large commercial operations, however, their economic impact has the potential to be substantial. In contrast, game bird operations, backyard flocks, and the pet bird industry provide the diagnostician with a wide variety of parasitic species and subsequent lesions.

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BROILER COCCIDIA DRUG SENSITIVITY PATTERN FOR WESTERN U.S.

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Introduction

Since the introduction of monensin in the early 1970s, drug resistance in *Eimeria* spp. of coccidia has been a rare field problem. Recently other anticoccidials such as halofuginone and salinomycin have been introduced to the U.S. broiler industry. With a choice of anticoccidials, many different anticoccidial programs have been implemented. However, during this time there has been little objective data presented to help define the efficacy of these anticoccidials in these programs.

The sensitivity test is one method of providing data for the determination of anticoccidial usage. It also can be used to define resistance trends and virulence patterns for geographical locations. The following study is presented as a part of an ongoing study of these two parameters.

Materials and Methods

Sampling

Eimeria spp. isolates were obtained randomly from broilers undergoing clinical examination. Oocysts were isolated for examination from intestines exhibiting gross coccidial lesions.

The samples were collected from December 1, 1987 to December 1, 1988. Forty-five isolates were obtained from four broiler growing regions in the Western U.S. The four regions in the Western U.S. include the following: the Arkansas region which includes Southern Missouri and Arkansas; the Louisiana region; the Texas region; and the Pacific region which includes California, Oregon, and Washington.

Twenty-two isolates were obtained from the Arkansas region, five from the Louisiana region, ten from the Texas region, and eight from the Pacific region.

Sensitivity Testing

For the sensitivity testing of each isolate, fourteen day-old broiler chicks were distributed in 10 pens in groups of 10 birds per pen. Two pens of chicks received each medicated feed treatment (see Table 1).

The treatments were given 2 days prior to the administration of the coccidia inoculum and continued until termination of the study, seven days after inoculation.

The challenge inoculums include 300,000 oocysts/bird for *E. acervulina*, *E. mitis*, *E. mivati*, and *E. maxima*. For *E. tenella* the inoculum dose is 50,000 oocysts/bird. At the termination of the study the following parameters were measured blindly with regard to medication identity, lesion scores, weight gain and feed conversion.

Results

The incidence of *Eimeria* species isolation for each region is noted in Table 2.

The results of the weight gains, feed conversion and total lesion scores are reported in Tables 3, 4, and 5.

Discussion

Of the 45 total samples from which *Eimeria* spp. were isolated, *E. acervulina* was not isolated from 2 samples. This 95.6% isolation rate indicates that *E. acervulina* is ubiquitous in broiler houses in the Western U.S.

In all four regions, salinomycin gave the highest weight gain compared with respective unmedicated infected control groups. In the Arkansas, Louisiana and Texas region halofuginone gave the second highest weight gain. Nicarbazin gave the second highest weight gain in the Pacific region.

In all regions, salinomycin gave the lowest feed conversion rates compared with respective unmedicated control groups. Halofuginone offered the second best feed conversion rates for all four regions. In Louisiana halofuginone tied with monensin in feed conversion rates.

Nicarbazin gave the lowest average lesion score for all four regions. The second lowest average lesion score was obtained with halofuginone. This indicates that the chemical

anticoccidials are better in controlling coccidial lesions than the ionophores. However, the chemical anticoccidials gave poorer results in live weight gain and feed conversion rates than salinomycin.

These data indicate that there are regional differences in coccidial challenge levels. Also they indicate that decisions concerning use of anticoccidial medications and shuttle programs can be based on results from sensitivity testing.

TABLE 1
Anticoccidial treatment levels

Anticoccidial	Level g/ton
Monensin	110
Salinomycin	60
Halofuginone	2.72
Nicarbazin	113.4
Unmedicated	—

TABLE 2
Eimeria spp. Isolation incidence by region

Region	E. acervulina	E. maxima	E. tenella
Arkansas	100*	72.7	31.8
Louisiana	100	100	0
Texas	100	50	20
Pacific	75	87.5	25

*Percentage of total number of samples
No *E.necatrix* or *E. brunetti* were isolated

TABLE 3
Weight gain by region in grams

Treatment	AR	LA	TX	Pacific
Salinomycin 60	309	264	329	310
Monensin 110	286	238	299	276
Halofuginone 2.72	299	254	309	293
Nicarbazin 113.4	287	230	306	303
Unmedicated	272	215	293	246

TABLE 4
Average feed conversions by region

Treatment	AR	LA	TX	Pacific
Salinomycin 60	1.55	1.47	1.55	1.58
Monensin 110	1.64	1.59	1.66	1.70
Halofuginone 2.72	1.60	1.59	1.64	1.62
Nicarbazin 113.4	1.63	1.66	1.65	1.64
Unmedicated	1.75	1.77	1.74	1.94

TABLE 5
Average total lesion scores by region

Treatment	AR	LA	TX	Pacific
Salinomycin 60	1.48	1.73	1.27	1.14
Monensin 110	2.04	1.87	1.84	1.83
Halofuginone 2.72	1.05	1.48	0.96	0.50
Nicarbazin 113.4	0.47	0.72	0.23	0.38
Unmedicated	3.23	2.63	3.06	2.59

FEATHER MITES IN DOMESTIC FOWL IN MEXICO

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Introduction

The feathers of fowl are an ideal place for the breeding of various organisms. Among them we find mites called feather mites.

The feathers called "of contour" form the visible plumage of fowls. These feathers present various forms and shapes depending on the region of the body in which they are inserted. Each feather has a central axis with two parts: the calmus and the axis. The calmus is void, transparent and cylindric, with an orifice on the top. The inner part of the calmus is divided into compartments through structures in the form of domes—called inner covers of the pulp. The axis is solid and holds a line of beards that receive the name of vexilo, whether internal or external, according to their placing—narrower on the outside than the inner one on the feathers called flight feathers.[4]

The beards hold a group of branches called barbules. Feather mites can be found in any of the regions of the body mentioned above. They constitute an extensive variety of families, genera, and species that have definite habitats.[1,3] Some of them live in or on the calmus, others on the axis, and others on the barbules. The harm they produce is of different sorts such as the occasional falling of the feathers, or the destruction of barbules or that of the inner covers of the calmus of the feather.[2]

In Mexico, for the past six years we have been studying which genera and species of mites can be found living on the feathers of hens to evaluate the harm they may produce.

Material and Methods

Our material has been feathers taken directly from the body of hens in various fowl enterprises in Mexico. We have taken, as a norm, a feather from each wing and another from the tail.

The method has been to first, carefully watch the feather itself, taking as a reference the region of the calmus, the axis and the vexilo and the barbules. The number of feathers taken has been different depending on the number of fowls in each enterprise. Samples have been taken both from technical enterprises and from domestic ones. After having observed them, mites have been detached and preparations have been made using Hoyer's liquid in order to prepare them for microscopic observation to determine genus and species, whenever possible.

The regions of Mexico in which samples have been taken are: Saltillo, Coahuila; Monterrey, Nuevo Leon; Hermosillo and Cumpas, Sonora; Guadalajara, Jalisco; Petaquillas, Guerrero; Colima, Colima; Puebla, Puebla; Veracruz, Veracruz; Pinotepa Nacional and Oaxaca, Oaxaca; Tuxpan, Veracruz; and Mérida, Yucatán.

Results

Families, genera, and species mentioned here, have been found at:

FAMILY ANALGIDAE	
genus and specie *Megninia ginglymura*	Regions of Mexico with mites Saltillo, Coah.; Monterrey, N. L.; Tuxpan, Ver.; Veracruz, Ver.; Mérida Yucatán; Pinotepa Nal., Oax.
Megninia cubitalis	Veracruz, Ver.; Pinotepa Nal., Oax.
Megninia ortari	Veracruz, Ver.

FAMILY PTEROLICHIDAE	
Pterolichus obtusus	Veracruz, Ver.; Colima, Col, Pinotepa Nal, Oax.; Petaquillas, Gro.
FAMILY DERMOGLYPHIDAE	
Dermoglyphus elongatus	Veracruz, Ver.

All these mite live on the surface of the feathers or among the spaces between the barbules or, as in the case of the Dermoglyphidae on the external basis of the calmus, feeding generally on scales of the skin.

We found in the calmus of the feather the following:

FAMILY SYRINGOPHILIDAE	
Syringophilus bipectinatus	Veracruz, Ver.; Colima, Col.; Cumpas, Son. Petaquillas, Gro.
FAMILY SYRINGOBIIDAE (Subfam. Ascouracarinae)	
Gallilichus hiregoudari	Pinotepa Nal. Oax.

Discussion

About mites found, *Megninia ginglymura* has been the most frequently observed; it has been isolated from five localities and, in at least two of them we have detected falling of the feathers because of the presence of these mites.[6]

Out of the mites found in the calmus of the feather, those of the *Syringophilidae* have been the most frequently found. These feed on the fluids of fowl by introducing their chelicerae through the keels of the feathers they inhabit.[5]

Gallilichus mites live in the calmus of the flight feathers and are among the largest (750 µm). This is only the second mention of these mites in scientific reports. The only previous report we know of was the original description by D'Souza and Jagannath who found them in 1982 in Bangalore, India, where they infested domestic hens.[7] Therefore, this is also the first report on the American continent.

Feather mites continue to be studied with the purpose of determining genera and species found in domestic fowls in Mexico and in evaluating the harm they produce.

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COLD-ADAPTED NEWCASTLE DISEASE VIRUS AS AN EXPERIMENTAL VACCINE FOR CHICKENS

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In recent years an increase in the severity of vaccine reactions of broilers following application of respiratory virus vaccines has directly contributed to a higher incidence of airsac disease and mortality. A number of different factors undoubtedly play a role in this trend. Some of these include infectious bursal disease virus, Marek's disease virus and perhaps other agents that may decrease immune function and overall resistance and in effect cause attenuated vaccines to be more pathogenic for immune-compromised chickens. Hatchery-related bacterial and fungal infections are also an important consideration as they predispose to more severe vaccine reactions in baby chicks. The wide acceptance and popularity of the coarse spray (aerosol) method for vaccine application has had a significant impact on vaccine reactions. Vaccines previously developed for use by eyedrop or drinking water were found to induce unacceptably severe vaccine reactions when given by spray. Although some biologics manufacturers responded by developing milder vaccines for spray, vaccine reaction problems still plague the broiler industry and the worst may be yet to come. The current trend in our industry is to discontinue the use of inactivated adjuvant-based Newcastle disease virus (NDV) vaccines in broiler breeders. Breeder hens vaccinated with inactivated vaccines confer high levels of maternal antibody to their chicks. NDV maternal antibody interferes to varying degrees with NDV vaccinations during the first week of life. We have also known for some time that chicks with low NDV maternal antibody titers experience more severe vaccine reactions. An outcome of the diminished use of inactivated NDV vaccine will be any increase in NDV-associated vaccine reactions and mortality in young broilers.

A common response of the poultry industry to increased early vaccine reaction problems has been to "cut" or dilute respiratory virus vaccines. Diluting vaccine is a risky practice that should be carefully evaluated particularly when mass vaccination methods such as coarse spray or drinking water applications are used. Diluted vaccine contains less vaccine virus on a per chick dose basis. Therefore, chickens vaccinated by coarse spray are less likely to receive an adequate immunizing dose of the vaccine virus. These "unvaccinated" chicks acquire the vaccine virus from a vaccinated flockmate. Bird-to-bird transmission of respiratory vaccine viruses increases virus pathogenicity[3] and contributes to extended "rolling" reactions since chicks become infected at different times. The normal decay of maternal antibody to NDV and infectious bronchitis virus (IBV) also plays a role in "rolling" reactions since chicks missed during vaccination become more susceptible to vaccine reactions the later they become infected.

Our laboratory has for several years been working on developing safer, less reactive, live NDV and IBV vaccines for broilers. Our approach has been to develop cold-adapted, temperature-sensitive (CaTs) viruses that would produce milder vaccine reactions than currently available commercial vaccines. The concept of using cold-adapted and/or temperature-sensitive viruses as vaccines is not new. The association of cold-adaption and/or temperature-sensitivity with virus attenuation has been used to develop and study human and animal experimental virus vaccines.[1,2,4] A temperature-sensitive herpes virus vaccine to control infectious bovine rhinotracheitis is commercially available. Temperature-sensitive strains have been developed from NDV. Some of these viruses were used in the study of viral genetics. Other temperature-sensitive NDV that were developed from highly virulent strains were found not to be suitable as vaccines because they were not fully attenuated.[4] A cold-adapted NDV with slight temperature-sensitive properties was developed from the LaSota strain, but never commercialized.[1,4] Below are some of our results obtained from a CaTs NDV developed in our laboratory from the commonly used B, strain.

Cold-adapted viruses are viruses that are adapted in the laboratory to grow at a lower than normal incubation temperature. The viruses that grow most efficiently at the lower incubation temperature often are also temperature-sensitive (Ts). Ts viruses are characterized by their inability to grow at high temperatures

such as 41°C, the internal body temperature of the chicken. However, viruses that are not cold-adapted and are not temperature-sensitive (non-CaTs) grow equally well at 41°C as well as the standard temperature of 37°C (Table 1).

CaTs respiratory viruses have considerable potential as vaccines for poultry because as with other animal and human viruses, CaTs viruses tend to be less pathogenic than nonCaTs strains. This is likely in part associated with a reduced ability of the CaTs vaccine virus to grow in internal organs where the temperature is 41°C. In chickens vaccine virus growth in internal organs such as spleen, liver, kidney, and oviduct ideally should be minimized to prevent possible damage to those tissues and a resulting heightened vaccine reaction. Commercial NDV B_1 strains vaccines tested to date do not exhibit a decreased ability to grow at the high temperature of 41°C and therefore are not temperature-sensitive. Theoretically commercial NDV B_1 strain vaccines have a greater potential to grow in internal organs and produce a more severe disease associated with vaccination.

The pathogenicity of CaTs B_1 NDV for young chickens was assessed under laboratory conditions. Tables 2 and 3 give the results of a trial in which one-day-old commercial broiler chickens were inoculated with 10^8 embryo infectious $dose_{50}$ (EID_{50}) of CaTs B_1 vaccine. Other viruses included in the study for comparative purposes were a commercial B_1 vaccine, the nonCaTs B_1 control virus, and the Hitchner B_1 virus originally obtained from the University of Wisconsin (Wisconsin B_1). The Wisconsin B_1 virus was the parent strain from which the CaTs B_1 and non-CaTs B_1 viruses were derived. The clinical disease response of the CaTs B_1-inoculated chickens was clearly milder than with the other viruses. Importantly, chickens given CaTs B_1 had fewer air sac lesions and a lower pathogenicity index.

In another pathogenicity trial, CaTs B_1 was compared to commercial B_1 strain NDV vaccines from seven manufacturers (Tables 4 and 5). Seven-day-old, specific-pathogen-free leghorn chickens were inoculated intratracheally with 10^6EID_{50} of each vaccine. There was considerable variation in the pathogenicity of the vaccines. The CaTs B_1 vaccine was found to be less pathogenic than the commercial B_1 vaccines. Again the "reactiveness" of the vaccines was best measured by the incidence of air sac lesions and the magnitude of the pathogenicity index.

A CaTs respiratory virus vaccine must induce protective immunity. The upper respiratory tract is the primary "target" for IBV and NDV live vaccines. Tissues such as the trachea, gland of Harder and others are important in the induction of local tissue immunity and vital to the development of protective immunity. The tissue temperature of the upper respiratory tract is lower than 41°C, the temperature of the deep internal organs, because of evaporative cooling from the respiratory mucous membranes. Consequently, CaTs respiratory viruses can grow in cells of the upper respiratory tissues and induce local tissue immunity. Table 6 shows results of a challenge of immunity study using different dosages of CaTs B_1 NDV for vaccination. Two-week-old SPF leghorns were vaccinated by eyedrop with different 10-fold dosages of CaTs B_1 ranging from $10^{2.5}$–$10^{6.5}EID_{50}$ of vaccine per chicken. At five weeks of age, the chickens were bled and challenged by eyedrop with 10^6–10^8ELD_{50} of the highly virulent Texas GB strain of NDV. Chickens given vaccine containing $10^{4.5}EID_{50}$ or more of the CaTs B_1 virus demonstrated a high degree of protection (≥93 percent).

Combination CaTs B_1 and IBV vaccines were given by eyedrop to seven-day-old SPF leghorn chickens and the birds were challenged with Texas GB and Massachusetts 41 IBV three weeks postvaccination. Single entity CaTs-vaccinated chickens were highly resistant to challenge whereas birds given combination CaTs + IBV vaccine containing different component dosages showed lower resistance (Table 7). NDV serum antibody production was also lower in birds receiving the combined vaccine. IBV immunity based on challenge of the upper respiratory tract with Massachusetts 41 also appeared to be lower in NDV +IBV vaccinates than in birds receiving IBV (Massachusetts type) vaccine alone.

The efficacy of CaTs B_1 vaccine for seven-day-old commercial broiler chickens was also studied (Table 8). Chickens were vaccinated by eyedrop with $10^{2.5}$ or $10^{4.5}EID_{50}$ of CaTs B_1 or a commercial B_1 vaccine. At four weeks of age, the immunity of the chickens was challenged by Texas GB eyedrop inoculation. Serum antibody titers were lower in the CaTs B_1-vaccinated chickens compared to the commercial B_1 vaccinates. However, most importantly, the CaTs vaccinated birds exhibited a high degree of protection following virulent NDV challenge.

Additional experiments involving vaccination of broiler chickens at various ages are planned.

In conclusion, CaTs respiratory viruses used as vaccines have significant potential to decrease the severity of vaccine reactions. However, we will need to learn how to best use these new vaccines to stimulate protective immunity.

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TABLE 1
Temperature-sensitivity of CaTs B_1 NDV. Infectious virus titer of CaTs and nonCaTs NDV B_1 strains titrated in embryonated chicken eggs at 37°C and 41°C

Virus	Titration temperature (C) 37	41	Virus titer
CaTs	9.2[a]	1.9	7.3
NonCaTs	9.9	9.3	0.6

[a]Log embryo infectious $dose_{50}$ per ml

TABLE 2
Clinical disease response of commercial broiler chickens inoculated by the intratracheal route with CaTs B_1 NDV at one-day of age

Virus Treatment	Day Postinoculation 1	2	3	4	5	6	7	8	9	10	11	12	13	14
CaTs B_1	0/20[a]	0/20	0/20	0/20	1/20	1/20	3/20	4/20	3/20	4/20	1/20	2/20	2/20	0/20
	0[b]	0	0	0	5	5	15	20	15	20	5	10	10	0
Commercial B_1	0/20	2/20	5/20	7/20	7/20	11/20	11/20	11/20	9/20	5/20	2/20	2/20	1/20	1/20
	0	10	25	35	35	55	55	55	45	25	10	10	5	5
NonCaTs B_1	0/20	0/20	10/20	17/20	17/20	18/20	18/20	18/20	18/20	8/20	3/20	3/20	3/20	1/20
	0	0	50	85	85	90	90	90	90	40	15	15	15	5
Wisconsin B_1	0/20	3/20	8/20	15/20	17/20	18/20	19/20	19/20	19/20	7/20	5/20	3/20	3/20	3/20
	0	15	40	75	85	90	95	95	95	35	25	15	15	15
None	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Number of chickens with respiratory disease signs (coughing, sneezing, tracheal rales, congestion or labored breathing) or death/total

[b]Percent of chickens exhibiting respiratory disease signs including death

TABLE 3
Pathogenicity of CaTs and a commercial B_1 NDV vaccine for one-day-old commercial broiler chicks inoculated by the intratracheal route

Virus Treatment	Mortality (%)	Airsac Lesions[a]	Pathogenicity (%) Index[b]
CaTs B_1	0/20 (0%)	1/20 (5%)	.075
Commercial B_1	1/20 (5%)	6/20 (30%)	.296
Non CaTs B_1	0/20 (0%)	13/20 (65%)	.478
Wisconsin B_1	2/20 (10%)	13/20 (65%)	.521
None	0/20 (0%)	0/20 (0%)	0

[a]Number of chickens with airsacculitis, perihepatitis or pericarditis/total
[b]Pathogenicity index calculated for 14 day postinoculation observation period. Pathogenicity index = sum of scores/number of observations
Scores: Morbidity = 1, mortality = 2

TABLE 4
Clinical disease response of SPF white leghorn chickens inoculated intratracheally with CaTs B_1 NDV and seven commercial B_1 NDV vaccines at seven-days of age

Virus Treatment	Day Postinoculation													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
CaTs B[1]	0/20[a]	0/20	0/20	0/20	0/20	2/20	3/20	0/20	2/20	2/20	2/20	0/20	2/20	0/20
	0[b]	0	0	0	0	10	15	0	10	10	10	0	10	0
Vaccine A	0/20	0/20	0/20	6/20	8/20	14/20	9/20	10/20	6/20	4/20	6/20	0/20	2/20	0/20
	0	0	0	30	40	70	45	50	30	20	30	0	10	0
Vaccine B	0/20	0/20	0/20	4/20	3/20	4/20	7/20	5/20	4/20	2/20	1/20	0/20	0/20	0/20
	0	0	0	20	15	20	35	25	20	10	5	0	0	0
Vaccine C	0/20	0/20	1/20	3/20	9/20	12/20	8/20	5/20	4/20	2/20	2/20	1/20	0/20	0/20
	0	0	5	15	45	60	40	25	20	10	10	5	0	0
Vaccine D	0/20	0/20	3/20	6/20	8/20	13/20	11/20	8/20	7/20	3/20	1/20	1/20	1/20	0/20
	0	0	15	30	40	65	55	40	35	15	5	5	5	0
Vaccine E	0/20	0/20	0/20	1/20	6/20	9/20	10/20	6/20	6/20	4/20	2/20	1/20	1/20	0/20
	0	0	0	5	30	45	50	30	30	20	10	5	5	0
Vaccine F	0/20	0/20	2/20	6/20	8/20	6/20	10/20	7/20	5/20	2/20	2/20	1/20	1/20	0/20
	0	0	10	30	40	30	50	35	25	10	10	5	5	0
Vaccine G	0/20	0/20	0/20	6/20	9/20	11/20	14/20	10/20	8/20	5/20	4/20	2/20	2/20	0/20
	0	0	0	30	45	55	70	50	40	25	20	10	10	0
Wisconsin B_1	0/19	1/19	2/19	9/19	9/19	10/19	9/19	8/19	6/19	5/19	2/19	1/19	1/19	1/19
	0	5	10	47	47	53	47	42	32	26	10	5	5	5
Neg Control	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20	0/20
	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[a]Number of chickens exhibiting respiratory disease signs (coughing, sneezing, tracheal rales, congestion or labored breathing) or death/total
[b]Percent of chickens exhibiting respiratory disease signs including death

TABLE 5
Pathogenicity of CaTs B_1 and seven commercial B_1 NDV vaccines of CaTs B_1 NDV for seven-day-old SPF white leghorn chickens inoculated by the intratracheal route

Virus Treatment	Mortality (%)	Airsac Lesions[a] (%)	Pathogenicity Index[b]
CaTs B_1	0/20 (0%)	1/20 (5%)	.046
Vaccine A	0/20 (0%)	8/20 (40%)	.232
Vaccine B	0/20 (0%)	6/20 (30%)	.107
Vaccine C	0/20 (0%)	8/20 (40%)	.168
Vaccine D	0/20 (0%)	9/20 (45%)	.221
Vaccine E	0/20 (0%)	8/20 (40%)	.164
Vaccine F	0/20 (0%)	7/20 (35%)	.178
Vaccine G	0/20 (0%)	14/20 (70%)	.253
Wisconsin B_1	1/19 (5%)	11/18 (61%)	.278
Neg. Control	0/20 (0%)	0/20 (0%)	.000

[a]Number of chickens with airsacculitis, perihepatitis, or pericarditis/total
[b]Pathogenicity index calculated for 14 day postinoculation observation period. Pathogenicity index = sum of scores/number of observations.
Scores: Morbidity = 1, mortality = 2

TABLE 6
Efficacy of CaTs B_1 NDV for SPF white leghorn chickens vaccinated at two-weeks of age and challenged at five weeks of age with the Texas GB strain

Vaccine Dose	Prechallenge Serology[b] ELISA	HI	Postchallenge Disease/Mortality
6.5[a]	260	11	0/15[c](100%)[d]
5.5	80	6	0/15 (100%)
4.5	79	5	1/15 (93%)
3.5	15	1	10/15 (33%)
None	29	0	15/15 (0%)

[a]Log embryo infectious dose$_{50}$ of virus per chicken
[b]Geometric mean serum enzyme-linked immunosorbent assay (ELISA) and hemagglutination-inhibition (HI) antibody titer
[c]Number of chickens with central nervous system signs or dead/total
[d]Percent protection

TABLE 7
Results of CaTs B,NDV and IBV combination vaccination trial in one-week-old SPF chickens vaccinated by eye drop with combination vaccines containing different dosages of CaTs B_1 and IBV and challenged by eyedrop three weeks later with Texas GB NDV or Massachusetts 41 IBV

Vaccines	CaTs B_1 dose	IBV dose	Pre-challenge ELISA serology NDV	IBV	Challenge protection NDV	IBV
CaTs B_1 + IBV combination	7.0[a]	5.0	41[b]	379	15/20[c](75%)[d]	14/20(70%)
	7.0	4.0	50	116	13/20 (65%)	15/18(83%)
	6.0	5.0	148	112	12/20 (60%)	13/20(65%)
	6.0	4.0	128	120	13/20 (65%)	18/20(90%)
CaTs B_1 alone	7.0	---	820	57	19/19 (100%)	ND[e]
	6.0	---	669	5	20/20 (100%)	ND
IBV alone	---	5.0	17	158	ND	18/20(90%)
	---	4.0	1	47	ND	17/20(85%)
None	---	---	20	56	1/19 (5%)	0/18(0%)

[a]Log embryo infectious dose $_{50}$ of vaccine per bird
[b]Mean enzyme-linked immunosorbant assay (ELISA) titer
[c]Number of birds protected against neurotropic NDV or Mass 41 infection of the trachea/total
[d]Percent protection
[e]ND = not done

TABLE 8
Efficacy of CaTs B_1 NDV for commercial broiler chickens vaccinated at one-week of age and challenged at four-weeks of age with Texas GB strain

Vaccine Treatment	Vaccine Dose	Prechallenge Serology ELISA	HI	Postchallenge Disease/Mortality
CaTs	5.5[a]	124[b]	2[c]	1/22[d] (95%)[e]
CaTs	6.5	245	1	1/21 (95%)
Commercial B_1	5.5	666	9	0/19 (100%)
Commercial B_1	6.5	782	12	0/21 (100%)
None	---	5	0	23/23 (0%)

[a]Log embryo infectious dose$_{50}$ of virus per chicken
[b]Mean serum enzyme-linked immunosorbent assay (ELISA) antibody titer
[c]Geometric mean serum hemagglutination-inhibition (HI) antibody titer
[d]Number of chickens with central nervous system signs or dead/total
[e]Percent protection

STUDIES OF A NEPHROTROPIC INFECTIOUS BRONCHITIS VIRUS—MW34

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Introduction

Infectious bronchitis (IB) was initially considered to be a disease primarily of the respiratory and reproductive tracts of chickens. However, infectious bronchitis virus (IBV) strains were later isolated from the kidneys of chickens experiencing IB outbreaks and they were found to induce nephritis and gout.[11,26] The effect of an IBV strain on the respiratory tract has been found to be independent of its effect on the kidney.[21] The nephritis–nephrosis syndrome was first described by Cumming and Winterfield and Hitchner.[11,26] In the ensuing years, IBV has been observed by numerous researchers to induce kidney lesions of varying degrees of severity.[24,25]

IBV exposure, inappropriate IB vaccination programs, and/or dietary calcium and phosphorus imbalances have been implicated in outbreaks of renal disease and/or urolithiasis in pullets and laying hens.[10,13,19,23,24]

Although several IBV strains are known to have variable effects on the fowl kidney, only two domestic IBV strains (Gray and Holte) have been reported to induce marked kidney lesions, and these have been placed in one serotype.[1,6,7,14,16,25,26]

This is a report of some physicochemical and biological characteristics of a virus (MW34) isolated from layers experiencing an outbreak of urolithiasis.[5]

Materials and Methods

Viruses

Reference IBV strains used in virus-neutralization (VN) tests and/or bird studies were Massachusetts 41, Connecticut, Florida, Clark 333, Arkansas 99, SE17, Delaware 2868, JMK, Holte, Gray, Iowa 97, Iowa 609, and New Hampshire.[17] The virus isolated from layers experiencing urolithiasis and whose characteristics are reported herein has been designated MW34.[5]

Stocks of IBV strains and MW34 were prepared in 10-day-old specific—pathogen-free (SPF) chicken embryos from three limit-dilution seed viruses.

Antisera

Chicken antisera to reference IBV strains were from department stocks. MW34 antisera was prepared in immunologically mature SPF chickens by standard procedures.[7]

Physical and Chemical Treatments

The MW34 isolate was sized by evaluating filtrates of 300, 220, 100, and 50nm average pore diameter (APD) Millipore filters. The morphology of MW34 was ascertained by examining a negatively stained preparation of concentrated virus with a transmission electron microscope (TEM).

Thermostability and divalent cationic stabilization tests were performed on MW34 with established methods.[22] Chloroform and trypsin susceptibility tests were also conducted on this virus using the methods of Feldman and Wang and Gresser and Enders, respectively.[12,18]

Virus-Neutralization Tests

The constant-serum, diluted-virus method of the VN test assayed in SPF chicken embryos was followed in this study.[3]

Cell-Culture Adaptation

A serial cell-culture-passage method of adapting embryo-propagated MW34 virus in primary chicken kidney cells (CKC) was performed as previously reported.[4]

Pathogenicity, Pathogenesis, Viral Persistence, and Cross-Protection Studies

Pathogenicity, pathogenesis, viral persistence, and cross-protection studies were conducted in groups of 15 to 90 SPF Single Comb White Leghorns (SCWL) of 1 day- to 28-weeks of age; the chickens being exposed to suspensions of IBV strains and MW34 containing 104 to 106 EID50/ml by ocular or oculonasal routes. All chickens were observed for signs of disease and mortality, and a variety of tissues and/or swabs were collected for virus assay and histopathology at various intervals of time post-inoculation (PI).

MW34 exposed chicks were evaluated for weight gain at 7 day intervals, commencing at 1-day-of-age and ending 27 days later. The collected data were statistically analyzed. Egg production and shell quality evaluations were made for mature hens inoculated with MW34 at 28-weeks-of-age.

Three to 5-week-old chickens used for cross-protection tests were challenged 4 weeks PI and the challenge was evaluated by attempting virus recovery from tracheal and cloacal swabs and kidney pieces collected 4 days post-challenge (PC).

Results

Physical and Chemical Studies

The MW34 isolate exhibited responses characteristic of IBV when exposed to various physical and chemical treatments.

MW34 passed through Millipore filters with APD of 300, 220, and 100 nm but not 50 nm, a finding comparable to that for IBV strains. Negatively stained preparations of MW34 particles showed them to be moderately pleomorphic, round or elliptical, and with widely spaced, club-shaped surface projections.

Exposure of MW34 to 56°C resulted in a complete loss of infectivity by 15 minutes. Divalent cationic stabilization tests demonstrated that MW34, like IBV strains, is susceptible to 50°C, whether in the presence or absence of magnesium chloride.

Exposure of MW34 to chloroform resulted in nearly complete loss of infectivity; an effect common to avian coronaviruses (enveloped viruses). Like IBV strains, MW34 was not affected by 0.25% trypsin for 30 minutes at 37°C.

Reciprocal Virus-Neutralization Tests

Reciprocal VN tests revealed marked two-way cross reactions with MW34 and JMK, Gray, and Delaware 2868 strains. Holte, SE17, and Iowa 97 strains of IBV were shown to be partly related (one-way crosses) to MW34.

Cell-Culture-Adaptation Studies

MW34 was found to replicate in CKC and produced a cytopathic effect (CPE) by the second serial cell-culture passage. Common IBV cytopathic responses (syncytia, degeneration, and necrosis) were observed between 18 and 24 hours PI in stained and unstained cultures infected with MW34. IBV-specific antigens were detected in infected cell cultures with a direct immunofluorescent assay.

Pathogenicity, Pathogenesis, Viral Persistence, and Cross-Protection Studies

Pathogenicity studies of MW34 revealed that mild respiratory signs could be readily induced in chickens 1 day- to 5-weeks-of-age, but not in mature chickens. Respiratory signs (sneezing and/or bronchial rales) were first observed 4 to 5 days PI and continued for an additional 3 to 5 days. Histopathologic evaluation of tracheas found them to have extensive mucosal deciliation with inflammatory exudate by 5 days PI. There was evidence of repair by 10 days PI.

MW34 induced mortality was restricted to very young chickens exposed to low embryo passage virus (Table 1). Mortality commenced as early as 5 days PI and continued through 15 days PI. A mortality high of 13.3% was achieved in the first trial.

Gross urinary tract lesions (observed through 15 days PI in Trials 1 and 2) of dead and moribund chicks included enlarged and urate engorged kidney lobes and ureters. Gross changes of less severity were also observed among chickens exposed to MW34 at 14 and 28 days-of-age. Histopathologic evaluation of kidneys from very young chicks revealed extensive degeneration of tubular epithelium and mineralization with interstitial inflammatory cell infiltration and cystic tubular dilation.

In Trial 2, chickens were observed for 30 weeks to evaluate viral persistence and the long term pathologic impact of neonatal MW34 exposure. No gross kidney lesions were found in these chickens for a period commencing 2 weeks PI and continuing through 16 weeks PI; samplings were evaluated at 3 and 4 weeks and then at monthly intervals thereafter. Gross kidney and ureter changes were observed in both males and females at 20 and 30 weeks, but not at 24 and 28 weeks.

An evaluation of viral persistence (Table 2) revealed (to date) that MW34 could be recovered from the kidneys of chickens, inoculated as neonates, up to 15 days PI and from tracheal and cloacal swabs (Trials 2, 3, and 4) up to 30 weeks PI. There appears to have been a period of viral latency between early (2–15 days PI) and late (28 and 30 weeks PI) virus shed in Trial 2; exceptions being single isolations at 4 (trachea) and 8 (cloaca) weeks PI. Mean HI titers for chickens in Trial 2 were found to decline from a high of 1:146 at 4 weeks PI to 1:20 at 12 weeks PI followed by a rising titer.

Two additional methods of evaluating MW34 pathogenicity were conducted; an examination of

its impact on growth rate and effect on egg production and shell quality. The weight gain study (Table 3) revealed that MW34 significantly reduced growth rate for a two week period commencing 7 days PI. Mature hens exposed to MW34 at 28 weeks-of-age exhibited minimal effect on egg production and no effect on shell quality. The egg production of infected hens was marginally depressed (8%), as compared to controls, the second week PI.

A limited pathogenesis study (Table 4) revealed that quantities of MW34 virus could be found in both the respiratory and urinary tracts as the result of oculonasal routes of exposure. The virus was found from 10 to 21 days PI in the organs of these tracts. MW34 was also recovered from a number of other organs including portions of the alimentary tract.

A cross-protection study (Table 5) conducted in 5 week old SPF chickens demonstrated that a mild Massachusetts strain vaccine could provide protection against an MW34 challenge and replicated an earlier finding (3 week old chickens) with a Massachusetts/Connecticut combination vaccine. The reverse was not true. Additionally, the data revealed that MW34 induced good protection against challenges with Gray or Holte strains of IBV. Holte induced some protection against MW34, but had limited effect against a Gray strain challenge. Because kidney infection rates were low for all IBV challenge strains except Massachusetts 41, interpretation of kidney virus recovery is difficult.

Discussion

An IBV isolated from commercial layers experiencing urolithiasis was found to exhibit responses characteristic of IBV strains exposed to various physical and chemical treatments.[9,13]

Interestingly, reciprocal VN tests revealed that MW34 is apparently closely related to JMK, Gray, and Delaware strains of IBV. Therefore, it is suggested that this IBV isolate be tentatively considered a member of the Delaware IBV serotype. The nephropathogenic IBV strains (Gray and Holte) are included in this serotype.[16,26]

MW34 was found to be somewhat atypical of IBV in that a low embryo passage of this virus produced an observable CPE in unstained CKC by the second serial passage.[6]

MW34 induced respiratory signs and tracheal pathology characteristic of IBV. Mortality was restricted to very young chickens exposed to low embryo passage MW34. The most severe gross and microscopic kidney lesions were also observed in the very young. These results lend support to previous reports which found that very young chickens (less than 2 weeks-of-age) had more severe renal lesions and higher mortality than older chickens.[1,4] Our finding of gross kidney lesions in older chickens (20–30 weeks-of-age) has also been reported by Alexander et al. for their long-term IBV pathogenesis study.[2] The transient reduction in growth rate observed among young chickens exposed to MW34 is not atypical of IBV.[26] The fact that MW34 was found to have minimal effect on egg production and shell quality was of considerable interest as this IBV strain was isolated from commercial layers experiencing a urolithiasis outbreak.[9] Despite this clinical problem, egg production in this commercial flock was not adversely affected.

Although an evaluation of viral persistence is in progress, our results to date have revealed a periodic shedding of MW34 recoverable from kidney tissue and tracheal and cloacal swabs over a period of 30 weeks. The data suggests that there was a period of viral latency of some 20 weeks. The late phase virus shed observed probably resulted from physiological stress associated with the onset of lay. The immune response (HI titers) of MW34 exposed birds was cyclic in nature and appeared to correspond somewhat to virus shed patterns. These results lend support to earlier findings of long-term IBV persistence with intermittent and erratic virus shedding.[2,15]

A limited pathogenesis study revealed quantities of MW34 in the respiratory, alimentary and urinary tracts following a natural exposure of chickens to this virus. The virus was found from 10 to 21 days PI in the organs of these tracts. A finding of some interest was the recovery of virus from the esophagus and proventriculus up to 14 days PI.

Contrary to anticipated results (based on reciprocal VN tests), reciprocal cross-protection studies revealed that a Massachusetts or a combined Massachusetts–Connecticut vaccine could provide good protection against a MW34 challenge. The reverse was not true. The finding that MW34 protects against Gray and Holte challenges lends support to the VN test results and its tentative placement in the Delaware serotype.

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TABLE 1
Summary of mortality and gross and microscopic kidney lesions in SPF White Leghorns inoculated with MW34 at various ages

Treatment	Trial no.	Number of chickens	Age of chickens (days/wks)	Specific mortality	Kidney lesions	
					gross	microscopic
7th EP	1	30	1D	4/30 (13.3)[a]	4/30	13/30
7th EP	2	90	1D	7/90 (7.7)	15/90	15/90[b]
4th EP	3	15	4D	1/1 (6.6)	3/15	5/15
14th EP	4	20	4D	0/29	0/29	0/29
8th EP	5	50	2W	0/50	4/50	10/50[b]
8th EP	6	39	4W	0/39	1/39	8/39[b]

[a]( ) = percent mortality.
[b]Evaluations not completed.

TABLE 2
Viral persistence and antibody response in SPF White Leghorns inoculated with MW34 at various ages

Trial no.	Age of chickens (days/wks)	Days/wks PI	No. chickens sampled	Mean HI titer	Virus recovery		
					Trachea[a]	Cloaca[a]	Kidney
1	1D	2–8D	12	--[b]	--	--	3/12
		10–15D	9	--	--	--	3/9
		17–21D	9	--	--	--	0/9
2	1D	5–9D	9	--	--	--	9/9
		10–13D	4	--	--	--	4/4
		3W	6	--	--	--	0/6
		4W	16	146	1/10	0/10	0/6
		8W	16	54	0/10	1/10	0/6
		12W	16	20	0/10	0/10	0/6
		16W	16	105	0/10	0/10	0/6
		20W	16	116	0/16	0/16	0/6
		24W	16	--[c]	0/16	0/16	0/6
		28W	16	--	4/16[d]	0/16[d]	0/6[d]
		30W	26	--	13/26[d]	12/26[d]	--
5	2W	5D	5	--	5/5	5/5	--
		3W	10	--	--	--	--
		4W	10	--	2/10	3/10	--
6	4W	5D	5	--	5/5	4/5	--
		3W	5	--	0/5	1/5[d]	--
		4W	9	--	2/9	4/9[d]	--

[a] Swab
[b] Not examined
[c] Evaluations not yet initiated
[d] Evaluations in progress

TABLE 3
Weight gain study in 1-day-old SPF White Leghorns inoculated with MW34

Treatment	No. of chickens	Days post-inoculation 7	14	21	28
Control	30	23.4[a]	48.7	68.3	69.0
MW34	30	23.5	42.8[b]	58.1[b]	65.7

[a]Mean weight gain in grams
[b]Values significantly different from that of control ($P<.05$)

TABLE 4
Location and titer of MW34 in 3-week-old SPF White Leghorns inoculated by a combination intranasal/ocular route

Days post inoculation	Log$_{10}$ virus titer per 0.2 gram organ homogenate: Trach	Esoph	Prov	Duo	Jej	Ile	Kid	Ceca	Bursa
5	2.7[a]	2.3	1.2	---[b]	---	---	2.8	0.5	0.9
10	1.4	---	---	---	---	---	---	1.3	---
14	---	0.7	0.5	---	---	---	0.5	---	---
21	---	---	---	0.5	---	---	---	---	---

[a]Average titer for 2 chickens
[b]No titer

TABLE 5
Summary of a Massachusetts strain vaccine MW34, Gray, and Holte cross-protection study

Immunizing virus	Challenge virus: Mass.	41	MW34		Gray		Holte	
Mass. vaccine	63[a]	80[b]	63	0	29	100	43	67
MW34	0	80	88	0	86	0	88	100
Holte	17	20	63	100	43	100	71	67

Percent protection as determined by challenge virus recovery from tracheal swabs,[a] and kidney[b]

INFECTIOUS BRONCHITIS VACCINATION STUDY AND DETERMINATION OF PROTECTION POST EXPERIMENTAL CHALLENGE

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Summary

One hundred and eighty two day-old SPF chicks were allotted to 13 groups of 14 each and raised on wire in Horsfall units. At 2-weeks of age 11 groups were vaccinated by eyedrop with one of the 11 infectious bronchitis (IB) commercial vaccines. The vaccinated birds and an unvaccinated control group were challenged with IB Mass41 strain using 75 µl of 106.4 embryo infectious dose by eye drop. One group was held as an unvaccinated and unchallenged control. Body weight gains, respiratory rales, tracheal ciliary activity, histopathology and virus isolation were the parameters measured to assess vaccination reaction and protection from immunity.

Seven vaccines elicited moderate to severe respiratory rales at 7-days post vaccination in some of the birds. No respiratory rales were present in birds vaccinated by vaccines C and D. Respiratory rales observed 7-days post IB-challenge were mild and present in some of the birds. Post IB challenge rales were not present in birds vaccinated with vaccine G.

Microscopic tracheal alterations marked to severe 7-days post vaccination were present in birds vaccinated by eight vaccines. Birds vaccinated by vaccine J had moderate tracheal alterations at 7-days post IB-challenge. Unvaccinated, IB-challenged control birds in treatment 13 had severe microscopic alterations.

Virus was not isolated post IB-challenge from birds vaccinated by 2 of the vaccines, and the unchallenged control birds.

Materials and Methods

One hundred and eighty-two straight-run day-old SPF chicks were wing banded, allotted to 13 groups of 14 each and raised on wire in Horsfall units. Eleven groups were vaccinated by eye drop using single doses of 11 Infectious Bronchitis (IB) commercial vaccines at two weeks of age. The commercial vaccines tested are given in Table 1.

Eleven vaccinated and an unvaccinated groups were challenged at 6-weeks of age with IB Mass 41 strain using 75 µl of 106.4 embryo infectious dose by eye drop. The challenge strain was supplied by the courtesy of Dr. Jack Gelb, University of Delaware. One group was maintained as an unvaccinated and unchallenged control.

Two birds were necropsied both one week post-vaccination and post-challenge to examine tracheal ciliary activity, for histopathologic evaluation and clinical evaluation of tracheal rales. Examination of tracheal ciliary activity was performed by evaluating 1–2 mm thick tracheal rings in organ culture under the inverted microscope using a scale of 0—5: 0—no ciliary movement, 5 = normal ciliary movement with intermediate scores of 1, 2, 3, and 4. Two tracheal rings were prepared from each bird. An average score was calculated from both birds and the results are given in Table 3. Microscopic tracheal alterations of mucosal thickening and cellular infiltrate present in the lamina propria were scored from 0—5: 0 = no change, 1 = minimal change, 2 = mild, 3 = moderate, 4 = marked and 5 = a severe microscopic change. The microscopic ciliary loss was scored on a scale of 0—5: 0 = absence of all cilia and 5 = presence of all cilia with intermediate scores of 1, 2, 3, and 4. Two tracheal sections from each of the two birds necropsied 7-days post vaccination and post IB-challenge were evaluated; scores are averaged and listed in Table 2. Clinical evaluation of respiratory rales was scored with + = mild rales, ++ moderate rales; +++ severe rales and – = absence of rales.

Tracheal swabs were collected from all birds 7-days post IB-challenge and inoculated in 10-day old SPF embryos via allantoic sac route. Results of virus isolation are given in Table 2. The birds were individually weighed at 2-weeks of age and 7-weeks of age. The weight gains were calculated by subtracting the weight of the 2-week old

bird from 7-week old bird. The average of weights at 2-weeks old, 7-weeks old and the weight gains are given in Table 1.

Results and Discussion

Severe respiratory clinical rales (Table 3) were present 7-days post vaccination with vaccine E. Vaccines A and B elicited mild rales; moderate rales were present in birds vaccinated with vaccines, F, G, H, I, J, and K. No respiratory rales were present in unvaccinated control birds in treatments 12 and 13 or in birds vaccinated with vaccines C and D. Results of respiratory rales 7-days post challenge are given in Table 3. Severe respiratory rales were present in 6 chickens from the unvaccinated challenged birds of treatment 13; 3 birds elicited mild rales. None of the birds in treatment 7 or 12 elicited rales. Mild rales were present in some of the birds in other treatments; complete details are given in Table 3.

Infectious bronchitis virus was not isolated from any of the birds vaccinated with vaccines A and D (Table 3) or from the unvaccinated unchallenged control birds of Treatment 12. Virus was isolated from 9 of 10 samples from unvaccinated but challenged birds of treatment 13. Virus isolation results of remaining treatments are given in Table 3.

Moderate microscopic tracheal alterations (Table 2) were present 7-days post vaccination in birds of treatments 3, 4, and 11 (vaccinated by vaccines C, D, and K). Seven-day post vaccination marked to severe tracheal alterations were present in birds vaccinated by vaccines A, B, E, F, G, H, and J. No tracheal alterations were present in treatments 12 and 13 indicating the absence of cross infection.

Moderate microscopic tracheal post challenge alterations were present in birds vaccinated by vaccine J. Severe post challenge tracheal alterations were present in unvaccinated but challenged birds of treatment 13.

Results of ciliary activity of tracheas evaluated both 7-days post vaccination and post challenge are listed in Table 3. The loss of ciliary activity 7-days post vaccination was severe in birds vaccinated with vaccines B, E, and F; moderate loss of activity was present in birds vaccinated by vaccines A, C, G, H, and K. A minimal loss of ciliary activity was present in birds vaccinated with vaccines I and J and in unvaccinated birds of treatments 12 and 13.

Good ciliary activity 7-days post IB-challenge was present in birds vaccinated with 10 commercial vaccines. Treatment 10 birds vaccinated by vaccine J had only mild ciliary activity. No ciliary activity was present in birds of treatment 13 that were unvaccinated but challenged.

A substantial decrease in body weight gain was present in birds of treatment 13 that were unvaccinated but challenged. No significant differences of weight gains were present in any of the other treatments.

TABLE 1
List of infectious bronchitis commercial vaccines

Company name		Other details
I	SELECT	Bronchitis vaccine, Massachusetts type, live virus
II	VINELAND	UniBrone, list #1222, B41 strain
III	CEVA	Bronchitis vaccine, Mass type live virus Mass Blen™ List #5736, 5737
IV	CEVA	Bronchitis vaccine, Mass type, live virus Mass Blen™—S, List #5961
V	INTERVET	Bronchitis vaccine, Mass type, liver virus, Mild vac-M
VI	INTERVET	Bronchitis vaccine Mass (Ma5) type, live virus
VII	SALSBURY	Bronchitis vaccine, Mass type, live virus
VIII	STERWIN	Newcastle–Bronchitis vaccine, B_1 type, B_1 strain, Mass type, live virus
IX	STERWIN	Newcastle–Bronchitis vaccine, B_1 type, B_1 strain
X	SCHERING ASL	Newcastle–Bronchitis vaccine, B_1 type, B_1 strain, Conn type, Monovax, live virus
XI	SCHERING ASL	Newcastle–Bronchitis vaccine, B1 type, B1 strain, Mass and Conn types LV, TWIN-VAX-MR

TABLE 2
Infectious bronchitis vaccination study in 2-week old SPF chickens: Results of body weights and microscopic tracheal scores

Trt	Vacc.	Chall.	2 wk old wt.(g)	7 wk old wt.(g)	Wt. gain[c] (g)	Microscopic Tracheal Change[b] 7 days post vaccination Muco. Thick.	Cellu. Infil.	Cilia Loss	7 days post challenge Muco. Thick.	Cellu. Infiltr.	Cilia Loss
1	A[a]	+	103	596	493	3	3.5	4	0.5	0.5	0
2	B	+	98	534	436	4	4	4.5	0.5	0.5	0.5
3	C	+	96	547	451	1.5	2	0.5	0.5	0.5	0
4	D	+	93	554	461	1.5	1.5	1.5	0	0	0
5	E	+	92	653	561	4.5	4.5	4.5	0	0	0
6	F	+	101	597	496	5	5	4.5	0	0	0
7	G	+	98	591	493	5	5	5	0.5	0.5	0
8	H	+	103	556	453	3.5	3.5	3.5	0	0	0.5
9	I	+	96	569	473	5	4.5	4.5	0.5	0.5	0
10	J	+	91	541	450	3	3	3.5	3	3	2.5
11	K	+	94	549	455	2	2	2	0	0	0
12	None	–	97	520	423	0	0	0	0	0	0
13	None	+	101	469	368	0	0	0	5	5	5

[a]Details of vaccine used are given in the Material and Methods section
[b]Microscopic change scored 0–5; 0 = no change, 1 = minimal change, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe microscopic change
[c]Weight gain = 7-wk old weights minus 2-wk old weights

TABLE 3
Infectious bronchitis vaccination results in 2-week old SPF chickens results of ciliary activity, tracheal rales 7 days post vaccination and post challenge

Trt	Vacc.	IB Chall.	Cilial Activity[a]		Tracheal Rales[b]		Virus[d] Isolation
			7 days post vaccine	7 days post challenge	7 days post vaccination	7 days post challenge[c]	
1	A	+	2	3.5	+	2/12+	0/12
2	B	+	0	4	+	4/12+	2/12
3	C	+	2.5	3.8	–	3/12+	4/12
4	D	+	3.5	4.3	–	2/12+	5/12
5	E	+	0	5	++	3/12+	0/12
6	F	+	0.8	4	++	3/12+	3/12
7	G	+	1.8	3.8	++	12/12	3/12
8	H	+	1.5	3.5	+++	2/12+	2/12
9	I	+	4.5	3.3	++	2/12+	4/12
10	J	+	4.3	2.0	++	3/10+	7/10
11	K	+	1.8	4.8	++	3/10+	4/10
12	–	–	4.3	4.3	–	7/7–	0/7
13	–	+	4.5	0	–	6/10+++ 3/10	9/10

[a]Ciliary activity was scored 0–5; 0 = no ciliary movement, 5 = normal ciliary movement with intermediate scores of 1, 2, 3, and 4
[b]Respiratory rales score; + mild rales, ++ moderate rales, +++ severe rales and – indicates absence of rales
[c]Number of birds with respiratory rales/total birds evaluated 7 days post IB challenge
[d]Number of samples virus isolated from/total samples tested

STUDIES ON INFECTIOUS BRONCHITIS AND BRONCHITIS CONTROL PROCEDURES IN COMMERCIAL LAYER FLOCKS

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Infectious bronchitis (IB) has been and continues to be a major disease problem to the commercial layer industry. Bronchitis expresses itself in layers in the form of decreased egg production, decreased egg shell quality and increased feed consumption. In light of today's changes in basic commercial layer management—i.e., in line multi-age complexes—drops in egg production and increases in feed consumption are often not easily detected.

IB virus has its effect on egg performance by hematogenous spread within the chicken. The results of challenge of susceptible and immune chickens (vaccinated with inactivated Newcastle-bronchitis) with virulent Mass-41 challenge virus are presented in Table 1. The data indicate the spread of the virus from the trachea (intraocular challenge) to lung, spleen, kidney, and cecal tonsil. Note that immune chickens still experienced virus replication in the trachea—but very little (5%) in the lung, kidney and cecal tonsil.

Translation of the effect of hematogenous spread into egg production was evaluated by challenge of susceptible (nonvaccinated) and immune (vaccinated with inactivated Newcastle bronchitis vaccine) layers with Mass-41 virus by eye drop application. Results of that study, presented in Table 2, shows a precipitous drop in egg production (20.4%) and a rise in serum antibody titer (IBV-VN, chick kidney cells) to Mass type IB virus.

Under field conditions, the effects of a bronchitis vaccination program are not always as absolute. Field studies with killed ND-IB vaccine have resulted in many trials depicting positive effects on egg production, feed consumption and egg shell quality. Field data, comparing two different vaccination programs—inactivated ND-IB vaccine versus a pullet live ND-IB vaccination—point toward the relative advantage of the inactivated vaccine program. The egg production curves presented in Table 3 show a 14% drop in the "pullet live program only" as compared to a 4% drop in the pullets which received the inactivated ND-IB vaccine. This "reduction of the impact of IB challenge" is more typical of the true effects of field IB challenge as no vaccination program is absolute.

Based upon these types of experiences a laboratory study was initiated to evaluate two different vaccination programs—pullet live ND-IB vs pullet live ND-IB followed by inactivated ND-IB vaccine. This study was conducted in cages with four replicates per treatments. Monitoring consisted of:

1. Egg production (% hen day)
2. Feed intake (lbs/100 birds/day)
3. Lbs. feed/dozen eggs
4. Lbs. feed/lb. eggs
5. Specific gravity
6. Livability
7. Cost/dozen eggs
8. Serological profile (ND-HI, Mass IB-VN)

Two different breeds of chickens were used for each of the two different vaccination programs. All other factors were constant as the chickens were housed under the same roof. Results, excluding the serological profile, are presented as Table 4.

As can be seen in the data, there is a difference in the cost to produce a dozen eggs. This difference is basically due to better egg production and decreased feed consumption on the part of hens receiving inactivated vaccine. Again, with the management systems of many multi-age in line complexes these differences are not apparent on a daily or monthly basis.

A popular method of controlling IB and ND in the field is the use of live virus vaccination on a periodic basis, i.e., 60 day booster program, to determine what impact this may have on egg performance. All chickens in this study were spray vaccinated with a "regular" live IB vaccine—typically used for such booster programs. As can be seen in the data presented in Table 5, there was an effect on egg production, feed conversion, livability and cost to produce a dozen eggs.

Results of serological profiling presented in Tables 6 and 7 depict the probable cause of the production problem. The rise in titer to Mass IB is very substantial. Again, under field conditions this type of pattern can be very difficult to detect, but may be typically occurring when a live virus vaccine boosting program is being used.

Control of IB in commercial layers is and will continue to be a major problem for the commercial layer industry. There is need for improvement in many vaccination programs. The problem is made even more difficult by changes in poultry management systems which make detection of IB problems more difficult.

TABLE 1

Vaccination	% IB Virus Recovery Rate in Chickens following challenge				
	Trachea	Lung	Spleen	Kidney	Cecal Tonsil
None	82	5	0	5	5
Inactivated Newcastle–Bronchitis Vaccine Only	95	95	80	90	90

TABLE 2
Egg Production and Serological Status of vaccinated (inactivated Newcastle-Bronchitis vaccine) and susceptible SPF chickens following challenge with Mass-41 IB virus

	Egg Production		MASS IB-VN-GMT	
	Prechallenge	Postchallenge	Prechallenge	Postchallenge
Inactivated ND–IB vaccine	85.5	83.7	37	111
None	83.8	63.4	2.5	111

TABLE 3
% egg production in field flocks on two different IB–ND vaccination programs

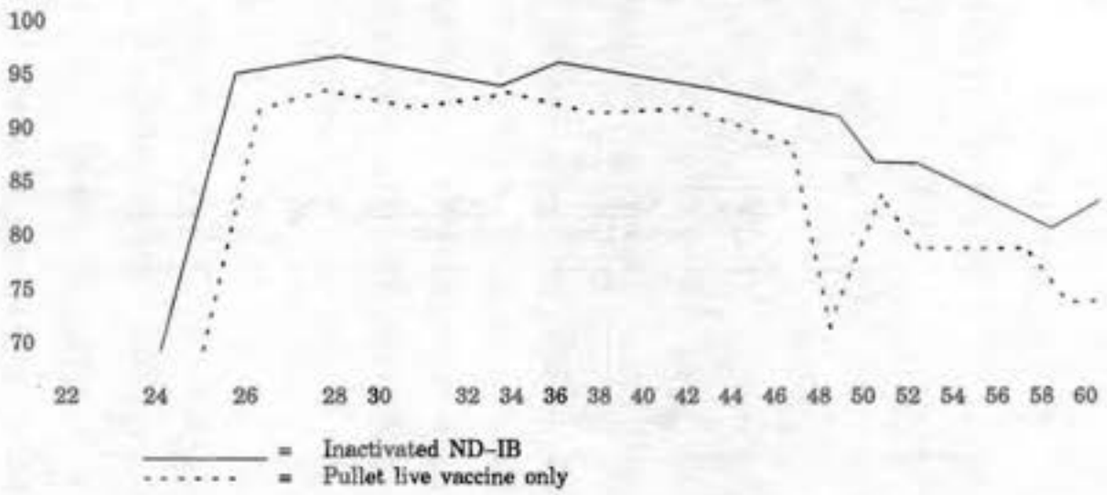


TABLE 4
Layer flock performance for life of flock (72 weeks of age)

	Breed I	Breed II	Inactivated ND/IB
Egg production (% hen day)	80.28	74.26	yes
	77.98	74.51	no
Feed intake (lbs/100 hens/day)	25.06	24.45	yes
	24.47	24.82	no
Feed/pound eggs (lbs)	2.41	2.51	yes
	2.45	2.54	no
Feed/dozen eggs (lbs)	3.75	3.95	yes
	3.77	4.00	no
Specific gravity score	1.0810	1.0803	yes
	1.0819	1.0804	no
Livability (%)	85.71	86.61	yes
	85.71	90.18	no
Cost Dozen	17.72	18.69	yes
	17.84	18.91	no

Study conducted in cooperation with Central Soya Feed Division Research

TABLE 5
Influence of spray vaccination for bronchitis on egg performance over a four week period

	Breed I	Breed II	Inactivated vaccine
Egg production (% hen day)	67.19	61.02	yes
	65.59	59.12	no
Feed intake (lbs/100 hens/day)	25.25	25.49	yes
	25.94	26.07	no
Feed/pound eggs (lbs)	2.78	3.03	yes
	2.78	3.12	no
Feed/dozen eggs (lbs)	4.52	5.13	yes
	4.79	5.32	no
Specific gravity score	1.0755	1.0754	yes
	1.0755	1.0750	no
Livability (%)	100.00	100.00	yes
	98.96	97.25	no
Cost/dozen eggs	22.20	25.17	yes
	23.52	26.10	no

Study conducted in cooperation with Central Soya Feed Division Research

TABLE 6
Mass IBVN and NDHI serological profile for Breed I

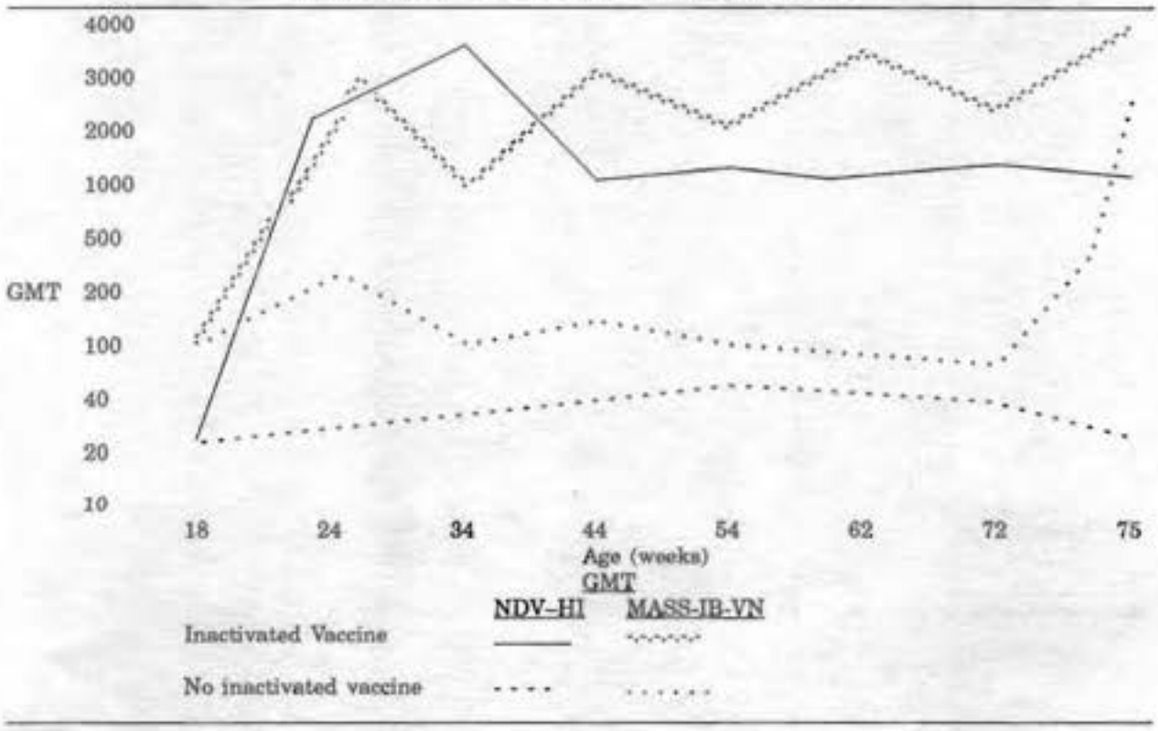
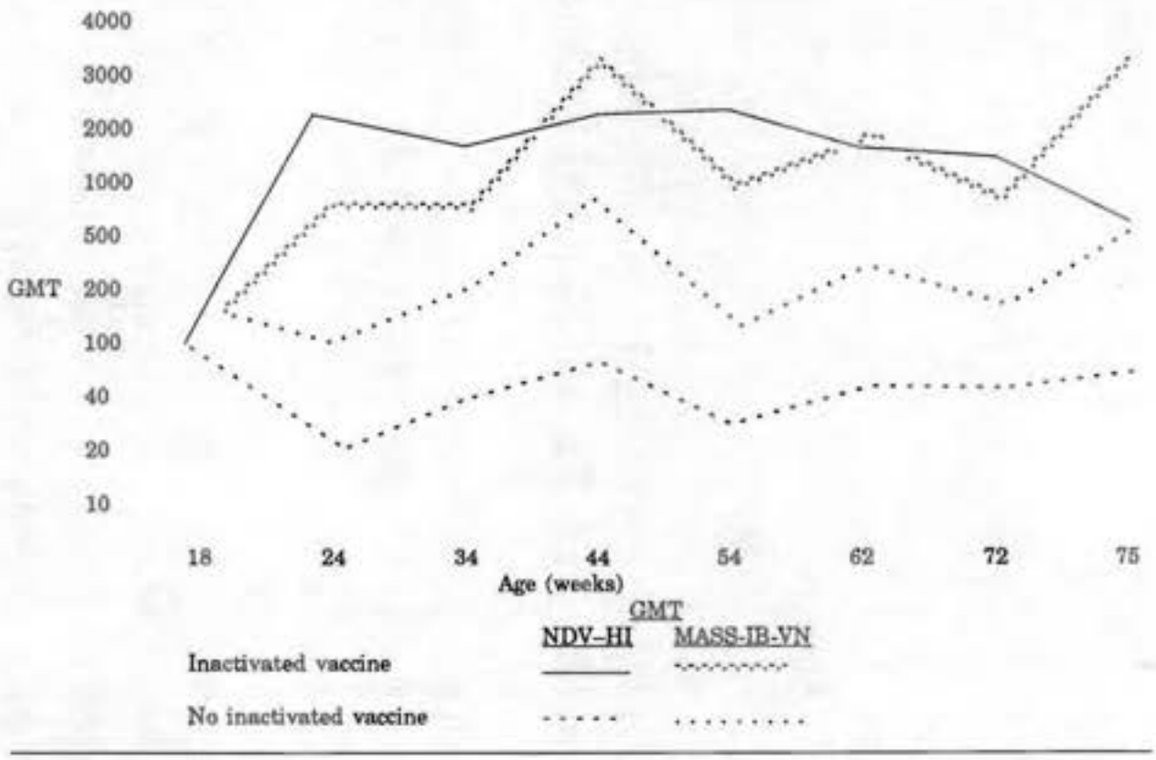


TABLE 7
MASS IB-VN and ND-HI serological profile for Breed II



COOPERATIVE INVESTIGATION FOR THE ISOLATION OF RESPIRATORY AGENTS ON PROBLEM CAGE LAYER OPERATIONS

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Introduction

In an area of Northern California from 1984–1986 a disease condition was causing egg production problems in vaccinated egg laying flocks. The egg production was close to normal in quantity, but the eggs had low internal quality. The eggs were examined and found to have low Haugh Units.

Egg shell quality was normal. In this area there are several egg producers with several ranches with a total population of approximately 500,000 layers in close proximity. This problem had occurred on more than one ranch in this area.

Transitive respiratory symptoms had been noticed on some farms with this problem. These companies raise their pullets far from this area. The symptoms were noticed a few days after moving pullets onto the layer ranches.

One company asked us if we could help to find the cause of this problem. We started with a serological survey of several flocks. Due to the history of egg production problems and respiratory symptoms, we tested for Infectious Bronchitis (IB) by hemagglutination inhibition (HI) using antigens for strains Mass, Conn, Ark, Cal 1 variant.[1]

The results of this survey were high titers to all strains tested. It has been reported that multiple vaccinated chickens will produce high level of antibody that reacts with HI antigens from serotypes to which the birds have not been exposed.[2] These chickens had been vaccinated several times with IB vaccines and may have had field exposure. Due to potential cross reactions we could not use the HI test on samples from vaccinated hens for a serological diagnosis.

A decision was made to use a sentinel bird surveillance program to try to find the cause of the egg production problems.[3,4] The particular ranch under investigation is J & W (Johnson and Weber). This ranch is a single age, 80,000 bird, cage layer operation.

This work had a three fold objective to determine whether we could isolate a significant virus from this flock. To determine if we could improve flock immunity using a new IB vaccine program (New Bronz™ Program Table 2) with products which have shown solid protection against standard vaccine strains and some protection against variant strains[5]. Continue protection studies with existing vaccines against newly isolated virus. If existing vaccines can provide protection then we could avoid proliferation of IB variant vaccines which are often demanded by the poultry industry.

In January 1986 a flock was placed on J & W Ranch which had been vaccinated using the New Bronz™ Program. SPF birds were obtained from SPAFAS and housed at the same time the hens were placed. The birds were observed from clinical symptoms and collection of samples was supervised by Dr. Richard Chin of Petaluma State Laboratory. The serological results are shown in Table 1.

The birds did exhibit respiratory signs and trachea swabs were taken on a number of occasions. The swabs were submitted for virus isolation but yielded negative results. As Table 1 indicates, the SPF birds were probably exposed to IB Cal 1 variant.

The hens had a rise in titer to IB Cal 1 variant but didn't meet our criteria for diagnosis. We feel that a serological positive flock must have a two dilution higher titer on an individual sample basis to the suspect strain compared to all other strains tested. The flock continued throughout its production cycle without any signs of damage to the oviduct as judged by the egg quality. The overall egg production was stated to be the best ever achieved on this farm.

Despite the good results of J & W Ranch there were still unexplained problems on other farms in the immediate area. The company made a decision in August 1988 to use the New Bronz™ Program for Newcastle Bronchitis con-

trol, but they wanted us to make another attempt to isolate any unknown virus. On this occasion we would attempt a modification of the previously described method.[5,6]

Methods and Material

The manager of J & W Ranch, Mr. Jim Carlson, collected tracheal swabbings and blood samples at routine times without waiting for symptoms to appear. The hens were placed in August 1988 and the SPF birds were obtained from Hyvac Laboratory Egg Company. The SPF birds were placed at the same time as the hens.

Tracheal swabs were taken using Precision Culture C.A.T.S. swabs with modified Amies media. The swabs were taken at 48 hour intervals and held in liquid nitrogen on the farm.

Blood samples were taken at 14 day intervals and were tested for IB by the HI test. The HI test was performed following standard methods.[6] The antigens and antisera used were provided by Salsbury Laboratories. Virus isolation was performed by standard methods using Hyline Hyvac SPF embryos.[7] Ten day old embryos were inoculated via the allantoic sac with swabbing suspension in tryptose broth with antibiotics and incubated for 5 days. The embryos were passed three times before embryo stunting and mortality was observed. The allantoic fluid was negative for hemagglutination activity. Virus neutralization studies were done by diluting the virus and treatment with specific antiserum to IB Mass, Conn, Ark, Cal 1. The treated virus was inoculated into ten day old SPF embryos and incubated for 5 days.

Results

The results shown in Table 3 indicates a rise in titer in the SPF birds to the Cal 1 variant, which met the standard we had set—that at least two dilutions above the other tested strains. After determination of the rise in titer described above, we went back to the swabs and selected the period we felt was most likely to yield virus (no obvious symptoms were noticed during this period).

A virus was isolated and identified by electron microscopy to be a coronavirus. In virus neutralization studies, the virus was neutralized with specific serum for IB Cal 1 but was not neutralized by specific serum to IB Mass, IB Conn, IB Ark.

Discussion

The serological and isolation data we have presented strongly suggests that we have isolated a IB Cal 1 variant from this flock.

We feel that the method used of regular sampling and not waiting for symptoms to appear has merit for practical farm use.

The cooperation of the farm owner/manager is essential but this is not difficult to achieve if benefits are explained. The training of such people is also possible with genuine interest on both sides.

We feel that the HI test (until better strain specific tests for IB appear) has great merit if due consideration is given to cross reactions.

This paper indicates a model in this case for what can be achieved by co-operation and communication between the farm, a local laboratory, a vaccine manufacturer and state authorities.

We aim to determine more details on strain identification, protection by present vaccines to lend the practical and constructive aspect to the whole exercise.

Acknowledgments

1. We would like to thank Mr. Jim Carlson of J & W Ranch without whose enthusiasm, excellent techniques and attention to detail this work would not have been possible.

2. Dr. Richard Chin for his work on the 1987–1988 flock without which this new attempt would not have been possible.

3. Dr. Antony Castro, Mr. Bruce Reynolds, CVDLS for further characterization of the isolate by electron microscopy.

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TABLE 1
Trial 1—SPF IB Cal 1 Variant HI Titer

Bird Number	Date Sampled 1-19-87	Date Sampled 1-30-87	Date Sampled 3-16-87
1	20	20	20
2	20	20	20
3	20	20	20
4	20	20	20
5	20	20	–
6	20	40	80
7	10	40	80
8	10	20	160
9	20	80	320
10	20	20	320
11	20	20	160
12	20	20	320

TABLE 2
New Bronz™ Program for Newcastle, IB Control Vaccine

Age	Vaccine
3 weeks	Newcastle B_1, IB Mass Conn
6 weeks	Newcastle Lasota, IB Mass II
12 weeks	Killed Newcastle, IB Mass II, New Bronz™

New Bronz™ is a product of Salsbury Laboratories

TABLE 3

SPF Sentinel Birds IB Cal 1 HI Titers

Bird Number	Date Sampled 8-17-88	Date Sampled 8-29-88	Date Sampled 9-14-88	Date Sampled 9-28-88
1	0	40	20	20
2	0	40	20	20
3	0	40	40	160
4	0	40	40	160
5	0	40	40	40
6	0	40	40	40
7	0	80	40	40
8	0	20	20	40
9	0	80	160	320
10	0	80	40	40
11	0	160	80	320
12	0	40	40	160

TABLE 4
Hens—IB HI Results—IB Mass, IB Conn, IB Ark, IB Cal 1

Bird Number	IB Mass	IB Conn	IB Ark	IB Cal 1
1	160	160	160	320
2	160	80	160	320
3	160	80	160	160
4	160	80	80	640
5	160	80	80	320
6	40	80	80	80
7	160	80	80	640
8	20	80	80	160
9	80	160	80	160
10	160	160	80	640
11	160	160	80	640
12	160	160	80	320

ISOLATION OF A NEPHROTROPIC STRAIN OF INFECTIOUS BRONCHITIS VIRUS FROM 3—4 WEEK OLD PULLETS FROM A COMMERCIAL FLOCK: A PRELIMINARY STUDY OF THE VIRAL PATHOGENESIS USING SPF CHICKS

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Introduction

The association of avian nephritis with infectious bronchitis virus (IBV) has been well established; furthermore, the virus has been isolated from the kidneys of chickens following a virus infection.[1-3] Nephrotropic strains of IBV have not been previously reported in poultry in California. This paper describes the isolation of a nephrotropic strain of IBV from young 3–4 week old pullets and the preliminary study of the pathogenesis of this viral isolate using SPF chicks.

Case Report

A nephrotropic strain of IBV was isolated from a submission of 20 pullets between 3 to 4 weeks of age which represented 5 houses consisting of 41,000 birds. Death losses from this disease outbreak ranged from 1.8% to 12.5% per week. Clinical signs in affected birds included swelling of the heads and watery discharges from the eyes and nostrils. At necropsy, kidneys from several birds were significantly swollen and some bursas were markedly atrophied. Histological examinations of the kidneys revealed moderate focal to diffuse interstitial lymphocytic and less prominent heterophilic infiltrations, and tubular dilations containing hyalin, mucinous, or granular casts. Some bursas were undergoing acute lymphoid necrosis with stromal edema and inflammatory cell infiltration. Other bursas had significant lymphoid depletion with atrophy of lymphoid follicles and plicae. Bacteriological cultures were negative from multiple tissues. A coronavirus was isolated from a suspension of pooled kidneys suspensions after the fifth passage in 10-day old chicken embryos. Inoculated embryos were stunted with evidence of urate deposits in their kidneys. Microscopically, various sections of kidney tissues showed occasional mineral deposits and a marked dilation of tubules.

Survivors of the affected group experienced loss of body weight, delayed coming into production, and 15 to 20% lower egg production than their sister flocks, which were raised and managed in a different region.

Immunology

Two young adult chickens were prebled and challenged intraocularly (IC) and intranasally (IN) with CAF obtained at the fifth embryonated egg passage. Both inoculated birds were bled at 29 and 33 days. Sero-conversion to IBV was demonstrated both by ELISA and hemagglutination-inhibition (HI). Sera at 29 and 33 days had HI titers of 1:128 and 1:512 for Ark 99; 1:512 and 1:512 for Mass. 41 and were negative for Connecticut and Calif.2 strains. The ELISA titers ranged between groups 2 to 4.

Inoculation of SPF Chicks

A CAF suspension of the virus obtained at the fifth passage in embryonated chicken eggs was instilled IN into twenty 4-week old SPF chicks. A group of 8 SPF chicks were used as controls. Before inoculation, blood was collected from all the 28 SPF chicks to ascertain their immunologic status. Each group was kept in a separate room. On post inoculation days, 4, 7, 11, 14, and 18, individual inoculated birds were sacrificed. Blood samples were also collected on days 7, 14, and 18. The bacteriology, virology, gross pathology, and histopathology findings are summarized in Table 1. Serum samples were tested against 5 different strains of IBV using the HI test (Table 2). The results of ELISA for IBV and the AGID test for antibody to Marek's disease virus are also summarized in Table 2.

Discussion

The isolation of a coronavirus from the submitted specimens from clinically-ill chickens, the necropsy lesions, the microscopic findings, and evidence of seroconversion by ELISA and HI to IBV in virus-inoculated birds indicated that the virus isolated was IBV. The results obtained

from the inoculation of the viral isolate into SPF chicks supports the conclusion that the isolate had nephropathogenicity. The bursal lesions seen are not unusual findings in recently vaccinated chicks and thus a reovirus isolated from the tissues probably reflected the vaccine of IBD-virus routinely administered to chicks at 15 days of age.

We have been unable to find a previous documented case in California of a nephrotropic strain of IBV. The significance of our findings may have major implications for the poultry industry in California, and thus demands further study of this virus. Currently, attempts are in progress to further characterize and serotype the virus by various laboratory methods.

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TABLE 1

Pathologic and microbiologic findings of nephrotropic IBV inoculated SPF chicks at 4, 7, 11, 14, and 18 days post inoculation

Evaluation Parameter	Days Post Inoculation 4	7	11	14	18
Gross Necropsy	Trachea: Mucoid exudates. Kidneys: No significant lesions. Bursa: No significant lesions.	Trachea: Mucus discharge in nasal and bronchi. Foamy airsacs. Swollen conjunctiva. Kidneys: Swollen. Bursa: NSL	Watery eyes; petechial hemorrhages in conjunctiva. Foamy airsacs. Kidneys: Swollen. Trachea: Few showed mucous exudates.	Trachea: No gross lesions. Kidneys: 2 kidneys were swollen.	Same as 14 DPI
Histopathology	Trachea: 2 out of 3 birds showed mild to moderate epithelial hyperplasia; mild lymphoplasmacytic infiltration in propria.	Trachea: Mild luminal mucopurulent exudation; complete epithelial deciliation; variable epithelial hyperplasia; moderate to severe lymphoplasmacytic infiltration in propria.	Trachea: Most appeared normal; few with luminal mucopurulent exudation; epithelial deciliation with variable hyperplasia. Lymphocytic infiltration in propria. Lung: Focal interstitial pneumonia. Eyelid & fascial skin: Mild cellulitis with scattered inflammation.	Tracheas: Mild subepithelial lymphoid infiltrates.	Same as 14 DPI
	Kidneys: NSL	Kidneys: Few to several interstitial lymphoplasmacytic foci; few hyaline casts and rare intratubular mineralized deposits. Lungs: NSL	Kidneys: Most sections had few to numerous focal areas of interstitial lymphoplasmacytic infiltration with scattered dilated tubules with tubular casts.	Kidneys: Mild to severe multifocal lymphoplasmacytic infiltrates, particularly severe around the medullary areas and also around the collecting tubules.	Same as 14 DPI
Bacteriology	No pathogens were isolated.	No pathogens.	No pathogens.	No pathogens.	No pathogens.
Virology	Dwarf embryos with typical of IBV stubby feathers and edema (kidneys & trachea)	Feather clubbings, edema, IBV isolated and identified by EM. Coronavirus (kidney).	Not available.	Dwarf, slightly hemorrhagic (kidneys, tracheas), typical of IBV.	Kidney (no visible lesions. Trachea slightly hemorrhagic embryos.

TABLE 2
Immunological responses at days 7, 14, and 18 of SPF chicks inoculated with IBV

No. of Birds Bled	DPI[a]	HI Titer vs Serotype of IBV					ELISA Group	Agid MAREK'S
		Ark 99	Mass 41	Conn	JMK	Calif. 2		
9	7	1:16	1:16	1:8	1:8	1:8	0	Negative
		1:32	1:64	1:8	1:8	1:8	0	"
		1:64	1:64	1:8	1:16	1:16	3	"
		1:18	1:16	1:8	1:16	1:8	3	"
		1:8	1:8	N	1:8	1:16	0	"
		1:64	1:32	1:64	1:64	1:64	0	"
		1:32	1:32	1:16	1:32	1:16	0	"
		1:256	1:512	1:64	1:64	1:64	0	"
		1:128	1:256	1:16	1:32	1:16	0	"
8	14	1:128	1:128	1:32	1:128	QNS	0	"
		1:64	1:128	1:8	QNS	QNS	2	"
		1:16	1:16	QNS	QNS	QNS	1	"
		1:128	1:32	1:64	1:64	QNS	0	"
		1:64	1:64	1:32	1:64	QNS	0	"
		1:64	1:16	1:32	1:32	QNS	2	"
		1:64	1:32	1:64	1:64	QNS	1	"
		1:16	1:16	1:8	1:16	QNS	0	"
4	18	1:128	1:64	1:64	1:128	1:32	0	"
		1:64	1:32	1:32	1:128	1:32	0	"
		1:16	1:32	1:8	1:64	1:16	0	"
		1:128	1:128	1:128	1:256	1:128	0	"
5[b]	14	1:8	1:8	N	1:8	1:4	1	"
		1:8	1:8	N	1:8	1:8	1	"
		1:8	1:8	N	1:8	1:4	0	"
		1:8	1:8	N	1:8	QNS	1	"
		1:8	1:8	1:8	1:8	1:4	0	"
2[b]	18	1:8	1:16	1:8	1:32	1:8	0	"
		1:8	1:16	1:8	1:64	1:8	0	"

[a]Days post inoculation
[b]Control birds

CAGE LAYER OSTEOPOROSIS/FATIGUE—A REVIEW

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Cage layer osteoporosis (fatigue) is the most significant disease of the skeleton in mature chickens used for egg production. The literature on the condition was reviewed by Riddell.[7] Recent reports indicate that the condition still is an important field problem in N. America[1] and Europe.[5] Poor bone structure in caged laying hens can cause considerable economic loss due to bone breakage when hens are processed.[4] Recently in Europe fractures secondary to osteoporosis in caged hens have been discussed as an animal welfare issue.[3]

The susceptibility of the modern laying hen to osteoporosis is related to the high demand for calcium required for eggshell formation. An excellent review on calcium logistics in the laying hen was written by Etches.[6] The laying hen contains approximately 20g of calcium, mainly in her skeleton, while the average eggshell contains 2.3g of calcium. Eggshell formation occurs in the 20 hours before oviposition. Maximum formation occurs during the middle of this period, in part during the night. The amount of calcium available for absorption from the intestine at night will be affected by the amount and type of calcium ingested late in the day. If the laying hen can select calcium she will ingest more near the end of a photoperiod. A large particle calcium source will persist longer in the intestinal tract than a finely ground calcium source. Calcium for egg shell formation is also obtained from breakdown of medullary bone. Medullary bone is a special woven bone which forms as interlacing spicules in the marrow cavity of many bones of the laying hen. It forms under hormonal influence in the 10 days before the first egg is laid. Skeletal weight increases about 20% during this period. Medullary bone breaks down or loses mineral during eggshell formation and is built up at other times. Medullary bone will form at the expense of cortical bone. The ability of different bones to provide calcium for eggshell formation is variable. Bones of the ribs, sternum, pelvis and spine are considered labile bones; bones of the skull, shank and toe are considered non-labile bones, while the femur, tibia and fibula are considered intermediate bones.

Clinical signs in cage layer osteoporosis are variable and include posterior paralysis and acute death with or without changes in egg production and shell quality. Paralyzed birds initially are alert. They may recover if removed from the cages and given ready access to feed and water. If left in the cages they will generally die from dehydration. On necropsy birds have fragile bones and sometimes fractures. The changes in the bones are generally most striking in the sternum and ribs. Collapse and infolding of the ribs due to fractures at the junction of the sternal and vertebral components are very common. Paralysis may be due to compression fractures occurring in the thoracic vertebrae. Recently avulsion of the patellar ligament has been described in affected birds as a possible cause of disability.[2] In some cases no lesions can be found to explain the paralysis or acute deaths. It has been postulated that hypocalcemia may be the cause of paralysis and acute death in these cases. Parathyroid glands are generally enlarged. Microscopic examination of bones from affected birds show thinning of and increased resorption cavities in cortical bone, but normal mineralization. These changes are typical of osteoporosis. The medullary bone, however, exhibits a lack of mineralization and in severe cases consists mainly of osteoid. These changes are typical of osteomalacia. The different microscopic changes in the two types of bone has led to some debate about terminology. Workers in the United Kingdom have described cage layer fatigue as osteopenia.[5] Osteopenia describes a decrease in bone tissue without specifying whether there is a loss of total tissue with normal mineralization or just defective mineralization.

Major factors which may cause or contribute to outbreaks of cage layer osteoporosis include inadequate skeletal development prior to egg production, a lack of exercise and deficient nutrition during egg production. Outbreaks have been associated with birds starting egg production at an early age when skeletons have been immature. Special pre-lay rations are desirable to provide adequate mineral for formation of medullary

bone. As the name of the disease indicates, housing birds in cages predisposes to cage layer fatigue. Experimental studies demonstrated that this may result from a lack of exercise.[9] Similar lesions of cage layer osteoporosis may occur from vitamin D, calcium, and phosphorus deficiencies.[5,8] Large particle calcium sources allows the laying hen to select the time of calcium ingestion and the amount of calcium ingested. This should result in more absorption of calcium from the intestine when eggshell is forming and reduce the amount of calcium withdrawn from the skeleton.

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PULMONARY HYPERTENSION SYNDROME AND ASCITES IN MEAT TYPE CHICKENS

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Introduction

Many broilers grown at high altitude die from ascites caused by portal hypertension following valvular insufficiency and right ventricular failure (RVF). Over the past several years there has been a marked increase in the mortality in broilers and roasters at low altitude from ascites caused by RVF.[1,12,13] The valvular insufficiency and RVF are secondary to right ventricular dilation and hypertrophy caused by the increased work-load of pulmonary hypertension (cor-pulmonnalae).

The right ventricle of birds is thin walled and responds rapidly to a pressure overload by hypertrophy. The right AV valve is a muscle flap and is a continuation of the wall of the right ventricle. When the wall hypertrophies the valve also hypertrophies. As the right ventricle dilates valvular insufficiency occurs and the resulting portal hypertension causes ascites. Although there are other causes of ascites, increased hydraulic pressure from portal hypertension is the most common in meat-type chickens.[2,3] Most of the fluid comes from the liver and leaks into the heptoperitoneal cavities. Liver ascites is high protein ascites and protein clots are frequently present in the fluid. Most of the lesions described in ascites are the result of portal hypertension from RVF[12,13] and are not associated with the cause which is pulmonary hypertension. Pulmonary hypertension develops because of insufficient pulmonary capillary capacity and small lung volume in meat-type chickens.[4]

Pulmonary hypertension may cause edema in the normally low pressure lung vasculature and may result in rapid death from respiratory failure. These birds may not have ascites and may be misdiagnosed as flip-over unless the heart is examined.

Causes of Pulmonary Hypertension

In the case of hypoxemia from the hypoxia of high altitude or phosphorus deficient rickets[6] the pressure overload is caused by polycythemia which makes the blood more difficult to pump through the lung. The PVC also rises markedly when RVF occurs following other causes of pulmonary hypertension increasing the problem of blood flow.

When ascites occurs in healthy fast-growing broilers at low altitude it is primary pulmonary hypertension with a volume overload resulting in a pressure overload because of insufficient pulmonary vascular capacity. The rapid metabolism and high oxygen requirement of rapidly growing broilers forces more blood through the restricted capillary space in the lung causing pulmonary hypertension.[4] Reducing growth rate, as with skip-a-day feeding in broiler breeders, results in an immediate decrease in ascites mortality.

Cold temperatures markedly increase the mortality from pulmonary hypertension syndrome in fast growing broilers[10] by increasing the metabolic oxygen requirement and blood flow to the lung. This is particularly significant at high altitude. Carbon monoxide[5] and other things which reduce the oxygen carrying capacity of the blood would result in an attempt to increase blood flow but would also cause polycythemia.

The reason that increased dietary sodium causes pulmonary hypertension[8] is not as obvious. Increased Na^+ does cause hypervolemia which would result in a volume overload. Increased Na^+ in the red blood cells may cause cell swelling making the cells more rigid, decreasing cell deformability and make them more difficult to pump through the small lung capillaries. Na^+ may also cause arteriolar vasoconstriction.

Pulmonary aspergillosis[7] and other agents that cause lung pathology[11] may interfere with lung development and oxygen exchange resulting in polycythemia or they may cause fibrosis or vascular damage which reduces the space for blood flow through the lung resulting in a pressure overload on the heart.

Summary

The lungs of birds are rigid and fixed in the thoracic cavity. The small capillaries can expand only very little to accommodate increased blood flow. Lung size as a percentage of body weight decreases as meat-type chickens grow. Increased blood flow results in primary pulmonary hyper-

tension and cor pulmonale with sporadic cases of RVF and ascites in fast-growing broiler chickens. Predisposing factors which increase oxygen demand, reduce oxygen carrying capacity of the blood, increase blood volume, or interfere with blood flow through the lung (by lung pathology which narrows or occludes capillaries by increased red blood cell size or rigidity or by polycythemia with increased blood viscosity) may result in flock outbreaks of ascites.

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ASCITES SYNDROME AND HEART FAILURE IN BACKYARD BROILER FLOCKS IN OREGON

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Ascites syndrome and heart failure is frequently reported as a cause of death in commercial broiler flocks.[4,8] The pathogenesis of this condition is poorly understood, although several factors have been associated with ascites syndrome including high altitude disease, salt, furazolidone, monensin, and other toxicities[1,2,3,5] as well.

This report discusses an unusual cluster of ascites and heart failure in backyard broiler flocks in Oregon and attempts to determine what common factors were involved in each case.

Case Report

History

Seven cases of heart failure and ascites were observed in backyard broilers in Oregon in 1988. These cases were submitted roughly in a one-month period from May until June. Geographically they originated from widely distributed areas of the state, including two from Northeast Oregon, two from Central Oregon, and three from the Willamette Valley area. The altitudes of these locations vary considerably with several near sea level.

The strain of chicken affected in each case was Cornish cross. Upon further investigation, it appeared that all broilers originated from one of two hatcheries in Oregon or Idaho. These hatcheries purchased broiler eggs from the same company in Arkansas. The exact breeder flock of origin, however, could not be determined, as the company obtained hatching eggs from many breeder sources. Interestingly, most of the commercial broiler eggs in Oregon also originate from the same source. Large numbers of ascites deaths were not reported from commercial stocks at this time.

These backyard flocks varied in size from 40 to 100 individuals. The housing situation in each case consisted of conventional deep litter indoor brooding with free access to range once the birds were fully feathered. The diets of these birds varied. Five out of seven flocks investigated were fed a commercial broiler-grower diet manufactured by a large national feed manufacturer. One flock was fed a similar broiler-grower diet produced by a different large feed manufacturer. Lastly one flock out of the seven was fed a specially formulated mix produced by a small local mill. In two out of the seven cases, other breeds of chickens including Leghorns, Rhode Island Reds, and Barred Plymouth Rocks were also fed the exact diets as the Cornish-cross chickens, with no apparent ill effects.

Excessive mortality was first observed by the owners at approximately four weeks of age. This mortality ranged from ten to sixty percent by five-to-six weeks of age. Symptoms in the broilers included dyspnea and acute death. Birds necropsied at the Veterinary Diagnostic Laboratory were all five-to-six weeks of age.

Necropsy Findings

Two birds from each case were submitted, although in one case, only formalin-fixed tissues were given. Prior to submission of the samples the referring veterinarians had also performed numerous necropsies and had reported ascites in the dead birds. In almost all the cases, the birds were well-fleshed, judging by developed pectoral muscle mass and abundant subcutaneous fat.

Large amounts of straw-colored fluid containing yellow clots were present within the abdominal cavity. The livers were swollen with rounded edges and contained numerous subcapsular cysts filled with straw-colored fluid. The lungs were dark-red, firm and edematous. The hearts were enlarged, and the pericardial sacs contain straw-colored fluid. All other organs within the coelomic cavity showed diffuse congestion. Bacterial isolation attempts in each case failed to demonstrate pathogenic organisms.

Histopathology

Microscopic changes in the heart were few and tended to be rather focal in distribution. Separation of cardiac muscle fibers was seen in almost all cases. Endomycial cell proliferation with small muscle hemorrhages and occasional fibrosis was observed. In the liver, diffuse dilitation of sinusoidal spaces was often observed. A mild cholangiohepatitis, characterized by mixed inflammatory cell infiltration was also seen. Fibrosis and thickening of the hepatic capsule was also a common finding. Congestion and

edema were the most prominent changes observed in the lung.

Results

Feed Analysis

Two samples of feed were collected from two of the growers and analyzed for salt, furazolidone, monensin, amprolium, lasalocid, cresol, PCB, organophosphate, and organochloride. Salt levels were within normal range. One sample contained 56 grams per ton monensin, which is an acceptable level for broiler feeds. Cresol was detected in slight amounts in each feed sample but was not considered excessive when compared with a control feed also analyzed. No other tests were performed.

Discussion

The mortality in these flocks appeared to be self-limiting after six weeks; however, most owners elected to slaughter the remaining birds between seven and eight weeks of age. No common factor could be attributed to the illness other than the strain of bird. Other breeds of chickens kept in the same environment remained healthy. No toxins could be detected in the feeds. Interestingly, no unusual cases of morbidity were submitted to the laboratory from commercial broiler flocks at this time, despite the fact that the origin was probably the same.

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FACTORS INFLUENCING THE INCIDENCE OF TIBIAL DYSCHONDROPLASIA IN COMMERCIAL BROILERS

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A total of 439 commercial broiler flocks in North Carolina, the Delmarva Peninsula and the Virginia Shenandoah Valley were evaluated for tibial dischondroplasia (TD) from October 1986 to October 1988. Five to seven birds were randomly selected from each flock, necropsied and scored for presence of absence of TD and bursal atrophy. Twenty-nine separate feed mills from eighteen different integrated broiler companies were included in the survey. Ages at time of necropsy ranged from 14 to 56 days of age.

The overall incidence of TD was 18.1% with a range of 0 to 100% of the birds affected within each flock. Table 1 gives a breakdown of the percentage of TD in each flock and the percentage of flocks which fall in that range.

Fifty-two percent of the flocks had no evidence of TD. The incidence of TD in those flocks that had at least one bird positive was 37.9% with the majority falling in the 21–40% range.

The incidence of TD was 9.8% in North Carolina while it was 21.7% on the Delmarva Peninsula and 21.1% in the Shenandoah Valley (sig. $P<.05$). There was also a significant ($P<.05$) company effect where the incidence of TD was a low of 4.4% (Company 4) ranging to a high of 33.1% (Company 10).

There was no significant effect of bursal score, age, season, or year on the incidence of TD.

TABLE 1

% TD in flock	No. flocks	% of flocks
0%	229	52.2
1–20%	53	12.1
21–40	89	20.3
41–60%	45	10.3
61–80%	18	4.1
81–100%	5	1.1

PRIMARY CENTRAL NERVOUS SYSTEM DISEASE OBSERVED IN CALIFORNIA TURKEY FLOCKS: A SUSPECTED OUTBREAK OF AVIAN ENCEPHALOMYELITIS AND ENCEPHALOMALACIA

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Suspected Avian Encephalitis

Turkey poults with nervous disorders were presented at ages ranging from 1 day to 3 weeks from 7 production sites in the Fresno area and from 1 ranch site in the Turlock area. Affected ranches represented 3 companies and cases were submitted over a 25 day period (July 25 to August 19, 1988). All affected poults derived from a common hatching egg source. Presenting neurologic signs included excessive mortality, lateral recumbency, ataxia, circling, and fine head tremors typical of avian encephalomyelitis (AE). In some poults there were also varying degrees of catarrhal enteritis, round heart disease, staphylococcal arthritis, crop mycosis, airsacculitis and other miscellaneous diseases but none of these disease entities were consistent through all accessions. Paratyphoid Salmonella were also cultured from the intestines of poults from several sites but the serotypes varied (*S. agona, S. indiana, S. arizona, S. hadar,* etc.). With the exception of a single poult that yielded *Salmonella arizona*, brain cultures were bacteriologically negative. The single finding consistent in all submissions was non suppurative encephalomyelitis characterized by lymphocytic perivascular cuffing and focal gliosis which were especially prominent in the brain stem, cerebellum and midbrain. Poults in some submissions had acute neuronal swelling and central chromatolysis, especially in neuronal nuclei of the brain stem, cerebellum and the ventral horns of the spinal cord. Most poults sampled early (1 to 7 days) had highs ELISA titers for Newcastle disease indicating substantial maternal antibody levels. Unfortunately, the only serologic tests available for avian encephalomyelitis were the chicken ELISA kit and an agar gel immunodiffusion (AGP) test. The chicken ELISA test titers for AE remained low to negative even in serum samples tested 3 weeks after the initial submissions (the maximum titer class observed was 4) and the AGP test results, though frequently positive, were questionable because of nonspecificity in the test system. Attempts to isolate the AE virus were not successful.

Encephalomalacia

This disease problem, while not unique, is presented here because of the frequency of its occurrence in the face of standard feeding practices that should provide adequate vitamin E and selenium levels. The disease is usually classical with postural or locomotor problems appearing at about 3 weeks of age. Affected poults have focal to massive malacia and hemorrhage in the cerebellum. Interestingly, in our diagnostic laboratories, this has been exclusively a problem of turkeys—the disease has not been seen in commercial broiler or egg-type chickens. The only exceptions have been cases of encephalomalacia in Bob White quail and in young fancy chickens and in both instances the affected birds were fed commercial turkey rations. Obviously there is a need to explore turkey feed formulation in an attempt to define those factors leading to the development of brain lesions in young poults.

CROSS PROTECTIVE PROPERTIES OF INFECTIOUS BURSAL DISEASE VIRUS

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Considerable progress has been made during this decade in understanding the antigenic and cross-protective properties of infectious bursal disease viruses (IBDV). Up to 1980, only one antigenic type of IBDV was recognized. In 1980, McFerran et al[2] reported on low relatedness values (30%) between some European serotype 1 strains. They also reported on the recognition of a second serotype of the virus. In the USA, a second serotype of the virus was recognized in 1982[3] and later that virus was shown to be related to the European serotype 2 isolate.[4] Although the American isolate of serotype 2 was obtained from turkeys, later studies indicated the widespread prevalence of serotype 2 infection in both chicken and turkey commercial flocks. Viruses of both serotypes are not cross-protective, could be distinguished by the virus neutralization (VN) test but not by the fluorescent antibody test. No known viruses of serotype 2 are pathogenic in either chickens or turkeys.

In 1987, we reported[1] on the antigenic diversity of serotype 1 strains of the virus based on the VN test. Six subtypes of serotype 1 were recognized and one of these subtypes included all the recent isolates from the US that are commonly designated as variants. These variant viruses were isolated in 1984[4] and 1985[5] from broiler chickens in the Delmava peninsula. They were designated as variants since the commercial vaccines available at that time did not protect against these viruses.

Considering the antigenic diversity of the viruses, we decided to examine the *in vivo* cross protective-properties of different IBDVs in SPF chickens. This was dictated by practical considerations related to the efficacy of vaccines made of the different subtypes in inducing protection against various antigenic types of IBDVs. Obviously, results from *in vitro* tests (specially the VN test) are useful for predicting the cross-protective properties of viruses but *in vivo* tests are essential to confirm these characteristics.

Our standard procedures consisted of producing inactivated vaccines in our laboratory that contained known amounts of virus and adjuvate with oil. Three week-old SPF chickens were vaccinated with the different vaccines and challenged two weeks later. The birds were observed for clinical signs and sacrificed at approximately 5 and 10 days following challenge. Protection was evaluated in terms of clinical signs, macroscopic and microscopic lesions, body weight and bursa-body weight ratios. Antibodies were titrated using the VN test.

A variety of experiments were conducted using different viruses for vaccination and challenge. The viral content of the vaccine and the challenge preparations were varied in the different experiments. Ten strains of serotype 1 IBDV belonging to the six subtypes were used in the different experiments. Some of these strains are those used in commercial vaccines, whereas others are the so called variants.

In our earlier studies, we found that higher doses (10^4 $TCID_{50}$) of any of the subtypes of serotype 1 were protective against challenge with 10^2TCID_{50} of a variant (E) virus. These results prompted us to use the different variations in vaccine and challenge doses. Two challenge doses 10^2TCID_{50} "similar to that used by the USDA to evaluate efficacy of commercial vaccines" and a higher dose of $10^{4.5}TCID_{50}$. Vaccine doses varied from 10^2 to 10^8 $TCID_{50}$.

To summarize the results, it was found that high vaccine doses of any of the commercial vaccine strains were protective against the low challenge doses whereas the same challenge doses caused disease in birds vaccinated with the low dose of the vaccines.

Based on these results, it is suggested that all serotype 1 viruses share a (minor) antigen(s) that elicit(s) protective antibodies.

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COMPARATIVE EFFICACY OF TWO LIVE IBD VACCINES IN BIRDS SPRAY-VACCINATED WITH OR WITHOUT THE PRESENCE OF LITTER

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Introduction

Infectious bursal disease (IBD) is a highly contagious disease of young chickens that has been a problem since the first occurrence in 1962.[1] The virus is very resistant to common disinfectants and is present in most of the poultry producing areas of the world. The majority of vaccinations in the past were administered in the drinking water. Recently, due to difficulty of vaccinating through closed water systems, coarse spray application of vaccine has become a common method of delivery.

Field observations indicate that it is not uncommon to experience low levels of seroconversion to IBD vaccination which may or may not be correlated with an absence of immunity to IBD infection. These experiments were conducted to determine if these incidents may be explained through environmental and/or vaccination equipment differences.

Materials and Methods

Vaccines

Two intermediate strength IBD vaccines were evaluated in all experiments. Vaccine A is licensed for use in drinking water or coarse spray in birds one day-of-age or older. Vaccine B is recommended for drinking water vaccination of birds seven days or older.

Vaccination Equipment

Experiments One and Three were conducted using a TURBAIR Vaccinator 240. This is a fine spray hand-held unit producing droplets of approximately 55 microns diameter. Experiment Two utilized a coarse spray vaccinator cabinet from Salsbury Laboratories, which produced droplet sizes of approximately 100 microns.

Serology

Circulating antibody titers were evaluated by ELISA (Salsbury Labs, Inc.) and reported as profile ranks; and by microtiter serum neutralization (SN) testing with vero cells.

Experimental Designs

In Experiment One, 60 2-week-old SPF white leghorn chickens were divided equally into three groups of 20 and housed in separate Horsfall Bauer isolation units. One unit was spray vaccinated with each vaccine using the TURBAIR fine spray unit. All air movement in the units were turned off prior to vaccination and for ten minutes following the application. The flooring used in this experiment was open mesh wire. Twenty days following vaccination, one-half of each unit was bled for serology. Challenge was performed by eyedrop on day 21 using USDA–APHIS material; and bursal lesions were scored on day twenty six.

Experiment Two parameters were the same as the previous test, except that spray vaccinations were performed in the Salsbury coarse spray cabinet. Each vaccine group remained in the cabinet for approximately five minutes following vaccination.

In Experiment Three, 280 2-week-old SPF leghorns were divided equally into seven groups of 40 and housed in separate isolation units. Two units designated for each vaccine were equipped with solid floors covered with sterile wood savings. The remaining three units had wire mesh flooring as in Experiments One and Two. Vaccine applications were performed with the TURBAIR fine spray unit. For each vaccine A and B, 20 birds were vaccinated in a wire mesh floor isolator, 20 birds were sprayed in a wood shavings unit, and for the remaining bedded unit, the birds were removed, vaccine was sprayed on the shavings, then the birds were replaced. Air movement was blocked off in the isolators as in Experiment One.

On days 3, 7, 11, 15, and 19 post-vaccination, ten chickens in each unit were bled for serology. Five birds were sacrificed in each isolator on days 5, 10, and 15 following vaccination and bursas were removed for histopathological examination. The remaining vaccinates and controls were challenged as described earlier on day 19. Bursal lesions were scored on day 23.

Results

Experiment One

None of the birds seroconverted as measured by the ELISA and SN tests. Also, none of the birds were resistant to standard challenge. These results were in conflict with earlier published results[2] and led to experiments two and three.

Experiment Two

Birds vaccinated with Vaccine A by spray cabinet had an SN GMT of 338, and were 100 percent protected against standard challenge. Birds receiving Vaccine B had an SN GMT of 97 and were also 100 percent protected.

Experiment Three

Birds vaccinated with Vaccine A in all treatment groups were 100 percent susceptible to IBD challenge. Birds given Vaccine B also showed zero percent protection, except for birds vaccinated and placed on litter, in which case one bird of 20 was protected. Some serology was noted in both vaccine groups, but involved only a few birds and was short lived.

Discussion

The lack of protection to challenge in birds vaccinated with the Turbair® machine is difficult to explain. The assumption was made that the particles are too fine depositing virus in the respiratory rather than the digestive tract. It was theorized that placing the birds on litter would allow the virus to cycle among the birds as it was picked up from the litter. This was not the case in this study. From the results seen here, it appears that particle size is of prime importance when immunizing birds to IBD by spray route and the larger particle (>100 microns) is preferred to the smaller (45 to 55 microns).

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ROLE OF K1 CAPSULE IN AVIAN COLISEPTICEMIA

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It has been shown that a large number of invasive *Escherichia coli* strains produce polysaccharide capsules that are important for virulence. One type of capsule—K1—was found to be associated with the majority of strains isolated from human septicemia, especially from newborns.

Since avian colisepticemia is an invasive disease, we have examined the possibility that K1 capsules play an important role in the pathogenicity of avian *E. coli* strains.

As in *E. coli* capsules can not be seen microscopically, their existence has to be shown either chemically or immunologically. We have studied a large number of strains isolated from colisepticemia in chickens and in turkeys and looked for their ability to make capsules, especially K1 capsules.

Our results showed a strong correlation between virulence and the ability to produce K1 capsules, especially in strains of serotypes O1 and O2. Furthermore, antibodies produced against K1 capsule gave full protection against these encapsulated strains.

CONGO RED DYE BINDING CHARACTERISTICS OF *E. COLI* ISOLATIONS IN TURKEY COLIBACILLOSIS FIELD CASES

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Introduction

Septicemia and associated lesions caused by *Escherichia coli* remain an important source of morbidity and mortality in turkeys. Berkhoff and Vinal[1,2] report that the ability of internal organ isolates of *E. coli* to either bind or not bind Congo Red (CR) dye is correlated to virulence in chickens. Gjessing, et al.[3] hypothesize that the subsequent occurrence of septicemia and/or airsacculitis in broiler chicks is related to aerosol inhalation of CR positive (CR+) *E. coli* while in the hatcher. In evaluating more than 1200 *E. coli* isolates from diseased turkeys, Jackson and Jensen[4] reported that about 45% took up the CR dye. The CR-binding characteristic is reported as a potential research tool in distinguishing between pathogenic and nonpathogenic *E. coli* in chickens.[1,2,3] Its role in differentiating between turkey *E. coli* isolates remains unclear. Neither has the potential usefulness of the CR-binding characteristic in evaluating turkey *E. coli* field outbreaks been adequately explored. The purpose of this report is to serve as a descriptive summary of the frequency distribution of the CR-binding characteristic in turkey colibacillosis cases. The results of this study are taken from internal organ isolates of *E. coli* recovered from cases of turkey colibacillosis in the Sanpete Valley of Utah. This was a preliminary study serving as a guide if further studies may be warranted in utilizing the CR-binding characteristic of *E. coli* isolates as a potential epidemiologic and/or diagnostic tool in assessing and characterizing outbreaks of turkey colibacillosis.

Materials and Methods

Selection and Determination of Colibacillosis Cases

Turkey necropsy submissions to the Moroni Feed Company Veterinary Laboratory were evaluated and classified as a predominantly *E. coli* infection by the following criteria: 1) Postmortem gross lesions of hepatitis, airsacculitis, pericarditis, or other lesions suggestive of an *E. coli* infection; 2) the majority of the turkeys in a given submission must exhibit similar lesions; 3) predominant or exclusive growth of *E. coli* from lesions showing gross abnormalities must be present on direct culture. The colibacillosis cases were subdivided into three general categories: 1) those exhibiting mainly respiratory tract pathology (airsacculitis being the major criterion), 2) cases in which enteric or septicemic pathology predominated, and 3) those cases where both respiratory and enteric/septicemic lesions were present.

Congo Red Culture Medium

Congo Red agar was prepared in a similar manner to that reported by Berkhoff and Vinal.[1] However in our work Tryptose agar (DIFCO) was used as the basal medium.

Selection and Determination of CR-binding Status of *E. coli* Isolates

Internal organs cultured and evaluated for growth of CR+ and CR– *E. coli* were dependent on lesions observed at necropsy. In general, for turkeys showing lesions of septicemia or enteritis, liver, spleen and pericardial sac were cultured. In cases where the respiratory system was predominantly involved, airsac, lung, and pericardial sac were cultured. All internal organ cultures were directly plated on Tryptose, MacConkey, Brilliant Green, and CR agars and allowed to incubate for 18 to 24 hours at 37°C. Colonies were observed at room temperature daily for 3 days. Colonies taking up the CR dye by 96 hours were classified as CR+. Representative CR+ and CR– colonies were removed from the agar plate and subjected to a battery of biochemical screening tests for *E. coli* determination. Further verification of isolates was carried out at the Veterinary Diagnostic

Laboratory, Utah State University. A total of 98 *E. coli* isolates were evaluated, representing 37 cases of turkey colibacillosis.

Results

We were unable to demonstrate clear-cut differences in the frequency distribution between CR– and CR+ *E. coli* isolates according to postmortem lesions (Table 1). The proportional isolation of CR– *E. coli* was greater from the liver, spleen, lung, and pericardial sac. The proportion of CR+ isolates exceeded CR– isolations only from the airsac (Table 2). However, when comparing organs of isolation in those cases where both CR+ and CR– *E. coli* were encountered, the airsac in most instances was the organ yielding the CR– isolate (data not shown). When comparing age groups of colibacillosis cases, a greater proportion in the 4 to 8 week old group yielded CR– isolates only. In the remaining two groups (i.e., 0 to 4 weeks and older than 8 weeks), there appears to be no definite trend in the distribution of cases according to the CR-binding characteristic of the recovered *E. coli* (Table 3).

Discussion

Because of inherent limitations in this report—particularly small sample size, sampling bias, diverse management practices, and possible inapparent concurrent disease, it is difficult to make firm conclusions based on one limited survey. However, within the limitations of this study, we were unable to demonstrate differences in the frequency distribution of the CR-binding characteristic distinct enough to be of consistent help in characterizing cases of turkey colibacillosis in our laboratory based solely on the CR-binding characteristic of *E. coli*.

Further studies are needed to characterize and define the significance of the CR-binding characteristic in turkey *E. coli* infections.

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TABLE 1
Lesion-specific frequency distribution of cases according to CR-binding characteristic of *E. coli* isolates.

E. coli CR-binding Characteristic	Frequency of Cases					
	Respiratory		Enteric		Resp. and Enteric	
CR– Only	4	(29)*	9	(69)	5	(50)
CR+ Only	5	(36)	4	(31)	1	(10)
CR– and CR+	5	(36)	0	(0)	4	(40)
Total	14	(100)	13	(100)	10	(100)

*Percentage from total number of cases with the specified postmortem lesion

TABLE 2
Frequency distribution of organ of isolation according to CR-bind- characteristic of *E. coli* isolates

E. coli CR-binding Characteristic	Organ of Isolation				
	Airsac	Liver	Spleen	Lung	Pericardial Sac
CR–	7 (44)*	22 (69)	9 (60)	9 (75)	15 (68)
CR+	9 (56)	10 (31)	6 (40)	3 (25)	7 (32)
Total	16(100)	32 (100)	15 (100)	12 (100)	22 (100)

* Percentage from total number of *E. coli* isolated from the specified organ

TABLE 3
Age-specific frequency distribution of cases according to *E. coli* CR-binding characteristic

E. coli CR-binding Characteristic	Frequency of Cases (Age in Weeks)		
	0 up to 4	4 up to 8	Older than 8
CR– Only	2 (22)*	11 (65)	5 (42)
CR+ Only	3 (33)	2 (12)	5 (42)
CR– and CR+	4 (45)	4 (23)	2 (16)
Total	9 (100)	17 (100)	12 (100)

* Percentage from total number of *E. coli* isolates from the specific age group

DIARRHEA ASSOCIATED WITH LONG SEGMENTED FILAMENTOUS ORGANISMS (LSFOs) IN THE SMALL INTESTINES OF TURKEYS, CHICKENS, AND QUAILS

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Introduction

Long segmented filamentous organisms (LSFOs) attach to enterocytes in the small intestines of mice,[1,4] rats,[5,6] and chickens.[2] Small-intestinal gross and histologic lesions usually are not present, and the pathophysiologic significance of LSFOs, if any, never has been established. The taxonomic identity of LSFOs remains not known.[1]

LSFOs never have been described in turkeys and quail, and clinical, pathologic, and epidemiologic data of LSFO infection in poultry never has been published. The purpose of this study was to describe the clinical and pathologic findings of LSFOs in Georgia and California turkeys, chickens, and quail.

Materials and Methods

For a 24-month period (1–1987 through 12–1988), laboratory reports at the Georgia Poultry Laboratory and at the Turlock Branch of the California Veterinary Diagnostic Laboratory System, School of Veterinary Medicine, University of California (Davis) were reviewed. Cases of LSFO infection were identified and laboratory reports and microscope slides from archives were retrieved for further study.

Results

Epidemiologic Findings

Fifty-two cases of LSFO infection were diagnosed during 1987 and 1988. Approximately one-half of these cases were diagnosed in Georgia (27 cases); the others were diagnosed in California (25 cases) (Fig. 1). Infection was relatively common in turkeys (46 cases) and was relatively rare in chickens (3 cases) and quails (3 cases).

Clinical Findings

Eighty-eight percent (46/52) of LSFO infections occurred in birds $\leq$ 3 weeks of age (Fig. 2). More cases seemingly were diagnosed during the Winter months than during the Spring, Summer, or Fall months (Fig. 3).

Pathologic Findings

In Georgia, cases always were diagnosed during histologic examination of submitted organs. In contrast, 96% (25/26) of California LSFO infections were diagnosed by cytologic examination of intestinal scrapings.

The most common presenting complaints and clinical signs associated with LSFO infection in turkeys, chickens, and quails generally were: diarrhea, huddling, and small birds in flocks where mortality rates were higher than normal (Table 1). The most common gross lesions in gastrointestinal tracts were: intestinal fluid/gas, litter, and retained yolk sacs (Table 2).

Histologically, LSFOs easily are recognized with a light microscope. These distinctive organisms stain blue with H&E and are PAS, van Orden, and Gram's positive. Variable numbers of LSFOs may be present (Fig. 4). Heavily-parasitized enterocytes often are vacuolated and the mucosa is inflamed. In well-preserved sections, clusters of infected enterocytes commonly are being extruded from the mucosa. Ultrastructurally, one end of each LSFO was buried into the brush border; the length of the organism protruded into the lumen and terminated at it's free end (Fig. 5). Secure attachment sites are formed between LSFOs and enterocytes and microvilli are displaced but the cell membranes appear intact (Fig. 6).

LSFOs were seen in all segments of small-intestine but were most likely to be found in jejunums and ileums. LSFOs never were seen in cecums or colons, other portions of the gastrointestinal tract, or in other organ tracts. Other infectious organisms sometimes were seen in or cultured from LSFO-infected intestines (Table 3).

Discussion

Long segmented filamentous organisms previously have not been described in turkeys and quails, and have been described only once in an asymptomatic chicken.[3] No morphologic differences between poultry and weanling laboratory mouse[3,4] and rat[2,3] LSFOs are visible.

LSFOs have no apparent pathological significance in rodents.[1] In this study, LSFOs always were associated with gastrointestinal disease; however, other enteric pathogens were present in some cases and, in others, work-ups for enteric pathogens were not complete. In our experience, LSFOs are not always seen in turkeys, chickens, and quails with gastrointestinal disease, but never have been seen in normal poultry or in poultry with diseases of other organ tracts. A cause-and-effect relationship between LSFOs and gastrointestinal disease can not be established in this study, and the significance of intestinal LSFOs in poultry remains not known.

LSFOs may be normal flora, pathogens, or commensal organisms that overgrow when certain gastrointestinal events occur. They easily are recognized by their distinctive cytologic and histologic appearance. Poultry diagnosticians and researchers should be aware that small-intestinal LSFO infections occur, and that gross and histologic lesions often are associated with LSFO infections. Although reportedly once grown in a medium not defined,[3] we and others have not been able to culture LSFOs.

Special efforts should be made to isolate LSFOs and determine their clinical and pathologic significance. In addition, isolation and identification of viruses and bacteria always should be attempted when infectious etiologies for gastrointestinal disease are suspected.

Acknowledgments

We sincerely appreciate the technical assistance of Dr. W. L. Steffens and Ms. Mary B. Ard, Electron Microscopy Laboratory, Dept. of Anatomy and Radiology, and Mr. John Turpley, Histopathology Laboratory, Dept. of Veterinary Pathology, College of Veterinary Medicine, University of Georgia, Athens, GA.

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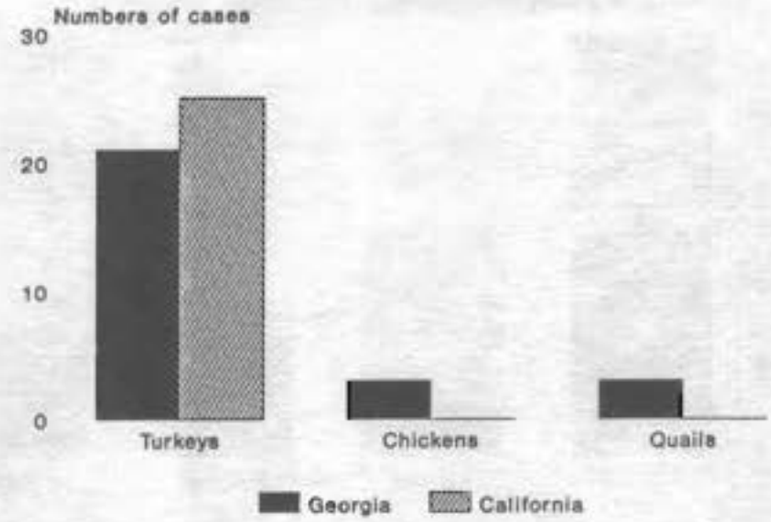

Figure 1. During 1987 and 1988, long segmented filamentous organisms were seen in small-intestinal segments from Georgia or California turkeys, chickens, and quails.

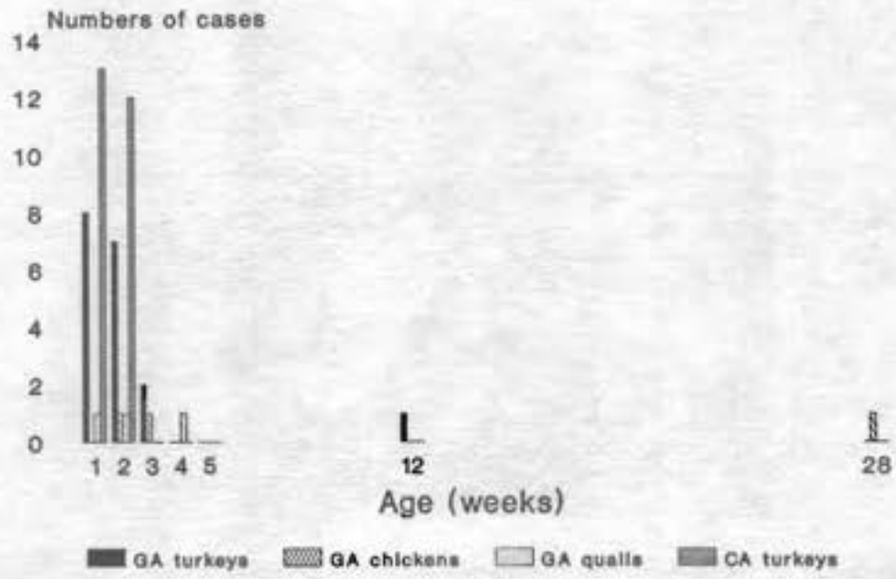

Figure 2. Long segmented filamentous organisms tended to infect the small-intestines from young poultry.

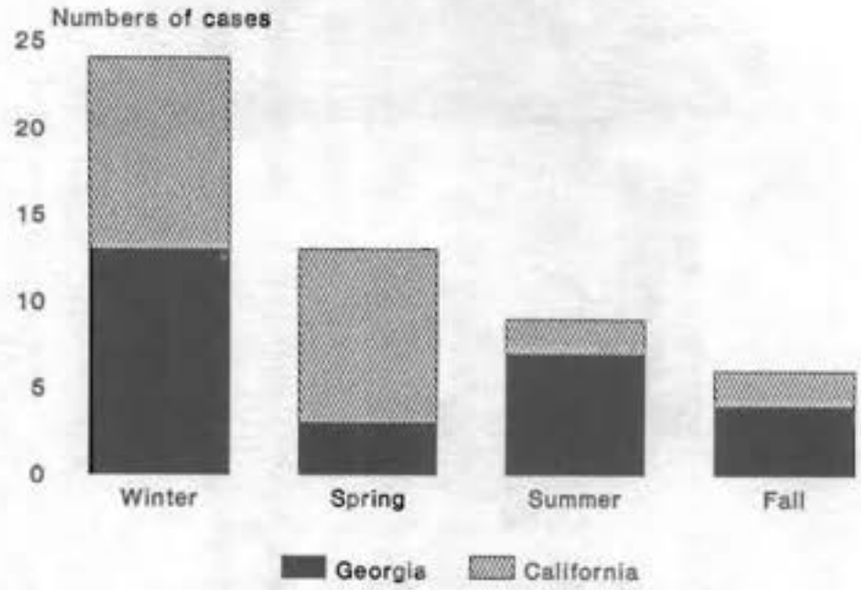

Figure 3. Diagnoses of small-intestinal long segmented filamentous organism infection were most common during the Winter months.

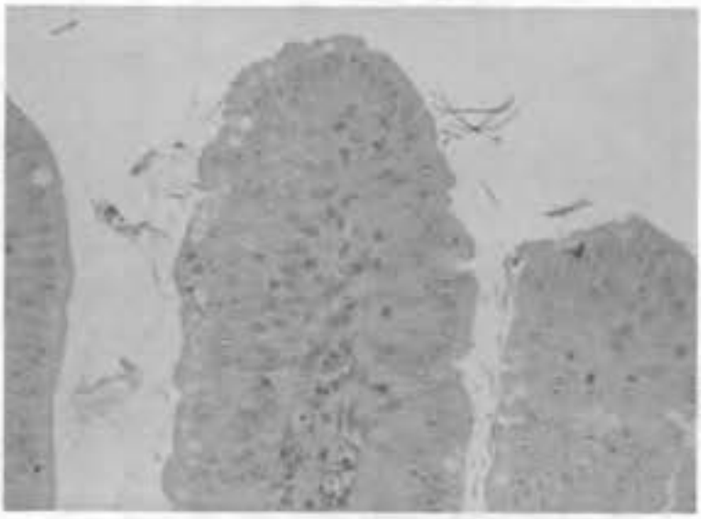

Figure 4. Long segmented filamentous organisms attach to enterocytes in a segment of small-intestine from a turkey. The mucosa is mildly inflamed H&E.

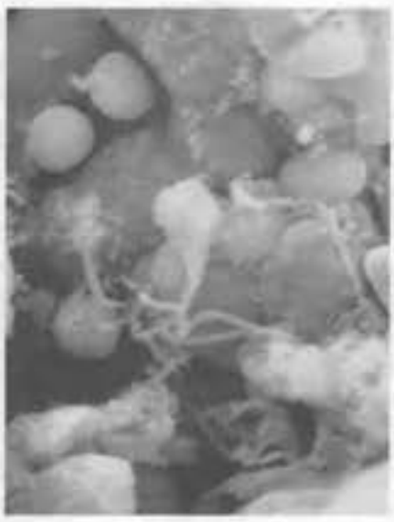

Figure 5. Proximal ends of several long segmented filamentous organisms are buried in enterocyte brush borders. The ends of the organisms dangle free in the lumen. Osmium tetroxide, gold-paladium.

Figure 6. Long segmented filamentous organisms are tangentially sectioned. They displace microvilli, form an attachment zone on the enterocyte, and distort the terminal web and apical cytoplasm of the cells. A large cytoplasmic bleb extends from one absorptive epithelial cell. Osmium tetroxide, lead citrate, uranyl acetate.

TABLE 1
Presenting complaints and clinical signs associated with intestinal LSFO infection in turkeys, chickens, and quails in Georgia and California during 1987 and 1988

Species	Complaint	Frequency (%)*
Turkey	diarrhea	50
	huddling	17
	small	13
	mortality up	13
	rough feathers	13
	droopy	9
	general check	9
	snicking	9
	nervous, inactive, not eating, pasted vents	≤5
Chicken	diarrhea	33
	lay on side, die	33
	out of production	33
Quails	diarrhea	66
	mortality up	33
	fail to thrive	33

*Signs were reported in 23 of 46 turkey cases, 3 of 3 chicken cases, and 3 of 3 quails cases

TABLE 2
Gross lesions associated with intestinal LSFO infection in turkeys, chickens, and quails in Georgia and California in 1987 and 1988

Species	Gross lesion	Frequency (%)*
Turkey	intestinal fluid/gas	100
	eating litter	23
	retained yolk sac	18
	swollen feet, joints	18
	pasted vents	11
	soft bones, small, peritonitis, round hearts	≤5
Chicken	intestinal fluid/gas	100
	duodenal hyperemia	33
	palor	33
Quail	intestinal fluid/gas	100
	incoordination	33

*Gross lesions were reported in 44 of 46 turkey cases, 3 of 3 chicken cases, and 3 of 3 quails cases

TABLE 3
In addition to long segmented filamentous organisms, other organisms sometimes were seen in segments of turkey, chicken, and quail small-intestine

Organism	Host species		
	Turkey	Chicken	Quail
LSFOs	23*	3	3
bacteria rods (Gram + or –)	8	1	0
Eimeria species	3	1	0
Spironucleus sp	1	1	1
Cryptosporidium sp	1	0	1
organism not identified	3	0	0

*Number of cases where the organism histologically was seen

CASE REPORT: FOWL CHOLERA IN BROILERS

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Introduction

Fowl cholera is a highly contagious disease caused by *Pasteurella multocida*, a small gram negative rod.[3] It usually appears as a septicemic disease resulting in high morbidity and mortality. It can persist as a chronic disease producing purulent arthritis, consolidated lungs and enlarged fibrotic wattles. Until recently, pasteurellosis has been a common problem only in turkeys six to ten weeks of age and in chickens over sixteen weeks of age.[14] There have been no previously published cases of fowl cholera occurring in broilers in the United States.

Many birds and most farm animals can be carriers of *Pasteurella multocida*, but not all of the *P. multocida* isolates from these animals are capable of producing disease in poultry.[4,6,12,15] In fact, with the exception of swine and possibly cat isolates, most are avirulent for fowl.[5,13] Once the disease is introduced into a flock, it spreads rapidly throughout the house. This occurs by contamination of open watering systems and cannibalism of contaminated carcasses.[5,11,12,13]

Strain classification varies with the testing method used. Swine isolates of *P. multocida* are generally serotyped according to their capsular antigens. Using this technique, most avian isolates would be classified as serotype A.[3] Heddelston's agar gel precipitation method detects somatic antigens which are lipopolysaccharides in the cell wall.[7] Using this technique there are sixteen serotypes detectable.[3] The most common isolates from turkeys are those possessing antigens of serotypes 3,4 and 3.[1,9,10] In chickens, the order of serotype prevalence is 3,4, followed by 1, and then 3.[9]

Case Report

Between April 12 and September 13, 1988 six flocks of broiler chickens were diagnosed at the Poultry Disease Research Center, University of Georgia, Athens, as having fowl cholera. The flocks ranged from twenty to forty-six days of age, located at four farms spread over an area of fifty miles.

The presenting complaint was an average increase in mortality of 1% and the birds were "getting down in the legs before dying." Post mortem findings included dehydration, purulent hock joints, femoral osteomyelitis, swollen livers and spleens with multifocal pinpoint pale areas. In addition the birds had airsacculitis, hydropericardium, pale kidneys, small bursas, and one had a purulent pleuropneumonia. Serology was performed on blood from only five birds representing two flocks, which is an insufficient quantity upon which to draw conclusions. However, the trends were low titers to Newcastle disease virus, infectious bronchitis virus, and high infectious bursal disease titers. Histology on samples from one flock revealed multifocal areas of coagulation necrosis in the liver and spleen consistent with bacterial septicemia.

Examination of housing conditions and management procedures on the affected farms revealed several problems. Outbreaks on one of the farms occurred in two different buildings on three consecutive growouts. The isolate from the first outbreak was classified as serotype 3. The subsequent outbreaks were the result of an isolate possessing antigens of serotypes 1 and 3 *P. multocida*. The grower on this farm worked in a hog parlor and maintained a flock of free roaming adult chickens. Feral cats and rodents were also a problem on the premises. There were four poultry houses on the property; two with nipple drinkers and two with open trough waterers. The outbreaks only occurred in those houses with the open watering system. Of the other farms suffering from outbreaks of fowl cholera, one reported that a dog had broken in and killed a number of chickens. All of the farms had rodent control problems. These farms had a variety of watering systems. No repeat outbreaks occurred on these premises, however. In those flocks where antibiotic therapy was administered, tetracyclines resulted in a decline in mortality.

P. multocida was isolated on blood agar from samples taken from the stifle and hock joints, liver, spleen, lung, bone marrow, pericardial sac and airsac. A *P. multocida* isolate from each case was serotyped by the agar gel precipitation method described by Heddelston. The frequency of serotypes found were as follows: three isolates were 1,3, two were 3,4 and one isolate was serotype 3.

In order to confirm that the clinical signs and postmortem lesions observed were, in fact, caused by *P. multocida*, two of the isolates were propagated and used to challenge susceptible broilers. The serotypes chosen were 3 and 1,3.

Day old broiler chicks numbering 140 were obtained from a local commercial hatchery and raised to five weeks of age, then randomly separated into six groups containing 22–23 birds per group. Three groups were housed in three adjacent floor pens and were separated by empty pens from the other three groups, which were also housed in three adjacent floor pens. Biosecurity procedures were followed at all times when moving between pens to prevent contamination of one group by another.

The two serotypes of *P. multocida* were grown separately on blood agar plates and then a single colony from each plate inoculated in brain heart infusion broth (BHI) and incubated at 37°C for 18 hours. One milliliter of that culture was transferred to 100ml fresh BHI and allowed to incubate for 4 hours at 37°C. These broth cultures were diluted in phosphate buffered saline (PBS) to obtain concentrations of $1x10^2$ colony forming units (CFU)/ml and $1x10^4$ CFU/ml for each serotype. Three pens of birds were challenged per serotype using the following methods. One pen was injected with one ml. of diluted culture containing $1x10^2$ CFU/ml intramuscularly (IM) in the breast, another with one ml. of diluted culture containing $1x10^4$ CFU/ml IM, and the third was challenged with the undiluted culture, $1x10^9$ CFU/ml, by introducing one drop in the eye, one drop in the nostril, and squirting 2ml into the palatine cleft with a blunt cannula.

The birds were observed for nine days. All dead birds were necropsied and samples taken for bacterial isolation. Several of the chickens in the group receiving the eyedrop inoculation had severe conjunctivitis. Birds which died acutely had enlarged, mottled livers and spleens, hyperemic lungs and purulent nasal discharge. Those birds that died near the end of the nine days had purulent synovitis and osteomyelitis. Of the surviving birds, several were lame and reluctant to move about. These birds were euthanized and the joints or bone marrow sampled for bacterial isolation. *P. multocida* was isolated from affected birds in each pen for a total of 87% positive cultures. The isolates were all serotyped 1,3. The degree of lameness and mortality varied between groups (Table 1).

Discussion

The natural route of entry of *Pasteurella multocida* is through the mucous membranes.[10] It was possible to reproduce the clinical signs and postmortem lesions by inoculation via this natural route as well as by IM injection, proving the pathogenicity of this organism for broiler chickens.

It is often impossible to ascertain the mode of introduction of fowl cholera into a flock. In these cases transmission from swine, rodents, cats, yard chickens, free flying birds and possibly a dog, could be potential sources of infection. In order to determine the possibility of transmission of *P. multocida* from the hog parlor where one grower worked, two *P. multocida* isolates, obtained from the lungs of swine, were serotyped for somatic antigens. Both cultures were 1,3 serotype which is one of the serotypes isolated on that farm. This indicates that swine were a potential source of infection in these flocks.

It is possible that some of the affected flocks had early exposure to infectious bursal disease with resulting immunosuppression allowing the birds to be more susceptible. Open trough waterers may have aided in rapid and complete spread of the disease through some of the houses.

It is interesting to note that on the farm with the recurring outbreaks the isolate from the first outbreak was serotype 3, whereas, isolates from subsequent outbreaks were serotype 1,3. Also, when birds were challenged with a culture derived by propagating one colony from the original serotype 3 culture, a serotype 1,3 was recovered despite strict biosecurity measures. This indicates that the original isolate was most likely a mixed culture containing serotype 3 and serotype 1,3. This theory is substantiated by the observation that the original isolate from that farm was serotype 3 and a later isolate was serotype 1,3. We have found previously that isolating mixed cultures of *P. multocida* from field material is not unusual.

Fowl cholera in broilers can be easily confused with other septicemic bacterial diseases of chickens. The gross lesions of early *E. coli*, salmonella and staphylococcal septicemias are similar to the lesions in birds with fowl cholera. In addition, the purulent synovitis often occurring as a chronic sequela to *P. multocida* septicemia is difficult to differentiate from synovitis caused by other bacteria. This case report documents the natural occurrence of *P. multocida* and its pathogenicity in broilers, therefore the avian diagnostician should consider fowl cholera as a diagnostic rule-out in cases of septicemia and purulent synovitis in younger chickens. Bacteriological culturing will establish the diagnosis, allowing for antibiotic sensitivity testing and proper antibacterial therapy.

Summary

P. multocida, the etiologic agent of fowl cholera, was isolated from six broiler flocks in Georgia during the summer of 1988. The flocks ranged in age from 20 to 46 days located at four farms spread over an area of 50 miles. Increased mortality and lameness was the clinical presentation of all affected flocks. Bacterial isolation and agar gel precipitation for somatic antigen serotyping revealed three of the cases were caused by serotype 1,3, two by serotype 3,4, and in one case serotype 3 was isolated. In order to prove the virulence of these organisms, two were selected to challenge five week old broilers. Mortality and lameness resulted from this challenge and *P. multocida* was re-isolated.

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TABLE 1

Challenge of susceptible five week old broilers with two isolates of *P. multocida* isolated from clinical cases of fowl cholera in broilers

Challenge serotype	Route of inoculation	Titer of challenge organism	Mortality	% Mortality	Number lame	% Lame	% Total affected
3	IM[a]	1 x 10[8][c]	1/22[d]	4	0/22[e]	0	4[f]
3	IM	1 x 10[4]	2/22	9	5/22	23	32
3	ORAL[b]	1 x 10[9]	2/22	9	3/22	14	23
1,3	IM	1 x 10[8]	5/22	23	3/22	14	36
1,3	IM	1 x 10[4]	12/23	52	6/23	26	78
1,3	ORAL	1 x 10[9]	11/23	49	3/23	13	61

[a]One ml injected intramuscularly in the breast muscle

[b]One drop placed in one eye, one drop in the nostril, and two ml squirted into the palatine cleft with a blunt cannula

[c]Colony forming units/ml

[d]Number dead/total in group

[e]Number lame at end of experiment/total in group

[f]Percent dead or lame

UPDATE ON BIOLOGICS REGULATORY ACTIVITIES

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During the period from October 1, 1987, to September 30, 1988, the United States Department of Agriculture licensed 11 new establishments to produce and distribute veterinary biological products. During this same period, five establishment licenses were terminated and the number of permits for firms to import and distribute biologics remained at four. The total number of licensed firms and permittees at the end of September, 1988, was 97 which was up from 91 twelve months earlier.

During this same period, 194 new product licenses were issued. This included 52 totally new products and 31 in a new form. Twenty six of the licenses were products for further manufacture, a procedure where a partially prepared product is moved from one manufacturer to another for further processing into final form and composition. During the fiscal year, 77 licenses were terminated. The 97 licensees and permittees distribute a total of 1,665 products which are used to prevent, diagnose or treat 121 diseases.

A total of 11,332 serials of veterinary biologicals were produced and presented to APHIS for release during FY 1988. Of this number, 2,273 (27 billion doses) were for use in poultry, and 9,059 serials (2 billion doses) for use in other species. The Agency withheld from the market 7 percent of the poultry serials and 5 percent of all other serials because they failed tests for purity, safety, potency or efficacy.

One problem that has come up in the last year is the presence of Chick Anemia Agent in the United States. USDA requires that vaccines be prepared from seed stocks and media that are free from adventitious agents. In order for vaccine manufacturers to determine that their products are not contaminated with this virus, reagents have been developed at National Veterinary Services Laboratory and made available to licensees.

Each licensee is responsible for determining the status of his master, working and production seeds. A condition for providing the reagents is that the manufacturer must advise Veterinary Biologics of the results of this testing.

Most poultry vaccines are grown in embryonated eggs or embryonic tissue cultures. The producers of specific pathogen free eggs for vaccine manufacture have committed to testing their flocks and ensuring that eggs used for the manufacture of biologics are free from this virus.

As more information becomes available about the transmission of CAA, particularly the role that vaccines may play, we will be taking appropriate steps to verify that poultry biologics continue to be free of unwanted viruses and that vaccines manufactured in the United States will continue to be recognized for their quality around the world.

DIFFICULTY AND IMPROVEMENT IN THE CONTROL OF MAREK'S DISEASE IN BROILER BREEDERS

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The vaccination against Marek's disease (MD) based on the use of bivalent or polyvalent vaccines containing homologous and heterologous serotypes of MDV has been of great success in the control of the disease. Nevertheless, increased incidence of MD or "breaks of immunity" have been recently reported in Mediterranean countries with highly concentrated poultry populations. Many factors have been considered to explain the vaccine breaks; inadequate preparation of vaccines, faulty vaccine administrations, exposure to field virus at early ages, the presence of MDV belonging to different serotypes, general stresses including other viral infections and genetic susceptibility of particular lines of chickens.

In Italy, we have recently noted rather peculiar situation; whereas in light hen stocks and their breeders, the disease incidence is less than 1% with the use of bivalent vaccines containing serotypes 1 and 3 MDV, the disease loss between 5 and 15%, sometimes as high as 40%, mostly occurring after 20 weeks of age, has been observed in "heavy" type breeder stocks. The virus isolated from outbreaks in "heavy" breeders showed normal virulence in 3 cases.

The method of vaccination that has been successful for reducing losses in "heavy" breeders is vaccinating birds at 1 *and* 7–10 days of age with a bivalent (HVT and MDV CVI–988) vaccine. The results of an extensive survey and field experiments are presented to support the above statement.

INTRA-BURSAL ROUTE OF ADMINISTRATION OF LIVE AND/OR KILLED VACCINES IN CHICKENS

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Experimental adjuvanted Infectious Bronchitis Virus (Ark + Mass) vaccine was prepared in our laboratory. STM (Ribi Immunochem Research, Inc.) was used as an adjuvant. Adjuvanted killed and live infectious bronchitis vaccine was administered intrabursally to two groups of one day old chickens. One half of each of these two groups also received a booster spray vaccination at 17 days of age. Another group of one day old chickens that were spray vaccinated at the hatchery, also received killed adjuvanted vaccine intrabursally on the same day. Half of this group was also boost sprayed at 17 days of age. Serum samples collected at 17, 31, and 45 days post vaccination were evaluated by hemagglutination inhibition (HI) test. Results of this study will be presented.

IMPLICATIONS OF REQUIRED TRIMMING OF LESION SITES IN FOWL CAUSED BY PARENTERALLY ADMINISTERED VACCINES

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The popularity of killed vaccines has increased over the last ten or more years. Their early use was mainly for immunizations of breeding stock; however recently they have been widely used by producers as well. Although they are generally more expensive to administer than their live counterparts, they usually assure greater uniformity of immunity, eliminate the risk of vaccine associated disease outbreaks, and in some cases, give a longer lasting circulating antibody response. Most killed vaccine products were originally designed to be administered by the subcutaneous route. Because of the difficulties vaccination crews were having with proper and safe administrations, the poultry industry requested that the vaccine producers provide products that could be injected into the breast muscle. In July, 1988, it had been estimated that as much as 17 percent of California's 30 million laying hens had received one or more vaccinations with killed product.

Killed vaccines have several advantages over live vaccines. Most reactions are minimal or all together absent. When properly primed, higher levels of circulating antibody result which in turn provides greater maternal antibody passage into the yolk. Due to the slow but long lasting exposure of the host immune system to the antigen, a prolonged immunity results. More consistent titers between birds within a flock are observed due to the individual administration methods employed. Broad spectrum or multivalent products are more easily manufactured using killed antigenic material. Probably most importantly, the threat of run away live vaccine viruses and/or bacteria, potentiating themselves as they move through and between flocks, is eliminated.

Killed vaccines have a few disadvantages compared with previously used live vaccines. They cost significantly more per dose. Since the vaccine material cannot replicate within the host or be passed from host to host, they must be parenterally administrated which adds significant labor costs. The manager must be concerned about the effects an additional bird handling may have on subsequent performance. Most recently, a serious economic consequence of muscle-vaccinated fowl using killed vaccines, has been tissue condemnations at the processing plant.

Killed vaccine preparations usually employ an oil adjuvant in order to improve both the level and longevity of the immune response. As a necessary part of this response, a localized inflammatory reaction occurs due to the migration of immunogenic cells to the site containing the adjuvant. Many times a granulomatous lesion results at or near the site of injection. The United States Department of Agriculture (USDA) Food Safety Inspection Service requires that when certain lesions are observed by their inspectors, the site must be trimmed away before passing into the human food chain. Examples of lesions that must be trimmed are: 1) granulomatous tissue; 2) caseous necrotic abscesses; 3) opaque or cloudy fluid-filled cysts and; 4) exudate. Examples of lesions that do not require trimming are: 1) clear fluid-filled blisters; 2) transparent amber, yellow or straw colored fluid-filled blisters; 3) corrective tissue; and 4) sites of needle puncture.

Over the past 16 months (since December 1987) field inspectors at fowl processing plants have detected numerous trimmable lesions resulting from killed vaccine injections, usually located between the superficial and deep pectoral muscles. As a result of complaints lodged by large commercial buyers of processed fowl objecting to breast muscle lesions, the USDA began requiring that ". . . mature fowl, light and heavy, must be screened for the presence of lesions resulting from post-vaccinal inflammation." This order for mandatory inspection was placed in effect on March 25, 1988 and will continue for one calendar year.

The inspection procedure requires that one or more cuts be made on each side of the breast of ten randomly selected fowl. If a post-vaccinal inflammatory lesion is detected, the inspector must require 100 percent inspection of that lot of birds. The directive allows for a second 10 bird sample, if necessary, to make a definitive disposition on the lot.

The required trimming of these lesions, and the inspection process itself, has had dramatic effects on processing plants, producers and

breeders, and vaccine manufacturers. At one point in time, two commercial processing plants in California refused to accept any breast injected fowl. Processors complained that the added inspection, slowed line speeds from between 80 to 90 to between 10 to 20 fowl per minute. Table egg producers with breast vaccinated fowl complained that they were adding one cent per dozen to their cost of production by having to sell their spent fowl to the renderer at 1.4 cents per pound, F.O.B. the plant, as opposed to 7 cents per pound in the cage. Vaccine manufacturers have also suffered serious losses. While it is difficult to determine the exact figures, some have estimated that certain killed vaccine sales have dropped as much as 50 percent. This drop in sales appears, however, to be transient since many producers are again using these products; but are administering them at sites other than the breast muscle.

The problems with post vaccinal inflammatory lesions has forced growers to make modifications in their vaccination programs. Some have responded by avoiding killed vaccines entirely, while a few (mostly breeders) have continued to use these products as before, using the breast muscle for the site of injection. Other producers have gone back to the cervical subcutaneous route in spite of its difficulties. A significant number of growers have changed the site of injection to the large muscle bellies of the leg. While the cervical subcutaneous and breast muscle routes are well proven, few if any long term studies have been done to evaluate the leg site in terms of the immune response. If the leg route can be shown to be efficacious, it would have significant advantages since the leg could be trimmed without causing severe economic losses, and would be a safer target than the cervical subcutaneous route.

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PROBLEMS ASSOCIATED WITH INJECTION OF OIL ADJUVANT KILLED VACCINES

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Oil adjuvant killed vaccines have become a popular alternative to live vaccines in the control of poultry diseases. Killed vaccines obviate disadvantages of live vaccines including necessity for multiple administration, reaction to and transmission of the vaccine virus, reversion to virulence and inaccuracy and unevenness of dosage. However, oil adjuvant killed vaccines have their own drawbacks including the necessity for individual inoculation and local reactions. Our laboratory has been involved with cases which illustrate problems associated with injection of oil adjuvant killed vaccines subcutaneously in the neck and intramuscularly in the leg and breast muscles.

The severe granulomatous reactions which may result from subcutaneous neck injection of fowl cholera bacterins in turkeys has been documented.[1] In cases involving neck vaccinated birds we have also noted severe inflammatory reactions. In certain instances vaccine was injected into neck muscle resulting in a diffuse granulomatous myositis. Vaccine has also been injected too close to the skull. Misdelivery of the vaccine in this way may result in neurological and/or musculoskeletal problems which impair the birds ability to eat.

Intramuscular injection of vaccine into breast muscle offers the advantage of insuring that vaccine remains in a localized region where adverse clinical reactions are not likely to occur. However, the recent ruling by the FSIS branch of the USDA regarding the trimming of breast vaccination lesions has severely decreased the popularity of this site in California. In our cooperation with producers and processors in our area we have begun an effort to more precisely characterize the nature of these lesions and how they change with time. From cases submitted thus far it appears that soon after injection a severe inflammatory reaction occurs with caseous necrosis and heterophilic infiltration and the eventual development of granulomas. However, with time these lesions resolved in birds examined 40 weeks or more beyond injection: lesions ranged from hardly noticeable to readily discernible amber to yellow colored cysts, focal white areas within muscle and scar tissue. Microscopic evaluation of these lesions showed them to be oil cysts, lympho-follicular aggregates and areas of fibrosis and mononuclear infiltration. Whether these more chronic lesions should be trimmed is a matter of controversy.

Many producers in California have now chosen intramuscular vaccination into the leg. Cases of leg vaccinated birds reveal that reactions can vary from an extreme inflammatory reaction to no clinical reaction at all. With severe reactions lameness may result. Underlying problems such as Marek's disease and Staphylococcus synovitis may make the reaction appear clinically worse but the swelling from the injection itself apparently resolves in a few weeks. Examination of birds at various times post injection suggests that immediately after injection most vaccine flows out along fascial planes and gravitates downward. This results in inflammatory reactions concentrated mainly on muscle fascia and subcutaneously above the hock. With increasing time post vaccination lesions become increasingly hard to find and much more subtle. Cases under investigation at this time suggest that leg vaccinations may offer an alternative route to breast vaccination without severe clinical side effects. However, whether this route is as efficacious as the breast route remains to be seen.

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UPDATE ON PARAMYXOVIRUS TYPE 1 INFECTION IN PIGEONS IN THE U.S.

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PMV–1 was first diagnosed in the USA in 1984. Pigeon flyers tried using live virus Newcastle Disease vaccine but were not getting protection. Late that year I was asked if I could produce an effective vaccine. At that time I found that most of the work had been done by Dr. Alexander in the U.K. who showed distinct differences between the pigeon virus and the chicken virus. The Newcastle disease virus had become adapted to pigeons. It causes a serious disease in pigeons but not in chickens. From his work, and from experience in Europe it was obvious that live virus Newcastle disease vaccine for chickens provided only poor protection in pigeons. Our work and experience in the U.S. has confirmed this. I decided that a killed virus vaccine would be most effective based on experience with the Newcastle disease—Killed Virus Vaccine in chickens.

I considered recommending our regular Newcastle disease Vaccine—Killed Virus. However, I considered the degree of contact exposure that pigeons are subjected to in races and shows, the high value of some pigeons, both monetary and sentimental, and that there were distinct serological differences between the chicken virus and the pigeon virus. It was decided that the pigeon isolate would provide the most effective vaccine possible.

The most common early symptom of PMV–1 is a watery diarrhea. Some of these birds become wasted or emaciated very rapidly. This is especially true of young birds still in the nest. The onset of disease varies greatly between lofts. In some cases many birds show nervous symptoms such as twisted necks, muscular spasms and leg and wing paralysis. However in most cases, the diarrhea may be seen, but the nervous symptoms are only seen in a few birds. Often these birds are killed and then it shows up in a few more. This can go on over a period of several weeks. In some cases over 50% show nervous symptoms before it runs its course. PMV–1 has a long incubation period—from 2 to 4 weeks. This is a characteristic of PMV–1 that makes it very difficult to control. A well intentioned owner may enter his perfectly normal appearing pigeons in a race, or a show or sell some. Although they may appear perfectly normal they could have been exposed and infected with PMV–1.

In avian medicine, certain diseases are controlled by security management. This is the prevention of any contact between flocks by people, by equipment and most important by movement of birds. However, pigeon fanciers do just the opposite. They bring together pigeons from many lofts for racing or for shows, which is ideal for disease transmission. Considering the long incubation period of PMV–1, normal appearing birds may be transmitting the disease. Many pigeons and lofts can become exposed from one infected loft during the racing season. The disease is often introduced by new pigeons being added to the loft. Pigeon fanciers are constantly buying and selling pigeons. This can transmit disease over very long distances and establish new foci of infection.

The incidence of PMV–1 in the USA is difficult to determine. Most cases are not taken to a diagnostic lab or to a veterinarian, so most cases are never reported. PMV–1 was first diagnosed in the New York City area in early 1984. During the 1984 young bird racing season it spread to CT, NJ, PA, and MD. Our inactivated PMV–1 vaccine was licensed in February 1985. Vaccination clinics were organized by the National Avian Disease Task Force for Pigeons and Doves and thousands of birds were vaccinated. However, most pigeon fanciers have resisted vaccination until the disease is present in their loft or in the immediate area, and then there is a rush to vaccinate. In 1986 and 1987 outbreaks were sporadic. We can generally determine the incidence of the disease by vaccine sales. The old bird racing season ends in June and sales peak in June and July. The young bird racing season ends in November and sales peak in November and December. In 1988 PMV–1 infection was seen in many parts of the U.S. during the old bird racing season. It continued to spread throughout the whole country during the young bird season. Sales of vaccine have been more than double over the 1987 sales. Unfortunately much of this vaccine has been used in emergency situations. Hopefully, after the bad experiences of this year, more flyers will vaccinate routinely.

In the beginning we recommended vaccination at 4 to 6 weeks of age and revaccination 4 to 6 weeks later. We now recommend that young birds be vaccinated one time about 4 weeks before potential exposure from racing (or training) or from showing. It is more convenient to do all young birds at one time. The second vaccination should be given about 3 or 4 weeks before the breeding season. At that time, all old birds should also receive an annual booster vaccination. If breeders have unvaccinated birds of any age and knows that PMV–1 outbreaks have occurred in the area, they should immediately vaccinate all unvaccinated birds and revaccinate 4 to 6 weeks later.

I have tried to follow the PMV–1 situation since we introduced the vaccine. To my knowledge, all outbreaks have occurred in unvaccinated birds. Many breaks of PMV–1 have occurred in the young unvaccinated birds but the old previously vaccinated birds in the loft have remained perfectly healthy. Unfortunately too many pigeon fanciers fail to understand or accept the need to vaccinate and wait until the disease is present in their loft or in the immediate area before they decide to vaccinate. Also, many breeders fail to continue on a vaccination program from one year to the next. The old birds are immune but the young birds are susceptible. As long as this is true, PMV–1 is not going to be controlled. It is necessary for the pigeon fanciers to set up a routine vaccination program and stick to it.

TETRACYCLINE DELIVERY: A REVIEW

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Introduction

Among the antibacterials employed in poultry medicine, the tetracycline class of antibiotics represents an ideal retrospective model of drug delivery systems as they have evolved with modernization of the industry that we have come to know today. Categorically, this drug family has enjoyed the longest track record of broad application dating back to the infancy of commercial bird production. Study of the three major dosage routes employed, injection, water, and feed, suggests an optimum rationale for therapeutic effect whether it be for utility or reformulation of existing approvals, or development of entirely new compounds for future application.

Discussion

Certain criteria must be met in order to best evaluate the usefulness of each chemo-therapeutic route and to successfully treat infectious bacterial diseases in poultry flocks. If a specific diagnosis, anticipated pathogenesis, and drug susceptibility of the suspect organism is known, and a favorable toxicological profile of the chosen drug exists, consideration is then directed toward economical dosage compatible with accepted husbandry practices, bioavailability of the active drug in its dosage form, and immunologic and physiologic aspects of the affected host.

Upon identification of tetracycline as the class of choice, selection must then be made from class members approved for avian medicine. Tetracycline, chlortetracycline, and oxytetracycline are available in water soluble; feedgrade and water soluble; and injection, feedgrade, and water soluble forms, respectively. These distinctions in themselves imply individual differences of use flexibility between the compounds and a "best-fit" approach to treatment of a specific disease entity. For example, only one formulation is licensed for the injectable route: oxytetracycline, up to a maximum of 25 mg activity per pound of body weight. All three tetracycline versions are applicable through water medication with nearly identical disease claim labelling.

Historically, mass medication via the needle has fallen into disfavor because of the obvious problem of increasing labor intensity with the upscaling of live production volume experienced particularly in the United States. Nevertheless, injection continues to be indicated among outbreaks of valued genetic stock, especially when the birds are being handled individually as a routine (e.g., AI in turkey breeders). Interestingly, a second potential niche for injection is in ovo where the individual (the embryo, in this case) is restrained within the confines of the eggshell and extra-embryonic membranes during the time of en masse transfer to the hatcher machines. Automated technology designed to realize this early infusion opportunity is currently nearing commercialization.[1]

Coincident with the trend away from the vial and syringe has been a refrain, in recent years, from controversial low-level feed-grade products in favor of less scrutinized treatment-level water supplementation. Recognizing this mounting popularity and the need to bring water usage in line with desired injectable dosage rates, regulatory guidance has taken the form of NAS/NRC (National Academy of Sciences, National Research Council) proposals to standardize labelling among all commercial water solubles (tetracycline: 25 mg/lb, all claims; oxytetracycline, and chlortetracycline: 25 mg/lb for organisms secondary to bluecomb enteritis, 400–800 mg/gal for other claims). These guidelines have not completely taken effect to date but are expected to be implemented in the near term.

Feed medication continues to be applied in great volume, though greatly reduced from years past. Clearances range from growth promotion programs to high-level disease claims (oxytetracycline: 5–500 g/ton; chlortetracycline: 10–500 g/ton).[2] In step with water medicants, feed preferences have shifted from low-level continuous towards aggressive treatment-level therapy, many times in adjunct to water treatment.

With a host of delivery options at one's disposal, selection of a dosage form then requires a knowledge of physicochemical, pharmacological, toxicological, dosing, and environmental variables loosely referred to as bioavailability. Ultimately, however, the goal of antibacterial therapy is attainment of adequate tissue levels to either kill a potential pathogen or at the least control its growth. Again, this can be expressed variously as area under the curve, related to minimum

inhibitory concentrations (MICs), etc., or in the broad sense as drug exposure. To measure drug exposure the traditional and most readily accessible tissue for analysis and the one generally recognized to be in equilibrium with other tissues is serum.

McMillan et al.[3] described serum profiles of commercial turkeys following intravenous injection or oral lavage of equivalent doses of tetracycline hydrochloride. Though on a much lesser scale than the injectable route, oral dosage showed appreciable peak activity within a comparable time frame of 2 hours and apparent half-life of 2.44 hours. More importantly, this comparison confirmed water as a "next best" dosing approach pharmacologically, reflecting the NAS/NRC basis for milligrams per pound accuracy conventionally associated with injectables.

Water is used routinely to monitor flock health status and is by far the most dynamic drug medium in that one can start, stop, or change quickly. Usually consumed better than dry feed by diseased birds, water treatment may be most appropriate for birds not seriously ill. As opposed to injectables, however, relative intake must be reckoned with in attempts to dose according to weight of bird. Daily water consumption falls predictably within a normal ambient temperature range (Figure 1) for chickens and turkeys as the bird ages. This, in turn, necessitates higher and higher antibiotic concentrations at the drinker and stock solution levels to maintain the same relative dose over the course of the flock growout cycle. Fortunately, dosing charts and slide rules analogous to a calibrated syringe can aid in calculating the amounts of water and powder required to assure accurate dispensation.

Compared to water solubles, feed medication is more economical, especially for prolonged therapy as less labor is demanded and it may better match a heterogenic health status within the flock. Pharmacologically, feed mimics a slow-release system resulting in less fluctuation *in vivo*. Of particular advantage is the storage of medicated feed in the crop from which there is a delivery of drug. Significant dose-responsive serum levels have been observed in commercial turkeys by Kula.[4] However, being even more restricted by intake relative to bird size than water, inclusion rates in feed would have to be greatly enhanced (600–2000 grams per ton) well beyond currently approved maximums in order to match the allowances through water (Figure 2). During the course of disease outbreak, inappetence of moribund birds exacerbates this inadequacy.

Having stated the case for bioavailability and drug exposure, success of treatment, nonetheless, cannot be automatically equated to these two key factors alone. As has been oft-stated, flock treatments are mostly too short and/or in too low of dose to be of any significant therapeutic effect. More germane to the issue is the question of timing in terms of both intervention and duration of therapy. A simplistic scheme showing the lateral transmission within a diseased flock illustrates the point of interface between a legitimate dose and an immuno-competent host (Figure 3). During a period of high morbidity and mounting mortality the flock can be roughly divided into three subpopulations: 1) unexposed, uninfected; 2) exposed, subclinical; 3) infected, clinical. With time progression, individuals within each group are being pulled into either the direction of mortality or survival as members shift dynamically between groups. Similarly, upon intervention with chemotherapy, probability of treatment success is subjected to the same factors in the individual bird. Successful abatement of disease syndromes ultimately rests with the immunological and physiological (water/feed consumption) integrity of the patient regardless of drug type or class employed. If the treatment is delayed or abbreviated, serious consequences in terms of bird loss or performance can be expected.

In summary, tetracycline delivery has emerged as a comprehensive strategy which should take into consideration the ideal formulation, dosage, and route of delivery. At the same time, drug activity should be optimized by recognizing host-pathogen interactions and by accordingly initiating and scheduling treatment.

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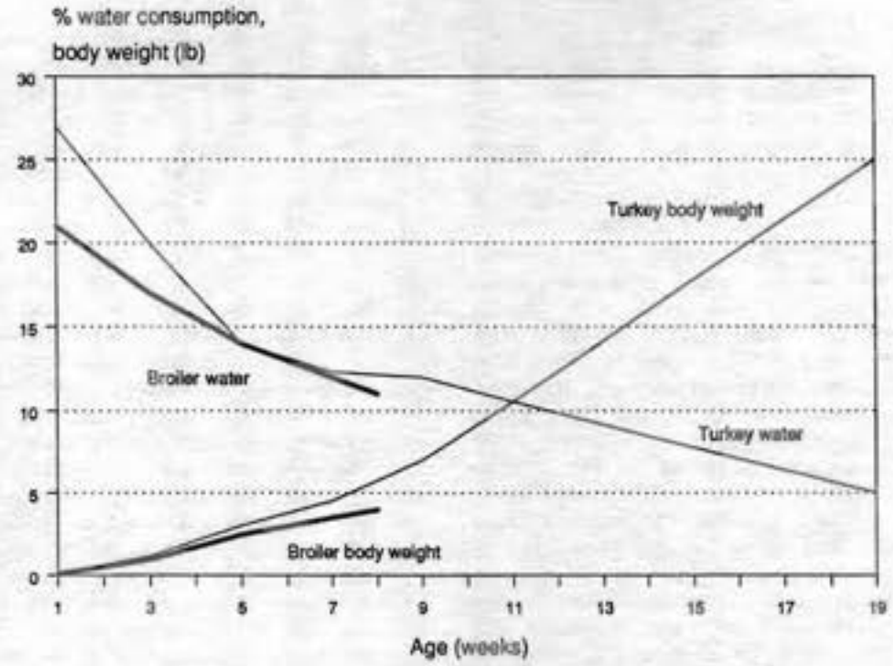

Figure 1: Water consumption (% body weight basis) and body weight of broilers and turkeys.

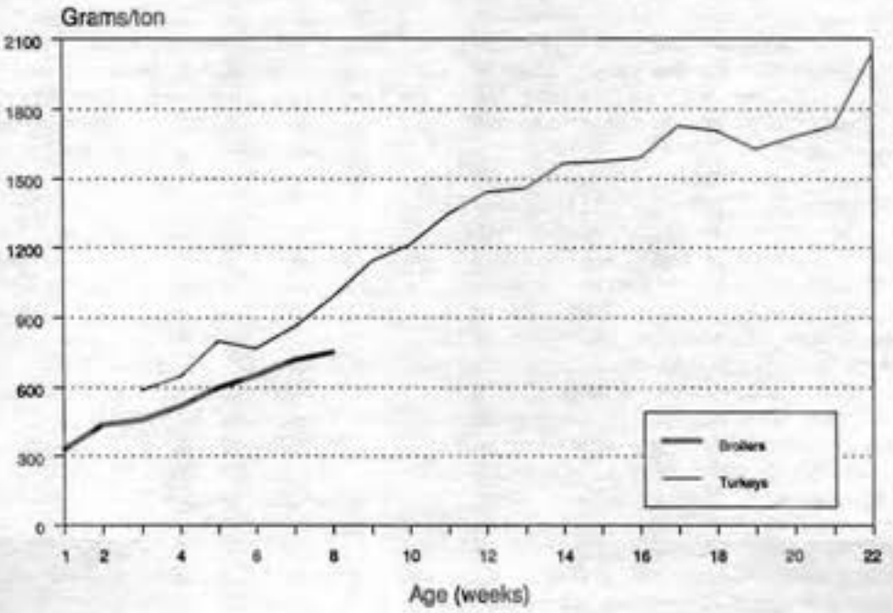

Figure 2: Grams of drug per ton of feed required to medicate broilers and turkeys at 25 mg/lb body weight.

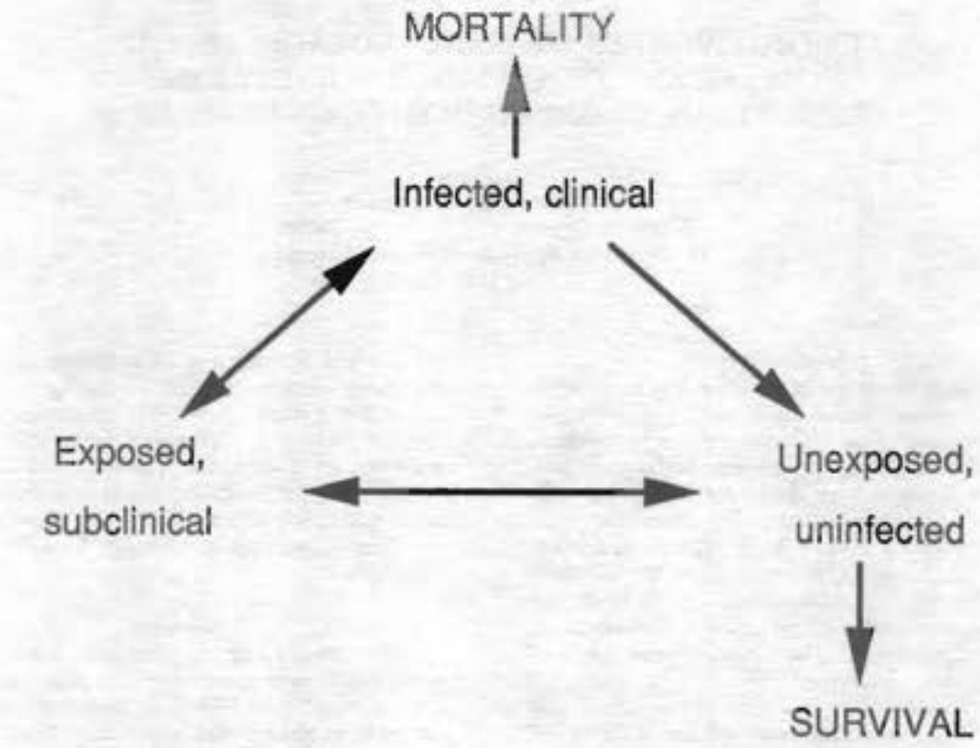

Figure 3: Disease dynamics in a poultry flock.

EFFECTIVENESS OF ENROFLOXACIN IN THE TREATMENT OF DIFFERENT BACTERIAL INFECTIONS IN BREEDER STOCKS IN MEXICO

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Introduction

Infectious diseases in breeder stocks produce severe economic losses as a result of increased mortality, drop in egg production, contamination, and low hatchability. Different pathogenic bacteria are prevalent in these problems: *E. coli, Mucoplasma gallisepticum, Pseudomonas spp., Klebsiella spp., Proteus spp., S. aureus,* and other genera.[1,7] Currently, the treatment of infections caused by most of these bacteria is difficult due to the development of multi-resistance to antimicrobial drugs. That is why these kinds of infections are increasing.[2,3,5]

Enrofloxacin (Enr) is a new chemotherapeutic agent, from the quinolonic group, with broad spectrum activity against Gram positive, Gram negative, and mycoplasma organisms.[1,2,7] It has been proved against different avian pathogens *in vitro* with excellent results. With therapeutical dosages there are no adverse effects on egg production, even in fertile eggs. This makes Enr a very good choice in the treatment of avian bacterial and mycoplasmal diseases.[2,4,5,6]

The purpose of this paper is to report the efficacy of Enr in field trials against *E. coli* and *E. coli*/*Proteus mirabilis* in Mexico.

Material and Methods

Animal

A 60% drop in egg production was observed in 5000 heavy breeders in floor pens. Previous egg production had been 92%. Water intake rose and scours were present which increased the dampness of the floor. The floor was removed. The problems of egg contamination with *E. coli* and *P. mirabilis* increased to 63% and hatchability dropped to 45%.

Tetracycline was given in the feed to the birds for 7 days, but the problem still remained. *Heterakis gallinae* was detected and a treatment with levamizol was carried out; but the scours remained uncontrolled. Birds were sent for bacteriological examination and *E. coli* was isolated from trachea, liver, kidneys, spleen, and ovaries of different birds. The antimicrobial susceptibility test showed a multi-resistant pattern and Enr was administered at 10 mg/Kg b.w. in the water for 3 days. After this treatment Olaquindox (Bayo-n-ox, Bayer) was given in the feed (100 ppm/Tbn for 7 days, 50 ppm/Tbn for 7 days, and 25 ppm/Tbn for another 7 days) to prevent a new outbreak. Bacteriological results from egg and embryos showed multi-resistant *E. coli* and/or *P. mirabilis.*

Results

After concluding the treatment with Enr, clinical signs were absent and a rise in egg production from 60% to 95% was observed. Egg contamination diminished drastically from 63% to 0.3% and hatchability raised from 45% to 85%.

Discussion

The results obtained agree with those reported by different authors who described excellent results in the treatment of infections caused by the most important avian pathogens.[1,2,4,7,8]

Enr could be a very good choice in the treatment of infections due to multi-resistant bacteria.

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EFFECTIVENESS OF ENROFLOXACIN AGAINST H. PARAGALLINARUM INFECTION UNDER CONTROL AND FIELD TRIALS IN MEXICO

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Introduction

Infectious coryza is a prevalent disease in broilers and laying hens in Mexico. The economic impact is very important due to high morbidity, production drop, mortality, vaccination and treatments. Currently, treatments are not so effective against the disease, maybe because there are some opportunistic bacteria which have developed multi-resistance against chemotherapeutic drugs being used.

Enrofloxacin (Enr) is a new chemotherapeutic agent from the quinolone group with a broad spectrum activity against Gram +, Gram –, and mycoplasma organisms. It has been tested in different animal species for the treatment of respiratory diseases caused by *Haemophilus spp.*

The purpose of this paper is to report on the effectiveness of Enr against *H. paragallinarum* under controlled and field trials in Mexico.

Material and Methods

Exp. 1. Dr. Hinz, Hannover

Animals. 216 Lohman Selected Leghorn pullets of 13 weeks of age were infected with a selected strain of 1–2 x 10^7 *H. paragallinarum* and treated with Enr at different dosages (15, 25, 50, and 100 ppm) in the water over 3–5 days (Table 1). Treatment started on days 2 and 3 post infection. Clinical signs were recorded up to 10 days post-inoculation. Pathomorphology and results of bacterial re-isolation were recorded 36h after the termination of treatment.

Exp. 2.

13,500 Hy-sex laying hens of 66 weeks of age located in a pen were affected by an outbreak of infectious coryza. Morbidity was approximately 30% and the mortality rate was 1%. Egg drop was reduced from 61.68% to 59.88%. Treatment with oxytetracycline was administered, but the problem continued. At week 67 Enr was given in the water (10 mg/Kg b.w.) during 3 days. Clinical signs and production was measured, comparing this with parameters of another flock, where there was no problem.

Results

Exp. 1.

100, 50, 25, and 15 ppm were highly effective against *H. paragallinarum* infections as demonstrated by the clinical signs. There was no exudate in the nostrils 2–3 days after the initiation of therapy (Table 2).

The re-isolation of *H. paragallinarum* was negative in all treated groups 36h after the end of treatment (Table 3). The pathological findings were in accordance with the clinical and bacteriological results.

Control groups were clinically affected and in all cases *H. paragallinarum* was recovered.

Exp. 2

At 48h from the beginning of treatment egg production increased to 62.98% and in week 68 rised to 64.91%. Clinical signs diminished and mortality rate remained at normal. Laboratory findings showed positive serology to *H. paragallinarum*, *P. haemolytica*, and *M. gallisepticum*.

Discussion

In both experiments the results were very good in controlling infection of *H. paragallinarum*. Enr could be a good choice in the treatment on infectious coryza.

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TABLE 1
Experimental design

Trial	Treatment with Enr (water)	
	experimental group	control group
1 27 birds/group light: 11 h/day p.i.)	100 ppm, 5 days (starting day 3)	–
2 27 bird/group light: 8 h/day	15 ppm, 5 days (starting day p.i.)	–
3 27 birds/group light: 8 h/day	25 ppm, 5 days (starting day 2 p.i.)	–
4 experimental group: 40 birds, control group: 14 birds; light: 8 h/day	50 ppm, 3 days (starting day 2 p.i.)	–

TABLE 2
Number of birds being clinically affected

Trial	Group	Birds/group	Days post inoculation 1	2	3	4	5	6	7	8
1	untreated control	27	9	15	22	22	18	18	16	16
	Enr 100 ppm	27	7	21	18*	9*	0*	0*	0*	0
2	untreated control	27	27	27	27	27	24	21	17	16
	Enr 50 ppm	27	26	26	26*	23*	19*	13*	6*	2
3	untreated control	27	27	27	25	25	24	20	17	
	Enr 25 ppm	27	27	26*	17*	15*	10*	6*	3	0
4	untreated control	14	14	14	14	10	7			
	Enr 50 ppm	20	20	13*	6*	5*	3	0	0	0
		20	20	15*	5*	4*	3	1	1	0

TABLE 3
Reisolation of *H. paragallinarum* and pathological findings (% of birds) 36 hrs. after end of treatment

Parameter		Average of 4 control groups		Treatment Enr A	B	C	D
Reisolation H. paragallinarum		89	(78–100)	0	0	0	0
reddish	nasal cavity	90	(81–100)	7	11	19	25
mucous	infraorbital	94	(79–100)	26	52	26	35
membrane	trachea	16	(0– 26)	0	4	4	0
exudate	serous	69	(57– 93)	0	19	7	5
into infra-	serous/	38	(21– 74)	0	4	0	0
orbital	fibrinous						
sinus	fibrinous	38	(0– 7)	0	0	4	0

A: 100 ppm, 5 days
B: 15 ppm, 5 days
C: 25 ppm, 5 days
D: 50 ppm, 3 days

COMPUTERIZED DRUG FORMULARY FOR POULTRY

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The computerized drug formulary was undertaken on the encouragement and advice of several poultry veterinarians and veterinary students who found it difficult and time consuming to assimilate the pharmaceutical data available for poultry.

This information was compiled from packaged labels and technical literature provided by the drug manufacturers. The data included avian species, drugs, dosage indications, method of application, required withdrawal time, and any warnings that are placed on the product.

Having this information on a database will enable one to retrieve the specific information requested. An example of this would be: You are treating a flock of broilers for Chronic Respiratory Disease (CRD) and wish to medicate the flock via drinking water. On the computer screen you will find those antibiotics labeled for CRD in broilers that can be administered through water. In addition, the information on dosages, withdrawal time, and warnings will also appear on the screen (Table 1). This information was originally placed on a data-base using a MacIntosh works software. This data has since been off-loaded to a Lotus 1-2-3 for IBM compatibility (Table 2).

The Lotus is somewhat limited in its data retrieval capabilities but by using the sort command you will be able to alphabetize a primary column (Avian species) and a secondary column (Indication) to crudely retrieve the information (Table 3).

A cost formulation spreadsheet has been developed that enables the user to compare the cost of several comparable products and methods of application. To use the spreadsheet, one finds the desired product in question and inputs the cost, dose, number to be treated, and if applicable body weight, water consumption, quantity of feed, etc.

The spreadsheet then calculates the total cost and cost per bird for the number of birds in question. It will also calculate the number of gallons of water and packages of product to be used. At the end of the spreadsheet there are several water and feed consumption charts.

Also included is a formulary text for those products that are mixed for water applications (see Table 4 for example). The text includes what products are available, the package size, and amount of a active ingredient. Dosage and mixing direction are included as well as any caution or warning concerning the product.

The purpose of this formulary is to provide quick access to fundamental information, such as indication of use, dosage level, method of application, and withdrawal time on each of the medications listed. The cost formulation and written text will provide additional information needed to medicate all types of poultry whether commercial or backyard. It is hoped that products used for medicating poultry will be used more efficiently and with less confusion.

TABLE 1
Products Available to Treat CRD in the Water
(Microsoft Works software)

Drug	Use Level	Indication	METH + W/DRAWL	WARNINGS
Chlortetraclcline	400–800mg/gal	CRD [MG, E. coli]	water	
Erythromycin	0.5gm/gal for 5 days	CRD (c) MG	water / 1 day*	
Erythromycin	0.5gm/gal for 5 days	CRD (c) MG	water / 1 day*	
Lincomycin–Spectomyc	2gms/gal 1st 5–7d age	CRD [MG]	water	
Lincomycin–Spectomyc	2gms/gal 1st 5–7d age	CRD [*E. coli*]	water	
Lincomycin–Spectomyc	2gms/gal 1st 5–7d age	CRD [MS]	water / none	
Oxytetracycline	100–200 mg/gal	CRD [p]	water	
Oxytetracycline	200–400 mg/gal	CRD [t]	water	oxytet/exclude eggs for food
Oxytetracycline	400–800 mg/gal*	CRD [t]	water / 5 days	
Spectinomycin	2gms/gal	CRD [MG]*	water / 5 days	
Spectinomycin	1gm/gal	CRD [MS]*	water / 4 days	
Tetracycline	25mg/lb bw/day	CRD [MG, *E. coli*]	water / 1 day	
Tylosin	50mg/lb bw 3–5 days	CRD (c) MG	water / 1 day	
Tylosin	50mg/lb bw 3–5 days	CRD (c) MG, stress	water / 1 day	
Tylosin	2000 mg/gal 3–5 days	CRD (c) MG	water / 1 day	
Tylosin	2000 mg/gal 3–5 days	CRD (c) MG, stress	water / 1 day	

*Refers to additional information in the Formulary Text File under the Product Name
[c] = Control
[p] = Preventive
[t] = Treatment

TABLE 2
Drug Formulary Data in Lotus 1-2-3 Format

Animals	Drug	Use Level
Broilers	Amprolium (#1)	113.5–227 mg/ton (continuous)
Broilers	Amprolium	72.64–113.5 mg/ton
Layers/rplm	Amprolium	113.5–227 mg/ton (continuous)
Layers/rplm	Amprolium	113.5 mg/ton
Layers/rplm	Amprolium	72.64–113.5 mg/ton
Layers/rplm	Amprolium	36.32–113.5 mg/ton
Layers/rplm	Amprolium	72.64–113.5 mg/ton

Indication	Meth + W/Drawl	Warnings
cocci [p]	feed / none	
cocci [p] E. tenella only	feed / none	
cocci [p]	feed / none	
cocci [s] up to 5 wks age	feed / none	
cocci [s] 5–8 wks age	feed / none	
cocci [s] >8 wks age	feed / none	
cocci [m] up to 5 wks age	feed / none	

[p] = Preventive
[s] = Severe
[m] = Mild

TABLE 3
Drug Formulary Data Organized by the Sort Command in Lotus 1-2-3

Animals	Drug	Use Level
Broilers	Novobiocin	200 gms/ton 5–7 da.
Broilers	Novobiocin	6–7 mg/lb BW 5–7 da.
Broilers	Sulfaquinoxaline*	15613.0 mg/gal
Broilers	Furazolidone	50 gms/ton (fed cont)
Broilers	Nitrofurazone	300 mg/gal
Broilers	Sulfaquinoxaline*	227 mg/lb
Broilers	Lasalocid Na	68–113 gms/ton (fed cont)

Indication	Meth + W/Drawl	Warnings
breast blister [staph]	feed / 4 da.	not for layers
breast blister [staph]	feed / 4 da.	not for layers
cocci [c]†	water / 10 da.	eggs not edible
cocci [c]	feed / 5 da.	not for layers >14 wks
cocci [c]	water / 5 da.	not for layers >14 wks
cocci [c]	feed / 10 da.	eggs not edible
cocci [c]	feed / none	

*Refers to additional information in the Formulary Text File under SULFAQUINOXALINE
†[c] = Control

TABLE 4
Example of Drug Formulary Test for Water Application

BACITRACIN METHYLENE DISALICYLATE

Water Application

AL Labs Solu-Tracin 50; 3.2 oz pack containing 10 gms activity of bacitracin
AL Labs Solu-stracin 200; 4.1 oz pack containing 51.2 gms activity of bacitracin

Mixing (Label)

One 4.1 oz. pack of Solu-Tracin 200 will medicate 50 gallons of water at 1,000 mg/gallon

Proportioner:

One 4.1 oz pk (51.2gms) added to 2 gallons water

	Proportioner settings	
Treatment dose	1 oz/gal	2 oz/gal
100 mg/gal	1/2 pk	1/4 pk
200 mg/gal	1 pk	1/2 pk
400 mg/gal	2 pks	1 pk

BOTTOM LINE MIXING

1) Compute water uptake or tank size
   a) # of birds/house
   b) House temp °F
   c) age (weeks) of the birds
2) Dosage of antibiotic in mg/gallon
3) Amount of antibiotic in package, i.e., mgs/pk

(Dose) x (water uptake or tank size) / (amount of BMD in pk [mgs]) = pks/house or tank

Example:

6 week old broiler in a house of 27,000 birds with an average daily temperature of 70°F in the house with enteritis

* 6 wk broilers in a house of 70° will drink – 49 gallons/1000 broilers a day (49 gal/day x 27 thousand = 1323 gallons/day)

[200 mg/gal] x [1323 gallons/day] / 51,200 mg/pk = 5.2. pks/house
[200 mg/gal] x [100 gallon tank] / 10,000 mg/pk = 1 pk/100 gal tank
10 gms = 10000.0 mg 51.2 gms = 51,200 mg

Proportioner Mixing

If rate is one oz/gal then 1 gallon of stock solution will medicate 128 gallons of water. [Dose] x [gallons medicated] / [mg/pk]

[200 mg/gal] x [128 gallons] / [51,200 mg/pk] = 0.5 pks/gal of stock

Caution

Administer for 5–7 days or as long as clinical signs persists, then reduce to prevention levels.
About 200 mg/gallon is equivalent to 100 gms/ton of feed.

HEMORRHAGIC ENTERITIS VIRUS INFECTION OF TURKEY EMBRYOS

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Virulent hemorrhagic enteritis virus (HEV) causes intestinal hemorrhage and death in turkeys. An intact bursa and spleen are integral in the production of this disease syndrome, but the exact nature of the interaction is unknown.[2,3] Attempts to propagate HEV in the laboratory in chicken and turkey embryos and fibroblast cell cultures have been unsuccessful.[1] Infection of dissociated spleen lymphocytes from chickens, pheasants, and turkeys has been reported, although demonstration of the production of infectious virus by the cultures during serial passage was unsuccessful.[3] The virus has been propagated, however, in two B-cell lymphoblastoid lines, MDTC–RP16 and MDTC–RP19.[4]

The goal of this study was to re-examine the turkey embryo, in particular the spleen and bursa of the turkey embryo, as a possible system for HEV growth. Infection of the turkey embryo in the latter stages of embryonation with HEV was established by detecting HEV antigen in the spleen and bursa by the indirect fluorescent antibody test. The route of inoculation—allantoic cavity or intravenous—had a significant effect on the time of appearance of viral antigens in the tissues. Additionally, evidence of infection appeared first in the bursa and then in the spleen, regardless of the route of inoculation or age of the embryo at the time of inoculation. The production of infectious virus in the embryo was not definitively established.

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TURKEY HEMORRHAGIC ENTERITIS: A FIELD VACCINATION AND CHALLENGE STUDY ON A U.S.D.A. APPROVED LIVE COMMERCIAL VACCINE (CAMBRIVAX™ HE)

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Introduction

The efficacy and safety of Cambrivax™ HE was evaluated in two commercial turkey flocks. Cambrivax™ HE is a U.S.D.A. approved live Hemorrhagic Enteritis Vaccine for use in turkeys. The strain is an avirulent MDTC-RP-19 cell line adapted Domersmuth strain.

This study was designed to confirm the efficacy of the commercial vaccine to protect against high challenge doses of virulent Hemorrhagic Enteritis Virus (HEV) strain. The conventional virulent field strain and an atypical strain isolated from a field case were used to experimentally infect field vaccinated birds.

Materials and Methods

Birds

Two commercial tom turkey flocks in Minnesota totaling 10,000 birds each were used in this trial.

Immunization

Vaccination was given as per label recommendation, orally via 2–4 hour supply of drinking water buffered with non-fat dried milk.

Challenge Strains

Challenge strains used were the conventional virulent field strain (T–1) and an atypical virulent strain (T–2) isolated from a field case. The dose was 1 ml of 20% splenic suspension administered gavage.

Sample Collection

Bleed — Prior to vaccination
Bleed — 3 weeks post vaccination
Bleed — 5 weeks post vaccination

Organs — 7 days post challenge the birds were sacrificed and observed for gross, pathological lesions. Organs collected were the spleen, intestinal loop with pancreas, and bursa of fabricius.

Serological Testing

Agar gel precipitation test (AGPT) was used to detect specific antibodies in the serum samples.

Experimental Design

Prior to vaccination, 50 birds were removed and kept isolated as the unvaccinated controls. Three weeks post vaccination another group of 50 birds (vaccinated) were pulled out from the farm. These birds (total of 100) were then grouped as follows:

A. T–1 strain group.
   1. 25 unvaccinates
   2. 25 vaccinates

B. T–2 strain group.
   1. 25 unvaccinates
   2. 25 vaccinates

These birds were housed separately in isolation and given the challenge material. At seven days post challenge the birds were sacrificed, necropsied and observed for gross pathological lesions. Spleens, intestines with the pancreas and bursa of fabricius were collected. Protection from virulent challenge was assessed using parameters of mortality, seroconversion and spleen weights.

Effect of vaccinations (safety) will be based on flock livability and market weight.

Statistical Analysis

Significance of the findings were tested using chi square test (HSD State program).

Results

Serology

AGPT on serum samples collected prior to vaccination were all negative. However, 3 weeks post vaccination 8% were AGP positive. At six

weeks post vaccination 100% of 25 samples collected were AGP positive (Table 1).

Mortality

A. T–1 strain group.
56% of the unvaccinated controls died within 4 days post challenge. However, there was no mortality among the vaccinated birds.

B. T–2 strain group.
There was no mortality among the non vaccinated and vaccinated birds (Table 2).

Clinical Signs

A. T–1 strain group.
The non vaccinates were listless and excessive bloody droppings (bloody vent) were observed. None of this was observed from the vaccinates.

B. T–2 strain group.
There were no noticeable clinical signs observed among the vaccinates and the nonvaccinates (Table 2).

Splenomegaly

A. T–1 strain group.
The non vaccinates that died had a mean splenic weight of 4.3 grams while the control birds which survived the challenge had a mean splenic weight of 6.47. The vaccinates had 6.28 mean splenic weight.

B. T–2 strain group.
The non vaccinates had a mean splenic weight of 6.3 grams while the vaccinates had about 3.5 grams mean splenic weight (Table 2).

Intestinal Lesions

A. Hemorrhagic intestinal mucosa and lumen filled blood were observed upon necropsy of the 14 non vaccinates that died. The eleven that survived also showed hemorrhagic intestinal mucosa without blood in the intestinal lumen. All the vaccinates had normal intestines.

B. T–2 strain group.
100% of the non vaccinates had hemorrhagic intestinal lesions but none had blood in the lumen. All the vaccinates had normal intestines (Table 2).

Production Data

The birds at market (17 weeks) weighed an average of 25.1 pounds. Liveability was 93.6% (Table 3).

Discussion

The AGP test is known to have low sensitivity in detecting specific antibodies to HEV. 8% of the birds had a detectable titers at 3 weeks post vaccination. Titers were detected in 100% of the samples collected at 6 weeks post vaccination.

The vaccinates were protected from challenge with the conventional virulent field strain (see data on Table 2). There was a significant difference between the mortality among the non vaccinates (56%) compared to the vaccinates (0%) ($P<.001$).

Based on the intestinal hemorrhagic lesions and clinical signs, there was a statistically significant difference ($P<.001$) between the non vaccinates and the vaccinates. The latter had neither intestinal lesions nor clinical signs.

The absence of splenomegaly in the non vaccinates which died within 4 days post challenge may be due to the acuteness of the disease. Birds with severe intestinal lesions did not have enlarged spleens. The splenomegaly observed in the vaccinates resulted from the enormous challenge given to those birds.

Protection from challenge with the atypical virulent strain of HEV was evident in the absence of splenomegaly and intestinal hemorrhagic lesions. Splenomegaly was observed among the non vaccinates. The difference was statistically significant between these two groups ($P<.001$). Intestinal hemorrhagic lesions were observed in 100% of the non vaccinates and no lesions were seen among the vaccinates resulting again in a statistically significant difference between these two groups ($P<.001$).

The data on production showed no decline in the performance of these two turkey flocks when compared to previous flocks. In fact, percent liveability and average weight gain per day to market was better than previous flocks.

Conclusion

Results from this trial confirmed the safety of the U.S.D.A. licensed HEV Vaccine (Cambrivax™ HE) in the field. Moreover, Cambrivax™ HE was shown to have the ability to protect from conventional virulent field strain and from an atypical virulent strain isolated from a field case.

TABLE 1
AGP Results

	Conventional Virulent Strain		Atypical Virulent Strain	
	Vacc.	Non-Vacc.	Vacc.	Non-Vacc.
3 Weeks Post Vacc.	1/25	0/25	3/25	0/25

Serum samples prior to vaccination—100% AGP(–)
Serum samples at 6 weeks post vaccination—100% (AGP(+)

TABLE 2
Mortality, Clinical Signs, Spleen Weights, and Intestinal Hemorrhagic Lesions

Groups	Mortality	Clinical Signs	Mean Spleen Weight (grams)	Intestinal Hemorrhagic Lesion
A. Conventional Challenge Strain				
1. Vaccinates	0 (0/25)	None Observed	6.28	0/25
2. Non-Vaccinates	56% (14/25)	Listlessness, Bloody Droppings	4.3[a] 6.47[b]	25/25[c]
B. Atypical Virulent Strain				
1. Vaccinates	0 (0/25)	None Observed	3.5	0/25
2. Non-Vaccinates	0 (0/25)	None Observed	6.5	25/25

[a]Spleen weights of birds that died within 4 days after challenge
[b]Spleen weights of birds that survived challenge
[c]14 birds that died within 4 days post challenge had whole blood in the intestinal lumen; survivors had none

TABLE 3
Liveability and Market Weights

Flock #	Age At Market	Liveability	Market Weight
4–88[a]	17 Weeks	93.6	25.1
3–88	16 Weeks, 6 Days	91.7	23.4
2–88 (hens)	---	---	---
1–88	18 Weeks, 2 Days	92.23	26.2

[a]Flock vaccinated with HEV

EVALUATION OF A MODIFIED LIVE ORAL VACCINE FOR HEMORRHAGIC ENTERITIS VIRUS OF TURKEYS AGAINST MARBLE SPLEEN DISEASE OF PHEASANTS *(PHASIANUS COLCHICUS)*

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Introduction

Marble Spleen Disease (MSD) is an enzootic disease of pen-reared pheasants *(Phasianus colchicus)* with epidemic presentation during late summer and fall. This disease has been reported in Europe, the United States, and Australia.[8,10] The disease is caused by an avian adenovirus type II serologically related to the etiological agents of hemorrhagic enteritis (HE) in turkeys and avian adenoviral splenomegaly in chickens. Previously, MSD had been identified in a 60,000 bird pheasant flock in California[7] and identification of an adenovirus from MSD-affected birds in the flock had been made.[1,9]

Two vaccination regimens for MSD have been reported in pheasants; namely, a live oral vaccine[2,4,5] and an inactivated subcutaneous vaccine.[5,8] In the United States and Italy, oral administration of splenic homogenates of pheasants with MSD have been used to induce an immune response in pheasant flocks.[2,5]

The purpose of this study was to evaluate in pheasants under controlled (trial No. 1) and field conditions (trial No. 2), the safety and efficacy of a modified live oral HEV vaccine (licensed for turkeys).

Materials and Methods

Trial No. 1

Pheasants. A total of 81 Chinese ringnecked pheasants *(Phasianus colchicus)* of 6 weeks of age were confined in isolated cages and divided into three groups of birds, as follows: a) group I consisted of 13 birds which were not vaccinated or challenged; b) group II comprised 19 birds which were not vaccinated but challenged, and c) group III was composed of 39 birds which were vaccinated and challenged. Pheasants in group III were subdivided into three subgroups and each subgroup was vaccinated with a different dose of the vaccine. Twelve birds received a half dose (0.5 ml) of the vaccine, 14 birds received the recommended dose (1 ml.), and 14 pheasants received a double vaccine (2 ml) dose. Eleven pheasants were added to group III as unvaccinated contact birds.

Vaccine. A commercial modified live oral HEV vaccine to hemorrhagic enteritis in turkeys was used.

Challenge virus. A virulent strain of MSD virus with a titer of 10^6 P.I.D. (obtained from Dr. A. Fadly of the Regional Poultry Research Laboratory in East Lansing, Michigan) was administered orally via crop intubation at 5 weeks following vaccination.

Serology. Serum samples for the AGID test were taken at 1 week prior and 5 weeks post-vaccination and at 6 and 13 days after challenge.

Necropsy. Of the pheasants from each group, 1/2 were euthanized and necropsied at 6 days after challenge, and 1/2 at 13 days after challenge. Splenic weight and length was done on each spleen. Splenic tissue for histopathology and the AGID assay were also taken. Spleen from each bird was assayed for adenoviral antigens by agar gel immunodiffusion (AGID).

Trial No. 2

Pheasants. Female pheasants of the same flock as trial No. 1 were selected as follows: 125 young pheasants at 10 weeks of age and 125 adult pheasants at 22 weeks of age. Forty pheasants within each age group, identified by clipped feathers on the wings and the tail, served as contact controls.

Vaccine. Birds in the vaccinated group were dosed orally with 1 ml. of the same HEV vaccine used in trial No. 1 using an intraesophageal tube.

Serology. Twenty-five percent of the birds were bled prior to vaccination, at 3 weeks post-vaccination, and at 1 week post-challenge to measure antibody levels to MSD virus by the AGID test.

Challenge. All 250 pheasants (vaccinated and contact controls) were challenged at 3 weeks post-vaccination. Prior to challenge, the vaccinates with their contact birds were divided into

2 groups of which one group was relocated to an animal facility where birds were held in isolated cages. Birds remaining on the farm were orally challenged with 2 ml of a strain of MSD (MSD–F) virus which consisted of a pooled splenic homogenate from pheasants with MSD. The virulent MSD (MSD–V) challenge of 1 ml was administered orally to each bird housed in isolated cages. Both challenge preparations were positive for viral antigen by AGID and by electron microscopy (EM) for adenoviral particles. No other viral particles were seen by EM in either preparation.

Necropsy. All test birds were euthanized and necropsied at the 7th day post-challenge. A blood sample was also obtained from each bird. Individual spleens were measured and their lengths recorded. Splenic specimens for histopathology and AGID test were also taken.

Results

Trial No. 1

By the AGID test, all sera taken prior to vaccination were negative for antibodies to HEV antigen. Vaccination of pheasants resulted in 100% seroconversion in all inoculated groups except those pheasants which received 1/2 dose (Table 1). Birds of group II, except for one, were all negative at 6 days post-challenge but 100% seroconverted by day 13. All the sera obtained after challenge from pheasants in group III were positive by day 13. All pheasants in group I remained seronegative. However, horizontal transmission from vaccinated to unvaccinated contact birds was evident by serology (Table 1). Mortality ascribed to MSD was seen only in group III with 2 deaths in birds which received 1/2 dose of vaccine and 3 deaths in the contact birds. Splenic weights and lengths are presented in Table 2. In only group II, at 6 days post-challenge, was found statistically significantly different ($P = <0.05$) for splenic weight and enlargement. This enlargement regressed to the average of the other groups by 13 days post-challenge.

Trial No. 2

Prior to vaccination, antibodies against adenoviral antigens (HEV) by the AGID test were detected in sera obtained from 71% of the tested birds in the young pheasant group. Detectable antibodies were not found by the AGID test in the adult pheasant group (Table 3).

Splenic lesions of varying magnitude were seen in all groups at necropsy; however, no birds showed the marked spleen lesions observed in field cases of MSD and no statistically significant difference were seen in splenic lengths among the groups.

Mortality due to MSD was seen only in adult pheasants. From the 125 adult birds used for this trial, 10 deaths were recorded throughout the trial. Three vaccinates died within 9 days post-vaccination. Three adult contact controls died between 18 and 21 days post-vaccination. Two vaccinates and two contact controls died within a week of challenge with the MSD–V. Splenic homogenates were all negative for MSD antigen by AGID, except for a spleen from an adult vaccinated and challenged with MSD–V.

Discussion

The low mortality of pheasants post-vaccination during both trials suggests that the turkey vaccine is safe for use in Chinese ring necked pheasants under both controlled and field conditions. The data implies that the vaccine virus was horizontally transmitted from vaccinated to unvaccinated birds as determined by seroconversion, thus, probably protecting the contact birds from significant splenic lesions at 6 and 13 days after challenge with MSD–V. In trial No. 1, the vaccine was effective in eliciting an antibody response against HEV antigens by 5 weeks post-vaccination. On the 6th day post-challenge, seropositive birds were protected against splenomegaly while seronegative birds showed a statistically significant splenic enlargement that rapidly regressed and was absent by 13 days post-challenge.

Trial No. 2 was designed as a further evaluation of the safety and efficacy of the modified live oral HEV vaccine under field conditions using pheasants of different ages from a pheasant farm with enzootic MSD. Some of the young pheasants were seropositive to HEV antigens by AGID at the start of trial No. 2; however, this enabled an evaluation of the efficacy of the vaccine in the presence of maternal or host-induced antibody to MSD virus. The pheasants in trial no. 2 were vaccinated and maintained on the enzootic premises. Possibly due to the high number (71%) of young birds with prior antibody, a high variability in serological profiles after vaccination and after challenge was evident in this group. In contrast, low number of adult birds had immune responses (30% of tested birds) from the group that did not have detectable antibodies (40 pheasants) at the beginning of the trial.

After challenge in trial 2, adults showed a significant rise in the number of seropositive birds while seroconversion in young birds remained at post-vaccination levels. Splenic lesions

were mild to non-existent in both adult and young pheasants. The results of the second vaccination trial under field conditions were difficult to evaluate due to the numerous variables (i.e., virus exposure or previous antibody) present in maintaining a flock in the field. However, the HEV vaccine appears safe for use in pheasants based on the absence of severe splenic lesions; furthermore, based on the number of seroconversion of vaccinates, multiple doses of vaccine may be required under field conditions.

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TABLE 1
Seroconversion of pheasants in Trial No. 1 Following inoculation with a HEV vaccine and challenged with a virulent strain of MSD virus

Group	No. of birds	Dose (ml) v	Dose (ml) c	Seropositives / Total 5[a]	Seropositives / Total 6[b]	Seropositives / Total 13[b]	MSD deaths
I	13	0	0	0/13	0/7	0/6	0
II	19	0	+	0/19	1/9	10/10	0
III	12	+	+	8/10	4/5	5/5	2
	14	+	+	14/14	6/7	7/7	0
	14	+	+	14/14	7/7	7/7	0
(CC)	11	0	+	0/8	4/4	4/4	3

[a]Weeks Post-vaccination V = Vaccinated
[b]Days Post-challenge C = Challenged
(CC) = Contact Controls

TABLE 2
Splenic weight and length of pheasants in Trial 1 following challenge by virulent MSD virus

Group	No. of birds	V	C	Spleen Weight Av in gm +/– SD		Spleen Length Av in mm +/– SD	
I	7	0	0	0.49	0.09	13.14	1.24
II	9	0	+	1.58*	0.46	19.44*	2.36
III	5	+	+	0.36	0.10	12.75	1.78
	7	+	+	0.34	0.11	12.30	1.27
	7	+	+	0.43	0.19	12.14	2.35
(CC)	4	0	+	0.48	0.26	12.00	2.54

*P=<0.05, Statistically significant; V = Vaccinated; C = Challenged
SD = Standard Deviation. (CC) = Contact Controls

TABLE 3
Seroconversion by age as measured by the agid test against HEV antigen in pheasants inoculated with HEV vaccine and challenged with MSD–V and MSD–F viral strains

Group	Bleedings: Pre-Vaccination % Pos. birds	3 Wks. Post-Vaccination % Pos. birds	1Wk. Post-Challenge % Pos. birds
Young Vaccinated	66	83	67 (MSD–V)[a] 80 (MSD–F)[b]
Young Contact Control	80	61	60 (MSD–V) 40 (MSD–F)
Young Group (av)	71		
Adult Vaccinated	0	30	69 (MSD–V) 57 (MSD–F)
Adult Contact Control	0	30	73 (MSD–V) 43 (MSD–F)
Adult Group (av)	0		

[a]Marble Spleen Disease virulent-MSD–V virus strain used as a challenge
[b]Marble Spleen Disease field-MSD-F virus strain used as a challenge

EPIDEMIOLOGIC INVESTIGATION OF INTERFLOCK TRANSMISSION OF *P. MULTOCIDA* USING RESTRICTION ENDONUCLEASE ANALYSIS

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Fowl cholera (FC) caused by *Pasteurella multocida* has been one of the costliest diseases in the California turkey industry in recent years. In the past 3 years, an average of approximately 60 FC outbreaks per year have been reported to and investigated by the California Turkey Project (CTP), USDA/Veterinary Services (VS), and/or California Department of Food and Agriculture personnel. The purpose of these investigations was to better understand the epidemiology and economics of this disease and its control. In order to do this it is important to identify reservoirs of the organism and potential modes of transmission. To date, it has been speculated that wildlife is an important reservoir of *P. multocida* and interflock transmission occurs either within or among companies, spread by personnel, insects, equipment or vehicles.

Although wildlife must still be considered a reservoir of *P. multocida*, based on differing serotypes obtained from FC-outbreak turkeys and neighboring wildlife, they do not appear to be the only reservoir. The purpose of this study was to examine the possibility of interflock transmission of *P. multocida* either within and among companies.

Isolates of *P. multocida*, from all confirmed FC-outbreak flocks in California between 1985–86, have been serotyped by the CTP at Davis. Isolates collected from 38 of the 41 reported FC outbreaks in 5 companies over an 8-month period were examined by somatic serotyping. Twenty-eight of these isolates, representing 24 FC-outbreak flocks, were further examined using restriction endonuclease analysis (REA) of whole-cell DNA. The potential for interflock transmission (between a source and target flock) was based on the following criteria: putative target flocks must have had reported FC outbreak at least one week prior to marketing of the putative source flock, identical source/target isolate serotypes, and identical source/target isolate DNA type.

Based on somatic serotyping, 20 of the 33 possible within company FC flock transmission, had common serotypes. Of these 5 of 8 (possible transmissions) occurred in company A, 1 of 4 in company B, 8 of 11 in company C, 4 of 6 in company D, 3 of 3 in company E. Examination of the FC-outbreak dates showed that these potential flock transmissions frequently occurred shortly after the putative target flock was marketed. The majority of these isolates were 3,4 serotypes.

More definitive examination of 28 of the isolates from 4 companies, representing 25 FC outbreaks was performed using REA analysis. Eight distinct REA types were reported, with the majority (18/28) being REA type 1.

Based on common serotypes and REA types, it appeared that within company transmission may have occurred 8 times and between company transmission 4 times. Several of the purported (based on serotyping information) within company transmissions were dispelled based on REA results. However, there appears to be sufficient evidence to conclude that 4 interflock transmissions did occur, 2 within and 2 between companies.

REA will eventually be performed on all serotyped isolates reported in this study. In addition, the potential for between company transmission will be more closely examined using company records.

A CASE REPORT: SEROTYPE 14 *PASTEURELLA MULTOCIDA* OUTBREAKS IN BREEDER TURKEYS

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In October of 1987 an outbreak of *Pasteurella multocida* (fowl cholera), serotype 14 occurred on a multiple age turkey growing/breeding complex. The initial outbreak appeared simultaneously in two breeder candidate flocks of turkeys at 26 and 27 weeks of age in environmental dark houses.

The affected flocks had been immunized with commercial live M9 *P. multocida* vaccine manufactured by American Scientific Laboratories (ASL) at 5, 10, and 16 weeks of age and received a commercial oil emulsion fowl cholera bacterin (Poultry Health Laboratory, California) at 20 weeks of age. The outbreaks occurred three days prior to fowl cholera booster injection with oil emulsion bacterin.

The clinical signs were of severe depression with a sudden increased mortality (Table 1). Pathological signs were of severe muscle congestion, severe unilateral and in many cases bilateral consolidation of lungs with abundant amount of yellowish fibrinopurulent exudate smothered the lung tissues. Enlarged and firm liver, mottled spleen and moderate degree of airsacculitis were also observed. Egg yolk peritonitis was a common finding in affected breeders.

During the early stages of the outbreak the immunization of other turkey flocks present on the same premises was intensified against fowl cholera using commercial live and killed fowl cholera immunizing agents. The dosage of the live M9 vaccine (3 x 4) was doubled and the breeders received additional administration of commercial (type 1, 3, & 4) aluminum hydroxide product with the thought that the serotype(s) causing the present outbreaks was one of the commonly occurring serotypes (types 1, 3, 4, or 3 x 4) in turkeys.

Pasteurella multocida isolates that had been recovered from the initial outbreaks were identified to be serotype 14 by the California Veterinary Diagnostic Laboratory System, U.C., Fresno Division[1] and were confirmed and were additionally identified capsular sero-group A by the National Animal Disease Center Laboratory, Ames, Iowa.[2]

Killed autogenous oil emulsion and aluminum hydroxide bacterins were produced by the Poultry Health Laboratory (PHL), Davis, California and the Maine Biologic Laboratories (MBL) for use. By the time the above information and the immunizing biologics became available to us the disease had become chronic and it was spread to other turkey flocks at various ages through contact and artificial insemination inflicting heavy mortality and production losses during acute and chronic stages of the disease (Table 2).

Eventually with the aid of repeated immunization using autogenous oil emulsion and aluminum hydroxide killed bacterins and the use of pure oxytetracycline (LA–200, Pfizer); spectinomycin (Spectam, CEVA); ceftiofur sodium (Naxcel, Upjohn); and sulfadimethoxine (Agribon, Roche) the outbreaks were brought under control.

The initial source of the infection was suspected to be dead bovine species disposed of at the same tallow factory where daily dead turkey mortality from this complex were also being disposed. The outbreak occurred one month after the farm personnel began using this particular tallow factory as a disposal site. Cross traffic was suspected in the extension of the initial infection to this complex.

Attempts[2] to reproduce the disease by intramuscular introduction of three strains of serotype 14 that had been recovered from these outbreaks into two weeks old turkey poults resulted in death at 7, 13, and 20%. Higher degree of mortalities at the field level were partly due to age and stress factors.

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TABLE 1
Mortality in turkey breeder hen flocks due to *P. Multocida* serotype 14 at the initial outbreak

Flk#	Pre-Outbreak(%)	Outbreak Age (Wk)	During Outbreak(%)	Post Outbreak To Market(%)	Total Mort.
017	4.8	27	17.3	4.4	36.5
019	3.4	26	31.2	17.5	52.1

TABLE 2
Mortality in turkey breeders due to *P. Multocida* Type 14

Flk #[1]	Pre-Outbreak(%)	Outbreak Age (Wk)	During Outbreak(%)	Post Outbreak To Market(%)	Total Mort.
A-1	7.0	17	40.8	5.5	53.3
B-2	4.8	27	17.5	4.5	26.8
C-2	3.4	26	34.6	17.5	55.5
D-2	3.8	22	14.3	8.4	26.5
E-2	9.9	48	73.3	0.0	83.2
F-2	2.3	7	25.4	31.8	59.2
G-2	23.7	46	34.9	1.9	60.5

[1] Numerical "1" stands for males; Numerical "2" stands for females; A through E are female line strains; F & G are male line strains

A COMPARISON OF FOWL CHOLERA VACCINATION PROGRAMS FOR TURKEY BREEDERS

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It has been more than 100 years since Pasteur developed the first fowl cholera vaccine. Since then, there have been many attempts at producing both live and inactivated fowl cholera vaccines. Inactivated vaccines or bacterins have the advantage of inducing an immune response without causing any vaccine reaction. However, bacterins will induce immunity only to the serotypes included in that vaccine.[3] On the other hand, avirulent live vaccines, such as CU or M-nine provide protection to multiple serotypes.[1] The major disadvantage to live vaccines is the risk of causing morbidity or mortality if the birds are stressed at the time of vaccination or the vaccine is improperly administered.

In a recent survey of *Pasteurella multocida* isolates it was found that 68% of 150 isolates submitted to the University of Georgia were of serotype 3 cross 4. In the past year 67% of the *P. multocida* isolated at the Cuddy Farms Inc. diagnostic lab have been serotyped 3 cross 4. The reason for this experiment was based on the fact that the major serotype of the field challenge *P. multocida* causing fowl cholera in the Cuddy Farms breeders was a 3 cross 4 and all previous challenge work for determining vaccine efficacy was done using single serotypes (i.e., type 1 or 3 or 4).

Seventeen week old Nicholas hens that had been vaccinated in the drinking water at 4, 8, and 12 weeks of age with live PM#1[4] were then given the following treatment:

Treatment 1	Serotype 3 cross 4 oil emulsion bacterin isolated from a Cuddy Farms Breeder flock.
Treatment 2	Serotype 3 cross 4 oil bacterin isolated from a second Cuddy Farms Breeder flock.
Treatment 3	Pabac (Salsbury Laboratory) containing individual serotypes 1, 3, and 4 in an oil emulsion.
Treatment 4	M-nine (ASL) given as a live vaccine in the wing web.
Treatment 5	Nonvaccinated controls.

All birds were challenged with a *P. multocida* serotype 3 cross 4 that was isolated from a fowl cholera outbreak in commercial turkeys by the University of Georgia. The method of challenge was by the squirt route.[4] The experiment was repeated a second time by the same procedure.

The results of both experiments can be found in Tables 1 and 2. The birds vaccinated with a commercial serotype 1, 3, and 4 vaccine were as well protected as those vaccinated with a serotype 3 cross 4 bacterin when challenged with a serotype 3 cross 4. The challenge strain caused at least 85% mortality in the nonvaccinated controls. While the wing web M-nine did afford some protection, it was much less than the birds vaccinated with the bacterins.

Our vaccination program for fowl cholera at Cuddy Farms has been revised at least 3 times in the past 12 months. Anyone who has had to deal with fowl cholera can understand that it takes trial and error to arrive at the vaccination program that best fits your cholera situation. Presently the program that has been most successful for Cuddy Farms is a combination of live and inactivated vaccinations. The live vaccinations are not only protecting the growing breeder but also are priming her immune system so that upon vaccination with a killed vaccine we get a much higher boost in antibody titer than by using the killed vaccine alone.[2] Unfortunately, no vaccination program will be successful in all instances or be successful for very long periods of time and I expect that I will again be looking for a new program in the near future.

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TABLE 1
Results of 3 x 4 Challenge Trial 1

Vaccine	Mortality/#Birds	% Survival
Serotype 3 x 4 Bacterin #1	5/25	80
Serotype 3 x 4 Bacterin #2	5/25	80
Commercial 1,3,4	2/27	93
M-nine (ASL) W.W.	12/25	52
Control	13/14	7

Challenge Type 3 x 4: 3.0×10^{10} CFU/ml (squirt). (4 weeks P.V.)

TABLE 2
Results of 3 x 4 Challenge Trial 2

Vaccine	Mortality/#Birds	% Survival
Serotype 3 x 4 Bacterin #1	2/31	93
Serotype 3 x 4 Bacterin #2	1/31	97
Commercial 1,3,4	2/31	93
M-nine (ASL) W.W.	10/36	70
Controls	13/15	13

Challenge Type 3 x 4: 3.0×10^{10} CFU/ml (squirt). (4 weeks P.V.)

MOLECULAR EPIDEMIOLOGY OF *PASTEURELLA MULTOCIDA* ON A TURKEY PREMISES WITH A HISTORY OF FOWL CHOLERA

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In epidemiologic studies involving *Pasteurella* and other bacteria, fingerprinting techniques utilized for the differentiation of strains of the same species have included serotyping, phage typing, determination of antibiogram, biotyping, plasmid DNA analysis, and whole cell protein analysis. All of these techniques have limitations because typing relies on traits which are inconsistently expressed, and the sensitivity level frequently is inadequate to accurately differentiate strains. An additional fingerprinting technique for epidemiologic studies is restriction endonuclease analysis (REA) of whole-cell (primarily chromosomal) DNA utilizing agarose gel electrophoresis. This technique has been shown to be sensitive and reproducible in a variety of bacteria. A major advantage of REA is the fact that one is examining the more stable genotype of the microorganism, not phenotype. A disadvantage is that, depending on the restriction enzyme used, profiles of digested and electrophoresed DNA may be difficult to interpret due to the large number of restriction fragments. One method for circumventing this problem is the use of an *E. coli* rRNA radiolabeled probe to highlight restriction site heterogeneity. This procedure has been referred to as ribotyping by some investigators.

To study the epidemiology of *P. multocida* on a premises with a history of fowl cholera, 25 isolates were fingerprinted by conventional and molecular techniques. Isolates were obtained over a 17-month period from turkeys dying of fowl cholera (6 outbreak flocks, 14 isolates) and from captured wildlife (11 isolates) on the premises. Epidemiologic markers examined and compared included: 1) serotype, 2) subspecies (biotype), 3) antibiogram, 4) presence of plasmid DNA, 5) whole-cell protein profiles following polyacrylamide gel electrophoresis, 6) REA patterns of whole-cell DNA, and 7) ribotype. The majority of the isolates were serotype 3,4 (80%), capsular type A (100%), subspecies multocida (84%), uniformly sensitive to the 8 antimicrobics tested, lacked plasmids (only 3/25 contained a plasmid), and possessed very similar proteins. REA and ribotyping proved to be a method for satisfactorily differentiating this fairly homogenous group of isolates.

REA and ribotyping revealed 7 different "ribotypes" within the 25 isolates. Four different types were found in turkeys from the 6 different outbreaks and 5 types in the wildlife. One ribotype accounted for 44% of all the isolates, and a second for 24%. Ribotyping, used in conjunction with serotyping, indicated: 1) the involvement of 4–5 different strains in fowl cholera outbreaks in the 6 turkey flocks present on this premises during the 17-month study period; 2) the presence of up to 3 different strains of *P. multocida* in one outbreak flock; 3) persistence of a single strain on the premises for over 11 months; and 4) the presence of common strains in turkeys and wildlife on the premises.

TRAPPING PROGRAM—CALIFORNIA TURKEY PROJECT

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As part of a large program to delineate the epidemiology of fowl cholera, a trapping program was initiated in 1985 to sample wildlife from the confines of participating turkey ranches. Primarily, wildlife on the ranches that had potential contact with the turkeys was sampled. Methods of collection included trapping and netting. Blood samples and oropharyngeal swabs were obtained from each specimen. The methods used to obtain wildlife samples and sampling procedures are illustrated. The relationships of the types of wildlife, environment and management procedures are illustrated.

An initial visual examination was performed on each ranch before traps or nets were placed on the ranch. This was to determine the type of wildlife present, their potential contact with the turkey flock, means of contact, harborages present and the overall environment.

Mist nets used for the collection of flying birds were placed in the flight pattern of the birds. Birds caught in the nets were manually removed and placed in a net sac preparatory to obtaining samples. Birds were released following sampling when possible.

Several kinds of traps were used for the mammals. Live catch traps were used on all ranches possible so that species caught could be released unharmed. These live traps varied in size from those appropriate for mice to the large carnivore size.

The highest level of biological security was maintained on each ranch. All traps and nets were disinfected before entry on the ranch and re-disinfected before leaving. Clean coveralls and disinfected rubber boots were worn at all times. No vehicles were driven onto the ranch. No materials taken onto the ranch were removed from the ranch unless it was disinfected. All waste material was disposed of on the ranch according to its waste management procedures.

A scientific collection permit was obtained from California Department of Fish and Game and the U.S. Fish and Wildlife Service before wildlife samples were obtained from the ranch. Care must be taken to be aware of any endangered or other protected wildlife species present within the trapping area.

A DUAL EPORNITIC OF TURKEY RHINOTRACHEITIS AND AVIAN INFLUENZA IN TURKEY BREEDING AND MEAT FLOCKS

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Introduction

Turkey rhinotracheitis (TRT) was first identified (retroactively) in Israel in 1978.[5] At present most of the meat type and breeder replacement flocks suffer at an early age (2–16 wks) and develop high titers of ELISA antibodies. Outbreaks in breeding flocks during the laying period occurred only during the first year of the epornitic (1978–79) with clinical signs of sinusitis, rales, and severe drop in egg production. In subsequent years, the disease affected flocks before coming into lay.

Avian influenza (AI) has affected turkey breeders only rarely in Israel (1979, 1 flock; 1982, 4 flocks).[3,4] In this presentation we shall discuss data concerning an epornitic affecting turkey breeding and meat type flocks during May and June 1988. These farms were all located within a 1.2 km radius in the Jezreel Valley. In 3 of the flocks TRT preceded influenza and intensified the clinical signs. In addition mortality was noticeably higher, egg production fell drastically and the birds failed to reach the projected levels of egg laying.

Materials and Methods

Tracheal and cloacal swabs were used as the material for virus isolation. The propagation of the field materials through embryonated eggs, titration of hemagglutinating (HA) agents, their passage, performance of neuraminidase (Nase) reaction, HA inhibition (HI) and Nase inhibition (NI) were according to Palmer et al. (1975).[2]

Hemagglutination inhibition test was carried out to detect avian influenza antibodies in the affected flocks. Antibodies to TRT were detected using ELISA as described previously.[5]

The influenza isolates were tested by intravenous pathogenicity index tests in twenty 6-week-old chickens and ten 8-week-old turkeys, as described.[1]

Results

The four hemagglutinating agents that were isolated from cloacal and tracheal swabs possessed neuraminidase activity and were not inhibited by either anti-Newcastle disease virus (NDV) or anti-Yucaipa virus reference antisera. While performing HI tests with a set of goat monospecific reference antisera against all the known types of influenza viruses, a partial HI with the antisera against H5 and H7 antigenic serotypes was observed (fluctuating around 30–70% of the full hemagglutination pattern). These results varied in the different experiments, especially when different passage levels of the isolates were used. The results of the NI test also showed partial inhibition: namely, the level of the inhibition fluctuated around 50% in a number of successive serial dilutions of the reference antiserum, starting from the very first dilution (1:10) and being the same until the endpoint. This was observed with both the anti-N3 and anti-N2 antisera. It was concluded that the isolate consisted of a mixed population of at least two viruses. Further passage of the isolate in embryonated eggs resulted in a viral preparation which was fully inhibited only by anti-H7 and anti-N3 antisera. This was interpreted as due to the survival of H7N3 virus as a result of the competitive interference with H5N2-containing component of the mixture. However, growth of the isolate in the embryonated eggs in the presence of an excess of the anti-H7 antiserum resulted in isolation of the H5N2 virus.

In the intravenous pathogenicity index test each strain gave an index of 0.0, i.e., no clinical signs in any of the birds over the 10-day observation period.

The influenza viruses were isolated from tracheal and cloacal swabs taken from 4 of the breeding flocks. No swabs were taken from two other flocks but evidence of AI was clearly present when HI antibody titers were examined.

In 3 flocks TRT infections preceded the outbreak of AI. These flocks had not previously been exposed to TRT virus during the growing period, and thus their ELISA titers were very low. Following the appearance of clinical signs the ELISA titers rose dramatically. In these 3 flocks TRT appeared 2–4 wks after the flocks were transferred from the dark houses. The clinical symptoms were identical to those described earlier,[5] namely sinusitis, rales and a strong drop in egg production. In the flocks suffering from AI the clinical signs were very simi-

lar. In addition there were many cases of egg peritonitis and the egg shells were white instead of their typical speckled appearance.

The flocks were Mycoplasma gallisepticum-free, and no isolates of *Pasteurella multocida*, Newcastle disease virus, or paramyxovirus Yucaipa were made.

In five meat type and one replacement breeder turkey flocks that were all located in the same village, evidence of AI infection was demonstrated in July 1988 when the birds were bled at time of slaughter and HI antibodies were found. All these flocks had tracheal rales, nasal discharge and were coughing.

Discussion

In this presentation we describe an outbreak of AI in meat type and breeding flocks. In some of these flocks TRT had appeared before AI was diagnosed. The losses from the dual infection were significantly higher than from AI alone.

On the basis of these data the four AI isolates made during the outbreak consisted of a mixed population of two strains: H5N2 and H7N3. Since the initiation in 1978 of our systematic studies in Israel on AI, no mixed population of AI strains had been previously reported from any of avian species including feral birds.

H5N2 was isolated in 1971, 1973, and 1978 from meat-type flocks, but H7N3 has not been isolated previously in Israel.

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TURKEY RHINOTRACHEITIS: FIELD OBSERVATION, AND SEROLOGY IN BADEN-WÜRTTEMBERG, WEST GERMANY

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Introduction

Turkey rhinotracheitis (TRT) is an acute highly contagious respiratory disease affecting turkeys of all ages. Although morbidity is often very high, mortality is extremely variable since it is influenced by many factors such as management, stress, ventilation rate, concurrent diseases and the types of 2ry bacterial infection.

TRT has been reported and become a problem in Europe, especially in the United Kingdom,[2,10] France,[1,5] and West Germany.[4] It has also been observed in South Africa[3] and Israel.[6]

Clinical Observations in Baden-Württemberg (West Germany)

In turkey flocks supervised from Poultry Health Service Stuttgart, respiratory disease outbreaks were observed many times prior to the end of 1986. These outbreaks occurred periodically and were characterized by a sudden onset of mild respiratory signs, rapid spread, high morbidity and low mortality.

Since Dec. 1986, several outbreaks have been observed with severe acute respiratory distress. Morbidity has been almost 100% with mortality varying from 3 to 15%. Clinical signs were seen in birds as young as 14 days old, but more commonly in birds between 3 to 9 weeks of age. Older turkeys and flocks in lay also succumbed to the disease. The condition appears to have become endemic, affecting all farms and every new restocking. Clinical signs are sneezing, nasal discharge, conjunctivitis, tracheal rales, sinusitis, and submaxillary oedema. The symptoms disappeared in most of all affected flocks within 7 to 10 days after antibiotic therapy directed to the 2ry bacterial infection (based on the results of sensitivity tests) supported with careful husbandry, improvement of ventilation and hygienic measures.

On post mortem examination, lesions depended upon the course of the disease. These include rhinitis, tracheitis and sinusitis. In many cases pericarditis, airsacculitis as well as congestion of the lungs and fibrinous exudate in the pleural cavity were observed. Histological examination revealed tracheitis with lymphocytes infiltration and hemorrhagic necrotic pneumonia.

Cultures for Mycoplasma and mycological examinations from the respiratory tract were negative. Monkey kidney cell line (Vero cells) inoculations showed negative results for virus isolation. Bacteriological examinations have yielded *Escherichia coli (E. coli), Moraxella* sp., *Pseudomonas aeruginosa, Staphylococci, Streptococci,* and *Proteus sp.*

Treatment Trials

During the period since Dec. 1986 a total of 23 farms have been observed. In five flocks, severe respiratory manifestations accompanied with high mortality rates were observed. All attempts adopted to keep the condition under control using drug treatment directed toward the 2ry bacterial infection, and based on the results of sensitivity tests, were useless. In two of the five flocks (Nr. 1 and 2) with about 18,000 two-week old poults, the mortality reached about 15 % within 5 days. Treatment with chloramphenicol and neomycin directed to isolated *E. coli* and *Pseudomonas aeruginosa* was of no value. The mortality could be stopped initially after application of Baytril® (0,5 ml/1 l) in water for three days. Baytril® (Bayer AG West Germany) is not licensed at this time in West Germany.

Similar conditions were observed in the other 3 flocks with about 29,000 poults aged 2, 5, and 9 weeks respectively. The mortality rates were 4.7% for flock Nr. 3 and 4.4 and 8.3% for flocks Nr. 4 and 5 respectively within 14 days after the onset of symptoms. In all cases, the mortality as well as the severe clinical signs could be kept under control after application of Baytril® in drinking water (Fig. 1–3). Furthermore, in flock Nr. 4 the highly morbid birds were injected with 1% Baytril® (0,5 ml/birds) i/m and 90% of the treated birds recovered. In flock Nr. 5 with installation of Baytril® in the crop of highly morbid birds, 60 out of 100 treated birds overcame the disease. It is interesting to note that in all 3 flocks the mortality rate of females was significantly higher than that of males kept in the same house separated with wire mesh and receiving the same medication. Additionally, the clinical symptoms in flock Nr. 5 were observed initially in male birds and later in females; but

the losses were very heavy in females despite treatment with chloramphenicol in drinking water as well as i/m injection of single birds. In the literature, there is no evidence that females are more susceptible than male turkeys.

Serological Examinations

Serological Survey

Serum samples from 35 meat turkey flocks reared in Baden Württemberg showing and/or recovered from outbreaks of respiratory diseases between 1982 and 1988 were examined for the presence of antibodies to TRT virus using indirect ELISA tests.

The sera collected between 1982 to 1987 were tested by a TRT-ELISA antigen which was kindly received from Dr. P. Giraud, Ploufagan, France.[6] Since 1988 we received TRT-BUT strain as well as negative and positive serum. The preparation of ELISA antigen and its calibration were carried out according to the method done in BUT-England.[7] A commercial preparation of goat anti-turkey IgG with peroxidase (Nordic) and a substrate chromogen (ABTS solution) with H_2O^2 were used. Sera with an optical density difference (ODD) of 0,2 or more were considered as positive.

The results showed that antibodies to TRT virus were detected in all 25 flocks which suffered from respiratory manifestations between 1986 and 1988, but not in any of 10 flocks which had recovered from respiratory outbreaks between 1982 and 1985 (Table 1).

Examination of Paired Serum Samples

Paired serum samples from three turkey flocks that suffered from typical rhinotracheitis signs in 1987 were collected shortly after the onset of the symptoms and 4 to 5 weeks later.

The results of TRT-ELISA are shown in Table 2. In all cases relatively low concentrations of antibodies were present at the time of the first sampling which then increased significantly by the second sampling.

Serological Profiles

In 1988 the serological profiles of 5 turkey flocks to TRT were studied. The birds were bled at 4 week intervals starting at the first day of age till 20 weeks.

The results obtained from flock Nr. 1 indicate that there are high concentrations of maternal antibodies at the first day of age which drastically decrease by 4th week of age (Table 1). After natural infection very clear seroconversions in all flocks were detected. The antibody level appeared to decrease 4 weeks later. In three flocks (Nrs. 1, 2, and 4) TRT infection began at the 4th week of age; in the other two flocks at the 9th (Nr. 5) and 13th (Nr. 3) week of age. Furthermore, in flock Nr. 1 an increase in antibody level at the 20th week of age could be detected without any remarkable clinical signs. In addition, antibody levels in flock Nr. 4 remained relatively low although severe respiratory distress at the 4th week of age was observed.

Discussion

The clinical signs which observed and described in turkey flocks accompanied with the detection of antibodies to TRT virus confirm the presence of TRT in Baden Württemberg, West Germany, since 1986. In the UK, no antibodies to TRT virus could be detected in sera collected from turkey flocks prior to 1985.[4]

The negative results for virus isolation till now, may be due to the late collection of samples after the onset of the clinical signs,[4] and the use of Vero cells for the initial isolation. Further trials using tracheal ring organ culture are in progress.

Treatment directed to 2ry bacterial infection in severely affected flocks using Baytril® seems to be of great value.

The results obtained by examination of paired serum samples as well as the flock profile results revealed a significant increase in TRT antibody levels after exposure to the infection and are in the most part in agreement with others.[4,5,7,8]

Acknowledgment

I want to express my deep appreciation to P. Giraud "Laboratorie National de Pathologie Aviaire"—Ploufragan, France for sharing the antigen and to G. P. Wilding, C. Baxter-Jones and M. Grant, British United Turkeys Ltd., Tarvin, Chester, UK for their continuous and excellent cooperation and for testing some sera to compare the results.

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TABLE 1
Results of the serological survey of turkey sera for TRT antibodies

Year	Nr. of Sera	Nr. of flocks	% positive sera	% negative sera	% positive flocks
1982	103	6	0	100	0
1984	40	2	0	100	0
1985	40	2	0	100	0
1986/87	211	8	82.5	17.5	100
1988	600	17	66.0	34.0	100

TABLE 2
Results of examination of paired serum samples for TRT antibodies

Nr. of flock	Nr. of sera	Age at signs (weeks)	Sampling time after signs (days/weeks)	Mean ODD
1	20	8	3 days	0.258
	20		4 weeks	0.406
2	20	9	4 days	0.332
	20		5 weeks	0.458
3	20	9	3 days	0.318
	20		4 weeks	0.609

TABLE 3
Serological profiles of turkey flocks for TRT antibodies

Flock Nr.	corrected mean optical density difference[b] at different ages (weeks)					
	0	4	8	12	16	20
1	0.550	0.012	0.895	0.255	0.300	0.558
2	n.d.[a]	0.119	0.741	0.186	0.144	0.109
3	n.d.	0.048	0.001	0.019	0.592	n.d.
4	n.d.	0.326	0.371	0.221	0.348	n.d.
5	n.d.	0.067	0.006	0.506	0.408	0.384

[a]not done
[b]No. of samples/week/flock = 20

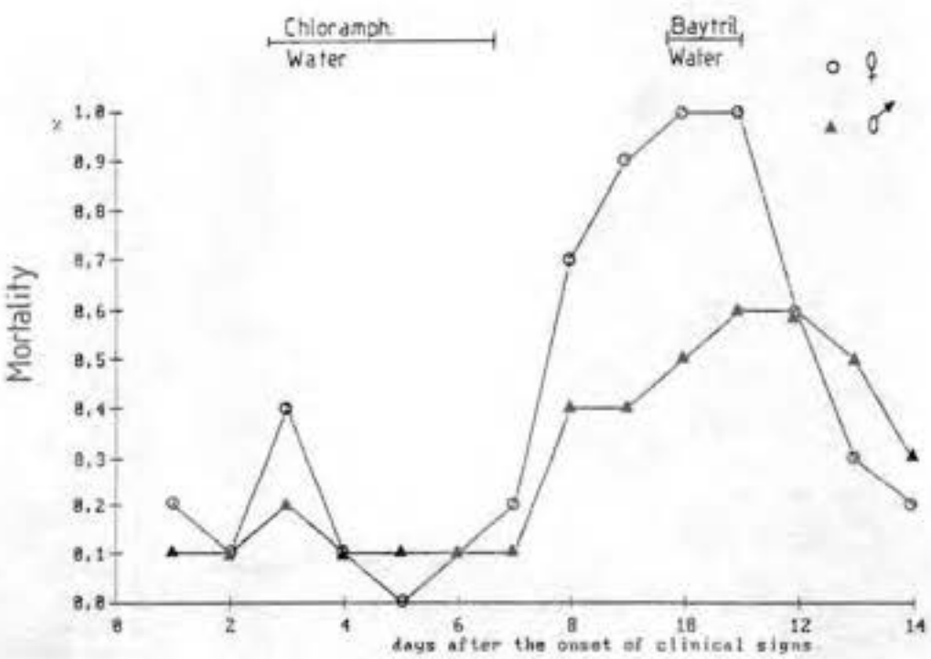

Figure 1: Treatment trials in flock Nr. 1.

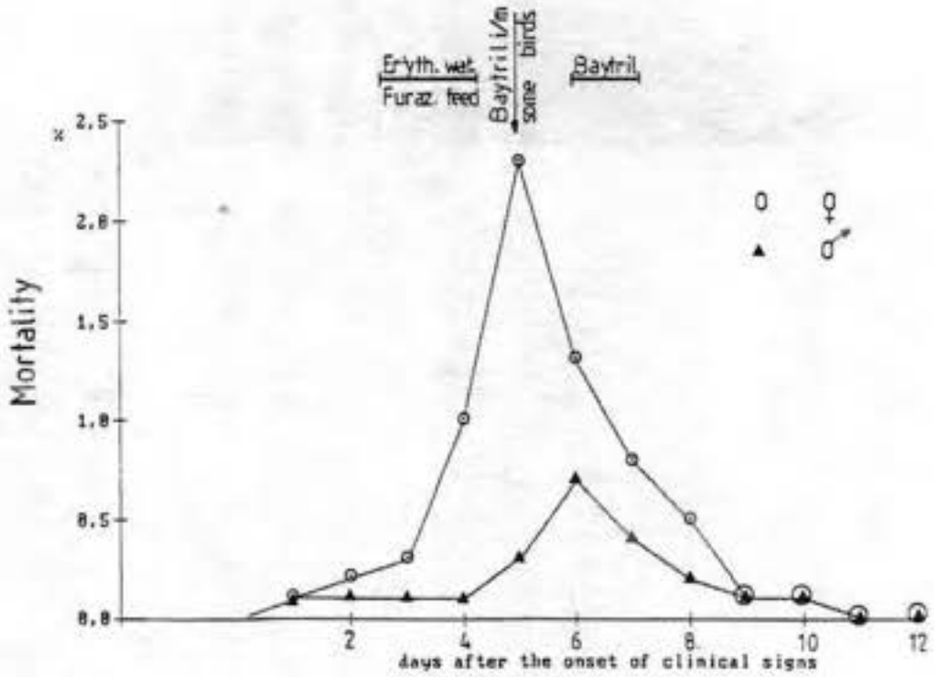

Figure 2: Treatment trials in flock Nr. 2.

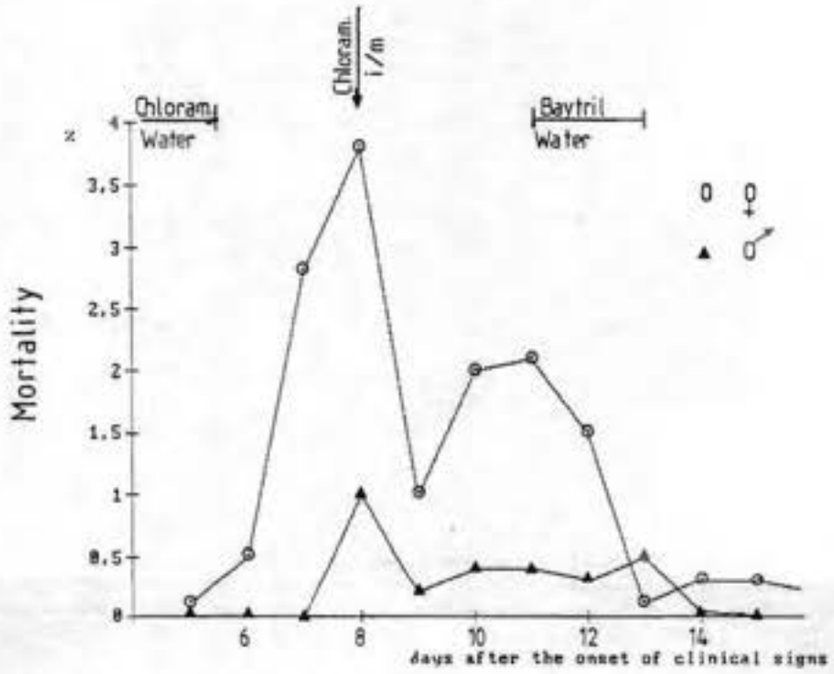

Figure 3: Treatment trials in flock Nr. 3.

MICROSPORIDIOSIS IN NESTLING BUDGERIGARS *(MELOPSITTACUS UNDULATUS)*

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Summary

Four cases of microsporidiosis in nestling budgerigars *(Melopsittacus undulatus)* from large breeding flocks are described. Microsporidia were demonstrated in the intestinal tract and liver. In the majority of submissions, microsporidiosis was associated with another infectious disease such as papovaviral infection or psittacosis. Clinical signs attributable to microsporidia were those of intestinal dysfunction characterized by malabsorbtion or maldigestion.

Introduction

Microsporidia are small, unicellular parasites belonging to the phylum Microspora. They are important pathogens of fish and arthropods and have assumed increasing importance in mammals, including man, and birds. They are common in rodents and lagomorphs where they usually cause mild disease or are latent. In some laboratory animals their presence interferes with research. More recently, infections of immune-compromised humans have been reported.[1] Among birds, microsporidiosis has been described in lovebirds and an Amazon parrot.[2,3,4,5,6]

Microsporidia are obligate, intracellular parasites that have two phases of development, namely schizogony, which is a proliferative phase, and sporogony, which results in resistant spores. The spore contains a polar filament or tube which lies coiled within one pole of the spore. During infection, it is everted and serves to penetrate the cell membrane of the new host cell. Transmission occurs by ingestion of food or water contaminated with urine or feces. Transplacental transmission has been demonstrated in laboratory animals.[1]

The most common microsporidian in the mammalian host is *Encephalitozoon cuniculi* the spores of which measure 2.5 x 1.5 µm. Encephalitozoon sp. is also suspected to be the genus of microsporidia described in lovebirds.[1]

Definite identification of microsporidia is usually accomplished by EM studies. In the cases reported here, they were tentatively identified by their characteristic size and oval shape, a greenish and slightly refractile appearance in wet preparations of intestinal mucosal smears, Gram-positive staining which was more intense on one pole, and intracellular location. Positive staining with Gram's stain is characteristic for microsporidia, all other protozoan cysts or spores being Gram-negative.[11] Further identification of this parasite was not attempted.

Case Report

Over a six year period, microsporidiosis was diagnosed in budgerigar nestlings from four large breeding flocks. It was the only finding in some submissions while in others it was accompanied by other, usually more significant diseases. The history was variable but included diarrhea, increased mortality and high morbidity (Table 1). Usually, a "bag-full" of nestlings of mixed ages from a few days old to three to four weeks of age was submitted. Routine necropsy procedure was carried out, including gross necropsy, bacterial cultures, cytology for chlamydia (Machiavello's or Gimenez stain), tissue culture or embryo inoculation for chlamydia when indicated, and histopath. H & E and Gram-stained sections were made. Intestinal mucosal smears (wet preparation and Gram stain) were examined when intestinal disorder was suspected from the gross lesions. To demonstrate incomplete digestion, fecal smears taken from the rectum were stained with Lugol's iodine and examined for purple-black bodies suggestive of starch granules.

Gross lesions are summarized in Table 1. In all four cases, numerous microsporidia spores were demonstrated in wet preparations of the intestinal mucosa.

Microscopic lesions in which microsporidia were found were confined to the intestines and the liver. In the liver, multifocal necrosis was often present. Foci varied from small coagulative foci to large foci having granulomatous centers surrounded by a rim of coagulative necrosis. Mild to severe, multifocal bileduct proliferation was prominent in some birds. Occasionally, large cyst-like structures containing eosinophilic, finely granular debris were within foci of proliferating

bile ducts. Microsporidia were demonstrated free or intracellularly within necrotic foci or proliferative lesions. They were not abundant.

In the intestines, microsporidia were present intracellularly in epithelial cells and macrophages within the lamina propria. Crypt and villous epithelium was affected to varying degree, some sections showing virtually every enterocyte of the lumenal one third of villi packed with spores while others showed occasional infected cells here and there.

Commonly, little or no pathology was associated with intestinal microsporidia. Occasionally, there was mild to moderate lymphoplasmacytic infiltrate into the lamina propria. In some birds, there was clubbing of villi, villous atrophy and fusion, and necrosis of enterocytes at the tips of villi.

Discussion

All cases were submitted during late spring, summer, and fall suggesting seasonal occurrence related to peak breeding season. However, flock IV bred all year under artificial lighting, perhaps indicating seasonal occurrence not associated with peak breeding.

History, clinical signs and gross findings varied, but in all cases diarrhea and caking of toes and vents with feces was a consistent finding and reflects interference of microsporidia with digestion or absorption of nutrients. Intestinal involvement was also reported in lovebirds, usually with no or minimal inflammatory reaction.[2,3,4,5] Liver lesions were also similar but kidney involvement as described in lovebirds was not demonstrated in these budgies. In lovebirds, numerous microsporidia were found in renal tubular epithelium and lumens.[2,3,4,5] Age, when given, was four to 6 months in lovebirds while all cases in budgies occurred in birds less than 4 weeks of age. In all four flocks, psittacosis or papovaviral infection was diagnosed either at the same time or a few months later, suggesting that microsporidia were not a primary pathogen. It is probable that microsporidia are opportunists in very young budgies with as yet immature immune systems or establish themselves in a host already weakened by other more virulent pathogens.

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TABLE 1
Microsporidiosis in nestling budgerigars—history, clinical signs, gross lesions, and other findings

Flock & Date	No. of Submissions	History	Gross Findings	Organs Affected	Other Diagnoses/Date
Flock I 7/82—10/82	3	Loose stools, low mortality, 20% morbidity. Incidence increasing as breeding progressed. Flock size: 1,500 breeders.	Pale hepatic foci, enlarged, pale intestine, passage of undigested feed, pasted vents, caked toes.	Intestine, liver	Papovaviral infection 10/82
Flock II 5/85—8/85	3	Increased mortality, soiled vent and feet. Flock size: 800 breeders.	Pale, voluminous intestines, watery intestinal content, splenic foci, hepatic foci, hepatosplenomegaly, airsacculitis, undigested feed in posterior gut.	Intestine, liver	Bursal cryptosporidiosis 5/85 Psittacosis 8/85
Flock III 7/86	1	Diarrhea, rough feathering, infertility, frequent molting. Flock size: 500 breeders.	Poor feathering, pale muscles, splenomegaly, pale intestines, mottled liver, incompletely digested feed in posterior gut.	Intestine	Papovaviral infection 7/86 Giardiasis 7/86
Flock IV 9/88	2	14% mortality. Flock size: 500 breeders.	Undigested starch in posterior intestines, soiled feet, hepamegaly with pale foci, pale pectoral muscle.	Intestine, liver	Myocardial degeneration 9/88 Psittacosis 10/88

EXPERIMENTAL REPRODUCTION OF PSITTACINE BEAK AND FEATHER DISEASE

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Since psittacine beak and feather disease was first recognized as a clinical syndrome in the early 1970s, numerous etiologies have been proposed for this disease. The list of reported etiologies includes endocrine abnormalities, nutritional deficiencies, toxins, environmental stress, and infectious agents. As information on the pathology of the disease began to emerge, basophilic intracytoplasmic inclusion bodies consistently identified in the feather follicles of suspected PBFD patients were found to be composed of viral-like particles. The repeated identification of these inclusion bodies from PBFD patients, limited epidemiologic observations, and a lack of other abnormalities consistently reported from PBFD patients lead most investigators to consider that PBFD had a viral etiology.

Work completed in 1986 by Wylie and Pass indicated that PBFD could be experimentally reproduced in budgerigars and galahs given suspensions of feather follicle homogenates from diseased birds and the B-propriolactone blocked infectivity suggesting a virus was responsible for the disease.[1]

In our study, virus isolated, purified, and concentrated from birds with PBFD was utilized to experimentally infect neonatal budgerigars and cockatiels by the oral and intracloacal routes. The infected birds exhibited signs of generalized stunting and retarded feather growth followed by eventual death. Intracytoplasmic inclusions bodies within follicular epithelium and necrosis of thymic and bursal tissue were considered consistent with histologic lesions previously described for PBFD.[2]

Electron microscopic identification of viral particles in the inclusion bodies, within the size range of the virus experimentally introduced to the birds, further suggest that this isolated, purified virus was responsible for early signs of PBFD. Positive results from immunocytochemical labeling techniques utilizing antibodies generated against the purified virus confirmed the transmission of psittacine beak and feather virus to the neonates.

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CHARACTERIZATION OF THE PSITTACINE BEAK AND FEATHER DISEASE VIRUS

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Since the 1970s a disease characterized by abnormal feather development and in some species by beak necrosis has been recognized increasingly and tentatively diagnosed in a number of different psittacine species. The syndrome called psittacine beak and feather disease (PBFD) is generally characterized by progressive relatively symmetric loss of down contour, crest, tail, and flight feathers. Gross feather pathology includes circumferential constrictions resulting in clubbed shortened feathers, retention of feather sheaths, and stress lines in the feather vanes.

Gross changes in the beak epithelium include palatine necrosis, as well as uneven wear, chips, enlargement, and longitudinal fractures of the beak. These changes are the clinical manifestation of histologic lesions indicative of epidermal cell necrosis.

Diagnosis of PBFD can be tentatively confirmed by biopsy of diseased feather follicles with histologic identification of basophilic intracytoplasmic inclusions within either epithelial cells or macrophage-like cells of the feather follicle epithelium and feather pulp.

Repeated attempts over the last decade by numerous laboratories to adapt the suspected viral agent to *in vitro* cell culture have been unsuccessful. Based on the historic difficulty in growing the virus in conventional cell culture systems, the identity and characteristics of the suspected viral agent have not been reported. Because we felt the virus had highly specific infectivity or growth requirements, an alternative approach to virus isolation was employed.

Virus was isolated and purified using conventional methods from birds diagnosed as having PBFD. The purified viral preparation was processed to an estimated concentration of 10^{14} virions/ml. The purified virus was than characterized based on ultrastructural configuration, nucleic acid content, and polypeptide composition. The isolated virus was confirmed to be the PBFD agent by infecting susceptible birds and inducing classically described histologic lesions in the follicular epithelium.

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CLASSICAL ACUPUNCTURE TREATMENT OF A YELLOW FRONT AMAZON EXHIBITING NEUROLOGICAL DEFICIT RESULTING FROM SEVERE HYPERTHERMIA

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Summary

A Yellow Front Amazon *(Amazona ochrocephala)* was exposed to extreme heat for an undetermined period of time. The subsequent hyperthermia resulted in a neurological deficit with partial paralysis, convulsions, and incoherent screaming. The Oriental diagnosis is an attack of Excess Heat (Yang) creating an Internal disharmony of Yin and Yang with a near collapse of Yin. Acupuncture therapy was initiated, utilizing specific acupuncture points to relieve the surface and extinguish the fire, tonify the Yin, and descend the Yang. After three consecutive days of treatment the patient was relieved of the most serious of the symptoms, and clinically normal within 13 days after treatment ceased.

Paco is a 9-year-old female Yellow Front Amazon parrot. She is housed in a large wire cage located in an unheated but comfortable garage in Portland, Oregon. On cold nights Paco was kept warm by means of a bare 250 watt heat lamp suspended over the cage, but, inadvertently, located directly over the birds' perch. The owner noted that Paco was in the habit of sitting under the heat lamp until well warmed, then moving on away down the perch to sleep. A few months earlier, the owner had noticed a large scab (3.5 x 3.0 cm) on the dorsum of Paco's head. Although she had never seen a sign of bleeding, she attributed the scab to Paco being pecked on by her mate, a male Yellow Nape Amazon, with which she was caged.

History

The night of December 28, 1987 was particularly cold and Paco apparently spent more than her share of time in the spotlight, so to speak. She possibly even went to sleep under the heat lamp which was, by actual measurement, only 16 cm above her head. At 4:30 A.M. her owner found Paco on the bottom of the cage, screaming and incoherent. The cage mate was normal.

At the time of examination the patient was laying on the bottom of the cage, firmly grasping a cage bar in her beak. Both legs and feet were in flaccid paralysis and wing muscle tone was noticeably reduced. At approximately 8-second intervals the patient would emit a distinct, loud, "barking" sound, accompanied by a single violent whole body spasm. Numerous small feathers around the periphery of the large thick scab on the head were curled and deformed, showing physical signs of heat damage. A delayed pupillary response was noted in the right eye, a normal response in the left. The skin was hyperemic and warm to touch. The body temperature (cloacal) was 107.8°F. Hydration was normal. Paco was totally non-aggressive, which was out of character for this particular bird. Paco was entirely oblivious and non-responsive to her environment. A diagnosis of extreme hyperthermia with accompanying neurological deficit was made. In Traditional Chinese Medicine (TCM), however, the diagnosis would be stated some somewhat differently—yet would have the same implications. In Oriental terms, Paco was suffering from an External attack of Excess Heat (which is Yang), progressing to an Internal invasion of Heat Pathogenic Factor, therefore creating an Internal disharmony of Yin and Yang, with a near total collapse of Yin.[2] The difficulties and dangers involved in the rapid cooling of the avian body by suggested Western methods were taken into consideration. Treatment by Traditional Chinese Medicine was elected.

Materials and Methods

The acupuncture needles used were 26 gauge—15 mm Hwato type, Chinese, silver handled with stainless steel shafts. The specific acupuncture points were selected to, in TCM terms, extinguish the fire, relieve the surface, and tonify the Yin, using the least number of needles possible, but using them effectively. Acupuncture points Hegu and Quchi were selected to relieve the excess Heat Pathogenic Factor. Sanyinjiao was used to tonify and strengthen the Yin of the body. Dazhui was employed to descend, or reduce, the Yang. All are designed to restore the critical equilibrium of life—the harmonious balance of Yin and Yang.[1]

Three treatments were given at 24 hour intervals over a 3 day period. Sanyinjiao was always needled first, to lessen the shock of needling the other points, for a period of 10 to 12 minutes; this was followed by Hegu and Quchi for a similar period of time. Dazhui was needled at the same time as Hegu and Quchi, but for a shorter period of only 7 to 9 minutes. Extreme relaxation of the body was achieved in all three treatment sessions.

Results

The patient responded rapidly and satisfactorily to the acupuncture treatment. By the end of the first 30 minute session on December 29th (Day 1) Paco was totally relaxed, but with near normal tone returned to the previously flaccid leg muscles. The "barking" was eliminated and the body spasms were markedly reduced in frequency and intensity. The body temperature was 105.4°F. On the morning of Day 2 the spasms were relieved entirely and Paco was somewhat aware of her environment. She was unable to perch due to general weakness and a tonic, clenched right foot. After treatment she ate fresh corn when coaxed and passed a large black stool, the first in over 24 hours. The sample tested positive for occult blood. The body temperature was normal at 104.2°F. By Day 3 Paco was eating on her own, perching with difficulty, and "talking."

Allopathic treatment with 25 mg Amoxicillin, per os, BID for 10 days, was initiated on Day 4. The patient was kept under close observation for 15 days. She continually showed improvement in her alertness and ability to perch. Upon release on Day 15 she was clinically normal except for the presence of the large scab on her head. Over a year later Paco is doing very well. Her voice is higher pitched than before the incident and she is non-aggressive, although quite protective of her owner. The scab is still present on her head. No attempt will be made to remove it.

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THE EFFECTS OF NAPHTHALENE AND P-DICHLOROBENZENE (MOTHBALL CHEMICALS) IN CANARIES AND FINCHES FED CUMULATIVE AMOUNTS IN CONTAMINATED FEED

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Case Report

Seven of fifteen canaries and finches ranging from three months to five years in age from a private aviary became ill and died within one month after introduction of several new birds. No quarantine had been in effect prior to incorporating the new birds into the established flock. The affected birds stopped perching, began to huddle with ruffled feathers, became anorectic and progressively weak. The birds were housed indoors in cages and fed a diet consisting of a millet seed mixture for the adults and a nestling feed mixture (to include cooked egg, corn, alfalfa) for the juveniles. Mothball products (naphthalene and p-dichlorobenzene) were customarily used inside the feed storage containers in small ventilated jars to minimize moth larval contamination.

Gross necropsy findings included moderate pectoral mass atrophy, marked hepatomegaly with tan mottling, and moderate splenomegaly with multiple tan nodules throughout the parenchyma. The liver was the predominant organ demonstrating histopathologic changes. Karyomegaly and cellular vacuolation were present in the hepatocytes. Periportal necrosis was seen with a mixed infiltrate of eosinophils, lymphocytes, and plasma cells. There was marked biliary hyperplasia and multifocal vacuolation of biliary duct cells. A moderate pneumonitis, serous atrophy of fat and diffuse vacuolation of pancreatic acinar cells were noted.

Pseudomonas aeruginosa was cultured from the lung, liver, spleen, and intestines. *Chlamydia psittaci* was not isolated in McCoy cell cultures or identified by fluorescent antibody stained impression smears. The birds were placed on antibiotic therapy but showed little improvement. Analysis of all the feeds showed contamination with naphthalene and p-dichlorobenzene (1.6—9.8 ppm and 0.5—4.7 ppm, respectively). OSHA standards permit inhaled naphthalene levels <10ppm daily. Higher levels are reported to cause eye, skin, and lung irritation with bronchiolar epithelial cell damage.[4] Chronic exposure to p-dichlorobenzene to 376 ppm in rats results in liver cirrhosis and focal necrosis.[4] No mycotoxins were identified.

Naphthalene has been reported to cause hepatic necrosis in mammals.[2] The toxic effects in birds have not been described but the liver is likely a target organ. Naphthalene is also known to cause hemolysis resulting in profound anemias.[1] Halogenated hydrocarbons, like p-dichlorobenzene, produce hepatic necrosis and may be immunoinhibitors.[5] Overall effects of exposure result in poor survival of the young, progressive cachexia, and complications by other diseases, usually infective.

The refractoriness to antibiotic therapy may have been associated with immune compromise induced by p-dichlorobenzene intoxication. Hepatic necrosis and associated parenchymal lesions have been attributed to both naphthalene and p-dichlorobenzene. Once the inciting agents were removed and the contaminated feed discarded, no additional birds became affected. Birds are highly sensitive to volatile organic compounds and the presence of these chemicals in the feed provided strong circumstantial evidence as to the cause of this flock's problem.

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THE EFFECT OF SELENIUM ON MALLARD DUCK IMMUNE FUNCTION AND DISEASE RESISTANCE

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Selenium, mobilized from soil into water by irrigation, is a significant environmental contaminant in many western states. Fly ash from coal combustion can also result in water contamination with selenium. Selenium has been reported to both increase and decrease immune function in rats and cattle. Our objectives were to determine if selenium, at levels found in the environment, affected immune function or disease resistance of migratory waterfowl.

Pairs of mallard ducks were held in pen on experimental creeks with water treated with 0, 10, and 30 ppb sodium selenite for 95 days. Selenium bioaccumulated through the food chain and plants, invertebrates, and a supplementary mash were available as food. Five parameters of immune function were measured in adult ducks. Two measures of non-specific host defenses were increased in ducks from the 30 ppb treatment group after 84 days exposure. These were "phagocytosis" of killed Pasteurella multocida by blood heterophils and monocytes, and monocyte concentration. Total leukocyte concentration and the concentration of other cells from the differential leukocyte count were not affected by selenium treatment. Antibody-mediated immunity after 95 days exposure to selenium was not affected by selenium treatment, as measured by the Jerne spleen plaque forming cell assay or hemagglutinating antibodies to sheep red blood cells. Cell-mediated immunity was not affected by selenium treatment for 85 days, as measured by delayed-type hypersensitivity to Mycobacterium bovis.

Ducklings, exposed to selenium via the egg and hatched and reared in the pens until 14-days-old, showed a significant positive trend of decreased disease resistance measured by mortality to a challenge with duck hepatitis virus 1 (DHV1), a picornavirus, when challenged at 15-days-old. Predation of eggs and "bred" ducklings reduced the numbers available, so additional ducklings were purchased from a game farm, exposed to selenium in the pens from 7- to 21-days-old, and challenged with DHV1 when 22-days-old. In this group of "purchased" ducklings mortality to DHV1 was not affected by selenium treatment, however, liver selenium concentrations were not as high as in the "bred" ducklings.

Predation disrupted reproduction, however, some parameters were monitored. There was no significant difference between selenium treatment groups in the number of hens laying eggs, or the percentage of hatched eggs per incubated egg. Embryos from the third egg laid were examined grossly after an estimated 18 days incubation, but no deformities were detected. There was no significant difference between selenium treatment groups in duckling body weights measured each week.

Exposure of ducks using sodium selenite treated water in this natural creek system resulted in liver selenium concentrations of 3.5 ppm Se dry weight, 6.5, and 11.1 for adults; 1.4, 3.6, and 7.6 for "bred" ducklings; and 1.8, 2.7, and 4.1 for "purchased" ducklings for the 0, 10, and 30 ppb Se treatment groups respectively. These levels are less than those found in waterfowl from areas where selenium is being recognized as an environmental contaminant. Selenium exposure of waterfowl can result in reduced disease resistance of ducklings to duck hepatitis virus 1. It is possible that the susceptibility of wild populations of migratory waterfowl to other infectious diseases could be increased by excess selenium exposure resulting in greater mortality.

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ORTHOBORIC ACID TOXICITY IN CHICKENS AND TURKEYS

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Orthoboric acid is an increasingly popular product used inside poultry houses for the control of darkling beetles. Because of the proximity of the birds to this compound, there is concern regarding the safety level of this chemical for chickens and turkeys.

Acute toxicity of the product was assayed by gavaging orthoboric acid to chicks and poults. In addition, a more natural route of exposure was tested in order to assess the toxicity of the product when applied on top of the litter at different levels.

Orthoboric acid proved to be of very low toxicity in young chickens and turkeys. Clinical signs and lesions described at the conference, and the toxicity level of orthoboric acid, as well as boron residues in the tissues of the birds presented and discussed at the conference, were not available when the proceedings went to press.

CASE REPORT: OLEANDER *(NERIUM OLEANDER)* POISONING IN TWO GEESE

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Oleander *(Nerium oleander)* is an ornamental plant that abounds in the tropical and subtropical area of the world. It originally came from the Mediterranean region. In the United States, it is found in the South, Southwest, and in California. In the latter, besides being common in hedges, yards, and parks, it is also frequently planted along the freeways. Oleander is a shrub that may grow up to 20 feet; has long narrow leaves which may measure up to 10 inches long; and, depending on the variety, forms clusters of red, pink, yellow or white flowers. It is claimed that its poisonous character, due to the digitalis-like glycoside toxin it contains, is not limited to the whole plant *per se* but also includes the smoke from its being burned as well as the water in which the flowers have been soaked.[1]

Cases of poisoning in humans, animals, and poultry from the ingestion of leaves or other parts of the plant have been reported in the literature.[1-6] The use of its twigs as meat skewers for barbecuing has also resulted in human poisoning.[6] The clinical signs of oleander poisoning in livestock include increased heart action, elevation of temperature, dilation of the pupils, inappetence, abnormal thirst, fetid diarrhea, profuse perspiration (horse), muscular collapse, coma, and death.[4]

As for its toxicity for poultry, Bardosi in 1939 found that doses of 6 grams and 3 grams of the dried leaves from the previous year's growth proved fatal to geese and ducks, respectively, while it took 15 grams of young leaves to kill hens. Hinshaw, in 1965, made mention of the 1939 findings of McNeil and Denny who reported the lethal effects in poults within 24 hours following insertion of oleander leaf sprouts into the birds' crops. Severe enteritis was seen on necropsy.[5]

Based on the reported precise dosages and method of administration, it appears that the data reported in poultry by the above workers were the result of experimental investigations.

The report presented herein is that of an actual clinical case of oleander poisoning in geese in San Bernardino County, California.

Case Report

On July 25, 1988 a privately practicing veterinarian (one of us: JM) in Victorville, California, called the senior author's office to request necropsy of a goose that had died early that morning at their hospital. The bird had been brought in by its owner for treatment on the morning of July 24, 1988.

According to the owner, they had 3 geese, breed unknown; an adult female that had been adopted as a 3-day-old stray in April, and two 7-week-old geese (one male and one female) which she had bought as goslings in the early part of June 1988, from a feedstore in Apple Valley, a neighboring community.

The geese were all apparently healthy till about midafternoon on July 23 when the young female goose was observed to be stumbling. Its condition rapidly deteriorated to a point where it could not walk and died about 6:30 P.M. The bird also manifested profuse salivation. It made attempts to drink water even though it was on the verge of dying.

The male young goose started showing the same clinical signs about 7:00 P.M. that same evening but when the owner saw the following morning that it was still alive, even though it was unable to walk around, she decided to bring it to a veterinary hospital for treatment.

At the time of presentation, the bird was showing profuse watery diarrhea, was prostrate,

and its neck completely laid out. A fecal examination by flotation method revealed no gastrointestinal parasitic problem.

Treatment

The bird was given 400 mg. of chloramphenicol IM and 2 mg. dexamethasone, also IM; 600 mg. prednisolone sodium succinate* intravenously and 60 ml. warmed Lactated Ringer's Solution (LRS) also IV. The bird was warmed utilizing a heating pad and supportive care was given throughout the day. Mild improvement was noted following administration of medication. The goose was able to regain a sternal position within approximately 30 minutes. It drank several times during the day.

A second dose of chloramphenicol and LRS was administered that evening. However, no further improvement was noted.

The goose was found dead it its cage the following morning (July 25, 1988).

Necropsy Findings

The bird was in good flesh. There was an excessive amount of mucus in the pharynx and upper one inch of the trachea.

Three fresh oleander leaves were found in the proventriculus. The gizzard contained much fibrous plant material as well as small fragments of what appeared to be oleander leaves.

Histopathological findings consisted of a mild clustering of hemosiderin-containing macrophages in the lamina propria of the tips of the villi of the small intestine.

The heart showed loss of striations. There was wide separation of myocytes in some areas of myocytoplasmic membranes were poorly defined, but specific swelling or hyalinization of myocardial fibers was not noted. Brain, gizzard, kidney, and liver were normal.

Other Laboratory Findings

The liver and lungs did not yield any pathogenic aerobes. The intestines were negative for *Salmonella*.

Remarks

An on-site investigation revealed the owner's premises to be practically devoid, other than a tree, of any vegetation. This is a general characteristic of many newly occupied desert residences. The owners of the birds lived in a mobile home and in an effort to beautify their surroundings, the owners had brought home earlier on July 23, 1988, 20 one-gallon size oleander plants. These were intended for planting along the fence of their front yard. (The newly-planted oleander plants were in much evidence on our visit on July 25th).

The young geese, which had the free range of the yard, noticing an abundance of leafy greenery on an otherwise parched environment in the middle of summer, eagerly partook and made a meal of the oleander leaves. The size of the plants complemented the opportunity for an easy ingestion of the leaves. Surprisingly, the oldest bird did not partake of the poisonous material; hence, it never got sick.

This report once again, reinforces the common observation that all it takes for a disease to occur is having the "right chemistry." In addition to the opportunity provided by the plants, the owner was apparently unaware of the poisonous character of the oleander plant.

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LEAD POISONING IN A FLOCK OF BACKYARD GEESE

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Case Report

Three 10-month old geese, one live and two dead, were submitted to the California Veterinary Diagnostic Laboratory System—Fresno Branch for examination. The owner reported that there was sudden onset of illness with the birds appearing listless, apparent paralysis, anorexic and subsequent death. Nine African geese had died within the past two days. There had been no prior losses. The flock consisted of approximately 150 geese, primarily African. They were purchased nine months previously as goslings and maintained as "pets" and for weed control on the owner's property. In addition, they were fed discarded vegetables obtained from the local grocery stores and surplus grain that was obtained free of charge.

Necropsy revealed minimal pathology with only mild to moderately swollen liver in one bird and marked splenic congestion in two birds. How-ever, all three birds had a small amount of metallic shavings in the gizzard that appeared to be copper. No significant bacteria nor viral agents were detected and all three sera were negative for avian influenza. Histologically there were moderate numbers of eosinophilic intranuclear inclusions in the renal tubules with no evidence of inflammation. Moderate to marked cytoplasmic vacuolation of hepatocytes, marked splenic congestion with lymphocytic depletion and moderate pneumoconiosis were also noted. Examination of brains, sciatic nerves, muscle and intestinal tract was unremarkable. Toxicological analysis revealed lead levels of 5.82 and 19.2 ppm in the liver and kidney, respectively. In addition, analysis of eggs submitted at a later time revealed lead levels of 13.8 to 15.2 ppm in the shell, but only 0.13 to 0.30 ppm in the egg contents.

Field investigation of the flock revealed multiple small "dump" sites which contained old batteries, solder, pipe, cable, paint and numerous other potential sources of lead. In addition, lead shot was reportedly used to shoot pigeons that were flying over the yard and adjacent fields.

BASELINE HEMATOLOGIC VALUES OF TURKEY BREEDING HENS IN EARLY EGG PRODUCTION

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A statement by a nutritionist employed by a major feed company prompted this study on the hematologic values of the turkey breeding hen. This statement concerned itself that turkey breeding hens consuming conventional diets are anemic during the initial stages of egg production thereby producing less eggs during this time than breeding hens fed diets supplemented with iron.

A search through the literature found a number of papers on the subject of normal turkey hematology,[2,4,5,8,9,12,14,15] with the work being done over 20 or more years ago.

This study was designed to determine the hematologic value occurring naturally in a turkey breeder hen during the initial stages of egg production and to establish normal standard values since current data is lacking.

Materials and Methods

Twenty-two Nicholas large white female line strain breeder hens *(Meleagris gallopavo)* were weighed and blood samples taken at four different age periods. The hens were randomly selected from a flock of 4000 hens as follows:

1. "Prelit": hens were 28 weeks of age and in a non laying period. The birds had been placed in an environmentally controlled dark out house at 22 weeks of age with six hours of light/day.
2. "Lit": hens were 30 weeks of age and had been moved into a curtain sided production house at 28.5 weeks of age. The hens were exposed to a light-stimulatory period of 14 hours of light/day, which was kept for the rest of the production cycle.
3. "First week of production": hens were 32 weeks of age and at the onset of egg production.
4. "Peak": hens were 36 weeks of age and at their peak egg production.

The birds were spray painted for identification during each period of blood sampling. An attempt to follow the same 22 hens throughout the study proved unsuccessful and only 12 of the original hens were bled repeatedly in each of the four sampling periods.

The turkey breeder hens were raised under a commercial breeder program that included a typical vaccination schedule and total confined housing. The birds were negative for MG, MM, MS, and free of any clinical signs of disease. The feed ration at the first sampling period was formulated at 2,850 Kcal/kg, 13% protein and 0.9% calcium. During the second period the diet was increased to 3,000 Kcal/kg, 17% protein and 3.4% calcium. These values remained constant throughout the rest of the study period.

Two milliliters of blood were drawn from the ulnaris cutanea or brachial vein of the wing[6] with a 0.2cc syringe and 22 gauge needle, placed in EDTA vacutainer tubes and transported in ice to the Laboratory of Hematology, Veterinary Medical Teaching Hospital, University of California Davis. Blood samples were taken consistently between 8:00 A.M. and 9:00 A.M. and analyzed within the next three hours. The parameters determined were: erythrocyte count (RBC), hemoglobin concentration (Hb), pack cell volume (PCV) and total plasma proteins (TPP). Hemoglobin concentration was determined using a modified cyanmethemoglobin method.

Values obtained from the repeatedly sampled 12 birds were analyzed using a "repeated measures" analysis of variance from SAS statistical procedures[7] with no grouping factor and one within factor time. Means for the dates were compared using "honestly significant difference" (HSD) test or Tukey's test.[7] All statements of significance are based on $P < 0.05$.

Results and Discussion

Results from the turkey breeder hens sampled repeatedly are summarized in Tables 1 and 2.

Red blood cells decreased in numbers from 28 weeks of age (prelit) to 30 weeks of age (lit) after the hens were transferred from the dark out house (6 hours light/day) into the laying house

(14 hours light/day), and two weeks before onset of egg production. The decrease in RBC at 30 and 32 weeks of age was significantly lower than at 36 weeks of age when the birds had been in egg production for 4 weeks. Packed cell volume (PCV) values changed significantly between age groups. Significantly lower PCV values were observed at 30 and 32 weeks of age compared to 28 weeks of age and increasing significantly at 36 weeks of age.

The lowered erythropoiesis observed during the 30 and 32 weeks of age, presumably could coincide with plasma estrogens that are at their highest concentration, as observed by Senior (1974) and Bacon et. al. (1980) for chickens and turkeys, respectively.

Mean values of RBS and PCV (Table 1) at the peak of egg production (36 weeks of age) are suggestive of a recuperating activity of the bone marrow in releasing erythrocytes into the blood stream.

Changes in hemoglobin concentration between age groups were insignificant (Table 1) although there was a faster rise in Hb from 30 to 32 weeks of age than observed from RBC and PCV. This could indicate an increased capacity of the bone marrow in incorporating Hb to the RBC, compensating for the lowered RBC at that time. This differs from what Paulsen et. al. (1950) described in a study with similar experimental design, where Hb values of turkey hens showed a distinct downward trend during the lay cycle.

The summary of erythrocyte indices are shown in Table 2. The indices are helpful in classifying certain anemias. They define the size and hemoglobin (Hb) content of the red blood cell (RBC) and the values are obtained from the RBC count, Hb concentration and PCV. The mean corpuscular volume (MCV) described the average volume of an individual erythrocyte in femtoliters. Mean corpuscular hemoglobin (MCH) describes the amount of Hb by weight in the average RBC in picograms. Mean corpuscular hemoglobin concentration (MCHC) characterizes the amount of Hb concentration in the average RBC in g/dl.

Changes in MCV were not significant between the age groups (Table 2). The sharp increase in MCV at 32 weeks of age helps explain the previous hypothesis of an increased capacity of the bone marrow in incorporating Hb at this particular time; there are fewer erythrocytes circulating therefore they are larger to keep carrying a normal supply of Hb.

The MCHC is probably the single most important hematologic index. It is used in the diagnosis of anemic conditions, since it reflects the capacity of the bone marrow to produce erythrocytes of normal size, metabolic capacity, and Hb content.[11] Changes in MCHC between the age groups were insignificant (Table 2). MCHC values remained fairly constant from 28 to 32 weeks of age because changes in PCV paralleled those observed in Hb. The more pronounced decrease was noticed at egg production and was due to an augment in PCV accompanied by a slight reduction of Hb.

Differences between mean values of age groups were significant for MCH (Table 2). The sharp decrease observed at the start of egg production (32 weeks of age), was caused by the return of Hb while RBC remained low.

The most noticeable change observed was in the total plasma protein (TTP) (Table 1). Total Plasma Protein values increased significantly during the light-stimulatory period and remained constant throughout the remainder of the study. The possibility of such increase due to hemoconcentration can be ruled out because of reductions in other hematologic parameters at 30 weeks of age. The most logical explanation to this abrupt increase arises from the effect produced by an elevated plasma estrogen concentration. Estrogens play a major role in growth and development of the ovarian follicle and possibly in the release of luteinizing hormone in mammals.[10] Endogenous estrogen is also responsible for the increase in plasma lipids, calcium, and proteins that occur during the time of sexual development, as well as for the increase in size and growth of the oviduct.[12]

Table 3 provides the mean hematologic values for the 22 hens at each of the four age groups during early egg production as a reference. These values closely parallel the 12 hens that were repeatedly bled through all four age groups.

Conclusion

It is unclear from the available references the values for hematologic parameters to determine the definition of an anemic state in adult hen turkeys. However, data observed in this study, particularly the values for MCHC, are suggestive of a normal healthy non-anemic status in turkey breeding hens during the early period of egg production.

It is also hoped that these values will be used as a baseline reference for future studies involving the hematology of the turkey.

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TABLE 1
Summary of hematologic values from turkey breeding hens sampled repeatedly in early egg production[1]

Age (Wks)	n	RBC ($x10^6$/ml)	Hb (g/dl)	PCV (%)	TPP (g/dl)
28	12	2.37±0.12[ab]	11.1±0.6[a]	39.5±2.0[a]	5.2±0.2[b]
30	12	2.25±0.10[b]	10.5±0.4[a]	37.3±1.4[b]	8.6±1.1[a]
32	12	2.26±0.11[b]	11.0±0.8[a]	38.8±2.4[ab]	8.4±2.4[a]
36	10[2]	2.39±0.16[a]	10.9±0.7[a]	40.2±3.4[a]	9.4±1.4[a]

[1]Values repesent the mean ± sd
[2]Two samples not evaluated due to clotting
[ab]Vertical means without a superscript in common are significantly different (P<.05)

TABLE 2
Summary of erythrocyte index values from turkey breeding hens sampled repeatedly in early egg production[1]

Age (Wks)	n	MCV (fl)	MCHC (g/dl)	MCH (pg)
28	12	166.6±7.4[a]	28.0±0.7[a]	46.7±1.8[ab]
30	12	165.6±4.4[a]	28.1±0.7[a]	46.5±1.4[ab]
32	12	171.7±5.4[a]	28.5±2.3[a]	48.8±3.2[a]
36	10[2]	167.8±8.4[a]	27.1±1.7[a]	45.4±2.4[b]

[1]Values represent the mean ± sd
[2]Two samples not evaluated due to clotting
[a,b]Vertical means without a superscript in common are significantly different (P<.05)

TABLE 3
Hematologic values observed in 22[1] turkey breeding hens sampled during early egg production[2]

	Units	Age (weeks) 28	30	32	36
RBC	(x10⁶/ml)	2.41±0.17	2.31±0.16	2.29±0.12	2.45±0.15
Hb	(g/dl)	11.2±0.7	10.6±0.5	11.2±0.8	11.1±0.9
PCV	(%)	40.0±2.4	37.5±1.9	39.2±2.7	41.6±3.1
MCV	(fl)	166.8±7.6	162.8±5.7	171.2±6.0	169.9±7.9
MCHC	(g/dl)	28.0±0.7	28.3±0.7	28.5±2.1	26.8±1.6
MCH	(pg)	46.7±1.9	46.0±1.7	48.8±3.4	45.4±2.5
TPP	(g/dl)	5.3±0.3	8.7±1.1	8.5±1.0	9.2±1.2

[1]Only 20 samples were analyzed at 36 weeks of age
[2]Values represent the mean ± sd

EXPERIENCES USING ELISA IN A RETICULOENDOTHELIOSIS OUTBREAK IN TURKEYS

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Reticuloendotheliosis (RE) is used to describe a complex of syndromes including acute reticulum cell neoplasia, a runting disease and a chronic neoplasia of lymphoid and other tissues. The etiological agent of RE is a type C retrovirus. Reticuloendotheliosis virus (REV) can naturally infect turkeys, chickens, ducks, geese, pheasants, quail and guinea keets.[4] REV was isolated in 1958[4] from a turkey with visceral lymphomas. The detection of antibody against REV in two turkey flocks was first reported by Purchase,[9] and indicated that this virus could be a potential cause of natural outbreaks in turkeys. The virus has been isolated from turkeys in a few natural outbreaks.[1,2,5,7,8,10,11]

REV is vertically transmitted in turkeys in small numbers mostly by breeders that suffer viremia and do not develop specific REV antibodies.[11] Poults infected as embryos or at 1 day of age by mechanical, horizontal exposure are the main source of REV in a flock. Therefore, eliminating viremic-antibody negative breeders from the flock is essential for the control of this disease.

In the summer of 1986, several turkeys showing lesions (lymphoid tumors) were submitted to the poultry diagnostic laboratory at New Bolton Center for diagnosis. Samples from these birds were sent to the Regional Poultry Research Laboratory (R.P.R.L.), E. Lansing, MI, where the diagnosis of RE was confirmed by virus isolation. Since that date, the farm experienced a syndrome characterized by acute diarrhea, runting, and mortality beginning the first or second week of age and persisting until the birds were 7 to 8 weeks of age. Around that age, the birds showed colibacillosis. Also, lesions from aspergillosis were constantly seen. Lymphoid tumors in the liver and spleen began to be noticeable around 12–14 weeks and mortality due to neoplasia persisted in commercial birds until they went to market at around 20–24 weeks of age. Neoplasia in breeders could be seen at a much older age. The mortality in infected flocks was as high as 30 percent and condemnation due to lymphoid tumors was as high as 5%.

In an attempt to learn more about the epidemiology, diagnosis and control of this disease, we have performed Enzyme linked immunosorbent assay (ELISA) to detect specific antibodies against REV[6] in commercial turkeys suffering from RE runting syndrome (3 different breeds that were progeny of infected breeder hens), commercial turkeys during a follow up for a vaccine trial, 20 week old commercial turkeys raised in the same pen that were progeny of exposed and non-exposed breeders, turkey breeders, 30–40 weeks of age, from the same farm, and artificially exposed turkey breeder hens and egg yolks from artificially and naturally exposed and non-exposed turkey breeder hens.

Commercial turkeys (0–20 weeks old) raised on the infected farm, from known exposed and non-exposed breeders, breeders from the infected farm, and eggs from artificially and naturally exposed and non-exposed turkey breeder hens, were used in these studies.

The Anti-REV ELISA kit was obtained from Agritech Systems, Inc., Portland, Maine.

Serum and egg yolk samples used in the AB ELISA were diluted at 1:500 in sample diluent, included in the kit, prior to testing. The criteria for considering the samples positive was a sample to positive ratio (s/p) > 0.5. Absorbances were measured at 490 nm.

The following observations are based on the results of testing different groups of birds on a single farm affected by RE and in a controlled experiment using the same breeds of birds from that specific farm. Results may vary from farm to farm depending on breed of bird, strain of REV, etc.

ELISA done on samples from 2 day old turkey poults (Table 2) or on egg yolks (Table 6) was useful in detecting REV maternal antibodies and gave an approximate estimation of the extent of horizontal exposure to REV in the breeders on this farm.

With a few exceptions, testing with ELISA between 2 and 9 weeks of age did not appear to detect many reactors for REV antibodies in birds that were suffering from RE runting syndrome (Table 1) or in birds that had been exposed to REV by contact exposure on this farm (Table 2). Most turkeys suffering from REV runting syndrome are probably viremic-antibody negative,

and therefore, could not be detected as reactors. Diagnosis of exposure to REV can only be accomplished by direct ELISA or virus isolation in this case. Horizontal transmission may have occurred slowly in the birds in the vaccine trial due to reduced numbers of virus shedders, lack of early mechanical transmission and/or improved biosecurity in that house.

Screening for REV antibodies using ELISA appeared to find more reactors in turkeys exposed to REV horizontally when testing at an age > 12 weeks (Table 2 and Table 3).

ELISA screening for REV antibody in breeders (Table 4) (30–40 weeks of age) found 91.3% reactors on this farm, thus, this test proved to be useful in determining the extent of horizontal exposure. Considering that most virus shedders are antibody negative, indirect detection of virus shedders among adult breeders by this test can be achieved without eliminating valuable, healthy birds, but only when the number of reactors is extremely high. Earlier research done on this farm by others showed that direct ELISA done on egg albumen proved to be the more consistent test for detecting RE virus shedders.[11]

Antibodies against REV are detected by ELISA between 11 and 21 days post-venereal exposure of a single dose of 40 µl of 10 plaque forming units of the CN22514 strain (R.P.D.L. East Lansing, MI) (Table 5).

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TABLE 1
ELISA for REV antibody of three different breeder progeny suffering RE runting syndrome

Age (Wks)	A Follow Up Breed 1	Breed 2	Breed 3
23	0/6[a]	0/5	0/4
30	0/4	0/5	1/5
37	0/3	0/5	1/4
44	0/3	2/2	0/4
51	0/3	0/3	1/4
58	2/3	0/4	0/4
66	1/4	0/4	2/4

[a]Number positive/total tested

TABLE 2
REV vaccine trial

Age	REV ELISA antibody test Control	% Positive	Once Vaccinated	% Positive	Twice Vaccinated	% Positive
2 Wk	1/25[a]	4.0	0/25	0	NT	NT
4 Wk	2/25	8.0	0/25	0	1/25	4
8 Wk	1/30	3.3	1/30	3.3	0/30	0
12 Wk	5/30	16.6	11/30	36.6	14/30	46.6
16 Wk	19/30	63.3	40/50[b]	80.0		

NT = Not tested
[a]Number positive/total sampled
[b]Vaccinated groups mixed
[c]Before vaccination, at 2 days of age, 82.5% of the birds tested positive with the REV ELISA kit

TABLE 3
Anti-REV ELISA (antibody)
Results on 20 week old commercial turkeys raised in the same pen and progeny from exposed and non-exposed breeders

Breeder Status for REV antibody	Positive/tested	% Positive
Negative	13/29	44.8
Positive	10/30	33.3

TABLE 4
Antibody ELISA done on turkey breeder hens and toms

Lot	Number Positive/Total Tested	% Positive	Mean Titer
1	183/188	97.3	10238
2	194/200	97.0	13685
3	263/269	97.7	12098
4	226/240	94.2	5728
5	135/179	75.4	3082
6	176/199	88.4	5703
7	235/278	84.5	4459
8	157/199	78.8	1657
9	80/117	68.4	1462
10	184/200	92.0	7868
11	262/272	96.3	7369
12	421/440	95.7	6399
13	353/360	98.0	5742
14	146/160	91.2	4674
Total	3015/3301	91.3	6440

TABLE 5
Anti-REV ELISA (antibody)

Results on adult turkey hens before and after venereal exposure to REV			
Days Post-exposure	Positive/tested	% Positive	Mean Titer
0	0/12	0	
11	0/12	0	
21	12/12	100.0	8687
32	12/12	100.0	7592
46	12/12	100.0	6182
55	9/11	81.8	5452

TABLE 6
Experiences using ELISA

Anti-REV ELISA (antibody) Results on egg yolk samples from artificially and naturally exposed and non-exposed turkey breeder hens		
Type of Exposure to REV	Status for REV ELISA antibody	Egg yolks Positive/Tested
Artificial	Positive	10/10
Natural	Positive and negative	7/12
None	Negative	0/10

PERIRENAL SUBCAPSULAR HEMORRHAGE IN TURKEYS

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Perirenal subcapsular hemorrhage (PSH) is also called "sudden death syndrome" and "hypertensive angiopathy." It is characterized by sudden death of large, rapidly growing and apparently healthy turkeys. PSH causes significant economic loss to commercial turkey operations; however, it occurs in backyard flocks and in small groups of research turkeys as well. Mortality up to 6% occurs usually in males at 8–13 weeks of age. PSH is primarily a problem of heavy and broiler turkeys. The most consistent lesion on postmortem examination is hemorrhage beneath the kidney capsule. However, the most significant lesion is pulmonary congestion and edema. Less consistent lesions are congestion of the liver and spleen and excessive amounts of mucus in the intestine. These lesions are seen by themselves in the majority of cases or in association with lesions of other conditions in a few cases. Histologically, angiopathy is inconsistently seen in turkeys with PSH and is also seen in apparently healthy turkeys.

PSH is primarily a problem of farms which market their turkeys between 22–29 lbs (age of 16–19 weeks) and is not a problem in farms marketing their turkeys between 11–17 lbs (age of 14–16 weeks). Average weight of turkeys at six weeks is higher in farms with PSH problems than in farms without it.

The incidence of PSH is not restricted to any feed source; however, it is higher in farms feeding high energy and protein diets. The majority of farms with PSH problems are semiconfined utilizing daylight and high intensity artificial light at night from day one until marketing. Birds in these farms are given a 24 hour free choice feeding. Farms with no PSH problem raise their turkeys in total confinement and utilize a "step up" lighting program. Feed intake on these farms is controlled because of this lighting program.

Because of excessive lighting, birds in barns with PSH problems are more active, could easily be excited, and have a high incidence of birds fighting. For this reason such barns tend to be dusty.

Further observation on mortality from PSH reveals that it starts 2–3 weeks after moving turkeys from brooding to growing houses at 5–7 weeks. Mortality is also noted if birds are fighting and is aggravated by hot and humid weather. Mortality from PSH occurs mostly early in the morning; however, occasional death occurs during the day as well. One farmer observed that mortality from PSH coincides with onset of sunrise. Another observed that turkeys dead from PSH are found in the brightest spots in the barn.

Factors such as temperature inside the barn, average number of turkeys in each barn, allocated space per bird, litter type, water or hatchery source and form of feed are seen with equal frequency on farms with PSH and without it. Presence or absence of difference antibiotics, coccidiostats and histiostats does not alter mortality from PSH. Various treatment regimens are tried; however, their significance in reducing mortality from PSH is questionable.

In conclusion, it seems that excessive and fast weight gain, a necessary economic strategy, is the major factor in this problem. Slowing down the growth rate of these birds may be the only way to prevent the occurrence of PSH at the present time.

A NATIONAL ANIMAL HEALTH MONITORING SYSTEM FOR MEAT TURKEY FLOCKS

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Introduction

The Veterinary Services section of the United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS) is in the process of establishing a National Animal Health Monitoring System (NAHMS) in the United States. The objective of NAHMS is to develop methods for accurately estimating the prevalence, incidence, trends, and economic impact of economically significant diseases and conditions of food-producing animals in the United States.[1]

In 1987, APHIS contracted with the Department of Epidemiology and Preventive Medicine (EPM), University of California, Davis, to develop a NAHMS program for turkeys based on the experience researchers in the EPM Department had gained from working with the California turkey industry, the USDA-APHIS Veterinary Services and the California Departments of Food and Agriculture and Fish and Game on the epidemiology and control of fowl cholera in turkeys. This report describes the NAHMS program that was developed for meat turkey flocks.

Materials and Methods

In order to estimate the level of various health parameters and their associated costs at the national and state levels, simple two-stage cluster sampling of the meat turkey population is used. From a list of all meat turkey premises in the state, a simple random sample of premises is drawn and for each of the premises selected, the flock placement schedule is obtained for the coming 12 months. One flock is then selected at random from the flocks to be placed on that premises.

For each flock in the sample, data are recorded on a weekly basis using a questionnaire. The data include clinical disease events, mortality, and disease prevention and control practices and their cost. Data on condemnations due to disease are obtained from slaughterhouse records. Blood samples are taken from 30 turkeys selected at random from each participating flock and tested for antibodies to various diseases. Each flock is bled at around the time that it leaves the brooder house and again towards the end of its life. At these same times and at one week-of-age, 8 dead turkeys are collected for pathological examination.

Data are entered into a database management package on a microcomputer for storage and analysis. To calculate standard errors of the population estimates for each of the variables of interest, standard statistical procedures are used.[1,2]

Cumulative reports pertaining to all flocks in the sample are compiled quarterly, while statewide estimates for the population are produced at the end of the sampling period. Information on individual flocks in the sample is produced for the company and premises management at the end of the flock's life and its performance is compared with that of the sample and population averages. No producer or company is individually identified on any report except for personal reports returned to the producer.

Data collected for a case-control study on fowl cholera were used to pre-test the NAHMS program. From May 1985 to April 1986, 49 flocks with fowl cholera (cases) and 43 flocks without fowl cholera (controls) were investigated for the case-control study. These flocks came from a population estimated at around 510 meat turkey flocks, placed on 148 premises in California and yielding a population of approximately 21 million turkeys. The NAHMS sample consisted of 27 control and 6 case flocks from 33 different premises. Blood samples were collected from 26 of the flocks. The control flocks were a random sample of the population while the 6 case flocks were selected at random from all the cases notified over the study period.

Results

All the premises in the NAHMS sample were located in the Central Valley of California. Eighteen (55%) of the premises had total confinement grow-out facilities, 10 (30%) had semi-confinement and 5 (15%) had range type facilities. Most of the premises (82%) held a maximum of 2 flocks at one time and placed an average of 3.5 flocks per year on the premises. The mean capacity in terms of total number of turkeys held on the premises at one time was 84,935 with a range of 20,000 to 250,000. The mean flock size was 41,000 turkeys with a range of 8,850 to 131,300.

The total mortality rate over the life of a flock was 10.4% in toms and 6.5% in hens to give an overall mortality rate of 8.6%. Colibacillosis was the most frequent disease seen in the flocks with 33% of flocks affected. Other diseases and conditions diagnosed in order of decreasing frequency were fowl cholera, salmonellosis, enteritis, leg weakness, coccidiosis, respiratory problems, hemorrhagic enteritis, staphylococcosis, aspergillosis and encephalomalacia.

None of the 26 flocks tested serologically had antibodies to *Mycoplasma gallisepticum*, *M. meleagridis*, *M. synoviae*, or avian influenza. Eleven flocks had titers to Newcastle disease virus. Two of these flocks had been vaccinated against Newcastle disease but 2 other flocks vaccinated against Newcastle disease did not show any serologic reaction. One flock had titers to Yucaipa virus.

Discussion

The purpose of NAHMS is to provide producers, veterinary practitioners, researchers, government agencies and importers of animal products with accurate documentation of the level and importance of animal diseases in the United States. To obtain accurate estimates of disease and their cost, a statistically valid system must be used for selecting the sample of flocks from the population.

In devising a sampling plan for meat turkey flocks, a number of factors peculiar to the industry had to be considered. Meat flocks have a lifespan of only 16 to 24 weeks, so each premises may raise several flocks during a year. As the incidence of disease may be seasonally influenced, it is important that data are collected over at least a full year. This means that flocks must be recruited into the program over a year, a condition fulfilled by the NAHMS sampling plan.

In drawing a sample from the population, it must be feasible to list all members of the population or, at least, the units in which they are clustered. To list all flocks to be placed in a state over a 12 month period was considered infeasible, so the premises became the enumeration unit. Flock placement schedules for the year must be obtained from each of the premises in the sample but this is much less work than obtaining them for the whole population.

The variables selected for inclusion in NAHMS had to satisfy the objectives of the program, be able to be measured reliably and be items producers were willing to supply. In poultry units, it is difficult to collect morbidity data on diseases; hence, emphasis has been placed on determining the incidence rate of diseases in flocks rather than in individual turkeys. Supporting data on the severity of diseases are provided by weekly mortality rates. As subclinical disease can also have a considerable impact on economic loss from disease, condemnation data from abattoirs have been incorporated into NAHMS. Serologic testing of turkey flocks is undertaken to gain information on exposure to disease agents, while pathological examination of dead turkeys enhances the quality of the clinical diagnosis of disease.

The feasibility of the NAHMS program was tested on data collected for the fowl cholera project. The turkey industry was able to supply values for all variables specified in NAHMS. However, economic data were not always complete, perhaps reflecting a reluctance of companies to divulge information believed to be of commercial sensitivity. Population estimates for California were determined for a number of factors in order to gauge whether a sample size of around 30 flocks would give reliable estimates. Many of the confidence intervals were large, so 30 flocks would be a minimum sample size for NAHMS in meat turkey flocks.

Based on the test run of NAHMS using data from the fowl cholera project, the NAHMS program described in this paper should be a feasible proposition and the information gained from it of use to all those involved with the turkey industry. A pilot trial of NAHMS in the field in California is now underway to further test the program.

Acknowledgment

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COMPUTER ASSISTED DIAGNOSIS AND INFORMATION MANAGEMENT PROGRAM FOR BIRDS—*CONSULTANT*

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CONSULTANT is a database for computer assisted diagnosis. The program was designed to provide differential diagnosis for clinical signs in birds and in other mammalian species. *CONSULTANT* is a user friendly program which has been used at the New York State College of Veterinary Medicine (NYSCVM), Cornell University for more than 4 years and is accessed by modem at 260 private practices and institutions in North America. Information on diseases and signs of birds has been recently added. The program was initially developed by creating an exhaustive and exclusive list of clinical signs used in veterinary medicine. A disease database was then developed by reviewing the veterinary literature. The user can search for a specific disease by entering a sign or group of signs or the name of the disease. Currently the program is loaded on NYSCVM computer and other 5 Veterinary Colleges in the US and Canada. The program on NYSCVM can be accessed by a modem from a remote location and it also can be provided for microcomputers for veterinary institutions.

MORTALITY AND DISEASE PATTERNS OBSERVED IN 76 REPLACEMENT PULLET FLOCKS IN CALIFORNIA FROM 0–20 WEEKS OF AGE

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Mortality in pullets can be a serious threat to the egg industry if it rises to such levels that forward planning is seriously hindered. Losses by death are commonly accepted as a necessary part of the cost of raising chickens. This is a preliminary report on a 5-year study on a pullet growing facility in California in which mortality (excluding culling losses) and disease patterns were observed in 76 replacement flocks from 0 to 20 weeks of age.

Material and Methods

Definition

For the purpose of this survey a flock means a group of White Leghorn day-old chicks, placed on a single pullet brooding facility within a few days, sharing the same air space in the same house, and marketed as a unit. An outbreak was tentatively identified by company representatives after an unusually high mortality rate (at least twice the expected rate) was accompanied by disease signs and/or lesions judged by the ranch manager and diagnosed by the California Veterinary Diagnostic Laboratory System (UCD), Turlock, California. An entire flock was considered affected by a disease if an outbreak was confirmed in one house.

Study Design

The population selected for the survey comprised 769,857 birds distributed as 76 flocks on one location under the same management and husbandry practices. Forty flocks were started in January and 36 in July during 1981–1985. The flocks arrived on the site within 3–5 days of each other in winter (January) and summer (July). Marek's disease vaccine was administered at the hatchery. All chicks were beak-trimmed and vaccinated under a standard schedule.

All the birds were housed in conventional open-sided buildings with curtains. All houses have concrete floors and new litter was used at each placement time. Brooding heat was supplied by gas hover brooders. An experienced and dedicated farm manager was responsible for the husbandry of the chicks during the five-year study period.

Data Collection

A questionnaire was designed to collect information on mortality, health, vaccination, medication, and body weights for each flock from company records during interviews with company representatives after the conclusion of each cycle. Flocks were identified for the purpose of examining daily/weekly mortality information.

Data Analysis

Statistical analysis of the data was performed using the MSTAT3 Statistical software (University of California, Davis, Calif.).

Results

Table 1 shows the number and size of flocks used in this study. January-hatched population consisted of 355,762 birds, with mean flock size being 9,880 (range = 5,616 to 15,329) birds. July-hatched population consisted of 414,185 birds, with mean flock size being 10,355 (range = 5,720 to 15,600) birds. Overall flock size ranged from 5,616 to 15,600 with mean flock size being 10,130.

Table 2 shows the mean weekly mortalities with the Standard Error of Mean (SEM) in the January- and July-hatched flocks and all the 76 flocks combined. In January-hatched flocks the mortality ranged from 2.1% to 9.57%, with an average of 4.61% (SEM±0.229). In July-hatched flocks the weekly mortality ranged from 1.69% to 11.04% with an average of 4.45% (SEM±0.336). The difference between January- and July-hatched were nonsignificant. Mean weekly mortality of all flocks ranged from 1.70% to 11.04% with mean being 4.53% (SEM±0.225). These mortality figures do not include culls, cockerels, or bleeders discarded. No relationships were found between flock size and mortality or hatch date and mortality.

Based on the laboratory reports, the total number of specimens submitted to the laboratory were 194, and 234 conditions diagnosed. Major diagnoses, expressed as a percentage of the total number of submissions made, are given in Table 3. Excluding those specimens for which the cause of death was not established, the principle

conditions encountered were coccidiosis (27.78%), Marek's diseases (19.66%), Staphylococcus infection (16.24%), ulcerative enteritis (11.11%), and Infectious bursal disease (6.41%).

Discussion

On the basis of weekly mortality, our data show a pattern of mortality with three peaks during a 20-week growing period. The first peak in mortality occurred during the first week in all the flocks studied. Early chick mortality was mostly associated with omphalitis, starveout, and malabsorption of yolk. The losses were much reduced during the second and third weeks. The second peak mortality was apparent during the 6th and 7th week and was mostly associated with coccidiosis and ulcerative enteritis. Heavy death losses were encountered in both the January- and July-hatched flocks.

The third peak in weekly mortality did not come in January- and July-hatched flocks at the same time as did the first two peaks. Instead, in January-hatched flocks the third sharp increase in mortality was evident during 11th week mostly due to Marek's disease and Staph infection. The July-hatched flocks reached their third peak in mortality in the 16th and 17th week associated with specific disease problems. Only limited comparison is possible with other published causes of mortality in pullets due to differences in record-keeping protocols, sampling techniques, system of husbandry, prevalence of diseases and types of stock.

The occurrence of coccidiosis in all flocks (27.78%) is of epidemiological interest and the fact that the condition tends to occur within certain time limits (see Table 9 and 10) may suggest that possibly continuous medication could be replaced by strategic therapy if and when the disease occurs. The possibility of drug resistance on the site has been considered in view of the recurrence of coccidiosis despite in-feed medication and (in winter) therapy. Although the losses from coccidiosis alone were not alarming, the secondary effects (ulcerative enteritis) must not be overlooked. Defective husbandry did not appear to play any part in the onset of coccidiosis.

All flocks had been vaccinated against Marek's disease, yet losses due to Marek's disease were sustained in vaccinated flocks showing gross neural lesions at postmortem examination. Losses from Marek's disease in vaccinated flocks may be of greater importance and need further investigation.

Staphylococcal infections in summer flocks were twice as much as in winter flocks (22.92% vs. 11.60%). Most necropsy reports suggested that an acute systemic infection had taken place and pathogens were recovered only from the joints. The comparison between winter and summer losses indicates that conditions such as coccidiosis, Marek's disease, Staph, ulcerative enteritis, and I.B.D. were more lethal in winter but the differences are not pronounced except ulcerative enteritis (18.12% in winter vs. 1.04% in summer). Conditions like Omphalitis, Colisepticemia, and riboflavin deficiency were diagnosed only in summer flocks.

TABLE 1
Number and Size of January- and July-hatched flocks Flocks

Description of flocks	Number of flocks	Number of birds			
		Min.	Max.	Sum	Mean
January-Hatched	36	5,616	15,329	355,672	9,880
July-Hatched	40	5,720	15,600	414,185	10,355
Both Combined	76	5,616	15,600	769,857	10,130

TABLE 2
Summary of Mean weekly mortality in replacement pullets

Age wks.	January-Hatched Mean(%)	±SEM	July-Hatched Mean(%)	±SEM	All flocks Mean(%)	±SEM
1	1.213	±0.140	0.999	±0.094	1.101	±0.083
2	0.358	±0.046	0.416	±0.040	0.389	±0.030
3	0.077	±0.015	0.045	±0.006	0.060	±0.008
4	0.039	±0.005	0.034	±0.005	0.037	±0.003
5	0.075	±0.011	0.072	±0.006	0.073	±0.006
6	0.513	±0.139	0.104	±0.009	0.298	±0.070
7	0.357	±0.104	0.108	±0.009	0.226	±0.051
8	0.169	±0.021	0.110	±0.015	0.138	±0.013
9	0.174	±0.017	0.180	±0.016	0.177	±0.012
10	0.181	±0.023	0.146	±0.015	0.163	±0.013
11	0.253	±0.039	0.199	±0.027	0.224	±0.023
12	0.313	±0.087	0.179	±0.017	0.242	±0.043
13	0.211	±0.023	0.157	±0.012	0.183	±0.013
14	0.233	±0.019	0.193	±0.016	0.212	±0.012
15	0.111	±0.018	0.090	±0.020	0.100	±0.014
16	0.118	±0.022	0.559	±0.185	0.350	±0.100
17	0.084	±0.013	0.527	±0.165	0.317	±0.090
18	0.045	±0.005	0.096	±0.016	0.072	±0.009
19	0.049	±0.006	0.070	±0.009	0.060	±0.006
20	0.057	±0.009	0.112	±0.018	0.086	±0.011
Total	4.614	±0.299	4.447	±0.336	4.526	±0.225

TABLE 3
Major diagnoses expressed as a percentage of the total number of specimens submitted in January- and July-hatched flocks

Conditions diagnosed	January-Hatched No.	%	July-Hatched No.	%	Combined No.	%
Coccidiosis	44	31.88	21	21.87	65	27.78
Marek's disease	31	22.46	15	15.62	46	19.66
Staph. inf.	16	11.60	22	22.92	38	16.24
Enteritis	25	18.12	1	1.04	26	11.11
I.B.D.	5	3.62	10	10.42	15	6.41
Omphalitis	–	–	10	10.42	10	4.27
Colisepticemia	–	–	10	10.42	10	4.27
Riboflavin def.	–	–	6	6.25	6	2.57
Others	17	12.32	1	1.04	18	7.69
Total	138	100.00	96	100.00	234	100.00

SEASONAL EFFECTS ON TABLE EGG LAYER PERFORMANCE

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The discovery of a poultry health or management problem is dependent upon the observation of flock conditions or performance departing from a level of "normalcy." Managers and their consultants must recognize the limits of normal conditions and be aware of the many factors which may alter expected performance.

It is obvious that consecutive flocks reared on the same farm, using what would appear to be similar management systems, fail to perform identically. Body weights for a given age fail to correspond; age at first egg and the age of the flocks' production peak are different; egg size fails to meet breeder standards; mortality is higher than normal and feed consumption appears to be low.

Most of us recognize that some degree of variability is expected, but a point may be reached when sub-par performance is not acceptable and the manager begins to look at the feed formula, the health status of the flock or to other possible causes. One of these other causes may be nothing more than the seasonal effects that reoccur with every flock with a similar hatch date.

The two environmental factors which have the greatest effect on performance are temperature and lighting. Temperature effects are well documented and may result in death or nutrient restriction due to low consumption. Temperature must be evaluated in terms of absolute temperature highs and lows, duration, night and day differences, acclimatization of the flock and relative humidity.

Lighting is the second environmental factor which can have a major effect on a flock's performance. Lighting programs are designed to give flocks sufficient hours and intensity with a pattern of light (change or constant) associated with the rearing or laying period requirements. Even though most poultry producers provide their replacement flocks with "step-down" or constant light patterns, flocks maturing in the spring and early summer months commonly begin egg production too early and this results in an increased amount of reproductive problems in the early stages of lay.

Body weight is strongly affected by the season in which the birds are raised. April to July hatches commonly weigh 5% to 10% below breeder standards as they are reared during the hot summer months. On the other hand, pullets hatched in October to January are commonly 5% to 10% above breeder standards.

Much of this variation in body weight can be avoided with the use of controlled environment housing. Temperatures are more uniform, feed consumption is less affected and body weights are more uniform from group to group.

Fall hatches are commonly associated with higher levels of mortality between 20 and 40 weeks of age. In some strains of birds, weekly rates may reach levels twice as high as for spring-hatched flocks. This problem requires specific strain-associated programs of light control and beak trimming if mortality is to be curbed. Such strains should definitely not be encouraged into early egg production.

Egg production rates are also affected by the season. The highest rate of lay to 60 weeks of age is associated with fall hatches. The peak rate of lay is 2% to 3% higher in fall hatches. Flocks hatched in February and September have a five to six egg per henhoused advantage over April to June hatched pullets to 60 weeks of age.

At specific ages, egg production may be from 2% to 4% different between months with the highest production, usually in the spring and the lowest production in the summer and winter. Molted hens usually perform poorest during the winter and the best in the spring and early summer. Differences may be as much as 5% to 9%. The highest rate of lay is associated with non-summer molting dates.

Egg size is affected by the season in two ways—the date of pullet housing in the lay house and the date in which the eggs are laid. The largest eggs are produced from hens housed in April and weighed in February. The smallest eggs are produced from hens housed in October and weighed in August. These dates coincide with the dates producing the largest and smallest 18 week pullets.

April housed pullets will lay eggs weighing .50 kg (1.1 lbs.) more per case than pullets housed in October. This makes a significant dif-

ference in egg mass in favor of April housed flocks.

On the average, spring hatched flocks will consume approximately 3% more feed than fall hatched flocks to 60 weeks of age. Winter feed consumption is 10% higher than summer. Housing temperature and dietary energy levels play an important role in moderating these differences.

In summary, performance is significantly affected by the season of hatch and the season in which performance is observed. Controlled environment housing is the best way of keeping these effects to a minimum. This requires both temperature and light control. Management must recognize the magnitude of these naturally occurring events and endeavor to reduce them. Temperature and light caused problems should not be confused with problems caused by disease.

TABLE 1
Effect of hatch date on 18 week body weight
(% of annual average weight)

Jan	Feb	Mar	Apr	May	Jun	Jul	Avg	Sep	Oct	Nov	Dec	Avg
103	99	99	95	97	101	90	100	107	102	106	107	100

113 flocks

TABLE 2
Effect of season of hatch on mortality rates
(% per week)

Age in Weeks	500 Flocks All Strains		101 Flocks Strain A		37 Flocks Strain B	
	Spring	Fall	Spring	Fall	Spring	Fall
21–25	0.232	0.339	0.224	0.245	0.225	0.246
26–30	0.286	0.414	0.291	0.403	0.221	0.202
31–35	0.308	0.384	0.240	0.455	0.203	0.242
36–40	0.298	0.329	0.288	0.335	0.237	0.230
41–45	0.285	0.272	0.265	0.269	0.257	0.231
46–50	0.262	0.226	0.220	0.217	0.227	0.225
51–55	0.287	0.220	0.230	0.202	0.283	0.239
56–60	0.293	0.244	0.242	0.199	0.381	0.295
Avg.	0.281	0.303	0.256	0.293	0.254	0.251

TABLE 3
Effect of season of hatch on rate of lay by age
(% hen-day egg production)

Age in weeks	Season of Hatch	
	Spring	Fall
21–25	18.6	22.9
26–30	71.9	75.3
31–35	83.4	83.2
36–40	81.5	82.0
41–45	78.8	79.6
46–50	76.1	77.2
51–55	73.1	74.0
56–60	70.4	69.8
Avg.	69.2	70.5

500 flocks

TABLE 4
Hen-day peak %, 20 to 60 week hen-day & and hen-housed egg production by month of hatch

Month of Hatch	Hen-day Egg Production		Hen-housed Eggs
	4 week peak	20–60 weeks	20–60 weeks
Jan	85.7	75.9	206
Feb	87.0	76.8	208
Mar	88.4	76.5	206
Apr	87.1	75.9	203
May	87.3	74.1	202
Jun	88.3	75.8	203
Jul	88.4	76.4	206
Aug	88.4	76.9	205
Sep	88.6	77.5	208
Oct	88.4	77.2	207
Nov	87.2	76.4	206
Dec	85.9	75.0	206
Average	87.6	76.2	206

487 flocks

TABLE 5
Hen-day % production at various ages relative to the month of the year

Age Weeks	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Avg
						First Cycle							
30	87	88	88	88	87	88	86	86	86	87	86	87	87
40	83	82	84	86	84	84	84	84	83	84	84	84	84
50	77	77	78	77	79	78	77	77	78	79	79	78	78
60	72	72	72	73	72	69	72	69	69	71	71	71	71
						Second Cycle							
Weeks into Cycle													
10	74	73	73	72	78	74	69	74	71	73	76	72	73
20	75	77	78	77	77	74	74	75	74	72	74	73	75
30	60	59	59	58	63	66	65	63	64	63	62	61	62
40	60	59	59	58	63	66	65	63	64	63	62	61	62

Number of flocks: 1st Cycle (866), 2nd Cycle (593)

TABLE 6
The effect of season on molt response (hen-day production %)

Weeks	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Avg
						Month of Molt							
Peak	81	81	80	80	80	79	78	79	80	81	81	80	80
+30	71	69	71	69	67	66	65	67	70	72	73	70	69
+40	64	60	62	60	59	60	59	64	64	65	63	63	62

887 flocks

TABLE 7
The effect of season of housing on egg weight (kg per case of 30 dozen eggs)

Month Housed	25	30	35	40	45	50	55	60	65	70	Avg
						Age (weeks)					
Apr	18.0	19.5	20.6	21.4	22.0	22.5	22.9	23.1	23.1	23.1	21.6
Oct	17.5	19.5	20.6	21.4	21.7	21.9	22.0	22.0	22.1	22.3	21.1
Year	17.8	19.5	20.6	21.4	21.9	22.2	22.5	22.6	22.7	22.7	21.5

TABLE 8
The effect of season of hatch on feed consumption relative to age (grams per day)

Season of Hatch	21/25	26/30	31/35	36/40	41/45	46/50	51/55	56/60	Avg
					Age (weeks)				
Spring	78	99	109	111	111	111	109	105	108
Fall	84	100	105	103	104	106	109	110	105
Year	81	99	107	108	108	108	108	107	107

407 flocks

85 YEAR HISTORY OF THE CALIFORNIA VETERINARY DIAGNOSTIC LABORATORY SYSTEM (CVDLS), 1903–1988, PARTS 1 & 2

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Part 1

Bryan Mayeda

Even though I was part of CVDLS for a 36 year tenure, 1949–1985, a gratifying 12 hour daily endeavor, I did not have the depth of appreciation for all that my supervisors and other predecessors had accomplished—and what a unique opportunity we have in life as diagnostic laboratory veterinarians, until I had devoted 125 hours (mainly in September '88 in between farm calls) into this fascinating and highly rewarding history project assignment by UC Davis. Perhaps it is "The years teach much which the days never know!"

This is a story of a small governmental unit, created by the needs of California's agricultural animal industries, and which responded to disease crises with efficiency and dedication—and in concert with regulatory veterinarians, research scientists and veterinarians, and private veterinarians and their staffs, preserved this important segment of California's economy and its public health.

For this proceedings, a capsule history of each laboratory will be presented in the chronologic order of its establishment.

Petaluma Laboratory

In 1898, poultry growers of Petaluma had sought aid from the state for their disease problems. Five years later the state appropriated $5,000 to create a poultry disease experiment station at Petaluma for UC Berkeley. Drs. A. R. Ward and V. A. Moore commenced the first veterinary work of the UC agricultural experiment station at this facility. Dr. Ward was a pioneer worker in the problem of fowl pox. The poultry disease station was closed in 1909. In 1914, Dr. J. R. Beach was hired as the first avian pathologist at a major U.S. institution of higher learning at UC Berkeley. Dr. Beach singlehandedly served the veterinary needs of the burgeoning California poultry industry until 1921, when he acquired his first assistant, Dr. C. Carpenter. Dr. Carpenter established his base of operations in a remodeled private residence at 627 F Street. He served as a one-man diagnostic laboratory and field veterinarian until 1923. Dr. D. Davis succeeded him until 1926, when he resigned to start the unique Chicken Pharmacy in Petaluma. Dr. G. Kernahan then occupied the position until 1930 when the facility was closed. Later that year, Dr. H. A. Hoffman was hired to reopen the laboratory. In 1931, the administrative control of this laboratory and the poultry laboratory at Los Angeles were placed in the California Department of Agriculture (CDA) to allow for UC to concentrate upon pressing disease research needs. In 1937, Dr. Hoffman was transferred to the Sacramento Laboratory and Dr. D. E. Stover took charge of this laboratory until 1944, when he was selected to be the first statewide chief poultry regulatory veterinarian. Dr. D. Splaver succeeded him for about a year and Dr. W. H. Armstrong took charge of the facility, 1945–1955. During Dr. Armstrong's tenure, the laboratory was relocated to its new, $265,000, 10,000 square feet quarters at 1500 Petaluma Boulevard South (near U.S. 101), which upgrading was mandated by a 1946 legislative enactment. Dr. D. L. Bristow then served as veterinarian in charge, 1955–1960. Dr. R. V. Lewis succeeded him for about a year and then Dr. E. L. Tieken took charge of the facility, 1962–1981. Dr. R. H. Hull then served as laboratory chief for about a year.

I had a thrill and pleasure of leading a fine team of 14 persons at this facility from September of 1983 until my retirement in October of 1985. Dr. B. C. Barr succeeded me in December of 1985 and served as laboratory director until September of 1988—when this historic laboratory was permanently closed.

Sacramento Laboratory

State Veterinarian Dr. C. Keane's lobbying for a general veterinary diagnostic laboratory at Sacramento finally bore fruit in 1917 when Dr. J. J. Frey was hired as a part-time laboratory diag-

nostician. His laboratory was in rented quarters at 9th & K Streets. In 1919 when the California Department of Agriculture was organized, the State Veterinarian's Office became the Division of Animal Industry (DAI) and the Ramona Hotel at 11th & K Streets was leased as headquarters of CDA and Dr. Frey's laboratory was moved to the new site—where diagnostic tuberculin was manufactured and collaborative work was performed with Cutter Laboratories in the development of the first effective vaccine for anthrax. Late in 1919, Dr. Frey was promoted to assistant chief of DAI and Dr. S. O'Toole was hired as his successor. In 1927, the laboratory was relocated to a building at 13th & L Streets, jointly occupied by the dairy laboratory and Dr. R. Mills succeeded Dr. O'Toole as the part-time pathologist.

In 1929 the laboratory was moved to new quarters in the basement of State Office Building No. 1 (SOB–1) (now the Unruh Building) at 9th & L Streets adjacent to the State Capitol. In 1931, Dr. A. G. Gierke succeeded Dr. Mills and became the first full-time pathologist at this general laboratory. Dr. Gierke retired in 1937 and Dr. Hoffman, head of the Petaluma Laboratory since 1930, took charge of this facility. In 1944, due to overcrowding, a separate pullorum blood testing laboratory was created at 5136 Folsom Boulevard. Dr. Hoffman died in July of 1947 and recruitment of his successor finally occurred in December of 1948 with the appointment of Dr. P. D. DeLay, who had previously headed the San Diego Poultry Diagnostic Laboratory, 1938–1940; served in the U.S. Army, 1940–1946; headed the Petaluma Laboratory, early 1946; and served with the Department of Veterinary Science at UC Berkeley, middle 1946–1948.

I began my state service at this laboratory in October of 1949 and Dr. DeLay was my first professional job boss. I was a 27-year-old fresh out of veterinary school at Michigan State College and a brief stint at the U.S. Army Medical Field Service School at Fort Sam Houston, San Antonio, Texas. I have vivid memories of Dr. DeLay and I dragging a dead steer off a truck bed in full view of startled onlookers on the sidewalk of busy L Street and of receiving complaints from CDA executives about "secretary wilting" malodors in their quarters above our laboratory.

In the near 7-year tenure at this laboratory, Dr. DeLay had many irons in the fire. In addition to administering the main general laboratory (the second one was created in San Gabriel in late 1949), he had collaborated with UC research personnel and sharpened the state of knowledge of Bluetongue, leptospirosis, Q Fever and salmonelloses; he had served as president of the California Veterinary Medical Association in 1953; and had aided Dr. A. G. Boyd, assistant chief of DAI, in the planning and implementation of the first major upgrading of CVDLS, mandated by legislation enacted in 1946, with construction of 4 new general laboratories at San Gabriel, Fresno, Petaluma, and Sacramento to eventually replace the outmoded laboratories at Petaluma, Los Angeles, and Sacramento. Dr. DeLay left CDA in 1955 to head the USDA's European Mission for Research on Foot-and-Mouth Disease. He was immediately succeeded by Dr. T. W. Jackson. In May of 1956 we relocated to new quarters at 3290 Meadowview Road, 10 miles south of SOB–1, which cost $317,476 and provided 10,000 square feet of space—and was the first laboratory equipped with an overhead chain hoist for adult bovines and equines.

During Dr. Jackson's tenure as laboratory director, pioneer work in avian mycoplasma culturing, agglutination antigen manufacturing and serologic testing procedures were perfected at this laboratory for CVDLS, with the solid support of renowned avian mycoplasmologists Dr. H. E. Adler and Dr. R. Yamamoto of UC Davis. Dr. R. W. Mitchell initially headed this priority work and after he left state service, this effort became part of my responsibilities. Dr. Jackson retired in 1967 and was succeeded by Dr. R. V. Lewis, who had previously headed the Petaluma and San Gabriel laboratories at different times.

Dr. Lewis retired in 1985 after a long and solid career with CVDLS and Dr. N. N. Storm succeeded him for about a year. Dr. B. R. Charlton then succeeded Dr. Storm as the acting veterinarian in charge until this laboratory was permanently closed in August of 1988.

Southern California Laboratories at Los Angeles, San Gabriel, and San Bernardino

The first poultry diagnostic laboratory was built in Southern California at 1451 Mirasol Street, Los Angeles, and dedicated on April 12, 1927, as culmination of a broad based joint effort by the poultry industry of the 7 southern counties, CDA, UC, and Los Angeles County. The poultry industry provided the site and erected a 3-room structure at a cost of $4,000; UC provided $1,000 worth of modern equipment; and CDA and Los Angeles County the personnel. Dr. H. W. Graybill, formerly of Rockefeller Institute, was appointed as a the first laboratory director.

The need for a Southern California laboratory had become increasingly apparent with the burgeoning growth of the poultry industry in that region and difficulties attendant in obtaining reliable veterinary help—due to the great dis-

tances to the laboratories at UC Berkeley and Petaluma.

The *Los Angeles Record* of October 27, 1927 reported: "More than 1,000 birds have been examined at Dr. Graybill's laboratory in the past 6 months and 25 diseases or conditions have been diagnosed in the modern, well equipped plant on Mirasol Street. Right now the laboratory is serving 110 localities extending from Santa Cruz on the north to San Diego on the south."

In 1936 Dr. Graybill retired and Dr. E. E. Jones, who had been an associate veterinarian at the laboratory since its inception, took charge of the facility. In 1940 a 960 square foot addition was constructed to the laboratory to accommodate a steady rise in work volume.

In December 1949 the laboratory was relocated to a new, 11,000 square foot quarters, built at a cost of $200,000 at 714 South Santa Anita Street in San Gabriel. This laboratory was the first of four laboratories authorized for construction by the 1946 legislative enactment to upgrade CVDLS. I received a most fortunate 10 months assignment at Dr. Jones' laboratory in 1950, which at that time had a staff of 15.

In 1961, Dr. Jones, who had served at the Los Angeles and San Gabriel laboratories for 34 years—25 years as head veterinarian, retired. In addition to being an outstanding laboratory director and poultry pathologist, Dr. Jones became prominent in the California Veterinary Medical Association and served as its president in 1939.

Dr. Lewis succeeded him as veterinarian in charge, 1962–1967; after Dr. Lewis' transfer to Sacramento, Dr. M. L. Murdock took charge of the facility, 1967–1971; after Dr. Murdock retired due to illness, Dr. J. S. Orsborn became head of the laboratory, 1972–1977; and with Dr. Orsborn's promotion to chief of Agricultural Veterinary Laboratory Services (a bureau of DAI) in November of 1977, Dr. R. S. Cooper then took charge of the lab.

The devastating outbreak of Velogenic Viscerotropic Newcastle Disease (VVND) which was first detected at the San Gabriel Laboratory in late 1971—resulted in a military type response by CDA and USDA to eradicate this highly lethal threat to the North American poultry industry. Dr. Orsborn was in charge of this laboratory, which had shouldered the bulk of the mountainous amount of diagnostic work generated by the first major outbreak of VVND in the U.S., during the entire period of this pandemic, which finally dissipated in late 1973, after destruction of 12 million birds (mainly chickens) and a total governmental expenditure of $65 million.

In September of 1981, the San Gabriel Laboratory was closed, after nearly 32 years of use as the principal CDA facility in Southern California, and operations moved to new, state-of-the-art, 15,000 square feet, $1,910,000 quarters in San Bernardino at 105 West Central Avenue on the Orange Show Fairgrounds. Dr. Cooper's administration of this newest CVDLS facility was distinguished with efficient isolation of Salmonellas from raw milk and Listeria monocytogenes from cheese—which zoonoses grabbed headlines, and impressive computerization of laboratory communications.

Dr. Cooper retired in 1987 after a solid 25-year tenure in CVDLS and Dr. H. Kinde took charge of this laboratory as the acting director.

San Diego Laboratory

In 1933, a CDA poultry diagnostic laboratory was opened at San Diego and functioned for 9 months, with Dr. C. Wallen in charge, and was then closed for lack of funds. In 1934, it was reopened in new quarters furnished by the San Diego Zoological Society in Balboa Park. Later that year Dr. Wallen resigned and Dr. Stover took charge of the facility until 1937, when he was transferred to Petaluma to head that station, and Dr. O'Toole was appointed as head of laboratory. With Dr. O'Toole's death in 1938, Dr. DeLay then took charge of the laboratory until he entered the U.S. Army in 1940. It was during Dr. DeLay's tenure that a large scale pullorum testing program was conducted with the turkey industry in the region, in collaboration with Dr. W. R. Hinshaw and Dr. E. McNeil of UC Davis. Due to a hiatus in records, it is not clear when Dr. H. Roberts took charge of the facility but he was in charge when the reins of this laboratory passed from CDA to the County of San Diego in July of 1947, and this unit became part of the San Diego County Livestock Department. Dr. Roberts died in July of 1948 and Dr. R. McFarland then filled the void for a half year period prior to Dr. E. R. Quortrup being appointed as county veterinarian in January 1949. At that time Dr. K. Schaaf headed the diagnostic laboratory, which had earlier accepted livestock accessions along with poultry work.

Dr. H. C. Johnstone joined the laboratory in 1961 and eventually took charge of the facility and later in July of 1968 was named as the San Diego County Veterinarian when Dr. Quortrup retired, following a distinguished career with the county. In 1963 this laboratory was relocated to its new 3,472 square feet quarters at 5555 Overland Avenue. In 1964 Dr. D. J. Thackrey and

Dr. Johnstone sharpened the knowledge of the diagnostics of avian vibrionic hepatitis. In 1965, Dr. Johnstone and Dr. J. D. Russell were the first to describe fowl pox as a cause of hemorrhagic tracheitis of chickens, which resembled the windpipe pathology of infectious laryngotracheitis.

During the 1966–1969 explosive outbreak of rabies at the southern border of the state with Mexico, this laboratory provided the critical diagnostic services which resulted in 119 positive cases within this time period.

Dr. K. Mahoney joined the laboratory as veterinary pathologist in 1977 and in that year Drs. Mahoney and Johnstone received national attention with their pioneering determination of the lethality for equines which had accidentally ingested cattle feed containing a growth enhancing antibiotic called monensin (also used as a common coccidiostat in chicken feeds). Dr. Mahoney is the current head of this laboratory.

San Joaquin Valley Laboratory at Turlock

The first CDA laboratory to be built in the San Joaquin Valley was opened for business in December of 1948, with Dr. W. W. Worcester in charge. This laboratory, like its counterparts at Los Angeles and Petaluma, was exclusively for poultry diagnostic work. This station was created by an appropriation of $20,000 provided by a 1947 legislative enactment. The laboratory is a 2,200 square foot remodeled Army barracks building hauled to the northern end of the Stanislaus District Fairgrounds. About half of the appropriation was spent for laboratory equipment. Dr. Worcester left this laboratory in 1951 to take charge of the new general diagnostic laboratory at Fresno. He was succeeded by Dr. S. Jamison.

On October of 1958 the facility was relocated to a new 2,800 square foot laboratory building constructed on the same site. Dr. Jamison resigned from state service in 1961 and Dr. Bristow took charge of the station. Dr. Bristow retired in 1983 and was succeeded by Dr. A. A. Bickford.

San Joaquin Valley Laboratory at Fresno

At a cost of $215,000, the CDA constructed a general regional diagnostic laboratory at 2789 S. Orange Avenue in Fresno, and it was opened for business in October of 1951, with Dr. Worcester in charge. This 11,000 square foot facility was the first diagnostic laboratory to be built in the lower San Joaquin Valley. At the opening ceremonies, Mr. M. Reiman, turkey grower of Planada, pointed out that the largest turkey producing area in the world is located here in the San Joaquin Valley with 4–5 million turkeys being raised within the area served by this laboratory.

The 1956 annual report of the laboratory described the increasing incidence of infectious bovine rhinotracheitis (IBR). At that time, a virus had been described in the literature as the cause of this respiratory disease in cattle. In 1959, through means of a bovine kidney cell-line monolayer tissue culture method introduced to the Fresno personnel by Dr. S. H. Madin (professor, UC Berkeley), this laboratory, under the leadership of Dr. G. N. Lukas and Shirley Wiedenbach, was able to routinely isolate and identify the IBR virus.

In February 1955 Dr. Worcester was promoted to be supervisor of CVDLS and Dr. Lukas took charge of the laboratory. Dr. Worcester's tenure as chief of CVDLS was distinguished with upgrading of capability in diagnosis of mycoplasmosea, chlamydiosis, IBR, and parainfluenza. He retired in 1977 and was succeeded as chief of CVDLS by Dr. Orsborn.

In 1964 the laboratory was remodeled to contain an isolated virologic unit to handle the burgeoning need for virology identification at all CVDLS laboratories. The 1972 annual report showed analysis of 40,000 tissue specimens, 125,000 diagnostic serological tests, and 155,000 program poultry blood tests. And out of 3,260 virus isolation attempts, 1,113 virus isolations were made, with 1,105 being viruses of IBR and Parainfluenza.

Dr. Lukas retired in 1978 after a long and distinguished career with CVDLS and was succeeded by Dr. M. Inverso. Dr. Inverso retired in 1983. Dr. Orsborn, although headquartered at Sacramento as chief of CVDLS, also served as acting laboratory director, 1983–1987, until Dr. Bickford was appointed as director of this laboratory—as an additional responsibility to his being the Turlock laboratory director.

Southern California Laboratory at Lancaster

The dedication ceremony for the $22,000 poultry laboratory at Lancaster in northern Los Angeles County was held in April 1955. This was the sixth new laboratory to be constructed in CDA's high priority post-war laboratory building program. A newspaper article reported: "Dr. Armstrong, formerly head of the Petaluma Laboratory, heads this laboratory, restricted to diagnosis of poultry diseases. The Lancaster area, which borders the southern boundary of Edwards AFB, has about 50,000 layer chickens, 400,000 turkeys, and a fryer chicken industry which produced around 12 million birds annually. The value of poultry and poultry products produced in

the area was estimated at about $16 million annually. The laboratory is located on the fairgrounds of the Antelope Valley Fair and Alfalfa Festival."

After serving at this facility for a time, Dr. Armstrong resigned from state service and Dr. R. T. Coffland took charge of this facility, until it was permanently closed in July 1968, due to a decline of more than 50% in work volume.

Part 2
The Restructured California Veterinary Diagnostic Laboratory System

Arthur A. Bickford

My arrival on the diagnostic laboratory scene on August 1, 1984 followed almost 5 years of intense study and planning directed toward upgrading veterinary diagnostic laboratory service in California. I was not involved in the evaluative and planning process and, therefore, cannot do justice to summarizing the extensive deliberations and exhaustive individual efforts devoted to defining future directions for the laboratory system. In this presentation I will restrict comments to my perception of progress toward a restructured administration and numerous major upgrades in personnel, physical plant, equipment, instrumentation, and technical capabilities.

As a result of agreements between the University of California, Davis (UCD) and the California Dept. of Food and Agriculture (CDFA), momentous changes have occurred which include:

1. Transfer of direct administrative responsibility from the CDFA to the School of Veterinary Medicine, UCD.
2. Establishment of a Central Laboratory on the UCD campus and closure of the Petaluma and Sacramento Laboratories.
3. Extensive expansion of staffing and technical capability through addition of discipline specialists and development of state-of-the-art laboratories in the Central Laboratory at Davis.
4. Assumption of all support services (personnel, purchasing, computerized Management Information System, billing, etc.) by the School of Veterinary Medicine—UCD.
5. Refurbishing and upgraded staffing plans for existing Branch Laboratories in San Bernardino, Fresno and Turlock.
6. Establishment of a mammalian diagnostic laboratory as part of the UCD Food Animal Teaching and Research Center in Tulare.
7. Establishment of a livestock industry advisory board to promote ongoing communication between the laboratory system and its clientele.

STATUS OF THE 1805 STRAIN OF *MYCOPLASMA IOWAE* (K COMPONENT OF SEROVAR I,J,K,N,Q,R) AND THE SA STRAIN OF *M. GALLOPAVONIS* (SEROVAR F)[1]

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Summary

The study reconfirmed the taxonomic position of two well-established strains of avian mycoplasmas, the 1805 strain of *M. iowae* and the SA strain of *M. gallopavonis*. It re-emphasized the long-recognized problems involved in working with and maintaining pure cultures of avian mycoplasmas.

Introduction

While early studies had established the identity of two avian mycoplasma strains, 1805 and SA, as members of the species *M. iowae* and *M. gallopavonis*, respectively, more recent reports have indicated that both strains were members of the species *M. gallinaceum*. In order to clarify these divergent findings, we examined three seeds of strain 1805 and two of strain SA from various sources. Following purification by filtration-cloning, the cultures were characterized by various biochemical and serologic tests and by SDS–PAGE.

Results and Discussion

Of the three seeds of strain 1805, one was found to contain a mixture of *M. gallinaceum* and *M. iowae* whereas the other two were pure cultures, respectively, of *M. iowae* and *M. gallinaceum*.

One of the seeds of strain SA was identified as *M. gallinaceum* whereas the other was found to be *M. gallopavonis*. The latter seed was from an original culture that we had maintained in our laboratory since initial isolation in 1958.

Some problems mycoplasmologists have encountered in the isolation, purification and maintenance of pure cultures of mycoplasmas are as follows. It is not unusual to isolate more than one mycoplasma species on primary cultures from field specimens. Despite picking of "single colonies" for transfer, the culture often remains "mixed." In mixed cultures, the faster growing nonpathogenic species outgrow the pathogenic species. It is also possible for a pure culture to become contaminated with another mycoplasma during serial passages. Mixed cultures may give spurious results in biochemical and serologic tests depending on their relative concentration in the culture. Finally, cultures may be mislabelled.

It appears that the confusion that has arisen regarding the taxonomic status of the 1805 strain of *M. iowae* and the SA strain of *M. gallopavonis* may be traced to one or more of the above problems. Thus, one of the seeds of 1805 that eventually was found to contain both *M. iowae* and *M. gallinaceum* gave a biochemical reaction pattern suggestive of *M. iowae;* however, the culture was negative by immunofluorescence using *M. iowae* immune serum, and the SDS–PAGE protein pattern was not typical of *M. iowae*. By growth inhibition, "breakthrough" colonies were observed which were suggestive of a mixed culture. *Mycoplasma iowae* and *M. gallinaceum* were separated from this culture only after careful cloning of the culture for a second time. Since several early studies indicated that strain 1805 was a member of *M. iowae*, it would appear that the *M. gallinaceum* that we have identified in two of three seeds of strain 1805 may have been a "contaminant." Likewise, *M. gallinaceum* seemed to be the "contaminant" in one of two SA seeds of *M. gallopavonis*.

However, with regards to the 1805 strain, it is possible that the original seed may have contained *M. gallinaceum*. Dr. Harry Yoder, who isolated this strain in 1964, described the culture as forming large colonies which is a characteristic of *M. gallinaceum*. In fact, it is the belief of Dr. Yoder that the original 1805 isolate was indeed *M. gallinaceum* and perhaps the *M. iowae* component was the "contaminant" (personal communication). This question cannot be unequivocally resolved without the availability of the original seed of 1805 as was the case for the SA strain.

Nevertheless, our study does point out that mixed cultures or even "displacement" of one species with another can occur in so-called established strains of avian mycoplasmas. The

study also emphasized the need to use more than one test to unequivocally prove the purity and identity of a mycoplasma culture.

The current classification of the avian mycoplasmas are presented in Table 1.

[1]This paper is taken from a manuscript that was accepted for publication in *Avian Diseases* (1989).

TABLE 1
Classification of avian mycoplasmas

Species	Serovar	Usual Host[a]	Biochemical reactions		
			Glucose	Arginine	Phosphate
M. gallisepticum	A	C/T	+	–	–
M. synoviae[b]	S	C/T	+	–	–
M. pullorum	C–O	C/T	+	–	–
M. gallinaceum	D–P	C/T	+	–	–
M. glycophilum	–	C/T	+	–	+/–
M. gallopavonis	F	T	+	–	–
M. gallinarum	B–M	C/T	–	+	–
M. iners	E–G	C/T	–	+	–
M. cloacale	–	C	–	+	–
M. anseris	–	G	–	+	–
M. meleagridis	H	T	–	+	+
M. iowae[c]	I–J–K–N–Q–R	T/C	+	+	–
M. lipofaciens	–	C	+	+	–
M. anatis	–	D	+	–	+
M. columborale	–	P	+	–	–
M. columbinum	–	P	–	+	–
M. columbinasale	L	P	–	+	+
Ureplasma gallorale[d]	–	C	–	–	+
Acholeplasma sp.[e]	–	C/T/D/G	+	–	+/–

[a]C = chickens; T = turkeys; D = ducks; P = pigeons, G = geese
[b]*M. synoviae* requires NAD and CO_2 for growth
[c]*M. iowae* grows in the presence of bile salts
[d]*U. gallorale* utilizes urea
[e]Several species of *Acholeplasma* have been isolated

DNA PROBES FOR EARLY DETECTION OF MYCOPLASMAS IN CHICKENS

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Introduction

The gene probe approach has been proposed as a possible solution in certain problematic areas of diagnosis in avian medicine. We have previously reported the selection and characterization of specific DNA probes for *Mycoplasma gallisepticum* (MG) and *M. synoviae* (MS).[1] Using dot-blot hybridization of tracheal swab suspensions from experimentally infected chickens, the MG DNA probe pMG4 detected as few as 105 mycoplasma organisms.[1] The probe enabled positive identification of MG at an early stage of infection, prior to the development of a serological response in the infected chicken.

The DNA probe pMG4 was also used for the detection of MG in tracheal swabs of breeder chickens and turkeys naturally infected under field conditions.[2] A major advantage of the method is the ability to obtain a positive answer in less than 4 days after sampling, even under conditions where serological diagnosis is ambiguous. Due to the importance of rapid diagnosis in order to confine infection and minimize the economic impact of an Mg outbreak, we have been using the method as a supplemental diagnostic tool in our MG control procedures in Israel. The reliability of the method has been repeatedly confirmed by comparison with currently recommended methods of diagnosis.[2]

This report relates to the application of the DNA probe method for diagnosis of MG infection in chicken broiler-breeder flocks prior to detection of serological reactions in the Rapid Slide Agglutination (RSA) screening test. These flocks were suspected of infection only due to their proximity to a flock undergoing an MG outbreak.

Methods

Specimens (approximately 20 samples per flock) were collected by swabbing the trachea with a dry cotton swab which was transported in an ice box to the laboratory and processed within several hours. The swabs were vigorously suspended in mycoplasma broth medium (0.6 ml), and kept on ice until tested. If not applied immediately to filters, samples were frozen at −70°C. Aliquots of 0.1 ml of the suspension of tracheal material were applied to a nitrocellulose filter (Scheichter & Schull) using a dot-blot apparatus. Standard methods for processing of filters and hybridization with the 32P-labeled probe pMG4 were previously described.[1]

Overnight autoradiography revealed the positive hybridization reactions when compared to positive and negative control spots containing MG and MS organisms respectively. Mycoplasma isolation and identification was carried out by standard methods, as described elsewhere.[3] The RSA test was carried out with commercial stained antigen (Intervet, Holland); hemagglutination inhibition (HI) was performed by standard methods,[4] using an antigen prepared from MG strain A5969.

Results and Discussion

Field Outbreak 1

Two flocks of 5 month old broiler-breeder chickens were maintained in adjacent units, separated by a two meter aisle. About two weeks after detection of positive reactions to MG in the RSA test, MG infection in the larger flock (9,500 birds) was confirmed by positive reactions with the DNA probe pMG4 and by diagnostically significant HI titers. Serological sampling of the adjacent flock (50 samples from 5,000 birds) showed no positive reactions in the RSA test, although a clear positive reaction was seen with the MG probe. With time in each flock, MG was isolated from about the same proportion of samples as were positive in the hybridization reaction, confirming the level of infection indicated by the DNA probe. Ten days later (at time of slaughter) only 3/25 samples showed a detectable RSA reaction.

Field Outbreak 2

At time of monthly serological screening, suspected positive reactions in the RSA test were detected in two out of 7 broiler breeder flocks (7 months old) located in conventional open buildings separated from each other by about 10 meters. Confirmation of the MG break was ob-

tained by HI results when tested 10 days later, at which time the DNA probe gave positive hybridization with tracheal swab suspensions from each bird tested. Probe hybridization of tracheal swab samples (12–16 each) of the other flocks in the complex indicated that MG infection was detectable at various levels in each one. However, serological results showed only 9/25 and 3/25 positive RSA reactions in two flocks, and no positive reactions in the other 3 infected flocks. Isolation of MG from each of the flocks supported results with the DNA probe.

The basis of the gene probe method of diagnosis is the specific reaction with DNA of the agent, unrelated to the serological response of the host. Thus, success is dependent in the first place on the number of organisms in the specimen tested. As has been shown in experimental infection models[1,2] tracheal levels of MG rapidly reach levels which are within the detection range of the MG probe. In the absence of interfering circumstances, isolation of MG is also readily accomplished at this stage of infection, as was found in these field outbreaks. However, the significant observation is that under field conditions the probe may detect MG in chickens 1–2 weeks before a serological response is detected. Rapid detection and elimination of infected flocks should be useful in confining the outbreak and minimizing losses.

Acknowledgment

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CHARACTERIZATION OF LOW VIRULENT *MYCOPLASMA GALLISEPTICUM* ISOLATES FROM COMMERCIAL TURKEYS

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Introduction

Mycoplasma gallisepticum (MG) infections of egg- and meat-type chickens that were difficult to diagnose based on expected clinical signs, routine culture procedures, and commonly used serologic tests have been described.[10] Isolates with these characteristics have been termed variant or atypical MGs.[5,10] Experimentally, these MG isolates resulted in infections of lower virulence, transmissibility, and serologic reactivity compared to typical field isolates.[5,6,10]

Infection of turkey flocks with atypical or variant MG has been suspected but infrequently documented.[4,8] Recently, two MGs (designated M876 and M35) were isolated by our laboratory from North Carolina market turkey flocks. Circumstances related to their isolation suggested they may be "atypical" MGs, and that they may have been transmitted from inapparently infected breeder turkeys. Herein we describe the clinical, cultural and serologic responses of turkeys exposed to these strains, compared to the virulent S6 reference strain; and attempts to further characterize these strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblots.

Materials and Methods

Mycoplasma gallisepticum Strains

Mycoplasma isolates, designated M876 and M35, were recovered from different flocks of young commercial turkeys exhibiting no signs of disease. These isolates were identified as MG by the direct fluorescent antibody (FA) test[7] with fluorescein-conjugated rabbit antisera kindly provided by Dr. S. Kleven (Department of Avian Medicine, University of Georgia, Athens, Georgia). MG isolates M876 and M35 were purified by filtration-cloning.[9] The S6 reference strain of MG was obtained from American Type Culture Collection (Rockville, Maryland).

Experimental Design

Day-old Large White female-line male turkeys (Nicholas Turkey Breeding Farms, Sonoma, California) were obtained from a National Poultry Improvement Plan monitored commercial hatchery and housed in isolation. Fifty 8-day-old poults were individually identified with numbered wingbands and randomly allocated to two groups of 25 in each of two trials. Trial 1 consisted of S6 and M876 exposure groups, and trial 2 consisted of M35 and sham-inoculated groups. At allocation all turkeys were cultured for mycoplasmas from choanal cleft swabs, and blood samples were taken for serology. Thirteen 21-day-old turkeys in each group were inoculated intranasally with approximately 10^8 cfu of MG strains S6, M876, or M35. Each inoculated group was moved into separate isolation rooms and placed in floor pens on wood shavings. The remaining uninoculated turkeys were maintained in brooders for 24 hours, and then placed in pens with the inoculated turkeys to serve as in-contact sentinels. Turkeys were observed daily, and sampled for mycoplasma culture and serology approximately every 2 weeks during each trial. Samples for mycoplasma culture were obtained from choanal cleft swabs. Sera were tested for MG antibodies by hemagglutination inhibition (HI) and ELISA (reagents for experimental use only provided by IDEXX, Corp., Portland, Maine).

Homologous and Heterologous ELISAs

ELISA methodology was adapted from previously established protocols,[1,2,7] using commercially available reagents (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland). Twenty-four hour broth cultures of MG strains S6 and M876 were used to prepare whole cell mycoplasma ELISA plate antigens. Optimal concentrations of ELISA reagents were determined by repeated checkerboard titrations using S6 antigen and serum pools from uninfected and S6-infected turkeys. Test samples consisted of S6- and M876-inoculated turkey sera from days 19, 62, and 95 PI. Absorbances (A) were determined spectrophotometrically (MR–700 Microplate Reader, Dynatech Laboratories Inc., Alexandria, Virginia). Corrected ELISA values were calculated

by subtracting the A values of background control wells from A values of test samples.

To examine the effects of antigen and serum source in homologous and heterologous ELISAs, analysis of variance was applied at sampling dates PI to ELISA values obtained using S6 and M876 antigens with sera from S6- and M876-inoculated turkeys. Split plot analysis of variance treating inoculum source as main plot factor with type of antigen in the plate as a subplot factor was used to test for main effects of inoculum source and an interaction between inoculum source and antigen. Data were analyzed using the SAS (SAS Institute Inc., Cary, North Carolina) statistical package. Significance levels of at most 0.05 were used throughout.

SDS-PAGE and Immunoblots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) analyses of mycoplasma proteins were performed in 10% resolving gels with 4% stacking gels. Ten μg of protein per well were run at 25 mA for approximately 1 hour and then at 35 mA for approximately 3 hours. Gels were stained with Coomassie Brilliant Blue.

For immunoblot (Western blot) assays, proteins were transferred to nitrocellulose, then strips were cut and blocked with gelatin solution. Strips were washed, reacted with primary antisera, washed, reacted with secondary antibody (horseradish peroxidase-conjugated goat anti-turkey IgG), and developed. Primary antisera were equivolume pools collected from uninfected turkeys, or turkeys inoculated intranasally with strains S6, M876, or M35.

Results and Discussion

MG was recovered on post-inoculation (PI) day 7 from 100% of the S6- inoculated turkeys and 92% of the M876-inoculated turkeys. However, culture positive rates at subsequent samplings declined more rapidly from M876-inoculated turkeys, and were much lower from M876 sentinels compared to S6 sentinels. Furthermore, only S6-inoculated turkeys showed clinical signs of MG infection. These results indicate that MG strain M876 was less pathogenic and transmissible than the S6 reference strain. MG was not recovered from turkeys in the M35 group and no antibody response was detected, indicating an apparent failure to initiate infection.

Serology indicated that ELISA was more sensitive than HI for antibody detection, and that both tests detected higher titers and seropositive rates in the S6 group compared to the M876 group. These results suggested that MG strain M876 was less immunogenic than S6, and/or that the serologic tests employed were less sensitive in detecting an antibody response to M876 compared to S6 infection.

Table 1 shows mean ELISA values of sera tested with S6 and M876 antigen-coated ELISA plates. Except for day 19 PI, statistical analyses indicated a significant main effect ($P < 0.05$) for serum source with mean ELISA values higher for sera from the S6-inoculated turkeys than M876-inoculated turkeys. Also, there was a significant interaction between serum source and type of antigen. This is evident in Table 1 where means were found to be greater for S6-inoculated turkey sera tested with S6 antigen (homologous test), than with M876 (heterologous test) antigen. Also, means for M876-inoculated turkey sera tested with M876 (homologous test) antigen were greater than those tested with S6 (heterologous test) antigen. These results confirmed that strain M876 was less immunogenic than S6, and that homologous ELISAs were more sensitive than heterologous.

SDS–PAGE analyses revealed that avian *Mycoplasma species (M. gallisepticum, M. synoviae,* and *M. meleagridis)* have distinct protein banding patterns at molecular weights less than 42 K, and that MG strains including M876 and M35 have similar patterns below 42 K, thus confirming their identification as MG. At molecular weights greater than 42 K, strains M876 and M35 had distinct protein banding patterns compared to each other, and the MG reference strain S6, the vaccine strain F, and the R strain.

Western blots showed that antisera from S6-infected turkeys recognized many proteins of strains S6, M876, and M35, indicating a high number of cross-reactive epitopes. Antisera from M876-infected turkeys recognized many proteins of strains S6 and M876, but relatively few M35 proteins. An increased understanding of the antigenic composition of MG strains is likely to be necessary for the development of improved serologic capabilities.

These studies add to the growing body of evidence that there are MG strain-related differences in infectivity, virulence, transmissibility, immunogenicity, and antigenicity which may present important problems to control and eradication programs in commercial turkey as well as other poultry production. Infections with less virulent and immunogenic MG strains may be especially difficult to detect and confirm based on expected clinical signs and serologic responses with commonly used tests. Continued study of factors responsible for MG strain-related vari-

ability will contribute to a greater understanding of MG pathophysiology and result in improved antigen and antibody detection systems.

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TABLE 1
Homologous and heterologous MG ELISA values of sera from turkeys inoculated with strains S6 and M876

Serum source[b]	ELISA plate antigen	Mean ELISA values[a] of test sera sampled on post-inoculation		
		Day 19	Day 62	Day 95
S6	S6	0.29(5)[a]	0.78(9)[a]	0.918[a]
S6	M876	0.19(5)[bc]	0.48(9)[b]	0.67(8)[b]
M876	S6	0.10(5)[c]	0.25(9)[c]	0.26(8)[c]
M876	M876	0.21(5)[ab]	0.48(9)[b]	0.58(8)[b]

[a]Mean of ELISA absorbances (number of sera tested). For each sampling date PI, means without a letter in common differ significantly ($P < 0.05$) using the least significant difference procedure

[b]Sera from turkeys inoculated intranasally at 3 weeks of age with approximately 10^8 cfu of MG strain S6 or M876

STUDIES WITH THE TS-11 VACCINE STRAIN OF *MYCOPLASMA GALLISEPTICUM*

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Strain ts-11 is a cloned chemically induced mutant culture of an Australian field isolate (80083) of *Mycoplasma gallisepticum* (MG). It was selected on the basis of its reduced ability to form colonies on agar at 39.5°C than its parent.

The safety and efficacy of ts-11 as a potential live vaccine strain have been investigated in pen trials and in the field. Strain ts-11 appears to be virtually apathogenic when inoculated directly into the air sacs of susceptible chickens or turkeys, or into the paranasal sinuses of susceptible turkeys. The vaccine has been used extensively in the field without obvious ill effect on production. The mechanism of the lack of pathogenicity is not known but is apparently not dependent on temperature sensitivity, since non temperature sensitive revertants selected in vitro were not pathogenic.

A significant safety feature of ts-11 is that horizontal transmission between chickens does not readily occur. Vaccination with ts-11 does not usually elicit a strong serum antibody response. However, vaccinated chickens were protected against the development of air sac lesions and loss of egg production after MG challenge. They were not protected against egg transmission of MG.

In the field, vaccinated layer flocks have produced up to 10 eggs/hen more than comparable non-vaccinated flocks. Restriction endonuclease analysis of the DNA extracted from about 30 Australian and American strains of MG has shown that ts-11 and its parent strain 80083 have banding patterns that distinguish them from all other strains tested. However, using the same technique it has not yet been possible to clearly distinguish ts-11 from its parent.

SALMONELLA ENTERITIDIS IN LAYERS—WHERE ARE WE—WHERE ARE WE GOING

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Salmonella enteritidis (SE) associated with Grade A shell eggs is of major public health concern. Although the problem appears to be more prevalent in the New England, Mid- and South Atlantic regions of the U.S., outbreaks have been increasingly reported from other regions. During each of the past 3 years, there have been about 30 confirmed outbreaks of *Salmonella enteritidis* (SE). During this 3 year period, there have been 14 deaths attributed to SE with 11 of these occurring in one nursing home. When compared to many other public health problems, SE might be considered as being relatively insignificant, but it is the perception rather than reality which becomes important. One need only to look at the recent SE scare in England where an ill-advised statement by a public official reportedly resulted in a 60% drop in egg consumption.

Although use of raw or partially uncooked product and improper product handling and/or food preparation is a significant factor in some outbreaks, a major responsibility for reducing this problem has been placed with the production segment of the poultry industry. At the present time, the owner of an infected flock has 2 options—depopulation or sending eggs to an egg breaking (pasteurization) plant. Either option can result in economic catastrophe.

The owners of these flocks have generally managed these flocks within established and acceptable industry standards. Having worked with this problem now for about 2 years, I am still uncertain as to whether the SE situation in implicated flocks has been significantly different from many other flocks or whether these flocks have been caught up in a process of random chance that resulted in part from the placement of a large number of eggs into the market place (large complexes) and sales to institutions and public facilities more likely to mix many eggs together and mishandle the product.

Salmonella enteritidis—the Organism

Salmonella enteritidis, as a paratyphoid type salmonella, can be found in many mammalian and avian species. Over the past 10 years, about 50% of the SE serotypes at the National Veterinary Services Laboratory were of mammalian and 50% of avian origin. Given the wide host range of SE, it will be important to determine if strains isolated from poultry differ in mode of transmission and pathogenicity and to develop a method of identifying such strains. Eradication of SE, as has been proposed by some, would seem an unrealistic goal.

Vertical Transmission

A possible explanation for the apparent increase in egg-associated SE problems is that SE has become a vertically transmitted disease. Vertical transmission would provide a potential source of infection for commercial flocks. In commercial egg-laying flocks, it could also provide a means to bypass the usual egg washing, sanitation, and grading procedures that have been effective in reducing the other paratyphoid type Salmonella shell contamination problems. Studies of experimental and naturally infected flocks have shown that some strains of SE are capable of causing ovarian infection. In some field cases, changes in individual ovum identical to those observed in pullorum disease have been present. In these discolored and often caseated ovum, SE can be isolated by direct culture. SE has also been isolated from normal ova, oviducts, albumin, egg yolks, and dead-in-shells.

Some epidemiological observations have cast doubt on the hypothesis that vertical transmission is the primary or only means by which flocks may become infected.

Sources of SE Infections

The evidence for potential vertical transmission has been discussed. Direct and indirect evidence would suggest that SE may have been present in at least 5 major breeds (strains). The significance of potential vertical transmission as an important source of infection for commercial egg flocks is uncertain. An intensive testing program to eliminate breeders as a potential source has been recommended and has been implemented in some states and by breeder organizations. In Pennsylvania, all breeder flocks are being tested serologically (300 at placement and 100

thereafter) and bacteriologically (dead-in-shells, litter, and nest box samples) each 90 days. The serological testing each 90 days is important to assist in detecting possible introduction of infection or an infection rate that may have been below detectable limits at time of placement.

Another source of potential infection for flocks is contaminated feed. Although SE has only rarely been found in feed, the limitations associated with frequency of testing and the isolation and identification of specific serotypes where multiple serotypes may be present, give one little confidence that feed could not serve as a potential source. Previously contaminated housing needs to be considered. SE has been shown to survive in the environment for at least 12 weeks. In England, the culturing of fan dust is reported to be an effective procedure. Salmonallae are resistant to drying. Rodents, wild birds and insects also are potential sources of SE. Some of these potential sources are being examined in current field research in Pennsylvania.

Prevalence of Flock Infections

With most potentially infectious organisms, the assumed prevalence rate depends on the intensity, methodology, and timing used in searching for the agent. The present limitations associated with both serological and bacteriological monitoring procedures, combined with an understandable reluctance to look because of the economic penalty extracted from a flock owner where an SE isolate is made, make any estimate of the true prevalence rate highly speculative. In Pennsylvania where monitoring activities over the past several years have included intensive testing of multiplier (parent) breeder flocks, serological and bacteriological testing of flocks involved in tracebacks of public health cases, bacterial culturing of pooled eggs submitted in conjunction with the avian influenza surveillance program (eggs are submitted monthly from each flock), flocks involved in research programs and cases submitted to the Diagnostic Laboratories, potential infections were found in 19 flocks. Pennsylvania has about 850 commercial egg laying flocks. In the New England and Mid- and South Atlantic regions, tracebacks or testing activity have also implicated flocks in 7 other states. In flocks confirmed to be positive (bacterial isolation), the serological positivity rate has ranged from 0.7–16%.

Clinical Syndrome

In general, there has been no clearly defined clinical problem in SE infected layer production, morbidity and mortality patterns. In one flock, a temporary drop in production (5%) and slight increase in mortality was thought to have been caused by SE. Ovum, similar to those seen in pullorum disease (discolored, caseated) were observed in 3 flocks. Other workers have reported production and mortality problems attributed to SE. In field problems the interacting complex of problems often make etiology specific diagnoses difficult. Limited experimental data suggest that infection could decrease egg production.

Egg Handling and SE

SE may enter the internal part of the egg either because of ovarian (yolk) or oviduct infection or by penetration from a contaminated shell. The significance of shell penetration is uncertain, but the risk is certainly increased when cracks are present. In 1981, the USDA Egg Products Inspection Act allowed for an increase in cracks from 5 to 7% in Grade A shell eggs. Shell quality standards were also relaxed and 1% dirties were allowed. Although these changes coincide with the beginning increase in SE infections in the Mid-Atlantic region, it is unlikely that they account for the increase in SE outbreaks because the effect should have been more national rather than regional in scope.

Several workers have also demonstrated the marked influence of temperature on SE multiplication in the egg. The FDA has indicated that they will publish requirements for the holding of retail eggs at 45°—the temperature required for other perishable foods. Most outbreaks of SE occur during the summer months. Warm temperatures may provide opportunities for SE to multiply in eggs during production, transport, storage or use.

Public Health Data Implications

Public health data suggests that the confirmed human infection rate has been about 5 times greater in the New England and Mid-Atlantic regions of the U.S. The New England rate appears to have been somewhat erratic but generally at this high level since 1973, and the rate in the Mid-Atlantic area began to increase in 1981–82. One inference that might be drawn from the public health data is that the SE responsible for outbreaks is not of recent origin. If this data is real, the reason(s) for such regional differences are uncertain. Various factors including the infection rate in supply flocks, different procedures in wholesale and retail egg handling and/or population differences in food preferences, work habits and even the human SE carrier status could be hypothesized.

Testing Procedures

At the present time, various serological blood tests, either singly or in combination, are being used to test flocks. These tests use either *S. pullorum* or *S. enteritidis* antigens. Any of the tests may be useful on a flock basis in selecting birds that should be examined bacteriologically. Little data is available on the sensitivity or specificity of these tests or how these may be influenced by stage of infection. In our experience, there was no advantage in using SE antigen over the available pullorum antigens. In plate agglutination testing using the Salsbury and Vineland polyvalent pullorum antigens, the Vineland antigen was less consistent in detecting potentially infected birds. In one series of 18 culturally positive birds, the serologic positivity rate for different tests was: Pullorum tube aggl 16/18; SE tube aggl 16/18; Salsbury plate aggl 15/18; Vineland plate aggl 7/18. On the other hand, the Salsbury antigen does detect more nonspecific reactions.

An ELISA test has been developed by Dr. Nagaraja (Minn.). This test may replace the tests presently in use.

There is some indication that potentially infected flocks might most efficiently be detected by culturing of yolk on egg belt collectors, manure, and dust.

Although culturing of as few as 10 eggs/month has occasionally detected an infected flock, the rate of egg infection in both experimental and natural infections is generally low. In repeated screening of several known positive flocks, the egg infection rate was less than 1 positive egg in 1,000 tested.

The infection rate is influenced by the stage of flock infection. There is some evidence that periods of stress might be followed by increased egg infection rates.

Vaccination

Work being conducted by Dr. Nagaraja (Minnesota) suggests that vaccination may be of value in reducing infection in exposed flocks.

The Model Control Program

Despite the dilemmas and uncertainties, the poultry industry was placed in the position of having to take action to try and reduce the potential for continued human outbreaks. In response to this need, the Salmonella Committee of the Northeast Conference of Avian Diseases developed a "Voluntary Model State Quality Assurance Program" for SE detection and control. The program has been revised several times and will continue to be modified as better information is gained. With minor modifications, the program has been endorsed by USDA–APHIS, CDC and FDA.

The major components of the Voluntary Model State Quality Assurance Program are: 1) Emphasis and initial testing activities are to focus on primary (grandparent) and multiplier (parent) breeder flocks. These flocks are to be retested each 90 days. Tests are to include blood serological tests for pullorum (cross reacts with SE) and/or *Salmonella enteritidis* (SE), and dead-in-shell and environmental samples for bacteriological culturing. 2) Flocks that are epidemiologically associated with a human outbreak must undergo extensive blood serological testing (300 samples/house in initial testing) and bacteriological culturing of any seropositive birds and the environment (manure and egg belts). 3) Testing of commercial egg-laying flocks has been given lower priority and is to be considered more experimental in nature unless involved in a traceback. It has been agreed that by January 1, 1990 all commercial egg-laying flocks will have been tested or have been repopulated with birds from breeder flocks tested and negative for SE.

The "Voluntary Model State Quality Assurance Program" is a recommendation of one possible approach to testing and control. Individual states will modify the program to meet their own particular needs and resources. A copy of the most recent version would be available from Dr. Everett Bryant, Department of Pathobiology, University of Connecticut, Storrs, Conn. 06268.

Some Public Health Related Questions

How can the public health risk that an infected commercial flocks presents be realistically evaluated? How does such risk relate to such flock parameters as prevalence of infection within the flock, length of time the flock has been infected (acute vs. chronic), and stress factors that might increase the shedding rate? Will it be technically possible to "eradicate" SE from commercial egg-laying flocks? If technically possible, will the economic cost to the industry and, ultimately, the consumer be prohibitive? How do we find more effective ways of articulating the concept and reality of cost vs. benefit (cost vs. risk) so that alternatives might be understood and acceptable?

Field Research Need

If the present SE problem is to be fully understood and rational solutions developed, there are some questions that will need to be answered by field-oriented epidemiological research. This research would involve testing that would be

much more extensive and intensive than is proposed in any present testing programs. The relatively small number of flocks that would be involved would also be participating in any required testing programs. Owners of flocks that participate in such research should not be penalized. If, because of penalties, such research becomes impossible, a full understanding of the SE situation will be delayed and the cause of public health instead of being helped will be hurt. It would seem important that governmental regulatory agencies (CDC, FDA, USDA-APHIS, State Depts. of Agriculture, State Depts. of Health), the poultry industry, universities, the press, and consumers clearly understand this problem and seek means to accommodate such research.

SALMONELLA IN POULTRY IN THE UK OBSERVATIONS AND ACTIONS

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During the past 10 years there has been a considerable increase in the incidence of *Salmonella* food poisoning cases in humans. Many of these have been associated by the health authorities with eggs or broilers. Considerable doubt still exists as to the undisputed number of incidents linked to the poultry industry without any other factors being involved.

The number of reported cases in food animals themselves has also risen in this period, mainly due to the implementation of the Zoonoses Order making it compulsory to report to the Ministry of Agriculture any isolation of a *Salmonella* in an animal, it's products or environment, including feedstuff.

Of all serotypes, those most commonly associated with human cases are *Salmonella typhimurium*, *S. enteritidis*, and *S. virchow*. In 1987 the relative incidences in humans were as in Table 1.

In 1988 *S. enteritidis* took over the major role of all *Salmonella* outbreaks according to Public Health Laboratory Service figures. It is now claimed to give rise to over 50% of all outbreaks, and up until the end of December 1988 had been implicated in over 12,000 cases of which Phage Type 4 was reported to be involved in over 8,000 (75%). This is the phage type most often correlated with poultry.

With some 33 different phage types of *S. enteritidis* it is notable how the incidence of PT4 has increased. It accounts for about 77% of the increase of *S. enteritidis* in total between 1981 and 1987 (Table 2).

In the same period *S. typhimurium* has also increased in human cases from just over 3,000 in 1981 to just over 7,000 in 1987, a doubling in numbers compared to a six-fold increase in *S. enteritidis*. Other serotypes of *Salmonella* have remained about the same during this time.

While *S. enteritidis* is principally associated with poultry, other food animals have also been shown to be contaminated. PT4 *S. enteritidis* has been identified in 18 bovine isolates in 1987.

In poultry it has been claimed that up to 20% of raw liquid egg destined for pasteurization which was sampled in 1987 yielded PT4 (184 samples), this being about one third of all *Salmonella* serotypes isolated.

Similarly 60% of all poultry sampled at retail outlets in 1987 was claimed to yield *Salmonella*, again with 1 in 5 having *S. enteritidis* PT4.

S. enteritidis is not, however, a new serotype in poultry. As long ago as 1947 Blount stated that it was, with *typhimurium*, the major problem in poultry after *S. pullorum* and *S. gallinarum*,[1] both of which are now virtually eradicated from the UK commercial egg flock.

To the end of December 1988 confirmed cases in poultry species in the UK were as in Table 3.

Clinical disease is not a feature in laying flocks, and broiler breeder outbreaks were backtraced from problems in broilers rather than any clinical signs in the breeders themselves. They arose from routine examination of chick box liners, dead-on- arrival day-old broilers or early mortality. Two commercial layer breeder flocks were also identified from yolk sac infection of replacement pullet chicks and in one case from examination of four week old pullets for coccidiosis.[2]

Broilers, however, have shown evidence of excessive mortality between 7 and 15 days, followed by uneven growth. At autopsy chicks with clinical disease have lesions identical to colibacillosis, except that more fluid is usually found in the pericardium and the fibrin strands have a yellowish coloration.

Examination of 40 broiler flocks exhibiting clinical disease, and in which *S. enteritidis* was shown to be the major factor involved, had one or more signs as noted in Table 4.[3]

When age of clinical disease was examined the number of flocks showing disease in one or more weeks was as in Table 5.[3]

There appeared to be a second peak of pericarditis and perihepatitis at 6–7 weeks of age just prior to slaughter and resulting in complete condemnation.

Pheasants and young geese showed similar lesions to broilers.

Although the majority of poultry isolates have been made from broilers, it was the association of human cases with shell eggs and products con-

taining raw or lightly cooked eggs that caught the attention of the media in the UK. Products involved were mostly mayonnaise or salad creams or mousses prepared for large functions, and where the care taken after preparation was in many cases dubious.

In the summer of 1988 one newspaper reported the *S. enteritidis* outbreaks in the U.S. and in August it was reported that there was a similar problem in the UK also, with *S. enteritidis* being associated with 4,424 human cases in the first half of the year.

Television took up the story and egg sales began to fall. Considerable ill-informed comments were put about regarding the feeding of hens back to other layers, which provoked much comment from the general public.

Then on December 3, 1988 a Junior Minister of Health from the Department of Health said on television "Most of the egg production in this country, sadly, is now infected with *Salmonella*."

These 14 words precipitated a catastrophic decline in egg sales from what had already been 15–20% down on summer sales to more than 50%. This resulted in a build-up of some 15 million eggs each day.

The Minister of Agriculture strongly denied the claims of the Department of Health and, in an attempt to put a bottom in the market, set aside $34 million to buy up surplus eggs for destruction at 54 cents per dozen, and point of lay pullets (18–30 weeks) at $2.72 per head for slaughter.

These prices contrast to the actual production cost of 81 cents per dozen for eggs and the cost of a point of lay pullet of some $4.50. The loss to the industry from depressed egg sales was running at about $1 million a day at this time.

Following the resignation of the Junior Health Minister because of the damage caused by those 14 words, and with the introduction of the support fund, and the issuing by the Ministry of Agriculture of an advertisement giving the facts about Salmonella and eggs to the public, egg sales began to recover, so that in January 1989 they were back to some 85–90% of former levels. But price per dozen was still depressed and producers were in a net loss situation still.

The speed of recovery of sales has resulted in only a limited uptake of funds for egg destruction and bird slaughter. Something over $5 million only was paid out to producers. Less young birds have been killed, but producers took the opportunity to clear out as many old birds as the processing plants could handle, and the national laying flock has been reduced as a result by 5–10%.

The situation has resulted in demands from consumer groups for stricter controls over egg production. The issuing of Codes of Practice by the Ministry of Agriculture for breeders, egg producers, and feed mills has been readily adopted by the industry and now involves a high degree of testing of birds and eggs for *Salmonella*. The amount of testing is well above levels in other EC member countries. It has increased production costs by some 5–9 cents per bird.

In our own Greendale laboratories we have cultured over 40,000 table eggs and an increasing number of carcasses and environmental samples from some 500 producers and as yet have isolated only one *S. enteritidis*, that being from an environmental sample.

This contrasts with the figures of 1 in 300 eggs being infected with *S. enteritidis* which were being widely claimed by some experts in the media.

Examination of the relationships between *S. enteritidis* and poultry and eggs have been increasing over the past few months.

It has long been known that up to some 3.5% of eggs lack cuticle, mainly over the large pole, and so penetration by *Salmonella* would be comparatively easy. Oiling, which is not permitted in the UK, has been shown to reduce penetration from 90% to 30% when the eggs are placed in a contaminated environment. This presupposes, however, that penetration had not taken place before oiling.

When 1,000 organisms were injected into the airspace of an egg, less than 100 organisms migrated into the albumen when stored at 4°C for 3 weeks. There was a vast increase in migration when the temperature was raised to that normally found in retail outlets.

In a series of cooking experiments varying survival rates of *S. enteritidis* was found in eggs.[3] Ten million organisms were inoculated into the yolk of each egg (Table 6).

Laying birds have been challenged by intravenous inoculation with *S. enteritidis* and 10 birds slaughtered and cultured each week for several weeks.[4] All eggs laid by the group during the trial were also externally sterilized and the contents cultured.

The organism could be easily recovered from all tissues of the bird including developing ova in the ovaries. But in no case was *S. enteritidis* isolated from the contents of an egg. This makes even more unsure the role of transovarian transmission of *S. enteritidis* which has been raised as being an important feature of the serotype and one which makes it potentially more dangerous as a food poisoning organism.

Eggs removed from the oviducts of the birds in the trial were found to have *S. enteritidis* on the shell in 6 out of 21 cases of birds at autopsy.

Varying levels of testing of commercial flocks are now being adopted by egg producers, and an example of a complete monitoring package undertaken by our laboratories would be as follows.

Replacement Pullets

—Chick box liners and dead on arrival at day-old
—Mortality between 7 and 15 days
—Mortality and litter at 3 and 5 weeks of age
—A sample of birds 5 weeks before point of lay
—Feed samples throughout rearing

Layers

—50 pooled cloacal swabs each month for the first 3 months of lay, every other month thereafter
—5 culls or mortality at peak lay
—10 environmental swabs or fan outlet dust every other month

Eggs

—Less than 10,000 birds in a house; 50 eggs per month
—More than 10,000 birds in a house; 100 eggs per month

These eggs are pooled in groups of 25 and samples of the resulting melange is cultured.

Blood testing is also available for those wishing to take that approach to control or eradication. This is seen as being more applicable to breeders. Research with challenged birds has shown that *S. pullorum* antigen used in whole blood Rapid Plate Tests identifies only one third of the reactors that are picked out by *S. enteritidis* antigen.

It is realized that elimination of *S. enteritidis* is unlikely to be 100% but the measures now being undertaken are aimed at reducing the overall incidence of contaminated eggs which are available for incorporation into salad creams or mayonnaise. It is hoped it will also put consumer confidence back into the egg sector of the poultry industry.

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TABLE 1
Serotype percentage of *Salmonellas* in 1987

Serotype	%
S. typhimurium	37.5
S. enteritidis	33.5
S. virchow	7.4
All other serotypes	21.6

TABLE 2
Relationship of Phage Type 4 to all *S. enteritidis*

Year	Total all enteritidis	PT4	%
1981	1087	399	37
1987	6858	4852	71

TABLE 3
***S. enteritidis* isolates in poultry species (1988)**

Species	Number isolates
Broilers	77
Layers	12
Broiler Breeder	2
Turkeys	3
Ducks	1
Geese	1
Pheasants	2
Quail	1

TABLE 4
Proportion of broiler flocks showing different signs

Presenting sign	Number flocks
Uneven growth	27
Septicaemia	20
Pericarditis	16
Perihepatitis	10

TABLE 5
Age of involvement

Week	Number flocks
1	27
2	15
3	10

TABLE 6
***S. enteritidis*—survival by cooking method**

Method	Average % survival
4 minute boiling	11
Sunnyside frying	75
Overeasy frying	Almost zero (10 organisms/g) Enrichment needed for recovery
Scrambled slow (73°C)	20
Scrambled fast (83°C)	Almost zero

A PROGRAM FOR SALMONELLA-FREE BROILERS IN SWEDEN

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The following program was presented at the International Congress on Animal Hygiene in Skara, Sweden, June 1988. Following the Congress, I visited a large feed mill that produces broiler feed under strict sanitary conditions. In addition, I visited a hatchery, breeder farms and the laboratory where broilers are submitted during the grow period to determine Salmonella status. Colored photographs will describe what was observed.

The Salmonella-free broiler program follows the general recommendations to be followed in a Specific Pathogen-free Bird Program that was developed at the University of Maine in 1963.

THE SWEDISH SALMONELLA CONTROL PROGRAM FOR BROILER PRODUCTION—A METHOD TO DETECT INFECTED BIRDS BEFORE SLAUGHTER

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Summary

Discussions between national veterinary authorities and the poultry industry in Sweden in 1975 resulted in a program with the aim to detect infections with Salmonella among living birds and to keep infected broilers and other types of poultry out of the slaughterhouses. To achieve this, the program was constructed to comprise all the different parts of the poultry meat production.

The participation in the Salmonella Control since 1984 is compulsory for any producer of broilers. If the economic part of the Control is to be valid, the producer must fulfill certain regulations. In return, the owner is guaranteed 90% of the birds' value if Salmonella is detected.

When Salmonella is detected among the broilers the National Agricultural Board decides if the birds are going to be killed or slaughtered under special hygienic measurements. An investigation is always carried out in order to find out how the infection reached the flock. Orders are also given that after cleaning and decontamination, an inspection is made of the building and the equipment to determine that no Salmonella persists. In the decontamination program a complete dismantling of the feed and water systems is always a part of the routine work.

Some of the experiences after eight years in practice are:

The control has contributed to a complete elimination of Salmonella in both grandparent and parent flocks as well as the hatcheries concerning broilers. The control has made it possible to quickly find out if the same source or *Salmonella* has been present in different flocks.

Even extremely low infection doses will very soon result in high morbidity in a flock of broilers. Accordingly, a flock reveals the presence of Salmonella in feed better than any known laboratory method. 0.15% of the birds are sent for laboratory control within 14 days before slaughter from each flock. This seems to be a safe method to detect Salmonella in the flock when Salmonella morbidity exceeds 5%.

Experiences further show the increased importance of feed as a source of Salmonella in broilers. Investigations made have shown possibilities for Salmonella originating from the raw feed material to survive in spite of the heat pelleting process. Very strict hygienic measures have been taken to improve the hygienic standard in the processing of pelleted feed.

PROTECTION OF BROILERS AGAINST SALMONELLA INFECTION BY SPRAY APPLICATION OF INTESTINAL MICROFLORA: LABORATORY AND FIELD STUDIES

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Summary

The Nurmi concept for diminishing salmonella infection of poultry by application of gastrointestinal microflora from adult birds into newly hatched chicks was tested in a field study in which 8 million broilers were involved. Half of the 284 flocks was treated in the hatchery by spray application of an undefined microflora propagated in SPF chicks.

A significant reduction in salmonella-contaminated flocks, as well as in the number of infected broilers in these flocks, was demonstrated.

No undesirable side effects were detected. It was concluded that hygienic improvements in transportation and slaughtering of broilers are needed in order to maintain the reduced salmonella contamination rate.

Introduction

It has been demonstrated that, under laboratory conditions, oral application of strictly anaerobically cultured intestinal microflora originating from mature specific pathogen free (SPF) chickens to day-old-chicks induces a very significant protection against challenge with high doses of salmonella organisms (10^4–10^9 c.f.u./salmonella/chick).[2,3] This confirmed the Nurmi-concept[4,7]

This protective effect is significantly influenced by the challenge dose and the sequence of events: although protection could be induced by treatments carried out 24 hours after challenge, the effect was better when the chicks were treated and challenged at the same time, and even better when the treatment preceded the challenge by 24 hours (unpublished data).

In other studies it was found that the same protection could be induced using intestinal homogenates of SPF-donors instead of bacterial cultures and, provided the microflora is protected against oxygen, it can be applied by spray. This procedure makes it possible to treat entire flocks while in the hatchers.[3]

Since, under field conditions, different factors (such as viral enteritis, coccidiosis, treatment with certain antibacterials, feed composition, housing, different stress factors) can interfere with the protective effect of the treatment, it was necessary to test this procedure in a large number of broiler flocks over a relatively long period.

This paper reports a 1 year study of 284 flocks (more than 8 million broilers) housed on 46 different farms (belonging to the same broiler farm integration). Besides the influence of the treatment on the occurrence of salmonella infection in the broilers, the performances of these flocks were studied.

Additional information was collected concerning skin contamination with salmonella before transport to the processing plant and salmonella infection of broilers elsewhere in the country.

Six months after the experiment, 57 additional untreated flocks from the same farms were examined for salmonella infection.

Material and Methods

Experimental Design

The study lasted from April 1984 to September 1985 and was conducted at 46 broiler farms, which received chicks from 3 hatcheries. The broilers were slaughtered in seven commercial processing plants.

The study was divided into two parts. Half of the participating farms received at least 4 treated and then 4 untreated flocks; the other half first received 4 untreated and then 4 treated flocks. In the first part 151 flocks (76 treated and 75 untreated) and in the second part 133 flocks (67 treated and 66 untreated) were involved. In total 284 flocks and more than 8 million broilers were included.

For each flock bacteriological examinations were carried out on the following samples:

— paperpads from 10 chick transportation crates;
— 5 litter samples collected from each broiler house before arrival of the chicks;
— 5 litter samples (with freshly produced faeces) collected from each broiler house at 2 and 5 weeks of age;

— 1 feed sample (1 kg) from each delivery;
— 5 faecal samples taken from the broiler transportation crates just before or after the birds were hung on the slaughter line;
— 100 caeca collected in the processing plant: 0.5–1 gram content of each caecum;
— 25 neck skin samples (10 grams each) collected after evisceration.

In an additional study the incidence of skin contamination with salmonella at the end of the fattening period (excluding contamination from transportation crates) was estimated. Skin samples were taken from 1000 broilers originating from 50 of the experimental flocks just before transportation to the processing plant.

During the last phase of the study a preliminary test was performed to estimate the incidence of salmonella contamination of broilers in other parts of the country: 25 flocks of broilers were sampled and approximately 100 caeca per flock were examined for presence of salmonellae.

Six months after the experiment, 100 caeca/flock from 57 flocks from the experimental farms were collected after slaughter, and examined for salmonellae.

At 2 weeks of age, 4 groups of 25 chicks collected at random from 4 different locations from the broiler house were weighed, and average body weight was calculated.

Technical date such as mortality, average body weight at 42 days of age, feed conversion and European Broiler Index were calculated according to the formulae given in Table 4.

Salmonella Isolation Procedure

Pre-enrichment in buffered peptone water at 37°C for 18 hours[4] was used only for the examination of feed samples (200 grams each). All other samples were directly cultured in selenite-brilliant green mannitol selective broth medium[4] at 37°C for 24 and 48 hours. The weight ration sample/medium was 1:10.

Thereafter the samples were inoculated on brilliant green-phenol-red-agar (oxoid CM 329), with 0.2 sodium-desoxycholate (Merck art. 6504) and cultured at 37°C for 24 hours.

After biochemical and serological identification (5) salmonella isolates were serotyped at the National Health Institute (RIVM, Bilthoven).

Method of Application

Intestinal Microflora

The intestinal microflora was an undefined flora, originating from mature SPF birds and propagated in young SPF birds, kept in isolators. These birds were inoculated orally at one-day-old; crops and intestines were harvested at 3 weeks of age. Homogenates of these organs were checked for avian pathogens (both *in vivo* and *in vitro*) and antibacterial drug resistant bacteria, and were demonstrated to induce very significant protection against oral challenge with up to 10^4–10^6 c.f.u. salmonella organisms/chick under experimental conditions.

Freeze-dried intestinal homogenates of SPF-birds were re-suspended in suspension-medium and sprayed on the chicks and eggs by a Gloria knapsack spray apparatus using 10 ml suspension per 100 eggs/chickens in the hatcher.[3] The spray produced had a Median Mass Diameter of 130u and geometric s.d. of 2.2 by 3 bar (Dr. J. H. H. van Eck, Dept. of Poultry Diseases, University of Utrecht, unpublished data). The treatment took place on the 20th incubation day at about 11:00 A.M. when about 30–40% of the chicks were hatched.

Statistical Analysis

For the statistical analysis of the results of the bacteriological examinations a chi-square test was used.

Results

Salmonella Isolations

Paperpads. Salmonellae of 10 different serotypes were isolated from 51 of 2,499 (2.0%) paperpads from chick transportation crates examined. From 263 flocks of one-day-old chicks, 20 (7.6%) were positive: salmonellae were isolated from 4.6% of the untreated and from 10.5% of the spray-treated flocks.

Feed samples. Salmonellae belonging to 9 different serotypes were isolated from 11 out of 901 (1.2%) feed samples examined. None of these serotypes was isolated from litter samples collected at 5 weeks of age, from caecal content or from skin samples.

Litter samples. 1,738 litter samples collected before arrival of the chicks were examined: only from 2 samples originating from 2 different farms were salmonellae isolated. In one of these farms, the same serotype was found in litter samples at 2 and 5 weeks of age, but not at the end of the rearing period in either caecal content or skin samples. In the other farm the serotype was not found in any other sample examined. 206 from 2,030 (10.1%) litter samples collected from 220 flocks at 2 weeks of age yielded salmonellae (11.1% of these positive samples came from untreated flocks and 9.2% from spray-treated flocks (Table 1)).

From the 220 flocks involved, 54 (24.5%) were positive: 26.2% of the untreated and 23.0% of the spray-treated flocks.

Salmonellae were demonstrated in 110 of 1,799 (6.1%) litter samples collected from 192 flocks at 5 weeks of age. The 7.4% incidence in untreated and 5% incidence in treated flocks were significantly different ($p \leq 0.05$)(Table 1).

34 of the 192 (17.7%) flocks examined were positive: the incidence in the untreated and treated flocks was 19.6% and 16% respectively (non-significant difference).

Caecal content. Salmonellae of 13 serotypes were isolated from 620 (2.2%) of the 28,499 caecal content samples.

In the untreated flocks the incidence was significantly higher (3.5%) than in the treated flocks (0.9%)($p < 0.01$).

From the total of 284 flocks examined, 55 (19.4%) were salmonella-positive (24.1% of untreated flocks and 14.7% of treated flocks ($p \leq 0.05$)(Table 2).

Within salmonella-positive flocks, the salmonella isolation rate from untreated and treated flocks was 14.3% and 6.4% respectively ($p \leq 0.01$)(Table 3).

Transportation crates. Salmonella examination of faecal samples from transportation crates revealed salmonellae in 67.3% of the untreated and 65.7% of the treated flocks (Table 5).

About 30% of the faeces samples were salmonella-positive.

Skin samples. Salmonellae were isolated from 11.4% of the neck skin samples taken during slaughtering, originating from (62.9%) untreated and (57.3%) treated flocks (Table 5).

Skin samples before transportation. Sampling of broiler neck skin just before transportation to the processing plant showed that 6 of 49 flocks (12%) were salmonella-positive, while sampling these flocks after slaughter demonstrated salmonella contamination in 46% of the flocks.

Caeca from elsewhere in the country. Salmonellae were isolated from caecal content originating from 11 to 23 flocks (47.8%) housed in farms located elsewhere in the country, while only 8% of the experimental flocks were positive in this period.

Salmonella incidence after the experimental period. Caecal content from 57 flocks, sampled 6 months after the last flora application in this broiler farm integration demonstrated salmonella contamination in 13 flocks (22.8%). The incidence of the salmonella contamination within these positive flocks was 17.6%, while the incidence even in untreated flocks at the end of the experiment was 5.2%.

There was a positive correlation between the rate of salmonella isolation from litter at 5 weeks of age and from caecal content at slaughter age.

Technical results. The average technical results are given in Table 4. No significant differences in average body weight at 2 weeks and at 6 weeks of age, mortality rate, feed conversion, and European Broiler Index could be demonstrated between untreated and treated flocks.

Discussion and Conclusions

The incidence of salmonella isolation from paperpads of transportation crates of untreated day-old-chicks was much higher than that of treated chicks.

The flora can not easily be held responsible for these findings since it was found to be free from salmonellae and other avian pathogens by direct examination, by examination of SPF chicks inoculated with this flora and serological detection methods (unpublished data). Since the hatching eggs as well as the hatcheries involved in this experiment were selected at random they also can not be responsible for this finding.

In 9 of the 20 salmonella-positive one-day-old flocks, the same serotype could be demonstrated in litter samples collected at 2 or 5 weeks of age. In only 2 of these flocks (1 untreated and 1 spray-treated) could the same serotype be isolated at the end of the fattening period from caecal content. On the other hand, in 5 flocks (3 untreated and 2 spray-treated) the same serotype was isolated from skin samples taken at the processing plant.

Although there was a heavier contamination of crates in which treated chicks were transported to the broiler farm, the broilers were better protected by the treatment, as can be concluded from the lower salmonella isolation rate from litter at 2 and 5 weeks of age in the treated flocks than in untreated flocks. At 5 weeks of age this difference was statistically significant ($p \leq 0.05$).

In general, the incidence of salmonella isolations from litter samples decreased from 10.1% at 2 weeks to 6.1% at 5 weeks of age. At both sampling times the incidence in untreated flocks was 2% higher than in the treated flocks. Also, the percentage of flocks with positive litter samples decreased with age: from 24.5% at 2 weeks to 17.7% at 5 weeks of age. In the two age groups the salmonella incidence was respectively 3.2% and 3.6% higher in the untreated flocks. At 101 farms (51 untreated and 50 treated) no salmonellae were isolated from litter samples at

2 weeks of age. At 5 weeks of age there were 113 of such flocks (51 untreated and 62 treated).

In both groups of flocks there was no correlation between numbers of salmonella isolates at the 2nd and the 5th week of age: in 23 of 54 flocks, positive at 2 weeks, no salmonella was isolated from litter samples at 5 weeks of age (Table 1).

Comparison of the results of salmonella isolations from caecal content showed that the protective effect even became very significant ($p < 0.01$) at the end of the fattening period.

A very positive correlation was found between the salmonella isolation rate from litter at 5 weeks of age and from caecal content (Table 6). These findings can be used to screen flocks before slaughter to enable processing plants to slaughter salmonella-free flocks prior to salmonella-contaminated flocks and to prevent cross contamination.

Over the total experimental period, it was found that besides a lower percentage of salmonella-contaminated flocks, a lower incidence of contaminated broilers in such flocks was also induced by the treatment.

It was further noticed that the percentage of salmonella-contaminated flocks in this broiler farm integration was reduced from 38.6% initially to 7.6% at the end of the experiment. The incidence of contamination within the salmonella-positive flocks was reduced from 15.9% to 5.2% during this period.

The salmonella incidence elsewhere in the country at the end of the experiment was, according to the additional study, much higher (47.8%), supporting the idea that the effects achieved are due to the treatment with flora. Moreover, when the situation in the same integration was studied 6 months after the end of the experiment, it was found that the incidence of salmonella contamination had increased significantly (from 7.6% to 22.8%).

These findings represent the situation at the end of the fattening period. However, after slaughter no significant difference in salmonella skin contamination of carcasses between untreated and treated flocks was found. This is undoubtedly due to the contaminating role of the transportation crates and the slaughter process.

Salmonellae were isolated from skin samples collected in the processing plant, although in 60% of the cases these flocks were salmonella-negative in litter and caecal content during the fattening period.

There was no correlation between salmonella isolation from caecal content and skin samples (Table 7). A much greater number of serotypes (28) could be isolated from litter samples at 2 and 5 weeks of age, in comparison with those isolated from caecal content at the end of the fattening period (13 serotypes). An increase of serotypes was found in faeces originating from transportation crates (22 serotypes) and neck skin samples taken during slaughter (26 serotypes).

A high degree of similarity in serotypes isolated from faecal samples from transportation crates and from skin samples was demonstrated, which is in contrast with the above mentioned lack of correlation between salmonellae isolated from caecal content and skin samples.

Salmonella contamination of feed samples found in this study was comparable with that reported from other regions in The Netherlands.[1] As none of the serotypes isolated from feed could be demonstrated in caecal content or on the skin at the end of the fattening period it can be concluded that in this study feed contamination was of no significance.

It can further be concluded that application of intestinal microflora to the newly hatched broilers may contribute significantly to reduced incidence of salmonella contamination of live broilers.

To maintain this effect, hygienic improvements in both transportation to, and slaughter at, processing plants are essential.

Separation of salmonella-negative from salmonella-positive flocks by bacteriological examination of litter samples (freshly produced faeces possibly from cloaca swabbing) approximately one week before slaughter in combination with the above mentioned hygienic improvements may lead to a significant reduction of salmonella contamination of the slaughter product.

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TABLE 1
Salmonella incidence in litter samples at 2 and 5 weeks of age

Flocks	Litter at 2 weeks of age		Litter at 5 weeks of age	
	n	%	n	%
Untreated	28/107	26.2	18/ 92	19.6
Treated	26/113	23.0	16/100	16.0
Total	54/220	24.6	34/192	17.7
Samples				
Untreated	109/ 979	11.1	63/ 852	7.4
Treated	97/1051	9.2	47/ 947	5.0*
Total	206/2030	10.2	110/1799	6.1

*significant difference $p \leq 0.05$

TABLE 2
Salmonella incidence in caecal content at the processing plant

Flocks	Caecal content	
	n	%
Untreated	34/ 141	24.1
Treated	21/ 143	14.7*
Total	55/ 284	19.4
Samples		
Untreated	486/14099	3.5
Treated	134/14400	0.9*
Total	620/28499	2.2

*$p \leq 0.05$
*$p < 0.01$

TABLE 3
Salmonella incidence in caecal content from salmonella-positive flocks

	Salmonella positive samples %	Total samples
Treated	134 (6,4)*	2100
Total	620 (11.3)	5499

*$p < 0.01$

TABLE 4
Average technical data of 46 broiler farms over 8 periods

	Untreated	Treated
Average weight at 2 weeks of age (g)	294	292
Mortality rate (%)	3.38	3.22
Average weight at 42 days (g)	1501	1496
Feed conversion (42 d)(a)	1.99	1.98
European Broiler Index (b)	175	175

(a) Feed conversion = $\frac{\text{total live weight—2\%*}}{\text{total feed consumption}}$

(b) European Broiler Index = $\frac{\text{(10\% mortality)* growth per day (g)}}{\text{feed conversion*}}$

*Includes compensation for not withdrawing feed and water

TABLE 5
Salmonella isolation from faeces samples collected from broiler transportation crates and from skin samples after slaughter

Samples	Faeces from crates		Skin	
	n	%	n	%
Untreated	158/ 534	29.6	363/3099	11.7
Treated	165/ 545	30.3	338/3074	11.0
Total	323/1079	29.9	701/6173	11.4
Flocks				
Untreated	72/107	67.3	78/124	62.9
Treated	71/108	65.7	70/122	57.4
Total	143/215	66.5	148/246	60.2

TABLE 6
Comparison of salmonella contamination of broilers at 5 weeks of age and at slaughter age in caecal content

5 weeks	slaughter age	number of flocks	%
negative	negative	122	65.2
positive	negative	17	9.1
negative	positive	28	15.0
positive	positive (the same serotype)	15	8.0
positive	positive (different serotype)	5	2.7

TABLE 7
Correlation between salmonella isolation from caecal content and skin samples collected in the processing plant

caecal content	skin samples	number of flocks	%
negative	negative	109	43
negative	positive	89	35
positive	negative	11	4
positive	positive	45	18

EFFECT OF A NEW PELLETING PROCESS ON THE LEVEL OF CONTAMINATION OF POULTRY MASH BY *SALMONELLA* AND *ESCHERICHIA COLI*·

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Summary

The efficiency of a new pelleting process in eliminating naturally-occurring *Escherichia coli (E. coli)* and *Salmonella* from poultry feed was evaluated by comparing the microbial load in raw and processed mash. The new process consisted of an Anaerobic Pasteurizing Conditioning System (APC System®) and a pellet mill. The APC System® is composed of a Vaporator® and an Original Vertical Conditioner®. Instead of being conditioned by steam produced in a boiler, mash pelleted by the new process was first thoroughly mixed in the Original Vertical Conditioner® with steam and other hot gases. The steam and hot gases were generated in the Vaporator® by igniting fuel in the presence of water.

E. coli was isolated from 72.0–100.0% of samples or raw mash in all eleven trials. Salmonellae *(S. senftenberg, S. bredeney, and S. mbandaka)* were isolated from 5.0–10.0% of raw mash samples utilized in three trials. The APC System® by itself, or in combination with the pellet mill, appeared to be 100.0% efficient in eliminating *Salmonella* and *E. coli* from poultry mash when mash entering the pellet mill from the conditioner had a mean temperature of 82.9 ± 2.1°C, a moisture content of 16.3 ± 1.3% and had been retained and treated with steam and hot gases for 4.3 ± 0.4 minutes.

·Some of the information presented here has already been submitted to and accepted for publication in *Avian Diseases*.

Introduction

Salmonellae are a common component of the commensal flora of the intestinal tract of animals. However, some species can be quite pathogenic. The diseases they cause in poultry can have adverse effects on the economy of the poultry industry. In humans, they are of great public health significance.[3,10,16] Poultry and other livestock are considered to be important sources of human *Salmonella* poisoning.[3,10]

Egg, environmental and feed contamination are the main sources of *Salmonella* infection in poultry. Technology has been available and utilized to eliminate the egg and environment as sources of infection.[8,11] On the other hand, technology to eliminate *Salmonella* from feed has been lacking. Attempts to eliminate *Salmonella* from poultry feed either by pelleting, or by sterilizing feed components of animal origin before their incorporation into feed, have at best resulted only in significant reductions in levels of contamination. Sterilizing only feed components of animal origin ignored the possibility of contamination from one or more of the other components. Pelleting should have been more effective since it involved treating the whole feed. However, the pelleting process is weighted heavily in favor of production of good quality pellets and conditions favoring the production of such pellets are not always the same as those detrimental to the survival of *Salmonella*. The main problem with current pelleting procedures

is that steam pressures which will generate enough heat to consistently kill all *Salmonella* present in feed also make the feed too moist for pelleting.[3]

Recently, an equipment configuration called the Anaerobic Pasteurizing Conditioning System® (APC System; VE Corporation Arlington, Texas) was introduced to the feed industry.[1,2] It was claimed to be capable of permitting the attainment of high mash temperatures without causing mash to become too moist. The APC System® consists of a Vaporator® and an Original Vertical Conditioner®. Fuel is ignited and combined directly with water in the Vaporator®, resulting in the generation of steam, nitrogen, carbon dioxide, and a trace of carbon monoxide. The hot gases are channeled directly into The Original Vertical Conditioner® through an opening in its lower end, and thoroughly mixed by paddles with feed introduced through the top (Fig. 1). This direct utilization of all the hot products of combustion is said to make it possible to control mash temperature independent of moisture levels.

Farm Service Elevator Co., a feed-processing firm purchased an APC System® for use in its program to produce pathogen-free feed. This is a report of experiments conducted to determine the effects of this new pelleting process on the microbial load of untreated (raw) poultry mash.

Materials and Methods

Effects of the new pelleting process on microbial load were determined by collecting samples of raw and processed mash during processing and comparing the levels of their contamination by *Salmonella* and *Escherichia coli (E. coli)*. Two experiments were conducted. The first consisted of seven trials and was carried out between February 10 and April 2, 1987. The second consisting of four trials, was conducted on December 8, 1987.

Sampling

Samples were collected into sterile "whirl pak" bags handled by samplers wearing disposable surgical gloves. The bags were introduced into the mash stream with their open ends against the direction of flow. Samples of raw mash were collected following batch-mixing, but before entry into one of the 25-ton storage bins above the conditioner (location A, Fig. 1).

In the first experiment, samples of processed mash were collected after the crumbler (location D, Fig. 1) and consisted of mash which had been conditioned, pelleted, cooled and, sometimes, crumbled by the new process. Samples were collected at regular intervals from beginning to end of processing in each of the seven trials. Within trial, equal numbers of samples were collected at the two locations. Between trials, the number of samples varied from 14–20. In each trial, four additional samples of conditioned mash were collected (location B, Fig. 1) for moisture determination.

In the second experiment, twenty-five tons of feed were processed during each of the four trials. Processing was completed in about one hour. Samples were not collected during the first and last ten minutes of processing. This was to allow the mill to be purged of any old feed and also permit the APC System® to attain full operational capacity. Ten sets of samples were collected at regular intervals during the remaining forty minutes of feed processing. Each set consisted of three subsamples weighing 75–100 grams. The first and second subsamples were utilized for microbiological analyses. The third was analyzed for moisture. Samples were collected of raw mash (location A, Fig. 1), conditioned mash (location B, Fig. 1) and pelleted mash as it exited the pellet mill but before entering the cooler (location C, Fig. 1). All samples were placed on ice after collection.

Microbiology

In the first experiment, samples of feed from six of the seven trials were examined microbiologically in the diagnostic laboratory of Willmar Poultry Company, Willmar, Minnesota. Samples from the seventh trial were analyzed in the laboratory of Campbell Soup Company, Farmington, Arkansas. Details of the micrological procedure followed at the Campbell Soup Laboratory were not made available. At the Willmar Laboratory, samples were analyzed for *Salmonella* and *E. coli* as describe earlier.[4]

Samples of feed collected during the second experiment were examined microbiologically at the College of Veterinary Medicine, University of Minnesota, St. Paul using the enrichment procedure. Subsample 1 was divided into five approximately equal portions weighing 15–20g. Each portion was transferred into a labeled, sterile plastic "whirl pak" bag. Tetrathionate brilliant green, an enrichment medium, was added to each bag and mixed with the feeds ample by kneading through the outside of the bag. Samples were incubated aerobically at 42° overnight, and then inoculated onto highly selective brilliant green agar (BGA) plates. The BGA plates were incubated at 37° for 24 hours and then examined for lactose-fermenting *(E. Coli)*, or non-lactose-fermenting organisms *(Salmonella, Shigella, Proteus, or Serratia)*. A few (1–5) of the non-

lactose-fermenting colonies were picked from each plate, inoculated into separate triple sugar iron (TSI) slants and incubated overnight. Suspect *Salmonella* cultures were transferred from the TSI slants for biochemical tests. Serological identification of *Salmonella* cultures was conducted using group specific somatic antisera and Spicer Edwards flagellar antisera. Lactose-fermenting colonies on the BGA plates were examined for *E. coli*.

The population of microorganisms present in feed samples was determined by adding 40.0g of feed from subsample 2 to 350.0 ml of 0.1% peptone water contained in a large, sterile plastic "whirl pak" bag. The feeds ample and peptone water were mixed and then homogenized using a stomacher. Serial dilutions of the homogenized sample were made and plated on MacConkey agar. A spreader was used to ensure that the inoculum covered the entire plate surface. After incubation overnight at 37°, any Salmonella or *E. coli* colonies present were counted. The number of colony-forming units of each microbe per gram of feed was estimated by dividing the number of colonies by 40 times the dilution factor.

The level of contamination of feed by *E. coli* or *Salmonella* was expressed either as the number of colony-forming units of each type of bacteria per gram, or as the percentage of all examined feed samples that tested positive for the organism. Levels of contamination before and after conditioning and pelleting were compared. The new pelleting process was regarded as being 100.0% effective against *E. coli* or *Salmonella* if the organism could not be isolated from pellets produced from mash known to be contaminated.

Retention Time, Moisture, Temperature

Retention time was estimated, and moisture and temperature of mash was determined as described earlier.[8]

Feed Type

The type of feed produced during each of the seven trials in the first experiment was either base feed or complete rations. Base feed is a premix containing relatively high concentrations of certain ingredients. It would need to be mixed with additional feedstuff before being fed to poultry. Only complete rations were produced during the second experiment. They ranged from turkey grower-finishers containing 16.0% protein to turkey starters containing 26.0% protein.

Statistical Analysis

Data from the first experiment were analyzed by multiple regression[12] using Stat View 512+ (Brain Power Inc., Calabasas, California 91302). The statistical tool utilized in the second experiment included analysis of variance[13] and Duncan's multiple range test.[12]

Results

Experiment 1

Results of the first experiment are summarized in Tables 1 and 2. *E. coli* was isolated from 72.0–100.0% of raw mash samples in all seven trials. *Salmonella* was isolated from raw mash samples in only three of the seven trials, and the rate of isolation was 5.0–10.0%

Salmonella was not isolated from pelleted mash in any of the seven trials. The new pelleting process was 100.0% efficient in eliminating *Salmonella* from feed. Conditions which ensured the elimination of *Salmonella* from mash used in trials 2 and 6, were inadequate against *E. coli* present in the same mash. *E. coli* was isolated from 12.5–56.0% of pelleted mash samples in four of the seven trials. Efficiency of the new pelleting process in eliminating *E. coli* from mash used in those trials ranged from 22.0–87.5%. No *E. coli* was isolated from mash pelleted in the other three trials, and efficiency was 100.0% The retention times, temperatures and nutrient compositions of mash used in trials in which efficiency was 100.0%, are compared, in Table 2, to those of mash used in trials in which efficiency was less than 100%. Among trials in which efficiency was 100%, mean retention time was 4.0 ± 0.7 and conditioned-mash temperature, moisture, protein and fat were 84.4 ± 1.3°C, 15.4 ± 0.8%, 26.7 ± 11.0%, and 4.9 ± 2.1%, respectively. The corresponding figures for trials in which efficiency was less than 100.0% were 3.7 ± 0.3 min, 86.3 ± 2.6°C, 13.4 ± 2.6%, 34.5 ± 7.0%, and 6.0 ± 1.6%. Efficiency thus appeared to vary with changes in levels of moisture, temperature, retention time, or fat and protein contents. Analysis of data by multiple regression showed that the impact of moisture level on efficiency of kill was significant (P = 0.14). The regression equation relating efficiency (y) to temperature (x^1), retention time (x^2), moisture (x^3), protein (x^4) and fat (x^5) was:

$$y = 239.42 - 2.65x_1 + 20.47x_2 + 14.74x_3 - 2.87x_4 + 20.17x_5$$

Experiment 2

Results of the second experiment are summarized in Table 3. *E. coli* and non-lactose-fermenters were isolated from raw mash in all four trials. Between trials, the mean number of

colony-forming units of microbes per gram of raw mash was 70.0 ± 41.2, and 92.0 ± 56.6 for *E. coli* and non-lactose-fermenters, respectively. The non-lactose-fermenters were confirmed not to be *Salmonella*.

No *E. coli* or non-lactose-fermenters was isolated from conditioned or pelleted mash samples. Microcidal efficiency of the APC System® by itself or in conjunction with a pellet mill, was estimated to be 100.0% against *E. coli* and non-lactose fermenters. Among trials, mean retention time was 4.6 ± 0.5 min., and temperature and moisture were 81.4 ± 2.3°C and 17.2 ± 1.0%, respectively.

Discussion

Williams[14] considered research into methods of producing and feeding *Salmonella*-free feeds an essential first step in any program to eliminate Salmonella from animals. According to the U.S. Advisory Committee on *Salmonella*[4] production of *Salmonella*-free feed was dependent on the implementation of research to identify optimum combinations of temperature, heating time, and moisture (optimum TTM) that will kill *Salmonella* contained in feed. Liu et al.[8] defined optimum TTMs for *Salmonella*, but lamented the lack of equipment that could achieve the desired temperatures under practical conditions. Cox et al.[3] also described recent attempts to utilize current pelleting process (a boiler-conditioner and a pellet mill) to eliminate *Salmonella* from poultry feed. They reported that the steam pressure which ensured a consistent elimination of *Salmonella* was not compatible with the production of pellets. The present study describes a new pelleting procedure in which the boiler-conditioner was replaced by an APC System®, and evaluates its microcidal efficiency against *Salmonella* and *E. coli* present in poultry feed. The optimum TTMs identified by Liu et al.[8] to be lethal for *Salmonella* senftenberg, the most heat-resistant *Salmonella*, were 87.8°C, 1.5 min., and 15.0%; or 89.4°C, 0.5 min., and 15.0%. Between all eleven trials of the two experiments described in the present study, mean optimum TTM was 82.9 ± 2.1°C, 4.3 ± 0.4 min., and 16.3 ± 1.3%. This optimum TTM was attained under practical conditions, appeared to be quite effective against *Salmonella*, and did not interfere with the production of good-quality pellets.

A uniformly high percentage of mash samples from all eleven trials were contaminated by *E. coli*, a common component of commensal flora of intestinal tracts of animals. The presence of *E. coli* is generally regarded as being indicative of fecal contamination and the probable presence of pathogenic microorganisms. The isolation of *Salmonella* in these trials lends further justification to this assumption. Certain genotypes of *E. coli* are quite pathogenic and associated with diseases that are of great economic importance to the poultry industry.[7] They are also of some public health importance.[10] The serotypes of *E. coli* isolated in the present study were not determined. However, it is not unreasonable to assume that feed so pervasively contaminated by *E. coli* represents a significant potential health hazard to birds consuming the feed. Since the general cycle of infection of *E. coli* is similar to that of *Salmonella*, utilization of the new pelleting process could enable the simultaneous elimination of *Salmonella* and *E. coli* from poultry.

The cooling rate of samples of conditioned and pelleted feed collected on ice in the second experiment was 1.5°C per minute. The residual heat resulting from slow cooling might or might not have enhanced the efficiency of kill of the new pelleting process. Further studies are needed and being planned to clarify this and to confirm results obtained in the present one.

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TABLE 1

Experiment 1: Effect of a new pelleting process[a] on the *Salmonella*[b] and *Escherichia coli* (*E. coli*) contents of poultry mash

Trial No.	*Salmonella* positive samples		Efficiency of process[c] against *Salmonella*	*E. coli*-positive samples		Efficiency of process[c] against *E. coli*
	Raw mash	Pelleted mash		Raw mash	Pelleted mash	
1	0	0	NA[d]	72.0	56.0	22.0
2	5.0	0	100.0	85.0	55.0	35.0
3	0	0	NA	100.0	12.5	87.5
4	0	0	NA	100.0	0	100.0
5	0	0	NA	100.0	0	100.0
6	10.0	0	100.0	100.0	20.0	80.0
7	10.0	0	100.0	95.0	0	100.0

[a]The new pelleting process consisted of an Anaerobic Pasteurizing Conditioning System[a] (VE Corporation, Arlington, Texas) and a pellet mill (Buhler–Miag, Minneapolis, Minnesota)

[b]*Salmonella* species isolated included *S. senftenberg* in trials 2 and 6, *S. bredeney* in trial 6, and *S. mbadaka* in trial 7

[c]Efficiency of the new pelleting process in eliminating the microorganism from mash was expressed as percentage of *Salmonella* or *E. coli*-positive mash samples that were rendered negative by pelleting process

[d]Not applicable

TABLE 2

Experiment 1: Effect of temperature, heating time and moisture, protein and fat contents of mash on the efficiency[a] of a new pelleting process[b] in eliminating *Escherichia coli (E. coli)* from poultry mash

Parameter measured	Efficiency of new process against *E. coli*	
	Partial	Full
Number of trials	4	3
Mean number of samples per trial	18	17
Mean efficiency (%)	56.1±32.5	100.0±0
Retention (heating) time (min)	3.7±0.3	4.0±0.7
Temperature of conditioned mash (°C)	86.3±2.6	84.4±1.3
Moisture in conditioned mash (%)	13.4±2.6	15.4±0.8
Protein content of mash (%)	34.5±7.0	26.7±11.0
Fat content of mash (5%)	6.0±1.6	4.9±2.1

[a]Efficiency was expressed as percentage of *E. coli*-positive raw samples that were rendered negative by the new pelleting process. The equation relating efficiency to the physical variables was:

$y = 239.42 - 2.56x_1 + 20.47x_2 + 14.74x_3 - 2.874x4. 20.17x_5$, where y is the efficiency of the new pelleting process against *E. coli*, and x_1, x_2, x_3, x_4, and x_5 represent temperature, retention time, moisture, protein and fat, respectively

[b]The new pelleting process consisted of an Anaerobic Pasteurizing Conditioning System[c] (VE Corporation, Arlington, Texas) and a pellet mill (Buhler–Miag, Minneapolis, Minnesota)

TABLE 3

Experiment 2: Effect of a new pelleting process[a] on the *Escherichia coli* and non-lactose-fermenting (lactose-negative) bacteria population of raw poultry mash

		Escherichia coli	Lactose negative[b]	*Salmonella*
Population[c] of microorganism (CFUx105 per gram of mash)	Raw mash	70.0±41.2	92.0±56.6	0
	Conditioned mash	0	0	0
	Pelleted mash	0	0	0
Efficiency of APC System and a pellet mill against microorganism	APC System[c]	100.0	100.0	NA[d]
	APC System and Pellet mill	100.0	100.0	NA

[a]The new pelleting process consisted of an Anaerobic Pasteurizing Conditioning System[c] (VE Corporation, Arlington, Texas) and a pellet mill (Buhler–Miag, Minneapolis, Minnesota)

[b]Includes non-lactose-fermenter like *Salmonella, Shigella, Proteus,* and *Sematia* spp.

[c]Mean of four trials. Average length of time during which mash was retained in conditioner, and the mean temperature and moisture of mash exiting the conditioner were 4.6 ± 0.5 min., 81.4 ± 2.3°C, and 17.2 ± 1.0%, respectively

[d]Not applicable

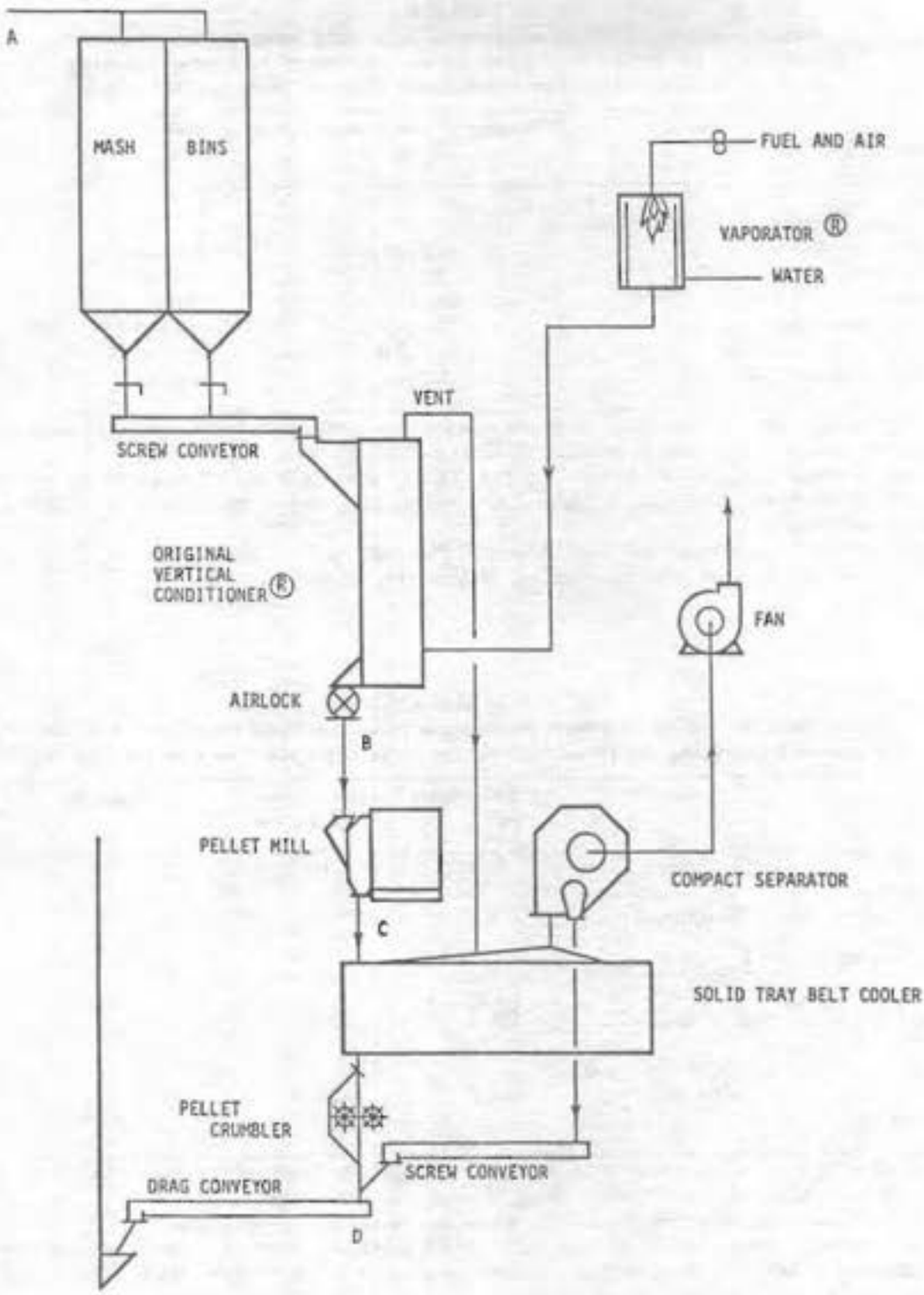

Figure 1: Diagram of new pelleting process showing sampling locations A, B, C, and D.

PROCEEDINGS OF THE MONOCLONAL ANTIBODY WORKSHOP

PRINCIPLES AND APPLICATIONS OF MONOCLONAL ANTIBODIES

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The general purpose of this presentation is to give a brief introductory overview of the subject of monoclonal antibodies.

For many there was a recognized need for producing homogeneous preparations of antibodies of defined specificity. However, it was not until the pioneering work in 1975 of Cesar Milstein and George Köhler at the Medical Research Council Laboratory of Molecular Biology in Cambridge that the technology became available to produce such preparations of homogeneous antibodies. Previous to this time, the only method of producing antibodies was in intact animals. As animals respond by producing antibodies against many of the antigens to which they are exposed in the natural course of their lives, all animals, regardless of how carefully they are raised, contain in their serum mixtures of antibodies of varying specificities. Any experimental attempt, using animals, to produce antibodies, even under the most stringent conditions, would result in preparations that contained not only the specific antibody being sought, but also all of the background antibodies found in the animal. Such antibody preparations are called polyclonal.

As our knowledge of the immune responses increased, the general rolls of the various white blood cells in these responses became known. One of the important findings was the concept of clonal selection. With regard to circulating antibodies, this means that a given B cell is able to combine with a specific antigenic determinant and in turn produce antibodies with specificity only against that some antigenic determinant. Once this B cell has been selected and programed to produce this specific antibody, it is then stimulated to proliferate into a large number of identical cells (clonal expansion), all of which produce this same specific antibody.

Preparation of Monoclonal Antibodies

Monoclonal antibodies are a pure preparation in which each antibody molecule is identical. They are produced by a culture of cells that were derived from a single cloned cell. Monoclonal antibody producing cells are produced *in vitro* and must be maintained *in vitro* if they are to produce antibodies that are completely free of background antibodies that are found in animals.

Two types of cells are fused together to form the monoclonal antibody producing cells. The first is a B cell, taken from the spleen of an animal, that has been programed to produce antibodies against a specific antigenic determinant. The second cell is a cancerous lymphocyte called a myeloma cell. The B cell can be induced to produce a specific antibody but cannot effectively multiply *in vitro*. On the other hand, the myeloma cell cannot be induced to produce a specific antibody, but it is able to multiply indefinitely *in vitro*. Monoclonal antibody producing cells are produced by combining the growth potential of a myeloma cell with the ability of a B cell to produce specific antibodies. This is done by fusing these two cells into a single cell called by hybridoma. An outline of a general method used to produce monoclonal antibodies from mice cells is shown in figure 1.

A mouse is injected with the antigen against which the monoclonal antibodies are to be produced. After several weeks, during which time the specifically programmed B cells have extensively multiplied (clonal expansion), the spleen cells are removed. The spleen cells and large numbers of mouse myeloma cells, that have been grown *in vitro*, are mixed together in a fusion chamber. Polyethylene glycol has been extensively used as an agent to facilitate the fusion of B cells with myeloma cells. Currently, a process called electroporation is also being used to fuse cells. This entails passing a high-voltage, short-duration pulse of electricity through the fusion chamber. Only a few out of several hundred thousand cells in a culture fuse to form hybridomas. Therefore, it is necessary to have a method of selecting these few hybridomas from the many unfused B cells, spleen cells, and myeloma cells. This is accomplished by using mutant myeloma cells that will not multiply in a medium that contains hypoxanthine, aminopterin, and thymidine (HAT medium). The cells from the fusion chamber are passed to HAT medium and there the unfused myeloma cells are unable to multiply and will die off in a few weeks. The unfused B cells are unable to grow *in vitro* and also eventually die out. Only hybridoma cells are able to multiply in HAT medium as their B cell component can utilize the HAT ingredients

and the myeloma components provide the capabilities to multiply *in vitro*.

The next task is to select the hybridoma that is secreting the desired antibody. This is done by subculturing the hybridomas in multi-welled microplates. A limiting dilution is used so that most wells will contain no more than one cell. After incubation, the supernatant from each well is tested for antibodies against the specific antigen. A sensitive assay, such as an ELISA, is used. A well that tests positive contains the sought-after monoclonal antibody producing hybridoma. The positive culture is again diluted and passed one or more times to make sure the culture is truly monoclonal. Usually, several months of intensive work are required to produce a clone of hybridoma cells that are secreting a pure monoclonal antibody.

The next step is to propagate these cells in large numbers. This is often done in an *in vitro* system where the antibodies are secreted into the medium uncontaminated by other background antibodies. When high purity is not critical, the hybridomas can be propagated in mice where antibodies will be found in high concentrations in the serum and other body fluids.

Applications of Monoclonal Antibodies

Monoclonal antibodies can be used in virtually all applications where conventional polyclonal antibodies have been used in the past. In addition, the purity and specific nature of monoclonal antibodies have made them valuable tools with many applications in medicine and all areas of research and industry involving any biologically derived or biologically active materials.

Monoclonal antibodies can be used to identify, locate, quantitate, purify, and destroy any biological substance that can act as an antigen. It is beyond the scope of this presentation to discuss the various applications of monoclonal antibodies.

Numerous books and periodicals contain information on monoclonal antibodies. In particular, Mary Ann Liebert, Inc., New York, publishes *Monoclonal Antibody News*, a forum for publication of information on new monoclonal antibodies, and *Hybridoma*, a journal with full-length papers on monoclonal antibodies. The *Journal of Immunological Methods* publishes a section listing new hybridomas. A good current coverage of this subject can be found in *Monoclonal Antibodies: A Manual of Techniques*, ed. H. Zola, CRC Press, Inc., Boca Raton, Florida, 1987.

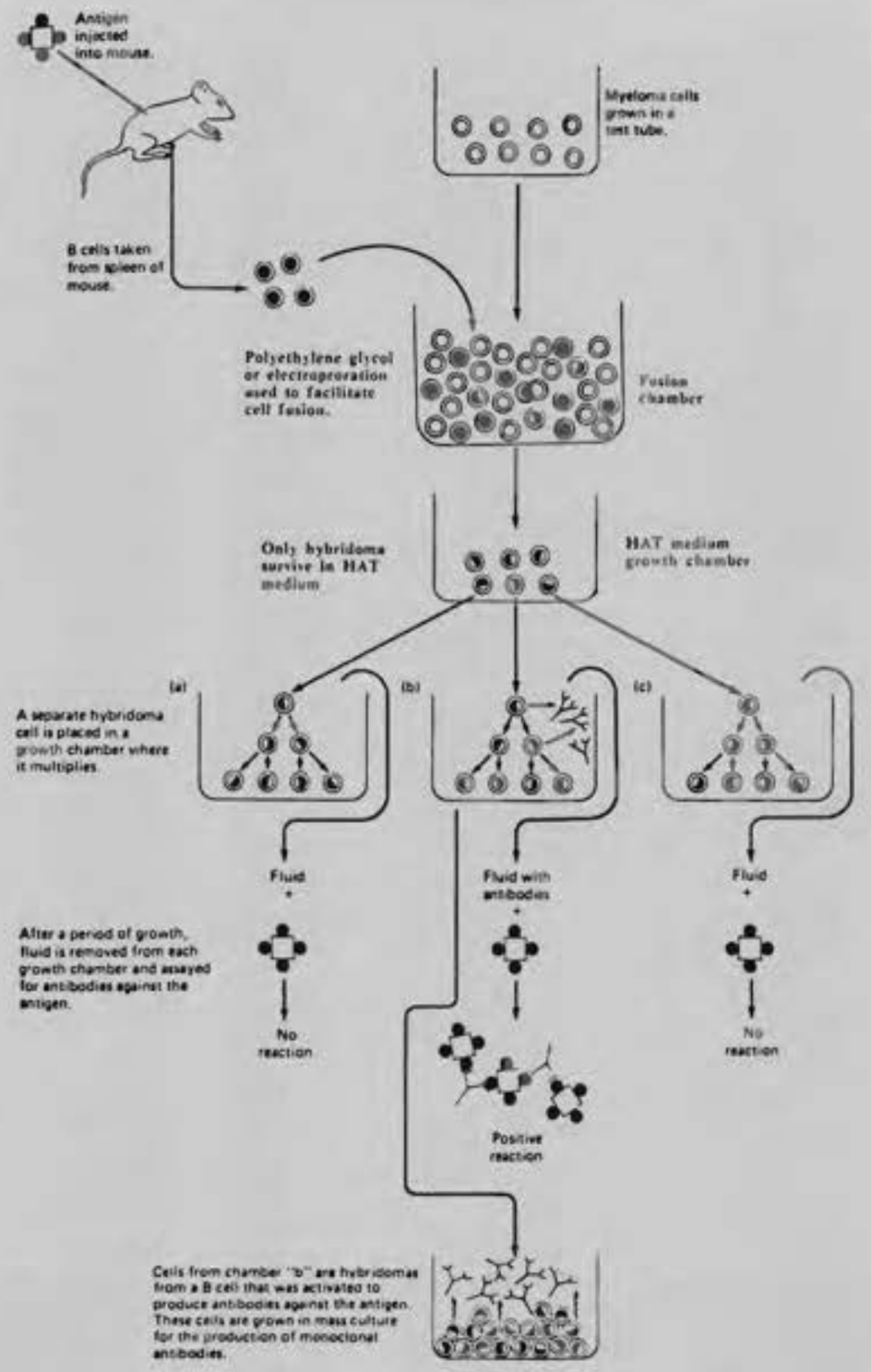

Figure 1: A schematic outline of a method used to produce monoclonal antibodies.

IMMUNODIAGNOSIS OF ENTERIC VIRAL INFECTIONS: APPLICATION OF ELECTRON MICROSCOPY AND MONOCLONAL ANTIBODIES

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Introduction

Enteric viruses are a frequent cause of diarrhea in the young of many species, including poultry. Their fastidious nature precluded routine isolation in embryo or cell cultures, techniques commonly used in diagnostic laboratories to detect and identify other types of pathogenic viruses. Only after electron microscopy (EM) was used to visualize these viruses directly in feces did their routine detection become feasible. However, even now the lack of cell culture systems and sensitive widely available diagnostic reagents and assays continues to hinder progress in detection, identification, and characterization of many newly described enteric viruses.

Several procedures are currently available for immunodiagnosis of enteric viral infections. Many of these procedures have been enhanced recently by the use of monoclonal antibodies (MAbs) alone or in conjunction with polyclonal antibodies for identification and differentiation of viral strains, serotypes, or serogroups. The following is a discussion of various immunodiagnostic techniques, including their use in diagnosis of enteric viral infections, and advantages and disadvantages of each technique.

Enteric Viruses

Properties

Recently, many viruses have been detected in association with diarrhea in man and animals, including poultry. These viruses include coronaviruses, adenoviruses, rotaviruses, caliciviruses, astroviruses, parvo-like viruses, and other unclassified viruses.[15-17,20,23-24] Many of the enteric viruses can be differentiated by EM based solely on their distinctive size and morphology. However, it is now apparent that antigenically distinct rotaviruses occur which are morphologically indistinguishable by EM. These serologically distinct rotaviruses are currently placed in 7 serogroups designated A to G.[17,18,24] Rotaviruses within each serogroup possess common group antigens, which are shared among rotaviruses isolated from different species.[24] Thus, serogroup A rotaviruses occur in most species, including poultry, and can be detected using antiserum which reacts broadly with common group A antigens.[9,15,17,24] At present, serogroup D, F, and G rotaviruses have been identified only in poultry,[17] whereas serogroup B, C, and E rotaviruses have been detected in other species excluding poultry.[15,24]

Another important antigenic property of rotaviruses or other enteric viruses is their serotype specificity. Within serogroup A rotaviruses, at least 7 distinct serotypes (designated 1–7) have been recognized, several of which occur in more than one species.[8] Multiple serotypes of group A rotaviruses exist in man, swine, cattle, and poultry.[3,9,17,20] Serotype specificity is an important characteristic to define since distinct virus serotypes usually do not cross-protect against one another.[3,20] Thus, an animal may become immune and develop cross-reactive, group-specific antibodies to one rotavirus serotype, yet still be susceptible to infection by a different rotavirus serotype. Similarly, animals immune to one rotavirus serogroup may be fully susceptible to infection by the other antigenically unrelated rotavirus serogroups.[7,20] Thus, it is important to develop rapid diagnostic assays which can differentiate not only virus families, but also virus serogroups and serotypes.

Epidemiology and Pathogenesis

Infections with enteric viruses often occur as enzootics in partially immune animals between 1–4 weeks of age.[15,17,22,23] Because most young animals have passively acquired maternal antibodies at this age, attempts to serologically diagnose these infections may fail to provide definitive diagnosis of the disease agent(s) involved.

Most enteric viruses replicate in villous/crypt epithelial cells and are shed in feces for variable time periods after infection.[23] Cytolytic infection of gut enterocytes leads to varying degrees of vil-

lous atrophy and consequently a malabsorptive diarrhea and dehydration in severe cases. The pathology and clinical signs induced by different enteric viruses cannot be distinguished. Thus, an etiologic diagnosis is needed to establish the cause of the diarrhea. Diagnosis is further complicated by the frequent detection of multiple viruses in association with the diarrheal syndrome.[16,17,20,23,33]

Antibody Reagents

Polyclonal Antibodies

Production of highly specific antibody reagents for immunodiagnosis of enteric viral infections requires use of at least semi-purified virus for hyperimmunization of seronegative animals. If no cell culture system is available for propagation of the enteric virus, specific pathogen free (SPF) or gnotobiotic (GN) animals may be necessary for additional amplification of the viral specimen to obtain adequate amounts of virus for purification. In addition, similar animals may be required to produce highly specific polyclonal antiserum, since use of conventional animals may lead to background reactions due to the presence of existing antibodies to other ubiquitous enteric viruses.[2,7,13,31,32,34] For example, because group A rotavirus antibodies are widespread in most species, including rabbits, goats, sheep, or conventional chickens commonly used to produce commercial antisera, such antisera may contain significant levels of group A rotavirus antibodies.[32] Guinea pigs usually have low levels of such antibodies and are often the species of choice (if GN or SPF animals are not available) to produce serotype-specific rotavirus antisera or antisera against other enteric viruses.[7,9]

Polyclonal antiserum has the advantage of being broadly reactive with rotavirus group antigens and may be especially useful for serogrouping rotaviruses and as detection antibodies.[21,24] However, they can be used for serotyping only following extensive cross-absorptions to remove the broadly reacting group-specific antibodies.[4]

Monoclonal Antibodies (MAbs)

Another option now available is production of MAbs for immunodiagnosis of enteric viruses. Monoclonal antibodies are commonly produced by fusion of spleen cells from virus-immunized mice with a nonsecreting mouse myeloma cell line.[4] Antibodies of various specificities can be produced and selected using the appropriate screening assays.[4,10,11,25,29] For example, broadly reacting serogroup-specific MAbs may be selected by using ELISA or immunofluorescence (IF) screening assays in which group antigens present on multiple strains of the same virus serogroup are recognized.[4,11,29] On the other hand, serotype-specific MAbs require use of virus neutralization or hemagglutination inhibition screening assays to identify antibodies which recognize only virus strains of a particular serotype, and fail to react with viral epitopes present on unrelated serotypes.[10,11,25]

After selection and cloning of hybridomas of the desired specificity, the hybridoma cells can be stored frozen in liquid nitrogen. MAbs are then produced as needed by growth of the hybridoma cells in cell culture and harvesting of the supernatant fluids, or by induction of ascites fluids in mice.[29] For use in diagnostic tests, the MAbs may require additional concentration from the supernatant fluids and purification from cell culture fluids or ascites.[4,26]

Immunodiagnostic Tests

Immune Electron Microscopy (IEM)

Diagnosis of enteric viral infections by EM or IEM is contingent upon the morphologic identification of virus particles or immune aggregates. IEM involves the incubation of diluted, filtered fecal specimens with optimally diluted antiserum, followed by ultracentrifugation to pellet the immune complexes or unreacted virus.[18,23] The specimen is then negatively stained, placed on an EM grid and examined for the presence of viral-antibody aggregates, using an electron microscope.[18] Alternatively, viral particles can be captured directly onto antibody-coated (directly or via protein A) EM grids in a solid-phase IEM technique.[1,12,14] Either procedure can be done within the same day or following overnight incubation of the virus-antibody mixtures. Although IEM procedures may require additional time compared with direct EM, IEM has distinct advantages for diagnosis of enteric viral infections. First, incubation of virus with specific antibody improves the sensitivity of virus detection and allows more rapid visualization of the larger virus-antibody aggregates formed.[18] This is particularly applicable for detection of the smaller (<35 nm) enteric viruses. Secondly, the aggregation of enveloped viruses, such as coronaviruses, with specific antibodies aids in their differentiation from morphologically similar membranous particles often present in feces.[1,7,18] Third, use of IEM permits the antigenic differentiation of virus serogroups or serotypes provided highly specific antibody reagents are used. We have used antisera prepared in GN or SPF animals to detect and distinguish the serogroups of rotavirus present in feces from pigs, calves, or poultry.[22,24] Others have used rotavirus serotype-specific anti-

sera in IEM to serotype human rotavirus strains.[8]

Recently, we and others[7,8] developed a protein A-colloidal gold (PAG)–IEM procedure to improve sensitivity for the detection and identification of enteric viruses from feces. This method is conducted as described for IEM, after which the immune complexes are re-suspended in a 10nm protein A gold solution which binds via the Fc regions of the IgG antibody molecules. The PAG–IEM improved the sensitivity of viral detection,[8] aided in the visualization of viral particles,[7] and improved the ability to serogroup rotaviruses present in fecal samples (Saif, L. J., 1988, unpublished).

Although in our earlier reports, only polyclonal antisera were used for IEM, we recently have examined the sensitivity and specificity of MAbs for IEM or PAG–IEM. At present, certain MAbs to rotavirus serogroups A & C appear useful as serogrouping reagents. Use of specific neutralizing MAbs in IEM to serotype rotaviruses directly from feces is also being investigated.[10,11]

Although EM techniques offer efficient and rapid procedures for diagnosis of enteric viral infections, access to an electron microscope and skilled personnel is required. Additionally, the presence of adequate amounts (approx. 10^6–10^8 PFU/ml) of recognizable viral particles are needed for diagnosis.[29] The time it takes to prepare and view specimens may also be a limitation, particularly if the daily examination of large numbers of specimens is required.

Immunofluorescence (IF)

Immunofluorescence microscopy is based on the principle that antibodies can be coupled with a fluorochrome such as fluorescein isothiocyanate (FITC) with retention of antigen binding properties. Such fluorescent antibodies, when stimulated by certain wavelengths of light, emit intense fluorescence (visualized using a fluorescent microscope) and provide highly sensitive probes to detect and localize antigens. Direct IF staining involves the use of antibodies which are directly coupled with fluorochromes. Specimens are incubated with labeled antibody, the unbound antibody removed by washing, and the stained specimen examined using a fluorescence microscope.[27,28] The advantages of this procedure are its speed, since only one incubation and wash step are required, and there is a decrease in possible background reactions if a single, highly specific antibody-fluorochrome reagent is used. However, a distinct disadvantage is that each antibody reagent used must be directly coupled to a fluorochrome. Although this may be readily accomplished using polyclonal antibodies, it is not uncommon that some MAbs may be inactivated by coupling with fluorochromes. To alleviate these problems, an indirect technique may be used in which a specimen is incubated with an unconjugated primary antibody (such as the MAb) washed and then incubated with a fluorochrome-conjugated antiglobulin directed against the immunoglobulins (Ig) of the species used for the primary antibody.[15,17,28,29] Indirect IF has the advantage that a single conjugated antibody may be used to detect primary antibodies prepared in the same species to many different viruses. In addition, indirect IF is more sensitive than direct IF and this application may be especially important with MAbs because fewer binding sites are involved. A possible disadvantage of indirect IF is the specificity of the secondary antibodies which may be obtained commercially but may contain background antibodies to ubiquitous enteric viruses (i.e., rotavirus).[15] Cross-reactions may also occur between antiglobins from various species, or non-specific binding of the secondary antibody may occur via Fc receptors. Proper controls are necessary to confirm the specificity of the reactions observed.

In our laboratory we have developed two main strategies for diagnosis of enteric viral infections by IF.[5,9,27,28] The first involves preparation of mucosal smears from the small intestines of infected animals. These smears are fixed in 100% acetone, washed, and stained by direct or indirect IF. Both polyclonal antisera and MAbs have ben used successfully in these assays, but the MAbs tested had a distinct advantage in the total elimination of nonspecific background IF staining often evident using polyclonal antisera. A more sensitive indirect IF procedure has been devised for detection of porcine coronavirus in intestinal smears or infected cells by using biotinylated MAbs followed by incubation with streptavidin-FITC.[28] Although the activity of several MAbs was destroyed by labeling with FITC, their activity was not adversely affected by biotinylation. Additionally, if more broadly reactive MAbs are desired for IF assays to detect group antigens present on multiple viral strains, it is possible to use a pool of MAbs with specificity for different viral proteins or epitopes.[28] If greater specificity is the goal, then a single MAb which recognizes only certain viral serotypes or strains may be advantageous.[10,11,28,29] It is necessary, however, to individually test the MAbs for use in IF, since it is possible that viral antigenic determinants may be destroyed by certain fixatives (especially aldehydes), and a particular MAb may therefore fail to react in IF.[5]

Advantages of IF staining of intestinal mucosal smears for disease diagnosis include rapid results (within 24 hours) and the ability to visualize viral antigens within the infected cells.[27,28] Disadvantages include the need to euthanize the animal to prepare the smears, the time required for their preparation, staining, and reading, and the need to obtain smears from animals in the early stages of diarrhea—prior to loss of the infected epithelial cells. Results are not easily quantified since visual readings are necessary which limits these procedures to smaller numbers of specimens.

To overcome some of these limitations, we adapted a cell culture immunofluorescence (CCIF) technique to detect and quantitate enteric viruses from feces.[2,17,23] This requires that the enteric virus induce at least a primary infection in cell monolayers seeded in microtiter plates. The virus-infected cells are then detected using standard direct or indirect IF staining. The numbers of infected cells are quantitated visually at each fecal dilution and viral titers can be expressed as numbers of fluorescent focus units/ml. Although this assay is applicable for screening large numbers of fecal samples, it still requires visual assessment of the results. However, the assay does permit detection and quantitation of infectious, fastidious viruses which might not serially replicate in cell culture or produce CPE or plaques under routine cell culture conditions.[27]

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA for antigen detection involves the sequential addition of capture antibody, antigen, and detector antibody reagents to a solid phase with appropriate wash steps to separate antibody bound to the antigen from non-bound antibody. Because of the high degree of automation possible, the entire procedure may be completed within a few hours. The assay is very sensitive, since it permits detection of intact as well as fragmented virus. Endpoint reactions may be read mechanically in a spectrophotometer (ELISA reader) permitting analysis of a large number of specimens within a day.

The principle behind ELISA is that an antibody molecule can be covalently linked to an enzyme to form a conjugate which retains both immunologic and enzymatic function. The antibody-enzyme conjugate can be bound to virus in the test specimen and then allowed to react with a chromogenic substrate to produce a visible color. A single enzyme molecule can react with many molecules of substrate without the exhaustion of enzymatic activity, accounting for the high sensitivity of enzyme-immunoassays. Most ELISA procedures for antigen (virus) detection involve coating microtiter plastic plates or beads with hyperimmune or pre-immune (control) serum as the primary capture antibody. The capture serum can be either the virus-specific polyclonal or monoclonal antibodies.[5,7,10,11,26] If a simple direct sandwich assay is used, then the antigen is sandwiched between two layers of the antiserum with the secondary detector antibody labeled with an enzyme. The advantage of direct ELISAs is that they require only a single antiviral reagent and one less incubation step. However, the direct ELISAs necessitate having a different enzyme-labeled antibody conjugate to detect each enteric virus. Another possible problem with the direct approach is that many MAbs may lose activity after conjugation, thereby necessitating use of a polyclonal antiserum as the detector antibody.

An alternative procedure is the use of an indirect sandwich ELISA.[7,21] In this case, antigen is sandwiched between antisera from two different animal species (primary and secondary antibody) and a tertiary detector antibody is used which consists of enzyme-labeled antiglobulins directed against the Ig of the species used for the secondary antibody. The advantages of the indirect ELISA include increased sensitivity, the commercial availability of the enzyme-labeled antiglobulins, and the use of a single enzyme-antiglobulin for different immunoassays, provided it is directed against the appropriate animal species (e.g., anti-mouse Ig if MAbs are used as secondary antibodies).[12,29] A disadvantage of indirect ELISA is that antiviral sera from two different species is required to prevent binding of the detector antiglobulin to the antibodies coated on the solid phase. Enzymes which are used commonly for ELISA include alkaline phosphatase and horseradish peroxidase with the corresponding substrates, p-nitrophenyl phosphate, and ABTS or orthophenylene diamine, respectively.[8] Additional sensitivity may result by using biotinylated antibodies and enzyme-avidin conjugates.[8,29]

In our laboratory, we generally use an indirect ELISA for detection of rotavirus or coronavirus antigens in fecal specimens.[7,21] Polyclonal antisera to these viruses are prepared in GN animals and used as capture antibodies. For detection of group A rotaviruses or coronaviruses, the secondary polyclonal antibodies are prepared in rabbits or guinea pigs, respectively. The specificity of the test is confirmed by a blocking step in which hyperimmune serum or pre-immune serum is added to the bound antigen in duplicate wells prior to addition of the secondary antibody.

Samples are considered positive if the mean absorbance values are at least 3 SD above the mean of the background absorbance values, and absorbance values are reduced by at least 50% by the blocking antiserum. Recently we have developed ELISAs to subgroup and serotype porcine rotaviruses by using subgroup or serotype-specific MAbs in place of the group A rotavirus antisera as the secondary antibodies.[12] These assays provide a rapid one-day test for subgrouping or serotyping rotaviruses.[5,10] However, the sensitivity appears to be lower than for detection of rotavirus group antigens and approximately 30% of porcine fecal samples positive in group A rotavirus ELISA failed to react in the subgroup or serotype ELISA.[12]

Summary

A number of assays are available for the immunodiagnosis of enteric viral infections. In many situations it may be advantageous to rely upon the results of more than one assay for diagnosis. EM has the advantage of detecting viruses which might be missed in other assays due to a lack of antibody reagents for diagnosis. Each assay has its particular strengths and weaknesses, depending upon its application.

With the exception of direct EM, these assays are contingent upon the production and availability of highly specific antisera to detect and differentiate the enteric viruses. It is likely that the development of MAbs specific for various enteric viruses may alleviate some of the current problems with antisera availability and specificity. However, the activities of the MAbs must be characterized carefully in the various assays and compared for sensitivity and specificity with their polyclonal counterparts. It is possible that pools of MAbs with specificity for different viral proteins and epitopes may be the reagents of choice for detection of multiple viral strains within a single virus family. On the other hand, individual neutralizing MAbs or several neutralizing MAbs against different epitopes on a single viral protein may be ideal for serotypic characterization or differentiation of viral strains. It is also important to recognize the possible limitations of the use of MAbs in various assays, including their possible loss of activity upon conjugation, their low reactivity in some assays due to their failure to bind to multiple viral epitopes, or to bind to protein A reagents (IgM MAbs), and their failure to react with multiple strains of a certain virus if a particular epitope is not highly conserved or is lost when viral mutants arise. However, the latter possibility may not necessarily be a limitation, and MAbs have been actively sought which can differentiate between strains of certain viruses. An example is the use of MAbs in a competitive ELISA which recognize enteric strains of a porcine coronavirus, but fail to react with respiratory strains of the serologically indistinguishable virus.[3]

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CURRENT TECHNIQUES IN IMMUNOHISTOCHEMISTRY INCLUDING THE USE OF MONOCLONAL ANTIBODIES: PRINCIPLES AND TECHNICAL ASPECTS

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Effective procedures for the identification and quantitation of specific biological molecules are required in a number of fields and have particularly important applications in research and diagnostics. Antibody-based detection systems have several advantages, perhaps the most significant being their potential specificity and sensitivity. Consequently, such systems represent invaluable analytical aids.

The evaluation and application of antibody reagents requires an appreciation of the flexibility and power of the immunohistochemical methods to which they may be applied. Slight modifications in methodology can often yield dramatically different results using the same primary reagents. Access to this flexibility is only limited to the lack of understanding with immunohistochemistry. In order to employ immunohistochemical techniques effectively, one must have a firm grasp of tissue processing requirements, reagent selection, and detection systems, as well as the interpretation of final staining results.

The use of immunohistochemical techniques for the localization of enzymes in tissue offers an alternative approach that is not dependent of the preservation of enzyme catalytic activity but on its structure.

Antigens are complicated, degradable proteins that are easily destroyed, denatured, or masked with routine processing. Fixation is probably the most important element in maintaining antigenicity. Ten percent neutral buffered formalin (NBF) is the most prevalent fixative but not necessarily the best one. Certain antigens will be masked due to aldehyde linkages with NBF, and the use of digestive enzymes is necessary.

Proteases break methylene bridges in antigenic molecules, restoring immunoreactivity. A selection of animal enzymes, those derived from bacteria or plants, are available for use. But, there is no such thing as a universal proteolytic enzyme for application in immunohistochemistry.

A frustrating complication of tissue proteolysis is the loosening of tissue sections from the glass slides. This problem may be surmounted by the use of chrom-alum gel, Elmer's glue, or poly-l-lysine[1] as bonding agents.

For many there is no substitute for the use of frozen sections in diagnostic immunohistochemistry. Glycolipid and lipoprotein determinants are usually degraded by liquid mordants, and can only be detected by analysis of fresh frozen tissue. Cell-surface immunoglobulins on lymphoid cells are also lost during standard processing. Frozen sections are especially relevant to immunoreactions done with some monoclonal antibodies, since many of these reagents react only with pristine specimens.

The success of a particular immunohistochemical procedure is predicated on the availability of an antiserum of high specificity for the particular antigen to be studied. Certain monoclonals may lack diagnostic specificity in spite of their absolute immunologic specificity. Regardless of this, monoclonal antibodies generally yield a more interpretable immunohistochemical result. One reason for this is that the hybridoma culture supernatant is essentially free of irrelevant antibodies in contrast to heteroantisera.

A polyclonal antiserum is capable of recognizing multiple epitopes on the same antigen. Unfortunately, the relatively large number of different determinants recognized by the antiserum enhances the likelihood that immunologic binding of macromolecules other than the target antigen will occur. With appropriate dilutions, these cross-reacting antibodies can be effectively reduced without loss of target labelling.

The basic techniques on which newer methods are founded are: the direct and indirect conjugate procedures, the labeled antigen method, the enzyme bridge method, and the peroxidase-anti-peroxidase procedure. New methods are developed either to enhance the sensitivity or specificity of these methods or to provide alternative approaches for use in special situations.

Exploiting the high affinity binding of avidin (a glycoprotein) for biotin has provided an alternative to the more usual labeled peroxidase anti-peroxidase methods. The utility of systems based

on biotin-avidin is limited by two major problems associated with the chemical structure and properties of avidin.[3] A new development in the design of indirect immunoperoxidase systems-the biotin-streptavidin system provides an alternative and offers the benefit of increased sensitivity. A broad spectrum of biological molecules, including antibodies and enzymes, can be efficiently biotinylated without significant loss of biological activity. Detection systems based on the biotin-streptavidin system do not suffer from the problems of non-specific binding associated with egg-white avidin but retain the flexibility and convenience associated with these systems.[3]

Another modification is Protein A, a cell-wall protein from staphylococcus aureus that binds specifically to the Fc portion of IgG from several mammalian species.[4] Two common immunoperoxidase methods using protein A are, the two stage, with protein A conjugated with horseradish peroxidase and the three stage technique utilizing protein A as an unlabeled bridge reagent. With protein A there are few limitations with respect to the species of origin of the unlabeled primary antibody. This gives an extraordinary degree of versatility, for protein A can be used with many different primary antibodies from many different species, with the sole provision that the Fc portion of IgG be able to react with protein A.

An immunohistochemical system must be supported by evidence of both method specificity and antibody specificity. Each antibody selected for use must provide an interpretable and useful result. To evaluate each new reagent, four phases of study are required: the selection of appropriate positive and negative controls; the determination of the optimum titer; the maximization of staining contrast at optimum titer; and the formal evaluation of antibody reactivity in tissue sections of known and unknown antigen content.

The many variations of the basic immunoperoxidase technique have been developed to meet some perceived need, for greater specificity, for higher sensitivity, or general utility.

In approaching an immunoperoxidase method, the uninitiated are advised first of all to define the requirements of the study, the types of tissues to be studied, and the reagents available for use. From consideration of these factors, coupled with some knowledge as to the nature and cellular localization of the antigen under study, it is possible to make a rational choice of technique to fit the occasion.

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INFECTIOUS BRONCHITIS VIRUS TYPING BY MONOCLONAL ANTIBODIES: RECENT PROGRESS

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Introduction

Infectious bronchitis virus (IBV) is the prototype of the family Coronaviridae. IBV, like the other members of the Coronaviridae family, posses surface projections called spikes or peplomers which give the virions their characteristic appearance in the negative-stained preparations. IBV contains three structural proteins, namely nucleoproteins (N), membrane or matrix (M) proteins, and the spike (S) proteins. Of these the M and the S proteins are partially exposed on the outer viral surface and the immune responses elicited against these are of both biological and economic interest.

In attempts to produce monoclonal antibodies (MAbs) useful in IBV typing, S proteins have been found to be most important. The S proteins form the viral peplomers. Each peplomer is composed of two or three copies of each of two structurally unrelated subunits, S1 [mol wt about 90,000 (90K)] and S2 (about 84K), derived by cleavage of a precursor glycoprotein.[1,2] The S2 molecule is anchored in the membrane, while S1, which forms the outside bulbous part of the peplomer, is noncovalently attached to S2. The S2 protein is highly conserved in sequences among coronaviruses, whereas S1 proteins are nonconserved. Viruses from which S1 glycoprotein was removed by urea treatment[3] were shown to no longer infect cells *in vitro* nor were they able to hemagglutinate. Also, viruses from which the S1 but not the S2 glycoprotein was removed did not induce protection or virus neutralizing (VN) and hemagglutination inhibition (HI) antibody in chickens.[3,4] However, intramuscular inoculation of monomeric S1 induced both VN and HI antibodies.[4]

Antigenic Diversity

One of the major concerns in the control of IBV in the field is the prevalence of many serotypes which antigenically differ from the vaccine strains. Such variant serotypes have been reported from all over the world and appear to periodically emerge in the field by the process of mutation or genetic recombination. Although such genetic changes may structurally alter any of the viral proteins, a change in the S1 protein is likely to result in the emergence of a "variant" virus. Such viruses possessing an altered antigenic site for neutralization will have selective advantage in vaccinated chickens and will spread rapidly through the flock.

Antigenic differences among IBV have been mainly demonstrated by VN and HI tests. Both of these procedures have been less than adequate in determining serological differences across the wide spectrum of IBV isolates.[4] Therefore, the search for a better method for serologic identification and classification of IBV strains continues. An approach that holds promise is the use of MAbs directed against virion surface proteins that would delineate strain differences and thus serve as a tool for identification and classification of IBV isolates.

Procedures for MAb Production

In preparing IBV antigens for mouse inoculation, care is exercised to preserve the integrity of S proteins. Allantoic fluid from IBV-inoculated embryonating chicken eggs is commonly used as a virus source. Mockett et al.[5] have described a procedure to prepare a spike-enriched preparation. They recommend NP40 to solubilize the virus followed by sucrose gradient purification and concentration of the spike proteins. Koch et al[7] have used virus concentrated by sucrose density gradient centrifugation, whereas Niesters et al.[8] have used sucrose gradient purified virus disintegrated with 1% Triton X-100. In our laboratory we first precipitate viral proteins with 7% (w/v) polyethylene glycol (MW 8000) prior to virus purification on a glycerol (30%)—Pot. tartrate (50%) gradient. This procedure has increased virus yield and was shown to preserve viral surface projections. Our immunization schedule of BALB/c mice includes intramuscular injection of purified IBV (in 50% Freund's complete adjuvant) followed by two additional injections *in* 50% incomplete adjuvant at 4-week intervals. Finally, purified virus without adjuvant is injected *via* the intrasplenic route (100 µg of viral protein in 50–100 µl quantity) and fusion is performed 4 days later.[9] Hybridoma supernatant fluids are

screened for antibody by an indirect enzyme-linked immunosorbent assay (ELISA) procedure. Cloning of positive hybridomas and preparation of ascitic fluids are performed using standard procedures.[8]

IBV Typing by MAb

Published reports as well as our own studies have all indicated that MAbs to epitopes on S1 protein offer the best possibilities for use in the typing of IBV isolates.[5,6,7] On the other hand, MAbs to S2 and M proteins, which are known to cross-react with a broad spectrum of IBV strains, are not suited for this purpose.

S1 has been shown to carry the sites important to both VN and HI. In addition, four other epitopes have been identified on S1 but no biological activities have been attributed to them.[4] Those MAbs that possess neutralizing activity generally possess HI activity as well. This could suggest that the VN and HA epitope(s) may be one and the same. However, there is evidence to suggest the sites involved in hemagglutination and neutralization are different. Mockett and Durbyshire[10] showed that HI and SN antibody responses in chickens after IBV infection were different. HI antibodies peaked early at 10 days postinfection (PI), whereas SN antibodies were late to appear and peaked around 23 to 30 days PI. The two MAbs that Mockett et al.[6] produced against S1 both had HI and SN activity, but one MAb had a higher HI than SN titer, and the converse was true for the other. This led Mockett et al. to suggest that the HI and SN sites on the S1 are either very close to each other or are overlapping.

Those MAbs that have shown SN and HI activity have mostly been strain-specific with little or no cross-reactivity with heterologous strains.[5,6] On the contrary, MAbs against the other epitopes on the S1 protein and those against matrix proteins have generally been cross-reactive against a wide spectrum of IBV strains.

Exploration of the IBV surface proteins with the aid of MAbs has just begun. Already, important information has been published on the role of S1 proteins in VN and HI and the fact that these sites are strain-specific. Future work is expected to bring out yet more discrete information which will lead to a more complete understanding of strain differences and help resolve problems associated with IBV strain identification and typing.

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ANALYSIS OF PROTEINS OF TURKEY HEMORRHAGIC ENTERITIS VIRUS BY MONOCLONAL AND POLYCLONAL ANTIBODIES

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Summary

A total of eleven polypeptides ranging from 14 to 97 kDa in molecular weight are detected in purified preparations of hemorrhagic enteritis virus (HEV) and lysates of cells infected with HEV as examined by immunoblot assay against polyclonal antisera from infected turkeys. Identical patterns were observed with preparations of marble spleen disease virus (MSDV). A number of monoclonal antibodies (MAbs) were selected for their reactivity in serum neutralization (SN) assay for identification of neutralizing epitopes of these two viruses. Several of these MAbs reacted with a 97 kDa capsid polypeptide in an immunoprecipitation assay, regardless of their reactivity in a SN test. Immunoprecipitation assay failed to detect low molecular weight polypeptides.

Introduction

Hemorrhagic enteritis (HE) is a contagious disease of young turkeys characterized by enlargement of spleen, hemorrhagic lesions in the intestine, and, incidentally, sudden death. The disease is caused by HEV, a type II avian adenovirus, which is antigenically distinguishable from all other type I avian adenoviruses. An antigenically related type II avian adenovirus causes marble spleen disease (MSD) in pheasants and is known as MSDV. Pathogenically, HEV is different from MSDV. HEV causes typical HE lesions and mortality in turkeys, whereas MSDV causes no HE lesions. MSDV has been used as an effective vaccine against HE in the field, and antibodies against either viruses effectively neutralize both viruses. Several serological tests such as immunodiffusion, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA) have been used for differentiation of HEV and MSDV without success. In this study, we applied the techniques of immunoprecipitation and immunoblot for identification of polypeptides of these two viruses with the use of hyperimmune turkey sera and monoclonal antibodies against these two viruses.

Materials and Methods

HEV and MSDV[1] were propagated in MDTC–RP19 lymphoblastoid cell line as reported earlier.[2] Procedures for ELISA using infected whole cell as antigen and serum microneutralization (SN) assay are as reported earlier.[3] Fluorescence antibody (FA) assay on infected RP–19 cells are those reported previously.[4] Monoclonal antibodies were prepared against HEV infected turkey spleen cells and MSDV infected RP–19 cells as described earlier.[5] Immune precipitation of ^{35}S methionine labeled HEV and MSDV against polyclonal turkey sera or monoclonal antibodies were according the published procedures.[6] Transfer of proteins to nitrocellulose filters for immunoblots were according to the procedures of Dunn.[7]

Results

A total of 34 MAbs were developed against HEV, of which all reacted with HEV antigen in ELISA, but only 30 reacted in the same assay with MSDV antigen. Of these 34 MAbs, 16 had neutralizing activity against HEV and only 15 against MSDV (Table 1). Of the 13 MAbs against MSDV all reacted with both viruses in ELISA, two reacted with MSDV, and only one reacted with HEV in the SN test. These data also indicate a possible antigenic difference between the two viruses. The two MAbs with specific activity against either virus, however, lost their neutralizing activity and could not be recovered after cloning of MAb producing hybridomas.

Six MAbs (Table 2) were selected for their high titers in serological assays used. One of these MAbs lacked SN activity while all other 5 had high SN titers. These were assayed in immune precipitation and polyacrylamide gel electrophoresis (PAGE) against ^{35}S methionine labeled HEV or MSDV antigens. Results indicated that some of the MAbs precipitated a single polypeptide of 97 kDa, which was identical with a similar polypeptide immune precipitated by hyperimmune anti-HEV turkey serum (Figure 1). The precipitation of this single polypeptide could

not be correlated with SN activity, as some of the neutralizing MAbs failed to precipitate this polypeptide, whereas the single non-neutralizing MAb did precipitate this polypeptide. Attempts to further analyze viral polypeptides reactive with MAbs by the immunoblot assay totally failed as none of the MAbs reacted in this assay.

Figure 2 demonstrates immunoblots of HEV and MSDV transfers reacted with a hyperimmunized turkey serum against HEV. Eleven polypeptides ranging from 14 to 97 kDa are detected in both preparations of HEV and MSDV. The highest molecular weight polypeptide appears detectable by both monoclonal and polyclonal antibodies, and is similar in size to capsid protein of other known adenoviruses.

Discussion

HEV and MSDV are members of type II avian adenoviruses and are antigenically distinguishable from all type I avian adenoviruses. Although there are several pathological and immunological differences between HEV and MSDV, it has not been possible to serologically distinguish one from the other. HEV is highly pathogenic for turkeys whereas MSDV does not show any major pathogenicity for turkeys, except some transient splenomegaly. MSDV, on the other hand is pathogenic for pheasants but is protective in turkeys against challenge with virulent HEV. Turkey sera against either virus has shown similar qualitative reactions in several serological assays, including SN test, but with some minor differences in SN titers (our unpublished data). Monoclonal antibodies offered some promise, in that a number of these reacted in ELISA with only HEV and not with MSDV. These require further evaluation.

We applied immune precipitation and immunoblot assays for identification of unique proteins of these viruses reacting with monoclonal antibodies. Three of the six MAb's studied reacted with a viral specific 97 kDa polypeptide that could be related to viral neutralizing epitope. These studies also showed that polypeptides of HEV, MSDV, and their molecular weights as studied by immunoblots against hyperimmune turkey serum were similar.

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TABLE 1

Reactivity of HEV and MSDV monoclonal antibodies (MAbs) in enzyme-linked immunosorbent assay (ELISA), fluorescent antibody (FA) and serum neutralization (SN) assays

MAbs Against	No.	ELISA HEV	ELISA MSDV	FA HEV	FA MSDV	SN HEV	SN MSDV
HEV	34	34/34	30/34	31/34	NT	16/34	15/34
MSDV	13	13/13	13/13	10/13	NT	1/13	2/13

TABLE 2
Reactivity of several selected monoclonal antibodies (MAbs) used in these experiments

HEV MAbs	ELISA	FA	SN
10B–95	+	+	+
10B–248	+	+	+
10B–403	+	+	+
10B–487	+	+	+
10B–514	+	+	+
10B–610	+	+	–

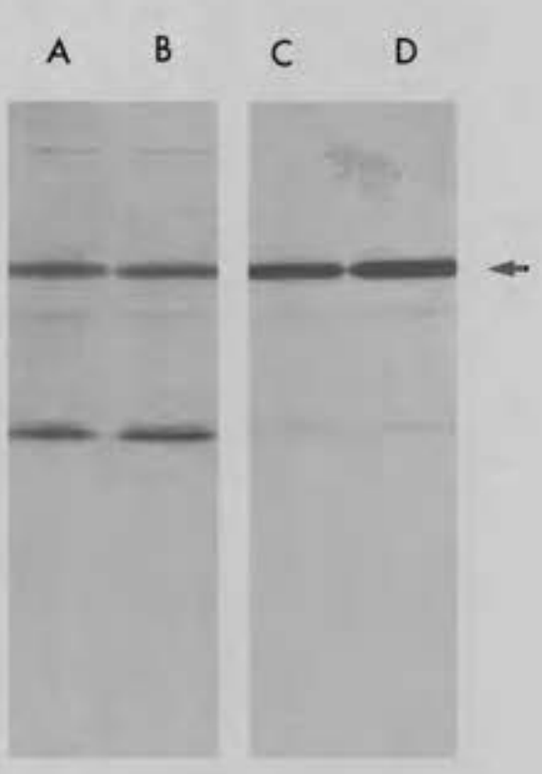

Figure 1: SDS PAGE immunoprecipitation of ^{35}S methionine labeled HEV and MSDV immune precipitated with anti-HEV turkey serum (A and B respectively) and anti-HEV MAb (10B–403) C and D respectively. Arrow points to a single polypeptide of approximately 97kDa common between both viruses and precipitated by both turkey serum and MAb.

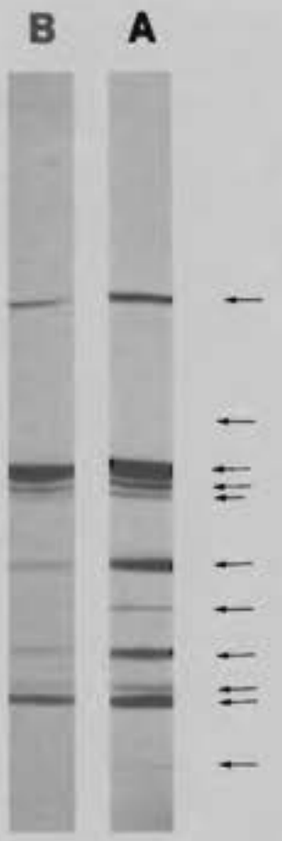

Figure 2: SDS PAGE immunoblots of (A:HEV) and (B:MSDV) lysates with anti-HEV turkey serum. Arrows point to specific polypeptides ranging in molecular weight from 14 to 97 kDa. All polypeptides appear in common between both viruses.

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST A TYPE 3 STRAIN OF *PASTEURELLA MULTOCIDA*

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Four monoclonal antibodies (MAb) designated as 6EE11, D7H10, C11H2, and E11E3 were developed by fusing SP 2/0 myeloma cells with spleen cells obtained from mice immunized with inactivated whole cells of *P. multocida*, strain P–1059. Supernatants of the hybridoma cells were screened with the surface protective antigen (2.5 S) derived from 2.5% saline extract and lipopolysaccharide antigen of P–1059 strain by an enzyme-linked immunosorbent assay (ELISA). The isotype of monoclonal antibodies were determined by an ELISA, indicating 6EE11 as IgG2b and the three others as IgG3 subisotypes. Monoclonal antibodies 6EE11 and D7H10 produced an identical pattern in the Western blot assay with 2.5 S antigen, while E11E3 and C11H2 produced another identical pattern different from that by the other two. Monoclonal antibodies 6EE11 or D7H10 reacted with a major band of the molecular weight of 35,500, whereas E11E3 or C11H2 recognized a major band corresponding to the molecular weight of 9,500 identified by the silver stain. When 2.5 S antigen was treated with periodic acid (10mM), E11E3 no longer reacted with the antigen, but 6EE11 still maintained the antibody activity. However, after the treatment with proteinase K, 6EE11 did not recognize the major band of 2.5% antigen. Colonies of fifteen serotypes of *P. multocida* were subjected to the indirect immunofluorescence assay employing MAb, 6EE11 and E11E3, and fluorescein-labeled goat anti-mouse IgG. Monoclonal antibody 6EE11 gave positive reaction with serotypes 3, 4, 9, 10, or 11, and, to a lesser extent, with type 12, but it failed to react with type 1, 5, 6, 7, 8, 13, 14, 15, or 16. E11E3 was positive only with serotype 3 or 10 strains. Both MAb were negative with *P. haemolytica, Escherichia coli, Staphylococcus epidermidis,* or *Salmonella typhimurium*. The monoclonal antibodies seem useful for studying the surface antigen of *P. multocida* and may be applied to identify the serotype in clinical specimen.

APPLICATION OF GENETIC FINGERPRINTING FOR AVIAN PATHOGENS

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Infectious agents differ in their capability to infect and cause disease in animals. The capability to infect is correlated to the intrinsic susceptibility of a host and whether or not the host is immune. Virulence is the ability of an infectious agent to cause disease in a particular host in terms of frequency and intensity. Pathogenicity and virulence are inherent properties of an infectious agent and are either phenotypically or genotypically conditioned.

Genetic fingerprinting is usually much more sensitive than serologic methods for recognizing genetic divergence among related strains. Once a main etiologic agent has been recognized, its description with regard to epidemiologically appropriate characteristics becomes an important objective. The first step is classification, because the agent may share properties with already known agents of the same class. The bacterial epidemic strains have conventionally been defined by genus, species, biotype, antibiotic resistance pattern, phage type, or serotype. These phenotypic determinations are based on specific assays for antigens, enzymes, toxins, and other gene products.

Sometimes, further tests are essential for additional subtyping of organisms, particularly when an outbreak of disease is produced by an organism with frequently occurring phenotypic characteristics. Identification of epidemic strains by genetic markers offers a more direct and specific evidence.

DNA fingerprinting involves: 1) isolation and purification of DNA; 2) cleavage of DNA with restriction endonucleases into discrete DNA fragments; 3) separation of DNA fragments in gels in an electric field; and 4) staining of DNA bands and their visualization under UV light. The electrophoretic patterns of DNA molecules cleaved with restriction endonucleases are called DNA fingerprints.

Chromosomal DNA can be isolated by many ways. However, the principles are almost the same. In case of bacteria the bacterial outer cell membrane is disrupted by treatment with EDTA. After disruption, the cell wall is removed by treatment with lysozyme. Internal membranes are lysed with a detergent; the proteins are removed by sequential extractions with phenol and chloroform; then DNA is precipitated, washed, and reprecipitated so that it will be free of cellular contaminants. Similarly, viral DNA can be extracted from virus particles using conventional phenolchloroform extraction methods.

Chromosomal DNA is digested with an enzyme (a restriction endonuclease) followed by electrophoresis to separate the DNA fragments by size. The gel is stained with ethidium bromide, which binds to the DNA and fluoresces when illuminated with ultraviolet light.

The restriction endonucleases recognize unique DNA sequence and split the double-stranded DNA at specific spots. The restriction endonuclease characterization depends on the presence of specific nucleotide sequences; therefore, the identical chromosomes would have identical targets and would be cleaved into identical DNA fragments.

Examination of genomic variation by polymorphism in nucleic acid fragments produced by restriction endonuclease digestion is a good method for understanding the epidemiology and pathogenesis of infectious diseases.

For the past two years, the researchers at the University of Minnesota are involved in examining the genetic relationship between strains among some poultry pathogens. Three poultry pathogens are being investigated. They include one DNA virus and two bacterial agents. The viral agent is Adenovirus type II and the two bacterial agents are *Pasteurella multocida* and *Salmonella enteritidis*.

The avian adenoviruses cause a variety of clinical and pathological conditions in chickens, quail, turkeys, pheasants, geese, and guineafowl. The splenomegaly in chicken (AAS), Marble spleen disease (MSD) in phensants, and Hemorrhagic enteritis (HE) in turkeys are all caused by a type II avian adenovirus.[1,2,3] The adenovirus type II of avian species are closely related and exhibit extensive serological crossreactivity. Conventional serological tests currently in use cannot

distinguish between AASV, MSDV, and HEV.[4,5,9] The DNA from these viruses were examined with five restriction endonucleases. Distinct differences in DNA cleavage patterns were found in these virus isolates suggesting genetic differences between isolates of adenovirus type II. This method was found to provide a method for distinguishing genetically different and yet serologically similar strains of avian adenovirus type II.

The genome from field isolates of *Pasteurella multocida* from turkeys and those of reference CU and M9 vaccine strains were analyzed and compared by cleavage with restriction endonucleases. The electrophoretic profiles obtained with DNA fragments from field isolates and vaccine strains of the same serotype were characteristic and reproducible. These features indicated the existence of heterogeneity among the isolates of the same serotype which cannot be differentiated otherwise with the presently available serotyping methods. However, several field isolates showed general similarity of electrophoretic profiles of cleaved DNA fragments with either CU and M9 vaccine strains.

We have concluded that restriction endonuclease fingerprinting of DNA genomes from *P. multocida* from turkeys provides the information for differentiation of field isolates or vaccine strains of the same serotype.

Currently, the chromosomal DNAs of *Salmonella enteritidis* isolates from chickens and human cases are being analyzed and compared for their possible relationship. The results of this investigation will have an important bearing on the epidemiology of infection by *Salmonella enteritidis*.

Twenty-eight isolates of *S. enteritidis* from animal origin were provided by the National Veterinary Service Laboratory at Ames, Iowa. These isolates originated from eleven species of animals and feed reported from fifteen states. The samples were isolated from chickens, turkeys, ducks, geese, cattle, horse, swine, cats, mink, monkey, rodents, and feed.

Seventy-four isolates from human and animal origin were provided by the Center of Disease Control at Atlanta, Georgia.

The nucleic acid (chromosomal DNA) extracted from *S. enteritidis* was examined using restriction endonuclease fingerprinting. A similar banding pattern was seen in the DNA from all chicken isolates except one isolate designated as Spain and one isolate designated as 87–7284. Both of these showed a different electrophoretic migration pattern from all others. In the Spain isolate, three extra bands (500 bp, 400 bp, 300 bp) were found which were not seen in other chicken isolates. In the isolate designated 87–7284, two extra bands (600 bp and 800 bp) were found. Thus, we inferred that three distinct types of *S. enteritidis* were recognized among the chicken isolates examined. The majority of the isolates belonged to Type I. The Spain and the 87–7284 isolates were distinct by themselves.

The Type I isolate designated above was then compared to the remaining isolates from other species. Identical nucleic acid banding patterns were observed between Type I and isolates from all other species received from NVSL.

The DNA extracted from the human isolates received from CDC was examined by the fingerprinting process. Three distinct migration patterns were observed. The majority of the human isolates had an identical banding pattern to the Type I of the chicken isolates. Isolates designated as NE86 from raw egg slurry and CDC–B2187 were distinct by themselves.

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USE OF A SYNTHETIC DNA PROBE FOR IDENTIFYING PENICILLINASE GENES IN STAPHYLOCOCCI ISOLATED FROM TURKEYS

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Introduction

DNA hybridization has become a routine procedure in many laboratories and has aided in the understanding of gene location, function, and expression. The procedure is based on the technology that utilizes a solid phase containing immobilized target DNA and a liquid phase probe. The reannealing of DNA probes with DNA immobilized on a solid support allows one to rapidly screen many samples of DNA for the presence of a specific gene. The power of this technology has been increased greatly by the advent of molecular cloning techniques and the invention of automated oligonucleotide synthesizers which can rapidly generate large amounts of oligonucleotides of a specific sequence.

This technology has directly aided us in our approach to producing a penicillin-resistant strain of *Staphylococcus epidermidis* that can be used as an interfering agent in the control of staphylococcosis in poultry. The currently used *S. epidermidis* strain 115 has been shown to be a nonpathogenic, bacteriocin-producing strain that is effective in reducing the incidence of staphylococcosis in turkeys.[1] However, *S. epidermidis* strain 115 has shown a high susceptibility to β-lactam drugs (penicillin, ampicillin, etc.). The ability to maintain protective colonization with *S. epidermidis* during the administration of penicillin would be enhanced if *S. epidermidis* could protect itself by producing β-lactamase (penicillinase). This could be accomplished by introducing the β-lactamase gene into *S. epidermidis 115*. A common source of the β-lactamase gene is *Staphylococcus aureus*. If the β-lactamase gene is stable on a *S. aureus* plasmid, then transfer of the isolated plasmid to *S. epidermidis* should confer resistance. However, if the β-lactamase gene is functional only when carried on chromosomal DNA, then procedures must be used that incorporate the gene into the chromosome of *S. epidermidis* rather than having it exist extrachromosomally on a plasmid.

Selection of Probe Sequence

The naturally occurring β-lactamase gene previously isolated from *S. aureus* has been completely nucleotide sequenced.[2] The gene is designated *blaZ* and codes for the β-lactamase designated type PC1. A computerized comparison of this sequence to the sequence of the β-lactamase gene TEM-1 from *E. coli* showed very little homology, thereby making it impossible to select a sequence suitable for synthesizing a probe that could detect both types of β-lactamase gene. This would have been especially useful as a positive control since the molecular weight marker DNA we use is constructed from *E. coli* plasmid pBR322 which carries the β-lactamase type TEM-1 gene. The following 20 nucleotide sequence was chosen to synthesize the probe because it contained a higher percent of guanosine (G) and cytosine (C) bases than any other possible length of 20 nucleotides in the gene: 3'-CCTAAGGGCGAATCTGAACC-5'. A high G+C content improves the specificity of the probe, since it allows for the use of a higher hybridization temperature which decreases the incidence of non-specific binding of the probe.

Automated Synthesis of the Probe Oligomer

Having chosen the sequence to be synthesized, a relatively large amount of the oligomer was synthesized using the Applied Biosystems Model 381A DNA Synthesizer. This synthesizer uses the efficient phosphoramidite method of oligonucleotide synthesis.[3] Briefly, the first step in the synthesis is treatment of the solid support matrix within the reaction column so it will bind the first nucleotide in the sequence. The column is then loaded with the first nucleotide and binding is allowed to occur. Excess nucleotides that are left unbound are flushed from the column. The column is then again treated to accept the second nucleotide, loaded with the nucleotide, and following binding once again flushed. As the

process is repeated, the oligomer continues to grow one base at a time in a programmable manner while one end remains attached to the column support. After the addition of the last nucleotide in the sequence, the oligomers are cleaved from the support, collected, and prepared for labeling.

The oligomer which is to be used as a DNA probe is either end labeled with radioactive phosphorous(^{32}P)[4], or labeled with photoreactive biotin.[5] Both of these methods are relatively simple and allow for subsequent detection of the probe when hybridized to the target DNA.

Blotting—Immobilization of DNA to be Probed

After culturing several field isolates of *S. aureus* from turkeys, the DNA from each isolate was extracted. Both chromosomal and plasmid DNA was extracted by first treating the cells with lysostaphin, an enzyme that digests the cell wall of most staphylococcal species. Complete lysis and purification of the DNA was accomplished using the SDS-centrimonium bromide method.[3]. Samples of DNA from each field isolate were then electrophoresed on 0.9% agarose gels which allowed for separation of the DNA by molecular weight.

DNA bands in the agarose gel were then transferred to nitrocellulose filters using Southern blot transfer.[7] The agarose gel was soaked in alkaline solution to denature the DNA bands and then layered with a sheet of nitrocellulose. Capillary transfer of the DNA occurs as high salt solution diffuses through the gel/nitrocellulose stack. Eventually all of the DNA is directly transferred from the gel onto the nitrocellulose filter which immobilizes the DNA in the same positions found in the gel.

Alternatively, the DNA can be electroblotted onto a nylon membrane. This is done by layering the gel with a nylon membrane and placing it in a electrical field perpendicular to the stack. DNA then migrates out of the gel and onto the nylon membrane. Because the biotin-avidin detection system is more sensitive on DNA bound to nitrocellulose rather than nylon, Southern blotting with nitrocellulose was the method of choice.

Hybridization

After the DNA has been transferred to the nitrocellulose, the presence of the β-lactamase can be detected by its ability to hybridize to the probe.[3] A solution containing the labeled probe was added to a sealable bag containing the nitrocellulose filter. This was then incubated at 37–42°C which is the optimal temperature range for hybridization of a 20 base oligomer. After allowing the hybridization to proceed overnight, the filter was washed several times at 45°C in low salt buffer. This washing removes any background or mismatched probe, leaving only correctly matched probe.

Detection

The detection system used takes advantage of the high affinity the avidin molecule has for biotin. Before beginning the detection procedure, a preformed complex was made by the addition of biotinylated horseradish peroxidase to avidin. Horseradish peroxidase is an enzyme that catalyzes the formation of a colored end-product in the presence of alkaline phosphatase substrate. The nitrocellulose filter was then incubated with the preformed complex. Under appropriate conditions, the avidin will bind to the biotin of the labeled probe. After sufficient time for binding, unbound avidin complex was washed from the filter. The filter was then incubated at room temperature overnight with the alkaline phosphatase substrate. Blue bands were present on the filter at those locations where the probe hybridized to the β-lactamase gene.

A probe labeled with ^{32}P can be detected by placing the filter over X-ray film and allowing exposure to proceed over night. Upon development of the film, bands will be visible where the probe had hybridized.

Results and Discussion

Our results indicate that the β-lactamase gene is most likely located in the chromosome of *S. aureus* isolates. Some difficulties in detection sensitivity were encountered when using the biotin-avidin system due to the shortness of the probe. It appears that during labeling one biotin molecule is associated with every 50 to 100 nucleic acid bases. Hence, with a probe of only 20 bases it is possible that not every oligomer is carrying a biotin molecule. This problem may be overcome in the future by using a higher concentration of biotin during labeling as a means of saturating the binding capacity of the oligomer. The probe labeled with ^{32}P gave good results. However, the half-life of ^{32}P is only 14 days which means the probe is not nearly as stable as the biotin labeled probe, which can be stored for over a year.

Attempts by our laboratory to transform *S. epidermidis* 115 with plasmids carrying the β-lactamase gene also show that the gene is unstable when carried on a plasmid in this strain of *S. epidermidis*. Efforts at transforming the

gene by fusion of *S. aureus* and *S. epidermidis* protoplasts[9] gave very poor results. This is most likely due to the fact that the β-lactamase gene is simply not carried on a plasmid in the *S. aureus* donor strains. Plasmids pWN101[10] and pJM13[8] have been constructed to include a copy of the β-lactamase gene and replicate in both *B. subtilis* and *S. aureus*. We have introduced these plasmids into *S. epidermidis* 115 by electroporation, a technique that creates transient pores in cell surfaces by application of a controlled electrical pulse. *S. epidermidis* cells carrying these plasmids either stop producing β-lactamase after several passages due to instability of the plasmid, or stop producing active bacteriocin while synthesizing β-lactamase.

Current research is aimed at constructing an integration plasmid from either pWN101 or pJM13. Integration plasmids have the capability of becoming incorporated into the host chromosomal DNA following transformation. This is because the plasmids contain an inserted fragment of the chromosomal DNA which allows them to enter the host chromosome through homologous recombination. Thus, a copy of the β-lactamase gene is carried into the chromosome where it should be stably maintained.

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