July 23 2000

AAAP Symposium

Molecular Identification and Epidemiology of Avian Pathogens

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Molecular Identification and Epidemiology of Avian Pathogens

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Symposium Goal: This symposium was designed to highlight only molecular diagnostic assays that are currently being used to diagnose disease agents in commercially reared poultry or are being used to study the epidemiology of avian pathogens. The presentations focus on the strengths and weaknesses of the molecular assays, how the tests compare with "gold standards" and most importantly, how the information generated can be used to improve poultry health.

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Acknowledgment:

Thanks to the AAAP Biotechnology Committee members for guidance and direction in the effort to plan and execute this Symposium.

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List of Speakers:

Blackall, P. J.	Animal Research Institute, Queensland, AUSTRALIA
Cookson, K.	Fort Dodge Animal Health, Overland Park, Kansas, USA
Danforth, H.	USDA-ARS-LPSI-Parasite Biology and Epidemiology Laboratory, Beltsville, Maryland, USA
Hoerr, F. J.	Alabama State Veterinary Diagnostic Laboratory, Auburn, Alabama, USA
Jackwood, D. J.	The Ohio State University/OARDC, Wooster, Ohio, USA
Jackwood, M. W.	The University of Georgia, Athens, Georgia, USA
Kapur, V.	University of Minnesota, St. Paul, Minnesota, USA
Keeler, C.	University of Delaware, Newark, Delaware, USA
Khan, M.	University of Connecticut, Storrs, Connecticut, USA
Lauerman, L. H.	Washington State University, Puyallup, Washington, USA
Ley, D.	North Carolina State University, Raleigh, North Carolina, USA
Nagaraja, K. V.	University of Minnesota, St. Paul Minnesota, USA
Stewart-Brown, B.	Perdue Farms Incorporated, Salisbury, Maryland, USA
Suarez, D.	Southeast Poultry Research Laboratory, Athens, Georgia, USA
Taxter, J.	IDEXX Laboratories, Inc., Westbrook, Maine, USA
Tripathy, D.	University of Illinois, Urbana, Illinois, USA
Wakenell, P. S.	University of California-Davis, Davis, California, USA
Zavala, G.	The University of Georgia, Athens, Georgia, USA

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8:00 AM **Opening Remarks and Program Overview**: Daral J. Jackwood

Coccidiosis: Moderator: Hyun S. Lillehoj

8:10 AM 1. Harry Danforth. Coccidiosis: Molecular approaches for the differential diagnosis of Eimeria species.

Mycoplasma: Moderator: Lloyd H. Lauerman

8:30 AM 1. David Ley. Avian Mycoplasma Strain Identification by Random Amplification of Polymorphic DNA (RAPD)

8:50 AM 2. Mazhar Khan. Pathogenic avian mycoplasma specific PCR and its possible application.

9:10 AM 3. Lloyd H. Lauerman, Sharon Levisohn, Eliana Icochea, Antonio Ramirez, Norma Noe, and L. Li. Field evaluation of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* polymerase chain reaction assays in various national and international laboratories.

9:30 AM **Break**

Bacterial Pathogens: Moderator: Mazhar Khan

10:00 AM 1. Vivek Kapur. 5' Nuclease assay for the detection of avian pathogenic bacteria.

10:20 AM 2. K. V. Nagaraja. SERE-PCR assay for the genetic fingerprinting of *Salmonella enteritidis*.

10:40 AM 3. Pat Blackall and Mazhar I. Khan. PCR and ERIC-PCR tests for the detection and differentiation of *Hemophilus paragallinarum* isolates and serotypes.

DNA Viruses: Moderator: Robert Silva

11:00 AM 1. Calvin Keeler. Infectious laryngotracheitis virus: Can we identify the bad guys?

11:20 AM 2. Deoki Tripathy. Molecular techniques for the diagnosis of fowlpox virus.

11:40 AM 3. Patricia S. Wakenell. Practical application of PCR for Marek's disease virus and chicken anima virus detection.

12:00 **Lunch Break**

RNA Viruses: Moderator: Mark W. Jackwood

1:30 PM 1. Mark W. Jackwood. Diagnosis of IBV using molecular techniques.

1:50 PM 2. Guillermo Zavala. Application of classical and molecular virology techniques to control Avian Leukosis virus infection.

2:10 PM 3. David Suarez. The use of nucleotide sequence to predict pathogenicity and for epidemiologic analysis of avian influenza virus outbreaks

2:30 PM 4. Daral J. Jackwood. Molecular diagnosis of infectious bursal disease viruses: Practical applications and significance of the results.

2:50 PM **Break**

Industry Perspective: Moderator: Daral J. Jackwood

3:20 PM 1. John Taxter. Commercial applications of molecular diagnostic techniques in veterinary medicine: A decade of experience at IDEXX Laboratories.

3:40 PM 2. Kalen Cookson. Using PCR analysis to monitor IBD status on broiler farms.

4:00 PM	3. Frederic J. Hoerr, Routine application of molecular diagnostics in the poultry diagnostic laboratory.
4:20 PM	4. Bruce Stewart-Brown. Practical Application of Molecular Diagnositc Techniques in Commercial Poultry.
4:40 PM	Adjourn

Coccidiosis

USE OF A POLYMERASE CHAIN REACTION-BASED TECHNIQUE TO IDENTIFY AVIAN COCCIDIA

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Avian Coccidiosis is an intestinal disease caused by protozoan parasites of at least 7 different *Eimeria* species in chickens, and is estimated to cost the U. S. egg and broiler industry over $300 million annually in lost poultry production. Identification of the coccidial species infecting the birds has relied on the traditional diagnostic methods of location and type of gross intestinal lesions and the microscopic morphology of the oocysts. These methods do have clear advantages in that they are relatively easy to learn, can be used in the field for quick diagnosis and can give a rather clear idea of what predominate species of coccidia (especially *E. acervulina*, *E. maxima* and *E. tenella*) are involved with an infected bird flock. However, these methods are not as reliable in detecting species that may be present in lower numbers, the lesser species (*E. burnetti*, *E. necatrix*, and *E. mitis*), and species such as *E. praecox*, which does not produce intestinal lesions. In addition, some species of coccidia do overlap in oocyst size and morphology, which makes for more difficult diagnosis with a mixed population of the parasites. Although the lack of sensitivity in coccidial species identification has not been a great problem to the poultry industry, the increased resistance of the parasites to anticoccidial compounds coupled with the growing interest in the use of live oocyst vaccines for coccidial control, may now call for a more precise technique in coccidial diagnosis. In this presentation, the use of a polymerase chain reaction (PCR) technique will be described, which can be used to specifically identify coccidial species within litter samples obtained from commercial poultry grow-out facilities.

PCR is one of the dominate techniques in molecular biology, and is based on the principle that the production and amplification of a target region of DNA from chromosomes can be done hundreds of millions times within a 2-3 hr time period in a small test tube. These large numbers of DNA can then be visualized by subjecting the reaction mixture to electrophoresis and staining the DNA in an agarose gel. The trick in developing this technique for coccidial species identification is to find the right DNA targets for amplification by PCR. In theory, any region or sequence would do, but some regions can be duplicated exactly each time and make million fold copies of the same sequence during amplification to give increased sensitivity to the test. The region focused on with the *Eimeria* is called the internal transcribed spacer 1 (ITS1). As the name implies, it is essentially a spacer that serves to separate important vital regions of the chromosomes of coccidia from each other. Therefore, changes in its specific DNA sequence through mutation does not harm the parasite as could possibly be the case if

DNA sequences within the vital chromosomes needed for coccidial survival were changed. This has made it possible for the ITS1 regions from different *Eimeria* species to evolve variations in both their length and DNA base composition. By isolating the ITS1 regions from seven different species of coccidia (*E. acervulina*, *E. brunetti*, *E. maxima*, *E. mitis*, *E. necatrix*, *E. praecox* and *E. tenella*), seven specific sets of reagents or primers have been prepared with each set corresponding to a stretch of DNA sequence that is unique to one species of *Eimeria*. Thus in a PCR reaction with ITS1 primers specific for only *E. acervulina*, DNA from only that species will be amplified and subsequently show up as a stained band after electrophoresis within an agarose gel. No other DNA from other *Eimeria* species will be targeted and a positive staining in the gel is definitive for that species.

Coccidial species identification can now be done by use of conventional techniques that takes from one to two days to complete. Oocysts are recovered from sieved litter samples obtained from poultry houses and cleaned by use of a clorox technique. The DNA is extracted by chloroform extraction, and PCR amplification is done in the presence of the specific ITS1 primers in a thermal cycler. Results are then visualised by agarose gel electrophoresis followed by staining with ethidium bromide. A positive stained band indicates the species of coccidia present in the litter sample. Results obtained from our laboratory have identified *E. acervulina*, *E. maxima*, *E. mitis*, *E. praecox* and *E. tenella* in the samples tested. These results are now being used to evaluate the effect of live oocyst vaccines and anticoccidial treatments on the variation of coccidial species population in litter samples.

Mycoplasma

AVIAN MYCOPLASMA STRAIN IDENTIFICATION BY RANDOM AMPLIFICATION OF POLYMORPHIC DNA (RAPD)

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ABSTRACT

The ability to readily identify the marked intraspecies (strain) variability among avian mycoplasma field isolates is key to 1) improving diagnostics, 2) outbreak and epidemiological investigations, and 3) recognizing and understanding virulence factors. Random amplification of polymorphic DNA (RAPD) is a PCR-based method of DNA fingerprinting that results in amplification of 'anonymous' stretches of DNA. Subsequent visualization of the amplification products by agarose gel electrophoresis results in characteristic banding patterns. Successful application of RAPD fingerprinting to field isolates of avian mycoplasmas, especially *Mycoplasma gallisepticum*, has validated the utility and importance of strain identification.

Mycoplasma spp. are important respiratory and systemic pathogens of poultry causing substantial economic losses worldwide. Mycoplasmas are cell wall-less bacteria that are small in size and also have minimal genomes. The functional result of these features means that these organisms do not survive for long outside a host, and they are nutritionally fastidious and may be difficult to isolate but do grow on cell-free media. Definitive diagnosis relies on 1) isolation of mycoplasmas in culture media and identification using species-specific polyclonal antisera in immunofluorescence or growth inhibition tests, or 2) species-specific polymerase chain reaction (PCR) tests. However, these tests are not capable of recognizing the marked intraspecies (strain) genotypic (DNA sequence) and phenotypic (e.g. antigenic, pathogenic, immunogenic) variation among avian mycoplasma field isolates. Intraspecies antigenic variability impacts our ability to correctly diagnosis infections using serologic or organism detection tests that rely on antigen-antibody reactions. Pathogenic variability is expressed by field isolates of a *Mycoplasma* sp. that range in virulence, some causing typical disease while others result in subclinical infections or exhibit low pathogenicity. An example of this maybe the so-called 'variant' or 'atypical' strains of MG that appear to be less virulent, transmissible, and immunogenic than typical strains. Flocks infected with these strains are difficult to recognize, and likely to yield equivocal or conflicting test results. The ability to readily differentiate avian mycoplasma field isolates from one another is key to 1) improving diagnostics, 2) outbreak and epidemiological investigations, and 3) recognizing and understanding virulence factors.

The ability to recognize and compare intraspecies (strain) differences within an avian *Mycoplasma* sp. has been an important and challenging research objective. Laboratory methods applied to avian mycoplasma strain identification have explored two approaches, 1) protein analyses (e.g. 1 and 2 dimensional gel electrophoresis, immunoblotting) and, 2) DNA analyses (e.g. restriction fragment length polymorphism, strain-specific polymerase chain reaction [PCR], random amplification of polymorphic DNA). Random amplification of polymorphic DNA (RAPD) is a PCR-based method of DNA fingerprinting that results in amplification of 'anonymous' stretches of DNA with one (or sometimes more) short arbitrary primers and subsequent visualization of the amplification products by agarose gel electrophoresis. Because this test is based on the polymerase chain reaction, which amplifies DNA, we need only small amounts of starting material, which saves both time and money. The amplified segments of DNA separated by gel electrophoresis result in characteristic banding patterns. Organism or samples with the same DNA will have the same banding pattern; if they are different their banding patterns will differ. RAPD banding patterns may be thought of as analogous to barcodes or fingerprints. Compared to other methods of avian mycoplasma strain identification, RAPD is fast, simple to perform, inexpensive, and does not require the use of radioisotopes.

However, there are disadvantages or limitations to RAPD fingerprinting that must be considered. Starting material for the test requires a pure culture of the mycoplasma isolate. This means that we must be able to grow the mycoplasma in broth media from sample material (clinical specimen or archived culture), identify the species (usually by immunofluorescence) and confirm that the culture contains only one *Mycoplasma* sp. and is not contaminated with other bacteria. This is because the PCR step of the RAPD test is not specific and will amplify any DNA that is in the reaction mixture. RAPD tests are known to have problems with reproducibility because they are sensitive to alterations in PCR conditions. Interpretation of RAPD banding patterns can also be challenging due to the aforementioned problem of reproducibility and the possibility of co-migrating bands. The 'challenges' of reproducibility and interpretation can usually be overcome by using a second primer set to confirm apparent relationships or resolve ambiguous results.

Work by Geary et al. and Fan et al. showed that RAPD could be used successfully for strain identification of avian *Mycoplasma* spp. (MG, MS, MM and MI). Strain identification gives us the ability to confirm the identities of reference strains. These are strains that are used by researchers for experimental purposes or as reagents in diagnostic tests. We can also use this capability to identify vaccine strains, and distinguish them from field isolates. This ability is especially important in the case of MG for which three different live vaccine strains are available, and for MS, which has one. Strain identification can also be used for outbreak investigation, e.g. for mapping the range of a given outbreak, tracking its spread, and possibly identifying its origin.

My laboratory gained extensive experience with RAPD fingerprinting during our investigation of MG conjunctivitis in house finches (*Carpodacus mexicanus*) and other songbirds. We compared RAPD fingerprints of MG isolates from this epidemic with each other, and with MG vaccine and reference strains and isolates from commercial poultry. RAPD analyses indicated the following results and conclusions. MG isolates from songbirds (house finches, goldfinches, and blue jay) had essentially identical RAPD banding patterns. Therefore, the epidemic in songbirds is caused by the same strain of MG suggesting a single point source for this outbreak,

probably first involving house finches and more recently goldfinches (infection in the blue jay was apparently acquired while the birds was in captivity). The MG 'finch isolate' RAPD banding pattern differed from RAPD patterns of reference strains, vaccine strains, and isolates from commercial poultry. Therefore, the epidemic of MG conjunctivitis in songbirds is not caused by a vaccine strain, and has not been linked to any reference strain or poultry isolate tested. Presently, we have no evidence that MG infection has been shared between songbirds and commercial poultry.

In commercial poultry, RAPD analyses have been most extensively and successfully used to identify strains and track outbreaks of MG. There has been some application of RAPD fingerprinting to outbreaks of MS, however reproducibility and interpretation of results have been more challenging than for MG. At the present time, RAPD analyses have not been commonly applied to other avian *Mycoplasma* spp.

The successful application of RAPD fingerprinting to avian mycoplasma isolates, especially MG, has validated the utility of testing methods capable of intraspecies (strain) identification. Future developments and improvements in strain identification technology can be anticipated and may include, 1) the use of computerized DNA fingerprint analysis and database system, and/or 2) new methodologies for molecular typing that improve on the performance of RAPD. Computer-assisted RAPD analysis systems can be used to correct, process and analyze gels in order to compare banding patterns, and are particularly effective in epidemiological studies. The power of a computerized RAPD analysis system resides in its capacity to compare every new strain with all previously analyzed strains, and to develop large databases for comparison. Additionally, there is the prospect that new molecular typing methodologies will be developed for avian mycoplasma strain identification that improve upon RAPD's requirement for growth and a pure culture, and problems of reproducibility and interpretation. In the mean time, RAPD fingerprinting remains the best method available for avian mycoplasma strain identification, providing timely and useful information for epidemiological investigations of avian mycoplasmosis.

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PATHOGENIC AVIAN MYCOPLASM SPECIFIC PCR AND ITS POSSIBLE APPLICATION

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Four species of mycoplasmas are known to cause economic losses in commercial poultry production. *Mycoplasma gallisepticum* (MG) causes chronic respiratory disease in chickens and infectious sinusitis in turkeys, *M. synoviae* (MS) most frequently occurs as subclinical upper respiratory infection and synovitis in chickens and turkeys, *M. iowae* (MI) causes a decrease in hatchability and high embryo mortality in turkeys, and *M melagridis* (MM) causes primary lesions in airsacs in the progeny, which leads to lower hatchability and skeletal abnormalities in young turkeys. On the other hand, multiple infections of the avain pathogenic mycoplasma are not uncommon in chicken and turkey flocks. Rapid identification of any of these avian mycoplasma organisms is of great importance to the poultry industry. Both serologic and isolation procedures have been used for the diagnosis of avian mycoplasmas. However, serological tests are often hampered by interspecies cross-reactions and non-specific reactions, while isolation of mycoplasma is difficult and time consuming. Molecular methods such as DNA probes and polymerase chain reaction (PCR) have been recently developed in an attempt to improve diagnosis of avian mycoplasma infections. These recent advances in the diagnosis of avian mycoplasmas have led to the development of commercial MG and MS DNA-based kits. These kits, which are needed to differentiate MG and MS, have resulted in doubling the cost of each clinical sample tested. The multi-species PCR using RFLP or species-specific DNA probes to identify the PCR products makes the procedure somewhat cumbersome and also expensive.

We have optimize and developed a multiplex PCR to simultaneously detect four pathogenic species of avian mycoplasmas. By using agarose gel electrophoresis we can detect and differentiate four pathogenic mycoplasmas in a single PCR reaction of 35 cycles. The mycoplasma-multiplex PCR products consisted of 850 bp (MM), 732 bp (MG), 299 bp (MI) and 207 bp (MS). Overall, the use of multiplex PCR assay for identification of pathogenic avian mycoplasmas is seems to be reliable and specific. The multiplex PCR, being specific, sensitive and cost effective, seems to be an attractive alternative to currently described PCR methods for detecting one or more species of mycoplasma in a sample.

FIELD EVALUATION OF MYCOPLASMA GALLISEPTICUM AND MYCOPLASMA SYNOVIAE POLYMERASE CHAIN REACTION ASAYS IN VARIOUS NATIONAL AND INTERNATIONAL LABORATORIES.

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ABSTRACT

The National Poultry Improvement Plan (NPIP) was implemented in the United States in 1935 to establish and maintain *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) clean flocks. The polymerase chain reaction (PCR) assays for detection of MG and MS were recently added to the NPIP test protocols. Species-specific and sensitive MG (MG13/14 primers) and MS (MSL primers) PCR assays were used and validated using thousands of specimens in numerous laboratories in the United States and around the world to detect the organisms in diagnostic specimens or to confirm the presence of the organisms in samples from poultry with positive serological tests, such as the rapid plate agglutination test, hemagglutination inhibition, or enzyme-linked immunosorbent assays. The PCR assays were used to identify MG or MS isolates and as a screening test in conjunction with other procedures in normal healthy primary breeder flocks or spike roosters to be moved to other flocks to give added assurance of mycoplasma negative status of the flock. These PCR assays have been demonstrated to be reliable, rapid and cost-effective for diagnosis of MG and MS.

INTRODUCTION

Polymerase chain reaction (PCR) assays have been researched in the Alabama Veterinary Diagnostic Laboratory (AVDL) as an aid for disease diagnosis for the past 10 years. The first PCR assay to be developed at the AVDL was for *Mycoplasma synoviae* (MS) in 1991 (Lauerman *et al.*, 1993). A PCR assay for *Mycoplasma gallisepticum* (MG) was reported in the scientific literature by Nascimento *et al.*, (1991) and the procedure was evaluated at the AVDL for specificity and sensitivity. These MS and MG PCR assays were demonstrated to be very specific for the respective species in cultures and field samples. However it was considered important to attempt to increase the sensitivity of the two test procedures. The following report describes the results of an attempt to improve sensitivity of the MS PCR assay by modification of the primers and an MG PCR by using a different set of primers.

MYCOPLASMA SYNOVIAE PCR ASSAY

The initial MS PCR primers were selected from the distal end of the 16S rRNA gene sequence using MacVector and a procedure of sequence alignment and were designated MS-1 and MS-2 primers (Lauerman *et al.*, 1993). These primers produced an amplicon of 207 bp in length and a sensitivity of approximately 100 colony forming units (CFU) at 50°C annealing temperature. The modification made to the MS-1 and MS-2 primers was to add 2 nucleotides to the 5' end of each of the primers to increase the annealing temperature to 55°C. The modified MS primers were as follows.
MSLF 5'-GAG AAG CAA AAT AGT GAT ATC A-3' (Lauerman, 1998)
MSLR 5'-CAG TCG TCT CCG AAG TTA ACA A-3' (Lauerman, 1998)
These primers produce an amplicon of 211 bp in length and a sensitivity of approximately 10 CFU at 55°C annealing temperature. The specificity of the two sets of MS PCR primers (MS-1/MS-2 and MSLF/MSLR) were the same. The conditions for the MS and MG PCR assays were described by Lauerman (1998) and were as follows. Prepare the PCR reaction mixture in a separate clean work station and wear gloves. The reaction mixture for one 50 ul PCR reaction contains the following, 35.75 ul ultra pure water, 5 ul of 10X PCR buffer, 1 ul of dNTP (10 mM), 0.5 ul of each primer (20 pmole/ul), 0.25 ul of Taq (5 U/ul), and 2 ul of $MgCl_2$ (50 mM). Dispense 45 ul of the reaction mixture into each PCR tube. Overlay the reaction mixture with 50 ul of light weight mineral oil if required and close the snap cap. Transport tubes to sample inoculation station and dispense 5 ul of sample into each PCR reaction mixture. Inoculate a positive and negative control for each run. Place the tubes in the thermal cycler and run the following cycles: 40 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec and 1 cycle at 72°C for 5 min and hold at 4°C. Visualize the PCR amplicon in a 1.5% agarose gel with ethidium bromide and record the results using UV transillumination and photography. The MS PCR assay using the MSL primers has been successfully established in numerous research and diagnostic laboratories throughout the United States (Alabama, Georgia, North Carolina, and Texas) and the world (Brazil, England, Hungary, Israel, and Peru). Difficulties were not encountered using the MSL primers by the various laboratories. The MS PCR assay is considered to be an essential tool for validating mycoplasma-free production systems and/or eradication programs.

MYCOPLASMA GALLISEPTICUM PCR ASSAY

A number of MG PCR species-specific primer sets were selected from the 16S rRNA gene sequence by sequence alignment using MacVector, as well as various published primers and evaluated for sensitivity. The MG13 and MG14 primers were demonstrated in comparative PCR and culture titration to be 100-fold more sensitive than the other MG primer sets evaluated. The nucleotide sequences of the MG 13/14 primers were as follows.
MG-14F 5'-GAG CTA ATC TGT AAA GTT GGT C-3' (Lauerman, 1998)
MG-13R 5'-GCT TCC TTG CGG TTA GCA AC-3' (Lauerman, 1998)
These primers produce an amplicon of 185 bp in length and a sensitivity of

approximately 10 CFU at 55°C annealing temperature. The MG PCR assay using the MG13/14 primers has been successfully established in numerous research and diagnostic laboratories throughout the United States and the world. Some difficulties were encountered using the MG13/14 primers by some laboratories and these were described under the Laboratory Evaluation section. However these difficulties do not hinder the ultimate sensitivity and specificity of the primers.

THREE COUNTRY COLABORATIVE RESEARCH

During the past 4 years a three country (Israel, Peru and the United States) research project was performed to transfer molecular diagnostic technology to Peru where the PCR assays were applied in the project supported Biotechnology Unit at the San Marcos University, Lima, Peru. The most significant result of the project was the establishment of the PCR assays as a rapid and reliable diagnostic test for detection of MG and MS infection in poultry which, for the first time, provided a rational basis for a mycoplasma control program. An extensive study was also performed to compare the PCR assay results with standard serological tests and the superiority of the molecular tests were demonstrated, as well as savings in cost and time.

LABORATORY EVALUATIONS

Ten laboratories were surveyed throughout the world that use the MSL and MG13/14 PCR primers to determine 1) the extent of use, 2) aid in diagnosis, 3) whether difficulties were encountered in their use, 4) if the primers cross react with other mycoplasmas, and 5) if the primers detected the new live MG and MS vaccines. The laboratories reported as follows. 1) Thousands of samples have been processed using the MSL and MG13/14 primers by the various laboratories over many years. 2) All of the laboratories reporting considered the MSL and MG13/14 primers to be useful as diagnostic tools. 3) Six of the 10 laboratories have experienced occasional samples that give multiple bands with the MG13/14 primers and this causes concern as to interpretation. When this has occurred the samples in question were retested with the MG13/14 primers in parallel with another set of MG specific primers. Another suggested approach was to use Gold Taq, Platinum Taq or Abgene Taq plus a hot start to reduce or eliminate the weak multiple bands observed in the gel. One of the 10 laboratories reported one MG PCR negative test while the sample was later determined to be MG positive by culture. Two of the 10 laboratories reported false positive MG PCR assays with samples from wild birds. It is advisable at this time to only use the MSL and MG13/14 primers on poultry samples. 4) The MG13/14 primers were demonstrated to react with *Mycoplasma imitans* (isolated from ducks, geese and partridge) and a penguin mycoplasma (Bradbury, 2000). To date there have been no confirmed reports of *M. imitans* or the penguin mycoplasma being isolated from chickens or turkeys (Bradbury, 2000). 5) Four of the 10 laboratories report that the MSL and MG13/14 primers detect the respective new live MG and MS vaccine organisms.

SUMMARY

Laboratory trials and diagnostic tests performed around the world in parallel with serology and/or culture clearly demonstrated the superiority of the molecular tests in cost and time efficiency, detection of early stages of infection, specificity, and less interference due to secondary infections or antibiotic treatment.

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Bacterial Pathogens

5'-NUCLEASE ASSAYS FOR THE DETECTION OF AVIAN PATHOGENIC BACTERIA

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ABSTRACT

We have initiated a program to develop rapid, sensitive, and specific nucleic acid detection methods to detect avian bacterial pathogens. These tests are based on the 5'-nuclease (TaqMan™) assay that enables the single-tube amplification and detection of nucleic acids in a quantitative manner. We here present preliminary studies standardizing the 5'-nuclease assay for the detection of avian mycoplasmas. Our preliminary results indicate that the enhanced sensitivity, ease and simplicity of this method will permit the analysis of hundreds to thousands of samples per day with high sample-to-sample reproducibility, and minimize cross-contamination and sample transfer errors that are inherent to traditional PCR-based approaches. This will enable high-throughput PCR-based screening for *Mycoplasma* species in clinical specimens in a sensitive and specific manner with automated data collection and analysis, and minimize the time, effort, and resources needed to detect infected animals in poultry farms. Our ongoing studies seek to devise multiplexed 5'-nuclease assays for the detection of avian pathogenic bacteria.

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INTRODUCTION

The sensitive and specific detection of infected animals is essential to the formulation of rational and effective strategies for disease control and eradication. In general, there are only two methods for detecting animals that are infected: bacterial culture or nucleic-based diagnostics. For many slow-growing organisms, bacterial culture-based methods are very time-consuming and often difficult to interpret (for example, due to overgrowth of other bacterial contaminants). Hence, nucleic-acid-based approaches are the methods of choice for the rapid detection of many different pathogens.

Although, the PCR (polymerase chain reaction) process has provided a facile means for the rapid amplification of nucleic acids specific to microbial pathogens, the DNA extraction and post-PCR product detection are time consuming, thereby presenting a considerable bottleneck in the routine application of PCR-based assays in a clinical setting. The TaqMan™ or "5'-nuclease" assay is a "one tube" or homogeneous assay, which is also quantitative, sensitive, specific and convenient for testing large numbers of samples (Heid et al., 1996). The 5'-3' exonuclease activity of *Taq* polymerase cleaves 5' terminal nucleotides of double stranded DNA, releasing mono- and oligonucleotides. A labeled oligonucleotide probe which hybridizes to the target sequence represents a substrate for exonuclease activity during PCR amplification, releasing the label in smaller fragments. In this assay, product detection occurs concurrently with target amplification, and requires little or no handling of the amplified sample. In the TaqMan™ assay, a nonextendible, dual-labeled fluorogenic hybridization probe is used. The probe contains a reporter fluorescent dye, such as fluorescein, attached to the 5'end, and a quencher dye, such as rhodamine, attached internally or at the 3'end. The fluorescence emission of fluorescein is quenched if the rhodamine is close enough to be excited through the process of fluorescence energy transfer. During the amplification of the target, the fluorogenic probe is displaced by the polymerase and then cleaved, releasing the reporter dye. The nuclease degradation of the probe releases the quenching of the fluorescein emissions, resulting in an increase in fluorescent emission. The fluorescent spectra can be measured continuously during the PCR amplification using a fluorescence detector system.

We here present a preliminary report of the development of TaqMan™ assays for the detection of mycoplasmas of poultry interest that are currently being developed in our laboratory. *Mycoplasma* diseases rank amongst the most prevalent and costly infectious diseases of poultry worldwide. Four species are known to be pathogenic in turkeys: *Mycoplasma gallisepticum, M. synoviae, M. meleagridis,* and *M. iowae*. *Mycoplasma gallisepticum* causes coryza, sneezing, moist rales, and sinusitis, and it impairs reproductive performance. *M. synoviae* causes depression, pallor, loss of condition, swelling of the joints, breast blisters, coryza, and sinusitis, and impairs reproductive performance as well. *M. meleagridis* is a turkey-specific pathogen and causes twisted necks and abnormalities in bone and feather growth, in addition to impairing reproductive performance. *M. iowae* impairs reproductive performance and has been shown to cause reduced growth, abnormal feathering, twisted leg, and chondrodystrophy in experimentally infected turkey embryos and day-old poults.

In general, *Mycoplasma* are difficult and expensive organisms to detect: it may take up to 4 weeks for a presumptive diagnosis to be made by traditional microbiological techniques. Several polymerase chain reaction (PCR) based strategies for the detection of *Mycoplasma* in clinical

samples have been described (Garcia et al., 1995, Silviera et al., 1996). These methods in general enable the relatively rapid (within 1-2 days of sample submission) detection of the organism. A commercial PCR-based test is also available for *M. gallisepticum* and *M. synoviae* (IDEXX Laboratories Inc., Westbrook, ME). However, there are several disadvantages to the currently available DNA-based diagnostic tests. For instance, they have low sensitivity, are relatively expensive, and require highly skilled personnel to perform the testing and interpret results. Further, as is often the case in any PCR-based test that involves substantial post-sample processing (e.g. pipetting and gel electrophoresis or hybridization), there is an increased risk of false-positive reactions due to sample contamination.

Our preliminary results indicate that the enhanced sensitivity and ease and simplicity of this method will permit the analysis of hundreds to thousands of samples per day with high sample-to-sample reproducibility, and minimize cross-contamination and sample transfer errors that are inherent to traditional PCR-based approaches

MATERIALS AND METHODS

Bacterial cultures. The cells were grown in *Mycoplasma* Broth Base (Becton Dickinson) containing enrichment solution (1% NAD, 10% cysteine, 10^5 U/ml penicillin G, 10% thallium acetate, 0.3% glucose) and 10% of sterile swine serum, at 37 C for at least one week, when a color change in the medium was observed.

DNA isolation. DNA extraction was performed with a commercial DNA purification system (Qiagen, CA) from pure cultures prepared by centrifuging 1 ml of the culture for 2 minutes at 13, 000 g. The supernatant was removed and discarded by aspiration.

DNA amplification. The target sequence for detection of *M. gallisepticum* was a 247 bp. region of the Trp-tRNA-UGA (Genbank accession number M32342). Both primers and the internal fluorescently labeled probe were designed using PrimerExpress (PE Applies Biosystems, Foster City, CA). PCR conditions were optimized for the amplification of low copy numbers of target DNA sequences. Several annealing temperatures (60 C – 65 C) were tested, as well as primer (100 nM- 1000nM), probe (50-300 nM) and $MgCl_2$ (1mM – 7.5 mM) concentrations and number of cycles (35- 40). The TaqMan™ reaction mixture (50 µl) contained: 1xTaqMan™ Buffer A (Perkin-Elmer Cetus, Norwalk, Conn.), 5.0 mM $MgCl_2$ (Perkin-Elmer Cetus, Norwalk, Conn.), 2.5 U AmpliTaq Gold (Perkin-Elmer Cetus, Norwalk, Conn.), 8% glycerol, 0.5 U Uracil-*N* – glycosylase, 600 nM each primer and 100 nM probe and 5 µl of template DNA. In order to minimize contamination due to PCR product carry-over, 0.5 U of uracil-*N*- glycosylase (AmpErase™ UNG, Perkin-Elmer Cetus, Norwalk, Conn) was added to the PCR reactions.

Data Analysis. Specific PCR products produced by the 5' nuclease assay were detected using the ABI PRISM 7200 or 7700 Detection System. The PCR plate was read on the 7200 TaqMan reader prior to PCR (pre-read) and following PCR (post-read). End-point data analysis was done according to PE Applied Biosystems instructions, and samples were assigned one of the following results: (+) positive, (-) negative, or no amplification. Real-time and end-point data analysis were done using the PE Applied Biosystems Sequence Detection Systems v1.6 software. Real time data analysis allows for the determination of the critical threshold (Ct) values, which represents the

number of PCR cycles at which the sample signal is greater than the background. Results were recorded as ΔRQ, which is the difference between the Rn (ratio of reporter emission to quencher emission) values of the sample and the no template controls. All samples were tested in duplicate, and a total of 6 NTC (non-template controls) were included in each assay. After the reads were performed, PCR products were also run on 1% agarose gels and stained with ethidium bromide for visualization to verify the results.

RESULTS AND DISCUSSION

The results of our studies show that the TaqMan assay is a sensitive and specific method for the detection of *M. gallisepticum*. The sensitivity of the test was shown to be 5-30 fg of DNA, corresponding to 5-30 copies of the amplified gene. Other PCR methods have been described with similar sensitivities. The sensitivity of a PCR assay does not only depend on the sequence copy number, primers and conditions used, but also on the visualization procedure. In this case, agarose gel/ethidium bromide staining was less sensitive (1 log difference) than the fluorogenic detection of the cleaved probe.

We used a dilution series of *M. gallisepticum* to further evaluate the sensitivity varied with the DNA extraction protocol: 0.85 color changing unit/reaction were detected when the Qiagen protocol was used. The specificity of the primers and probe was also tested, obtaining no positive signals with any other of the microorganisms used besides the *M. gallisepticum* strains.

The development of the polymerase chain reaction (PCR) represented a new approach to rapid diagnosis of viral, bacterial and parasitic infections. The ease, sensitivity and widespread use of this technology make it a very attractive tool in a diagnostic laboratory. However, PCR techniques, as used today, are still laborious, time consuming and difficult to automate. TaqMan™ based tests have two main advantages: tests are amenable to automation and, hence, likely to result in the development of rapid, inexpensive, and high-throughput diagnostic tests from pathogens. Furthermore, contamination problems are reduced because amplification, hybridization of the probe and emission measurement take place in a same closed tube and the use of uracil-*N*-glycosylase (AmpErase™ UNG) can also be used to prevent PCR product carry-over to further minimize PCR product contamination.

The TaqMan™assay developed in this research was shown to be specific and rapid for the detection of *M. gallisepticum*. Ongoing studies in our laboratory are designed to evaluate the use of the *M. gallisepticum* TaqMan assay for clinical specimens, either from early infection or from chronic cases, when the microorganism is present in lower numbers and is not detected with other techniques such as culture.

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SERE –PCR ASSAY FOR THE GENETIC FINGERPRINTING OF *SALMONELLA ENTERITIDIS*

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Salmonellas continue to be one of the main causes of foodborne illness worldwide; in many countries salmonellosis is the most frequently reported foodborne disease. Though circumstantial evidence links poultry *Salmonella enteritidis* isolates with human salmonellosis, continued efforts are needed to define this relationship.

Salmonellas are gram-negative, motile, rod-shaped bacteria that can grow both aerobically and anaerobically. The organism is ubiquitous among domestic and wild animals and all serovars have the potential to cause diseases in humans. Some serovars are adapted to certain species and these serovars are much more virulent for the species to which they are adapted than for other species. For example, the avian-adapted *S. pullorum* and *S. gallinarum* cause severe disease in poultry but very rarely infect humans. SE Phage type 4 causes an invasive infection of poultry that leads to septicemia and subsequent chronic infection of various organs. When the ovaries become infected, subsequent infections of eggs result due to transovarian transmission.

A variety of identification methodologies have been developed to identify the relatedness of salmonella isolates in an epidemiological investigation. Recently, DNA fingerprinting has been carried out on SE isolates from various sources. [9,11] Antimicrobial susceptibility testing, and the molecular epidemiology of antimicrobial susceptibility from various animals and human cases of salmonellosis have also been investigated.[3,11,13] Phage typing has been widely used in England and the US as a reliable strain marker. [6] Within the serotype, phage typing provides a rapid method of strain discrimination which is useful in epidemiological investigations. However, in the US, the predominant phage types identified (accounting for approximately 90% of the SE's isolated) are 8,13,13a and 14b.[4] It is evident from this data, that phage typing does not allow for sufficient differentiation among SE isolates. Plasmid profiling would allow for the subdivision of SE phage types for better tracking of given bacterial clones. [15] Plasmid analysis have been carried out on various SE isolates, with many investigations of human salmonellosis traced by plasmid profiling.[2,3,10,14,15]

Plasmid profiling does provide a methodology capable of aiding in further subdivisions of SE, however, there are many isolates which contain only one (usually the VAP) or two plasmids. Thus, further differentiation is necessary. A valuable method for doing this is restriction endonuclease digestion of the VAP of SE. Only one study has been done on the restriction analysis of the VAP of SE, back in 1985 on only four unrelated isolates. The results showed that the samples shared almost all fragments when digested with Eco RI, Hind III and Bam HI. They went further to conclude that all VAP's of SE are identical. [12] Perhaps further work needs to be conducted to conclusively demonstrate that such a claim is accurate. It is true that virulence is a multifactorial orchestrated event and the VAP is only one player. However, scientific data strongly supports the idea that this plasmid plays a major role in virulence, since curing of the plasmid results in a decrease in virulence. [1,5,7,8,16]

Repeat sequences are present in the genomes of all organisms. Several short, non-coding, intercistronic repetitive DNA sequences have been identified in prokaryotes [17]. The highly conserved nature and the widespread distribution of these repeat sequences in the bacterial genome have facilitated their use in the genetic fingerprinting of bacteria -- for example, repetitive sequence-based PCR (rep-PCR), in which a DNA sequence, similar or identical to the repeat sequence, is used as primer to generate a specific pattern or genomic DNA fingerprint [17,18,19]. In previous studies, rep-PCR generated fingerprints have been found to be species- or strain-specific and have proved useful for strain typing [18-21]. Due to its ease of use and discriminatory power, rep-PCR has become an important tool with which to fingerprint bacteria involved in outbreaks of disease and to determine the sources and vehicles of transmission [1,20-22].

In this presentation , the presence of a novel, highly-conserved repetitive DNA element located within the regulatory region of the *sefABCD* operon of the Enteritidis genome and its application in a PCR assay (SERE-PCR) for the genetic fingerprinting of isolates of Enteritidis and other serotypes of *Salmonella* from diverse O-serogroups will be described.

In our study a SERE-based polymerase chain reaction (SERE-PCR) assay was developed to fingerprint 54 isolates of Enteritidis representing 9 distinct phage types and 54 isolates of other *Salmonella* serotypes. SERE-PCR identified 5 distinct fingerprint profiles among the 54 Enteritidis isolates; no correlation between phage types and SERE-PCR fingerprint patterns was noticed. SERE-PCR was reproducible, rapid, and easy to perform. The results of this investigation suggest that the limited heterogeneity of SERE-PCR fingerprint patterns can be utilized to develop serotype and serogroup-specific fingerprint patterns for isolates of Enteritidis

The five distinct SERE-PCR fingerprint patterns identified among isolates of Eneteritidis examined were very similar. This limited heterogeneity of fingerprint patterns among Enteritidis isolates, recovered from various distinct host species and geographic locations, may reflect either a relatively recent origin of serotype Enteritidis or a widespread dissemination of one highly virulent Enteritidis clone. These hypotheses are consistent with the findings of a multilocus enzyme electrophoretic analysis of Enteritidis isolates that revealed a restricted genetic diversity in this serotype and showed that most (93%) Enteritidis isolates from worldwide sources were represented by one clone, En1 [23]. Analysis of Enteritidis strains from sporadic cases and outbreaks by pulsed field-gel electrophoresis and ribotyping have also indicated the limited genetic diversity of this serotype [24].

In conclusion, the results of our investigation show that the paucity of heterogeneity in SERE-PCR fingerprint patterns may be utilized to develop species and serogroup-specific fingerprint patterns for isolates of *S. enteritidis*

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PCR AND ERIC-PCR TESTS FOR THE DETECTION AND DIFFERENTIATION OF *HAEMOPHILUS PARAGALLINARUM*

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SUMMARY

This paper reports on studies performed in both China and Australia on the development and validation of a PCR test for *Haemophilus paragallinarum*. As well, we provide details of ERIC-PCR based typing of *H. paragallinarum*. The *H. paragallinarum* PCR has been shown to consistently outperform traditional culture-based methods when used in China on typical field submissions from suspect coryza outbreaks in commercial chickens. As well, laboratory trials, in both Australia and China, using chickens infected in pen trials have shown that the PCR test is more robust than traditional culture. Samples can be successfully stored for up to 180 days for PCR and only three days for culture. With ERIC-PCR typing, we found that the 11 serovar reference strains were allocated to ten different ERIC-PCR types. The sixteen Australian field isolates formed a single ERIC-PCR type while the nine Chinese isolates were allocated to seven different ERIC-PCR pattern types.

INTRODUCTION

This paper will provide an overview of our experiences with two PCR tests, both developed initially at the Animal Research Institute in Australia. One of the tests is a species-specific test for *Haemophilus paragallinarum*, the causative agent of infectious coryza of chickens (2) and the other is an ERIC-PCR method for subtyping *H. paragallinarum*. Full details of the species-specific PCR test for *H. paragallinarum* described in our formal publication (6). The technology used in the ERIC-PCR typing is a standard method that has been described in detail in studies on other bacterial species e.g. *Haemophilus somnus* (1). In this paper, we provide an overview of the experiences we have gained with the use of these assays. There is an emphasis, for the *H. paragallinarum* PCR, on practical application of the PCR in both China and Australia.

TRADITIONAL DIAGNOSIS OF INFECTIOUS CORYZA

The traditional definitive method for the diagnosis of infectious coryza requires the isolation of the suspect bacterium and then an extensive biochemical characterisation to confirm the identity of the isolate (2). This is a challenging set of requirements. *H. paragallinarum* is a fastidious, slow growing organism. Hence, the organism is often overgrown by other faster-growing commensals. Biochemical characterisation requires the availability of specialised, expensive media that can support the growth of NAD-dependent bacteria - such media are often beyond the resources of diagnostic laboratories, particularly those in the developing countries where coryza remains a pressing problem.

THE *H. PARAGALLINARUM* PCR

The difficulties and limitations of culture stimulated us to develop a PCR test for *H. paragallinarum*. This PCR is specific for *H. paragallinarum* and rapid, with results being available within 6 hours compared with days for conventional techniques (6). In testing performed in Australia, all 41 *H. paragallinarum* isolates tested were positive in the PCR, including the NAD-independent *H. paragallinarum* from South Africa and the variant Page serovar A isolates and the unusual Page serovar B isolates from Argentina (6). The PCR, termed the HP-2 PCR, has given negative reactions with many closely related bacteria. In particular, the NAD-dependent forms of *Pasteurella avium*, *P. volantium* and *Pasteurella* spp as well as *Ornithobacterium rhinotracheale* have been shown to give a negative reaction in this PCR when tested in a collaborative study performed in Australia and South Africa (9).

While the HP-2 PCR was originally developed in Australia, the test has now been successfully transferred to China. In comparing traditional culture and the HP-2 PCR in China, it has been shown that the PCR is better than traditional culture when both tests are used on routine diagnostic submission. In one study, we used the HP-2 PCR test and traditional culture in parallel to investigate suspect infectious coryza outbreaks on eight commercial farms in China (5). The HP-2 PCR gave 15 of 39 birds and 6 of 8 farms as positive while culture gave only 8 of 39 chickens and 4 of 8 farms as positive (5). The two farms that were not positive by culture, but were positive by PCR, had chickens showed typical clinical signs - thereby providing further evidence that the culture results were false-negatives. The submitted chickens from the two farms that were negative by both culture and PCR did not show typical clinical signs of infectious coryza when received at the central laboratory (5). In further recent work in China, we have examined a further 77 chickens from a range of suspect coryza outbreaks in a number of provinces of China. From these 67 chickens, the HP-2 PCR gave 14 positive results while traditional culture gave only 4 positives (Chen and Blackall, unpublished data). The problems of poor samples, delayed transport and poor quality (but expensive) media mean that culture will have a higher failure rate in developing countries than in developed countries.

Recent work has shown the robust nature of the HP-2 PCR. In further work performed in China, it has been shown that samples can be stored for up to 180 days at 4 C or -20 C and the majority of known positive samples will remain positive in the PCR. In contrast, culture failed to detect *H. paragallinarum* after 3 days of storage at 4 C or -20 C (7).

Overall, the HP-2 PCR represents a significant step forward in diagnosing infectious coryza. While PCR technology appears at first glance complex and expensive, the validation/evaluation work of the HP-2 PCR in China is demonstrating that the technology can be used, and can give significantly better results than traditional culture, in developing countries. Even in a well developed country like Australia, there are considerable advantages to the HP-2 PCR – the test is now the preferred diagnostic option for the Bacteriology Research Laboratory at the Animal Research Institute (a laboratory that functions as a national and international reference laboratory for infectious coryza). To our knowledge, the HP-2 PCR is now in routine

use in Australia, China, India and South Africa while laboratories in Mexico and Peru are looking at establishing the test. Other laboratories may also be using this test.

ERIC-PCR TYPING OF *H. PARAGALLINARUM*

Traditionally sub-typing of *H. paragallinarum* is performed by serological methods. There are two main serotyping methods – the Page scheme (10) and the Kume scheme (8). PCR based typing methods are an attractive alternative method. They are rapid and are universal in applicability. In contrast, only those laboratories with access to high quality, high titre antisera can perform serotyping.

We have examined the ability of ERIC-PCR typing to differentiate amongst a varied collection of *H. paragallinarum* isolates. Amongst the 11 reference strains and 25 field isolates used in this study, a total of 17 different ERIC-PCR patterns were recognised.

The available knowledge of serovars and source of origin indicates that, with the exception of the Australian strains, the other nine reference strains share no connection and should give unique, distinct patterns. This was indeed the case – with the nine non-Australian reference strains yielding unique ERIC-PCR patterns. The two Australian strains (HP14 and HP60) shared the same pattern, despite being different Page serovars. This lack of detectable genetic diversity, despite serological diversity, has also been found in Australian *H. paragallinarum* isolates using an entirely different typing method termed multi-locus enzyme electrophoresis (4).

The 16 Australian field isolates all had the same ERIC-PCR pattern as the Australian reference strains of HP14 and HP60. This occurred despite the isolates belonging to two different serovars and three different restriction endonuclease profiles (3). In contrast, a total of seven different ERIC-PCR patterns were recognised amongst the nine Chinese isolates.

Clearly, ERIC-PCR has potential as a typing tool for *H. paragallinarum*. We have shown that ERIC-PCR typing cannot be used to assign an isolate to a Page or Kume serovar. However, ERIC-PCR can be used to determine if isolates have a common source. It should be noted that in areas where is limited genetic diversity amongst the *H. paragallinarum* isolates, such as Australia, there is limited application for ERIC-PCR typing. It is important to note that the Australian situation with regards to limited diversity of *H. paragallinarum* appears to be unique, meaning that ERIC-PCR typing should be valuable in most regions.

CONCLUSIONS

This paper has reviewed our experiences with the species-specific PCR for *H. paragallinarum* as well as the ERIC-PCR method for sub-typing *H. paragallinarum*. The species-specific PCR has been extensively evaluated for use on isolated colonies and been shown to be specific and suitable for routine use. The *H. paragallinarum* PCR has also been adapted for use directly on sinus swabs – and shown to be superior to culture. Our experiences with the *H. paragallinarum* PCR test in China have repeatedly confirmed that PCR is capable of being used in a developing country. Further, in the case of infectious coryza, PCR repeatedly and consistently outperforms

traditional culture based diagnostic methods. While culture-based diagnostic methods will always have some role to play, we feel that for selected diseases, such as infectious coryza, PCR can and should be adopted as the routine diagnostic test of choice, in both developed and developing countries.

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DNA Viruses

INFECTIOUS LARYNGOTRACHEITIS VIRUS: CAN WE IDENTIFY THE BAD GUYS?

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ABSTRACT

Infectious laryngotracheitis (ILT) causes an acute upper respiratory infection in chickens. Mild forms of the disease can sometimes be difficult to diagnose. The presence of intranuclear inclusion bodies in tracheal or conjunctival tissue is pathognomonic for ILT but may not be observed late in infection. ELISA results can also be inconsistent because of the lack of a consistent humoral immune response to the virus. PCR based assays have been developed which can readily detect the virus. However, techniques are not readily available to determine whether specific ILT outbreaks are caused by unique field strains, vaccine strains, or latent virus.

OVERVIEW

Infectious laryngotracheitis virus (ILT) was first described in 1925 (May and Tittlser, 1925). In its acute form, ILT is characterized by signs of respiratory distress in birds, accompanied by gasping and expectoration of bloody exudates (Bagust and Guy, 1997). In addition, the mucous membranes of the trachea become swollen and hemorrhagic. The epizootic form of the disease spreads rapidly and although severe forms of the disease cause high morbidity (90-100%), mortality generally averages 10-20%. Milder forms of the disease can sometimes provide a diagnostic challenge. As with many upper respiratory diseases, mild forms of ILT are characterized by watery eyes, conjunctivitis, and persistent nasal discharge. Generally, birds recover within two weeks. The etiological agent for ILT is infectious laryngotracheitis virus (ILTV), an alpha herpesvirus. Like other herpesviruses, ILTV can also establish a latent carrier state in recovered animals. Virus shed after the latent period is another source of virus capable of causing disease in susceptible birds. Fortunately, ILT is a slowly spreading, controllable disease, which does not normally exhibit the mortality and condemnation statistics associated with some other avian respiratory disease agents. Unfortunately, infections of broiler flocks with field strains of ILTV can result in the loss of poultry exports to foreign markets.

The clinical symptoms of an acute outbreak of ILT are easily recognized. However, in the absence of acute symptoms, confirmation of ILT must be obtained in the laboratory. Virus can readily be isolated from tracheal or lung tissue and the demonstration of intranuclear inclusion bodies in tracheal or conjunctival tissue is pathognomonic for ILT (Cover and Benton, 1958). Unfortunately, inclusion bodies are usually observed only during the early stages of an infection (van der Heide *et al.*, 1967). A variety of procedures have been described for the rapid diagnosis of ILT. These include PCR techniques, enzyme-linked immunosorbent assays

(ELISA) and indirect fluorescent antibody (IFA) procedures. Virus neutralization tests for ILTV are difficult to reproduce (Andreasen *et al.*, 1990) and IFA tests are difficult to run on large numbers of samples. PCR amplification of the ILTV thymidine knase gene can detect ILTV from infected trachea, chorio-allantoic membranes or infected cell culture supernatants (Abbas *et al.*, 1996; Scholz *et al.*, 1994). Commercial ILT ELISA kits are readily available. These kits can be effectively used to monitor vaccinated flocks. However, they are not as effective when used as a surveillance tool (Leong *et al.*, 1994). The serological response of birds exposed to ILTV in the field is sporadic and inconsistent. Individual birds can be infected but have low ELISA titers. This phenomenon is due to both the biological and immunological properties of the virus. ILTV infection is usually limited to the upper respiratory tract and viremia is rarely observed. Furthermore, the humoral immune response, including secretory and maternal antibodies, and levels of neutralizing antibodies do not correlate with protection. Instead, protection seems to be mediated primarily by the cellular immune response. ILTV is a prime example of an infectious agent which induces a mucosal, cell-mediated, protective immune response.

Vaccination for ILT has generally been used only in areas where the disease is endemic, since vaccination can result in the occurance of long-term "carrier" birds due to the virus' ability to enter a latent state in the trigeminal ganglia. Furthermore, current vaccines are themselves mildly pathogenic, with a resulting economic "cost". There is justifiable concern over the negative performance (growth, mortality, feed conversion) associated with current ILT vaccines. Some investigators (Guy *et al.*, 1991) have reported that modified live ILT vaccines increase in virulence by mutation during bird-to-bird passage in the field. This has led to an additional reluctance to vaccinate for ILT unless a region is faced with an active outbreak of the disease. Other groups contend that vaccine strains of ILTV are genetically stable (Keeler *et al.*, 1993). However, by spreading into flocks that may contain birds of different ages and with a different immune status, the incompletely attenuated modified live vaccine strains of ILTV may manifest a clinically more severe disease. Consequently, the diagnostic lab is often faced with the difficult question of whether an outbreak of ILT is caused by a field strain of the virus or by a vaccine virus.

Uncertainty regarding the source of an ILTV outbreak results from the difficulties observed in differentiating strains of ILTV. There is only one serotype of the virus, and biological characterizations can be laborious and time-consuming. Several groups have tried to differentiate strains of ILTV on the basis of restriction fragment length polymorphisms (RFLP). In the traditional application of this technique, the virus in grown in tissue culture (chick kidney or chicken embryo liver cells). The infected cells are then lysed and the viral DNA is purified by centrifugation. The purified viral DNA is then digested with restriction endonucleases and the resulting products are electrophoresed on an agarose or polyacrylamide gel. Due to the large size of the ILTV genome (~155 kb), traditionally restriction enzymes with six nucleotide recognition sequences and generating 10-40 fragments (bands) are utilized in this assay. In the United States, Andreasen *et al.* (1990), Guy *et al.* (1989), Keeler *et al.* (1993) and Keller *et al.* (1992) have all reported that most field isolates of ILTV have restriction patterns which are identical or very similar to those observed for embryo propagated vaccine strains of ILTV. However, they can differentiate embryo propagated vaccine strains from tissue culture adapted strains and from the standard challenge virus. With the exception of Guy *et al.* (1989), the other groups have all reported the presence of field isolates of ILTV that exhibit unique restriction patterns. In all

cases, these unique strains represented less than 10% of the isolates examined. Consequently, the similarities observed in ILTV DNA restriction patterns makes it difficult to determine the source of the virus causing field outbreaks of ILT. As a result of this inability to clearly and easily differentiate ILTV, in most cases diagnostic laboratories are unable to state with certainty whether specific ILT outbreaks are caused by unique field strains, vaccine strains, or latent virus.

Recently, Dr. Garcia at the University of Georgia has combined the use of the polymerase chain reaction (PCR) with RFLP to improve the sensitivity of a DNA-based molecular assay described by Clavijo and Nagy (1997). In this modification, specific oligonucleotide primers are used to amplify a limited region, typically 3-5 kb, of the viral genome. This region of amplified viral DNA is then digested with restriction enzymes and the fragments are visualized after agarose gel electrophoresis. Since a smaller fragment of the genome has been amplified, restriction enzymes that generate more fragments can be utilized. Using this technique, Dr. Garcia has been able to differentiate tissue culture adapted vaccine strains of ILTV from chicken embryo propagated strains. Our efforts to use this technique to examine a different region did not reveal any restriction enzyme polymorphisms.

ILT is a serious upper respiratory disease of poultry, which presents a significant diagnostic challenge. The challenge comes not in determining whether a flock is infected with ILTV but in determining the source of that infection. Current methodologies are not able to clearly make that distinction. In the future, one approach will be to use live attenuated vaccines, which have been genetically engineered to contain a marker, or reporter, gene. A simple colorimetric or PCR-based assay would then be able to distinguish this vaccine virus from a field virus.

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MOLECULAR TECHNIQUES FOR THE DIAGNOSIS OF FOWLPOX

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ABSTRACT

Three molecular techniques which have been used in the diagnosis of fowlpox virus infections are: restriction fragment length polymorphism (RFLP), DNA hybridization and polymerase chain reaction (PCR). While all these molecular methods are sensitive and specific, only PCR is simple, less time consuming and cost effective. It is also useful in differentiating field and vaccine strains in relation to reticuloendotheliosis virus (REV) integration and thus predicting the pathogenicity of field isolates. Moreover, since only REV long terminal repeat (LTR) sequences of variable length are retained in the genomes of vaccine viruses, PCR can also be useful in the differentiation of vaccine viruses.

INTRODUCTION

A tentative diagnosis of fowlpox is usually made when proliferative skin lesions (cutaneous form) appear in the unfeathered areas of the skin of chickens and turkeys. However, the disease can also be manifested by development of lesions in the oral and upper respiratory mucosa (diphtheritic form). In the later case, it can be confused clinically with other respiratory virus infections, especially infectious laryngotracheitis. Although the large size of the viral particles (elementary bodies) enable their detection in stained smears of lesions, definitive diagnosis is usually made by histopathological examination of infected skin or diphtheritic lesions for the presence of cytoplasmic inclusion bodies. The virus is isolated by inoculation of the chorioallantoic membranes (CAM) of developing chicken embryos.

Some of the molecular approaches that have been used in recent years for diagnosis of fowlpox are briefly described below:

a. Restriction Fragment Length Polymorphism

Comparative genomic analysis of various members of the Avipoxvirus genus by restriction fragment length polymorphism (RFLP) has been useful in their genetic characterization and differentiation. For example, the RFLP profiles of quailpox, canarypox, mynahpox and recent pox virus isolates from Hawaiian forest birds are different from those of fowlpox virus (Schnitzlein et al, 1988, Ghildyal et al, 1989, Tripathy et al, 2000). On the other hand, restriction fragments of the genomes of most strains of fowlpox, pigeonpox and turkeypox strains show similar comigration pattern with only minor differences. Interestingly, a virus recently isolated from an outbreak of pox in

ostriches had profile similar to that of fowlpox virus. While this method can be useful in epidemiological studies, its routine use as diagnostic tool is limited because for viral DNA isolation either (a) purified virions from infected CAMs or (b) adaptation of the virus to avian cell culture becomes necessary. Thus the entire process can be time consuming and expensive.

b. DNA Hybridization:

Radioactively-labeled cloned genomic fragments of fowlpox virus have been used to detect the presence of virus specific DNA in the samples suspected of harboring pox virus. In this case, the crude DNA isolated either from the skin or diphtheritic lesions is first transferred to a nitrocellulose membrane, hybridized with the labeled probes and then exposed to Xray film (Tripathy and Radzevicius, 1991, Tripathy, et al 1992). Positive results are seen only when fowlpox virus specific DNA is present in the test sample. The test is simple and specific but its utility as a routine cost effective diagnostic method is not feasible because of the use of radioactive material, and the cost of other reagents. However, the utility of this method was demonstrated in its application for detecting a natural dual infection by pox and infectious laryngotracheitis viruses (Fatunmbi et al, 1995). In this case virus isolation would have been more expensive, inconvenient and time consuming.

c. Polymerase Chain Reaction

Currently, polymerase chain reaction (PCR) is one of the most simple and quickest methods for fowlpox virus diagnosis. Initially, primers representing specific regions of the thymidine kinase (TK) genes of fowlpox and infectious laryngotracheitis viruses were used since clinically it is difficult to differentiate between these pathogens (Tripathy, et al 1992). In both cases, only specific amplification occurred. Moreover, all four primers could be combined in a single PCR reaction without affecting the specificity (Kohrt and Tripathy, 1996). Since then, the relative sensitivities of virus isolation and PCR to detect fowlpox virus infection have been compared (Tripathy and Spangler, 1998). In this case, tracheal lesions from suspected diphtheritic cases of fowlpox virus were used. Since the sensitivity of virus isolation was higher than that of PCR, it is likely that biologically inhibitory substances in the tracheal mucus may have prevented amplification in those cases which were positive for the virus. Recently, we have used PCR to differentiate vaccine and field strains of fowlpox virus with respect to presence of integrated reticuloendotheliosis virus (REV) sequences (Singh et al, 2000). In this study, field isolates were obtained from diphtheritic lesions in birds which had been vaccinated either with fowlpox or pigeonpox virus vaccines. The biological companies provided the vaccine viruses. While all the vaccine viruses tested were found to have variable lengths of REV long terminal repeat (LTR) sequences, the field strains were shown to retain nearly intact REV provirus. Thus by suing combinations of primers corresponding to fowlpox DNA regions flanking the REV insertion site, the REV LTR and the REV envelope gene, vaccine and field strains can be differentiated. Moreover, since most field strains contain the full length REV sequence and are more virulent than currently used vaccines, PCR can help in the initial identification of fowlpox virus strains with respect to REV integration and potential pathogenicity.

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PRACTICAL APPLICATION OF PCR FOR MAREK'S DISEASE VIRUS AND CHICKEN INFECTIOUS ANEMIA VIRUS DETECTION

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ABSTRACT

The polymerase chain reaction (PCR) assay is not commonly used by diagnostic laboratories for the diagnosis of Marek's disease virus (MDV) or chicken infectious anemia virus (CIAV). Currently, diagnosis of MDV by gross postmortem examination and histopathology is still more practical than PCR since PCR offers no clear advantage. If PCR could determine MDV pathotype rather than just serotype or degree of attenuation, it would become more advantageous. In contrast, using PCR for the diagnosis CIAV is more sensitive and specific than standard techniques and should become more common in the future.

INTRODUCTION

The intent of this paper is to discuss the advantages and disadvantages of using the polymerase chain reaction (PCR) in the diagnosis of Marek's disease virus (MDV) and chicken infectious anemia virus (CIAV). Both of these diseases are immunosuppressive and can impact the poultry industry through clinical disease and interference/interaction with vaccines and other diseases. Diagnosis of MDV requires differentiation from avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) whereas CIAV can complicate the diagnosis of infectious bursal disease virus (IBDV).

MDV: In the past, age was used as one of the major criteria for differentiating between tumors caused by MDV and those caused by ALV in both the broiler and layer industry. Before 14 weeks of age, it is unlikely to encounter lymphoid tumors caused by ALV[7,14]. Since the majority of tumors in this age range are caused by MDV, gross evidence of visceral and/or peripheral nerve tumors has generally been considered sufficient for a presumptive diagnosis of MDV[2]. However, it has been recently apparent that multicentric histiocytosis and tumors caused by REV may have been erroneously attributed to MDV, particularly at processing where only gross diagnosis is used. Serology may be useful for flocks that have single infections with REV but is less practical for the ubiquitous MDV[10,15]. Polymerase chain reaction (PCR) conducted on the tumor tissue or blood may aid in diagnosis and is faster and less labor intensive than virus isolation[1,3]. Attributing tumors to REV, MDV, or ALV in the presence of dual or triple infection can be difficult. Since MDV can persist indefinitely in the blood, PCR assays conducted on tumor tissue will also identify blood borne MDV. Generally, REV tumors lack the tumor-associated surface antigen found in MDV tumors which can also aid in diagnosis[13]. An additional

frustrating problem with MDV is the inability to ascertain the different pathotypes of the serotype 1 virus *in vitro.* Although PCR can distinguish amongst the serotypes of MDV and between an attenuated serotype 1 virus and a non-attenuated, live virus; it cannot differentiate between a virulent MDV and a vv+ MDV[9]. Currently, pathotyping *in vivo* against known standard pathotypes is the only way to determine the strain of serotype 1 MDV. In addition to being expensive, time consuming and labor intensive, the pathotyping of MDV strains may not distinguish between two strains of the same pathotype, i.e. RB1B and MD5. Before the pathotyping can done, the MDV must be isolated and purified from the vaccine virus(es). Isolation of MDV is not available as a routine diagnostic laboratory service. Therefore, while PCR is useful as a quick way to determine if a virus isolated from the field is MDV, ALV or REV, it cannot determine if the tumor is caused by one of those viruses in cases of multiple infection, and it cannot ascertain what is the MDV pathotype. PCR is practical when used as a research tool to characterize an MDV isolate as to serotype and attenuation (vaccine versus virulent virus). However, gross postmortem and histopathology are still the most useful tools for diagnosing MDV in the field.

CIAV: Diagnosis of CIAV in the field is becoming more important in order to determine its role in vaccine failure and in exacerbation of other diseases[12]. Since CIAV can cause both immunosuppression and atrophy of the thymus, it also must be considered as a differential in cases of virulent IBDV. There are two important situations where PCR is useful as a tool in the diagnosis of CIAV; as an adjunct to serology in field diagnosis and surveillance, and in determination of CIAV contamination of vaccines and seed stocks[4,5,6,8,11]. Currently, CIAV PCR is most commonly used in the vaccine industry and academia in order to ensure seed stock purity[8]. CIAV contamination of vaccines can be disastrous, particularly with in ovo vaccines where the host is injected at its most susceptible age. It is also crucial for analysis of challenge virus stocks used in research. Contamination of challenge virus stocks is common, particularly with MDV and IBDV isolates. Many of these challenge stocks are harvested directly from tissues that can harbor CIAV, i.e. liver, bursa or spleen, or are propagated in tissue culture that can support CIAV growth. Contamination of challenge stocks with CIAV can completely alter the pathogenesis of the intended virus and thus invalidate research results. Serology is more commonly used in the field for CIAV diagnosis and/or surveillance. However, recent research (personal communication, Dr. Carol Cardona) has demonstrated that chickens can be serologically negative for CIAV while still PCR positive, particularly in gonadal tissue. PCR can be conducted on fresh tissue or tissues submitted for histopathologic examination either after extraction from paraffin blocks or as in situ PCR[5]. In situ PCR can be complicated and time consuming so is not generally used. The best tissues to submit for CIAV PCR are thymus, liver and spleen. Since virus isolation can be technically difficult, time consuming and less sensitive than PCR, it is rarely used by diagnostic laboratories. Although many diagnostic laboratories do not currently offer CIAV PCR, its availability should increase because of its simplicity and better sensitivity and specificity than standard techniques.

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RNA Viruses

DIAGNOSIS OF INFECTIOUS BRONCHITIS VIRUS USING MOLECULAR TECHNIQUES

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ABSTRACT

Infectious bronchitis is a highly contagious, economically important upper-respiratory disease of chickens. Diagnosis of infectious bronchitis virus (IBV) and identification of IBV serotypes is important for controlling the disease because a live attenuated vaccine against one serotype does not cross protect against another serotype. Traditionally virus-neutralization tests in embryonating eggs and cross-protection experiments in birds were used to identify IBV serotypes. Now, two molecular based tests are being used to identify the serotype of IBV isolates; a serotype specific reverse transcriptase-polymerase chain reaction (RT-PCR) test or a RT-PCR/restriction fragment length polymorphism (RFLP) test. Those tests are used to examine the S1 gene of the virus because the sequence of that gene correlates with serotype. Molecular based serotype identification tests can be used to identify many isolates in a short period of time, are relatively easy to conduct, are extremely accurate, and can detect more than one serotype in a single sample. This is a tremendous improvement over traditional serological or vaccination and challenge methods.

INTRODUCTION

Infectious bronchitis is an economically important disease in commercial chickens. It is a highly contagious upper-respiratory tract disease that is caused by a coronavirus. Coronaviruses are enveloped viruses that contain a single stranded RNA genome approximately 23 kilobases in length. By their nature, coronaviruses can change rapidly due to point mutations, insertions, deletions, and recombination events. In addition to this ability to change, numerous serotypes of IBV exist making identification of the virus very challenging. Diagnosis of IBV is important because vaccines do not cross-protect between different serotypes or "variants" of the virus.

Virus isolation in embryonating eggs followed by cross virus-neutralization testing is traditionally conducted to identify the serotype of IBV isolates. In addition, vaccination and challenge experiments in chickens are used to determine cross-protection between isolates of IBV. Although still the gold standard for identification of IBV serotypes, those methods are very labor intensive, and can take weeks to identify the serotype of just one virus.

Molecular techniques have greatly enhanced our ability to identify IBV and rapidly determine the genotype/serotype of the virus. Glycoprotein spikes on the outside of the virus are responsible for determining the serotype. They are made up of two subunits designated S1 and S2. The S1 subunit makes up the distal portion of spikes and contains serotype specific and virus neutralizing epitopes. Identification of IBV serotypes using molecular techniques is possible because the sequence of the gene (genotype) that codes for the S1 glycoprotein, correlates with the serotype of the virus.

MOLECULAR BASED DIAGNOSTIC TESTS

Even though identification of IBV has become faster and easier, viruses must first be isolated from the field, which is still difficult. The most successful method is to place sentinel birds on the problem farm. Sentinel birds are collected at 5 and/or 10 days after placement and virus isolation from the trachea is attempted. To conduct molecular identification of IBV, the virus is first propagated in embryonating eggs. Typically, tracheal swabs or tracheal scrapings in antibiotics are inoculated into the chorioallantoic sac of 9 to 11 day-old embryonating eggs. The virus is passaged from 1 to 3 times, and allantoic fluid containing the virus is collected for testing. Two types of molecular based diagnostic tests for IBV are currently being used. Both tests utilize RT-PCR to amplify all or part of the S1 glycoprotein gene.

Serotype specific RT-PCR. The serotype specific RT-PCR test (Keeler, *et al.* 1998) utilizes serotype specific PCR primers designed to amplify a hypervariable region in the S1 gene. Currently that test can be used to identify the Arkansas, Connecticut, Massachusetts, DE072, JMK and California serotypes of IBV. Different sized amplicons are generated for each of the serotypes, which are identified by agarose gel electrophoresis (figure 1). Primers for more than one serotype can be included in the same reaction to decrease the number of tests conducted on each isolate. An additional benefit is that more than one serotype can be identified in the same sample.

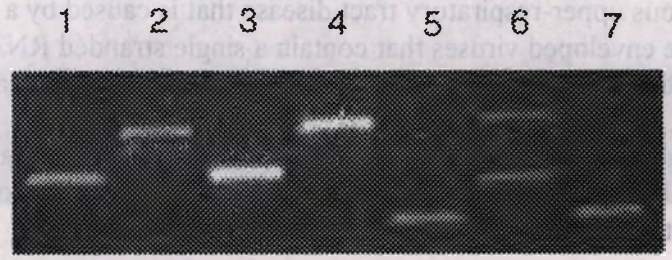

Figure 1. Serotype specific RT-PCR test. The serotype and size of the amplicons are indicated on the right side of the figure.

Serotype identification by RT-PCR/RFLP. The serotype identification by the RT-PCR/RFLP test uses one set of primers in the RT-PCR to amplify the entire S1 gene from all of the IBV serotypes. Then, restriction enzymes, which, cut DNA at specific

sequences called recognition sites, are used to digest the amplified gene and the different sized DNA fragments are visualized on an agarose gel (figure 2). This technique yields a pattern of DNA bands unique for each serotype of the virus, and can be used to identify

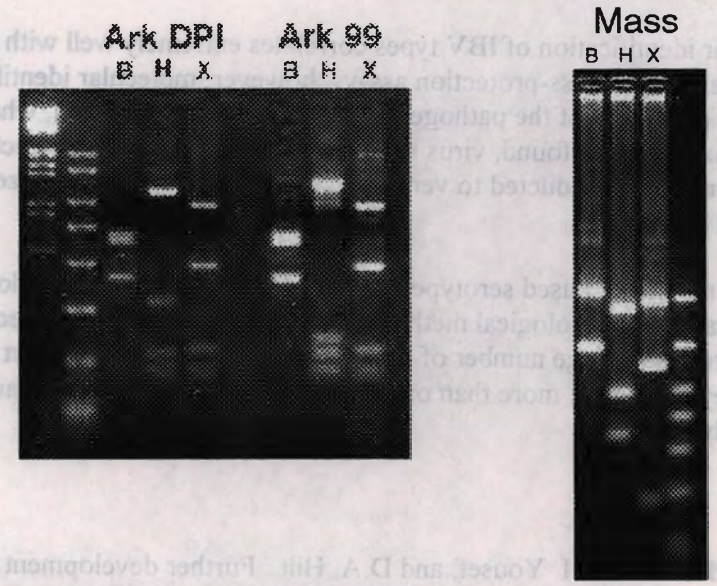

Figure 2. Serotype identification by RT-PCR/RFLP. Restriction enzymes are B= BstYI, H= HaeIII, and X= XcmI. All other lanes are molecular weight markers.

all known serotypes and variants of IBV. Like the serotype specific RT-PCR test, the RT-PCR/RFLP test can be used to identify more than one IBV type in a single sample.

Advantages and disadvantages. The biggest advantage of molecular based serotype identification tests over traditional serological tests for IBV is that they can be used to identify many isolates in a short period of time. Another advantage of molecular identification of IBV is that killed virus also works in the test. Virus in allantoic fluid is killed with an equal volume of buffered phenol (pH 4.5), then viral RNA is extracted and the RT-PCR/RFLP test is conducted. Viruses killed with phenol can be imported from outside the USA with the appropriate permits. In this way IBV isolates from all over the world can be examined and compared to USA isolates. Other advantages are that they are relatively easy to conduct, they are extremely accurate, and they can detect more than one serotype in a single sample.

Disadvantages of the tests are problems inherent in any RT-PCR reaction. These include, failure to amplify the viral genome because of contaminants that inhibit the reaction, poor quality viral RNA template, or reaction conditions that are not conducive to amplification. Of greatest concern is when the reaction fails to amplify the viral genome because of a change in the sequence of the S1 gene. When this occurs, the primers, which direct the amplification of the gene, no longer anneal to the viral RNA. If

this occurs, the change in the viral sequence needs to be determined and new primers must be designed. As a safeguard against false negative results, both molecular based tests utilize a universal set of PCR primers against a highly conserved region of the viral genome. Those primers amplify all IBV types.

The molecular identification of IBV types correlates extremely well with the virus neutralization assay and with cross-protection assays, however; molecular identification tests do not give any indication of the pathogenicity of the virus. In addition, when a new previously unrecognized virus is found, virus neutralization assays and or protection experiments in birds must be conducted to verify that the previously unrecognized virus is indeed a new serotype.

In summary, molecular based serotype identification tests are a tremendous improvement over traditional serological methods to identify IBV serotypes, because they are fast, accurate, and a large number of viruses can be examined in a short period of time. In addition, they can detect more than one virus type in the same sample and variant viruses can be quickly identified.

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APPLICATION OF CLASSICAL AND MOLECULAR VIROLOGY TECHNIQUES TO CONTROL AVIAN LEUKOSIS VIRUS

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ABSTRACT

Classical virology and serology techniques have been used successfully to control avian leukosis virus (ALV) infection in poultry breeding stock. A combination of virus isolation, complement fixation for avian leukosis (COFAL) and direct antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) contributed to the success of the table egg layer breeding industry to control ALV. Control methods for more recently discovered ALVs such as subgroup J ALV (ALV-J) have also involved classical virology and serology, but molecular methods have been added to improve the efficacy and speed of reduction and eradication programs. Besides their application in diagnostics, molecular methods have also facilitated a rapid progress in understanding the biology and epidemiology of ALV, which are essential aspects for success in eradication. Some applications for common conventional and molecular detection methods are described, with emphasis on comparative diagnostics, and their use in epidemiology and pathogenesis studies. Only current diagnostic techniques are discussed.

MOLECULAR METHODS OF VIRUS DETECTION

By convention, "classical" techniques in ALV detection involve virus isolation and serology, while "molecular" techniques involve work with nucleic acids (DNA, RNA, and cDNA). For practical reasons, we will describe virus isolation and all serological methods as "classical" techniques, while any work involving nucleic acids will be considered "molecular". Protein assays are excluded, since they are used more commonly in research rather than diagnostics.

Common molecular methods for virus detection include the polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR). The former detects proviral DNA, while RT-PCR detects viral RNA. In its free (infectious) form, the ALV genome consists of two identical copies of an RNA molecule. Upon cell infection, the viral RNA is transcribed into DNA (cDNA) prior to its integration into the cellular genome. An integrated ALV is known as a "provirus". Proviral DNA is detectable through PCR, which involves the amplification of specific regions or sequences within the viral genome. The regions within the ALV genome that appear more specific to each subgroup involve primarily the envelope gene, but other sequences can and have been exploited for molecular diagnostic purposes. Several PCR systems have been developed to differentiate endogenous from exogenous ALV's, or to

identify specific subgroups. In sampling birds for PCR or RT-PCR assays, it is important to consider the biological cycle of the virus, its forms of transmission, and its likely location within tissues or fluids. It is also relevant to contemplate immunological aspects and the likely evolution of the virus within birds as they age. For instance, young birds are more likely to harbor higher concentrations of virus in the blood. Therefore plasma and serum are suitable samples for viral RNA detection through RT-PCR, whereas a variety of tissues and blood cells may be used for RNA and/or DNA detection using RT-PCR or PCR respectively. Viremia tends to decrease as age increases, while the proviral DNA remains present in multiple tissues. Hence, in older birds PCR has a better application for proviral DNA detection in tissues, tumors, or white blood cells, whereas RT-PCR is more suitable for fluids, although it may also be used to detect viral RNA in tissues. Variations of molecular methods have been extremely useful in confirmatory assays, such as *in situ* PCR and riboprobes. *in situ* PCR permits the detection of ALV genetic sequences in fixed tissues, an approach that may be used as a confirmatory diagnostic assay, or as a tool to locate infected cells in pathogenesis studies. In essence, *in situ* PCR is a PCR assay where DNA is amplified within the tissues where the virus is present. Riboprobes allow the identification of ALV-infected cells or tissues, and thus they can also be used in diagnostics or pathogenesis studies. Riboprobes are genetic sequences that have been chemically labeled, so that when they bind to specific ALV sequences in cells or tissues they serve as a reporter molecule that allows visualization of infected tissues harboring ALV genetic material. An additional variation of PCR and RT-PCR is a competitive PCR, which makes possible the molecular quantitation of viral nucleic acids. Although they are not really practical for diagnostics, quantitative molecular assays are powerful tools for pathogenesis studies, since they permit a better understanding of the conditions that favor virus replication. Our laboratory is currently using this technique to quantitate ALV-J genomes in immunosuppressed birds.

CLASSICAL METHODS FOR ALV DETECTION

Commonly used classical methods include virus isolation and enzyme-linked immunosorbent assays (ELISA). Virus isolation in chicken embryo fibroblasts is currently considered the "gold standard" in ALV diagnostics, and it serves as a reference for all other virus detection methods. While most genetic lines of chickens are permissive to virtually any ALVs from subgroups A,B,C,D,E and J, embryo cells from specific genetic lines are used to selectively propagate endogenous or exogenous viruses. For instance, C/O cells are used to propagate any of the known subgroups of ALV (A-J). C/E cells from line 0 chickens are used for the selective isolation of exogenous viruses. For some applications, it is important to determine if birds are infected with only endogenous viruses (which are ordinarily of no consequence), or if the bird or flock is infected with exogenous viruses, which are frequently associated with detrimental effects. Other types of cells are available for specific applications. C/J cells (developed at the ADOL/USDA, East Lansing, MI) selectively propagate ALV's other than ALV-J, and are therefore useful for differential subgrouping of field isolates. A standard method for virus isolation uses chicken embryo fibroblasts infected and incubated 7-9 days. Viral proteins (gsa or p27) are detected using an antigen-capture ELISA system (AC-ELISA). The gsa protein is shared by all replication-competent ALV's, but it may also result from transcriptional activity of endogenous virus genes encoding gsa. Therefore, ALV subgrouping still requires additional methods. A practical approach is to

rule out or rule in the various subgroups of ALVs that are most common in commercial poultry (subgroups A, B, E,and J). This may be accomplished by combining virus isolation in selectively permissive cells (C/E, C/O, or C/J), AC-ELISA, and molecular methods such as PCR and RT-PCR. One major disadvantage of virus isolation is that some viruses such as ALV-J spread extremely rapidly within young flocks. Often laboratory results cannot be generated quickly enough to eliminate infected birds from pedigree flocks before the virus spreads to naive birds. Still, virus isolation is very commonly used because of ease, reliability and cost.

ELISA AND AC-ELISA

An ELISA system that detects specific viral protein is known as an antigen-capture ELISA (AC-ELISA). As indicated above, AC-ELISA is used to detect a virus-produced structural protein known as "group specific antigen" (gsa, also known as p27). AC-ELISA can also be used to directly detect gsa in a variety of samples, including as cloacal or vaginal swabs, egg albumen, feather pulp and chick meconium. gsa may be present in such samples as a result of an active infection with exogenous virus or from infection with endogenous viruses carrying gsa-coding sequences. Endogenous viruses are usually non-detrimental to flock health, they are often non-infectious, and for the most part, they have no economic significance. Hence, direct detection of gsa (p27) should be complemented with other methods for an objective evaluation of the status of infection and its significance in a given flock. Direct AC-ELISA on serum or plasma is not recommended, since an excessively high number of positive reactions are induced by endogenous virus protein. ELISA systems that detect antibodies against subgroups A/B/D, or against ALV-J are also available. The usefulness of antibody tests is limited, because of the difficulty in interpretation of results. A positive identification may signify previous exposure, or a response to endogenous viral protein, without necessarily being infected with economically significant exogenous viruses. Absence of antibodies might be due to lack of exposure, or immune tolerance induced by congenital or very early infection. In addition, the antibody titer does not reflect a certain level of protection. For these and other reasons, antibody responses definitely should not be used as a single diagnostic criterion, but they may be used to screen flocks or genetic lines expected to be free of specific ALVs, and therefore free of specific antibodies.

APPLICATION OF CLASSICAL AND MOLECULAR METHODS

Figure 1 illustrates an example of a comparison of different diagnostic approaches for field samples. A variety of samples were obtained from 92 birds from a 34-week-old broiler breeder flock. The samples were held and processed following different protocols adopted in various diagnostic or monitoring laboratories. It can be observed in Figure 1 that there is a significant discrepancy between the different methods, and that all virology and serological methods rendered a higher number of "positives" than the "gold standard" (virus isolation in C/E chicken embryo fibroblasts). This does not necessarily mean that all the procedures other than virus isolation on C/E cells are better or more specific. In this case, a higher frequency of positive results is likely to be due to endogenous virus infection. In addition, the correlation of positives and of negatives was considerably low (data not shown), which is usually due to significant differences in sensitivity and specificity.

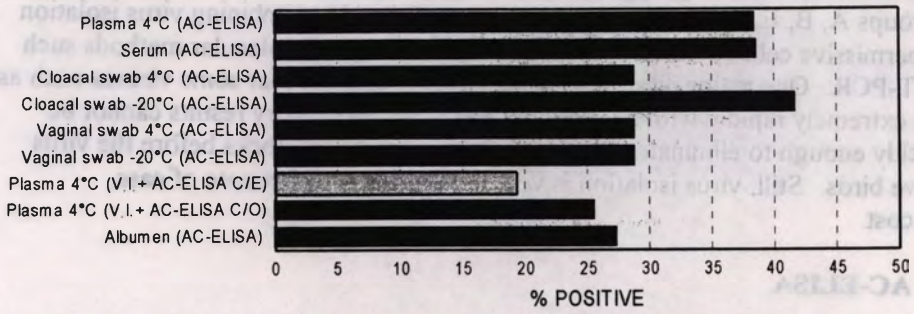

Figure 1. Comparison of classical methods for ALV detection in a 34-week-old broiler breeder flock with high mortality with tumors (n = 92). The samples were either frozen at –20°C or held at 4°C overnight before processing. Virus isolation was accomplished from plasma inoculated into C/O or C/E chicken embryo fibroblasts and incubated/7 days. Cell culture fluids were assayed through AC-ELISA after sequential (3X) freezing/ thawing. Zavala, G., G. Zavala, L. Dufour-Zavala, and P. Villegas (unpublished).

It is not possible to review here all the reasons for the vast differences in the results observed. It should suffice to say that among classical methods, the most repeatable and accurate results for an evaluation of viremia and shedding were obtained through virus isolation in C/E chicken embryo fibroblasts, and when the plasma samples were refrigerated overnight and processed within 24 hours. It should be noticed that in this case no molecular methods were used.

COMPARATIVE DIAGNOSTICS

Comparative studies between classical and molecular methods have consistently rendered a higher frequency of positive results using molecular methods. PCR on tissues is a particularly sensitive assay, and should be expected to detect infected birds whether they have developed clinical disease or not. Both PCR and RT-PCR are extremely sensitive and specific. Results generated using RT-PCR are not precisely equivalent to results obtained using virus isolation, but they can be considered similar in some ways, since both assays detect viable (replicating) virus, while PCR detects proviral DNA, whether the virus is replicating or not. *in vitro* studies performed in our laboratory have confirmed that both PCR and RT-PCR for ALV-J can detect viral nucleic acids earlier than virus isolation.

USE OF CLASSICAL AND MOLECULAR METHODS FOR ERADICATION PURPOSES

Most primary breeding companies rely heavily on a combination of virus isolation and AC-ELISA for the identification of infected birds. Multiple samplings during the life of a pedigree bird are sometimes necessary. One reason is that the virus may be present in very low concentration in blood, egg albumen or other samples, or simply, the sampling age may anticipate age of infection. Also, because of errors or problems in sampling, sample processing, virus isolation methods, and intrinsic imperfections of virus isolation systems, it is necessary to sample birds multiple times to increase the likelihood of positive identification of infected breeding stock. Direct AC-ELISA may be performed on egg

albumen, chick meconium, and cloacal and/or vaginal swabs. Thus, a pedigree (elite) or multiplier bird is tested multiple times and through different methods before it qualifies to be used for reproductive purposes in order to produce the next generation. Breeding stock must also be tested during the egg production period to insure that congenital shedding does not occur. Despite multiple efforts to positively identify infected stock, it is not uncommon to overlook some infected birds because of problems inherent to detection systems, and also due to the fact that birds may be intermittent shedders, or they might still be susceptible to infection at a relatively "late" age. Because of these and other limitations, molecular methods have been incorporated into eradication programs. PCR and RT-PCR are frequently used as confirmatory assays, but they can also be used as diagnostic tools. Although it would be economically and logistically very difficult to test every bird through molecular methods, it is possible to test birds on a "pedigree family" or "small group" basis. In this case, PCR or RT-PCR can be successfully performed on pooled samples such as feather pulp (at 7-10 days of age), or plasma or serum (at any age). Our laboratory has performed studies to evaluate the usefulness and reliability of PCR on feather pulp samples in young birds. The correlation of positive results approaches 100% when compared to virus isolation on C/E cells. In our studies, PCR on feather pulp appeared to be more sensitive than virus isolation in birds under 3 weeks of age.

USE OF VIRUS DETECTION METHODS FOR EPIDEMIOLOGICAL STUDIES

One crucial step in conducting epidemiological and pathogenesis studies on ALV infection is to formulate precise hypothesis to be tested. For instance, if there is a need to know exactly how many birds have been exposed to ALV-J, PCR is perhaps the most suitable assay, since it will detect proviral DNA, whether the bird is viremic or not, and whether it is shedding virus or not. If there were a need to know the rate of infectious virus shedding, RT-PCR and virus isolation would be more suitable. If there is an interest in finding out the frequency of infection with endogenous vs. exogenous viruses, then parallel virus isolation on C/O vs. C/E cell cultures, or PCR for endogenous or exogenous viruses should be used.

SUMMARY

None of the virus detection methods hereby described agglutinates all the desirable criteria for an optimal assay. Some of the assays are time-consuming and expensive, and some exhibit a significant compromise in specificity in exchange for convenience and speed. Successful eradication programs require a combination of detection methods, with virus isolation and AC-ELISA ("classical methods") being the most commonly used. Molecular methods are used as confirmatory tests, and in some cases they are used as diagnostic tools, especially in situations where samples can be pooled, which is when birds are tested on a "group basis" rather than on an individual basis. Both classical and molecular methods have a direct application in diagnostics, eradication and research of avian leukosis viruses. Classical methods do the volume; molecular methods do the fine work.

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THE USE OF NUCLEOTIDE SEQUENCE TO PREDICT PATHOGENICITY AND FOR EPIDEMIOLOGIC ANALYSIS OF AVIAN INFLUENZA VIRUS OUTBREAKS

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ABSTRACT

The direct determination of the RNA or DNA sequences from different pathogens is becoming a widely available and necessary tool for most research laboratories, and with a better understanding of what genetic factors are involved with disease, sequencing of clinical samples is becoming more common. Although sequencing technology has become more widespread and the cost has decreased in the last five years, this technology still remains too expensive to replace most routine diagnostic tests. However, the determination of sequence information promises to be the single most important prediction factor as to the pathogenicity of viral and bacterial diseases and remains the definitive test for epidemiological analysis. The current and future uses of sequencing as a diagnostic and epidemiologic test for avian influenza will be highlighted in this presentation.

METHODS

Influenza viruses are segmented RNA viruses that can be commonly isolated from tracheal or cloacal swabs for mildly pathogenic avian influenza isolates, and also from internal organs from highly pathogenic avian influenza (HPAI) isolates. The RNA from the virus needs to be purified and then is reverse transcribed into DNA and amplified to larger numbers using the polymerase chain reaction (RT-PCR). The RT-PCR DNA product is then usually directly sequenced. Influenza viruses can be directly amplified and sequenced from clinical samples, but unless the virus is being excreted to high titer, you cannot routinely amplify enough DNA to sequence most samples. Typically, influenza viruses are first isolated in embryonated chicken eggs or in cell culture, and then the virus is RT-PCR amplified and sequenced. The sequence obtained is then compared in one of several computer programs to make the necessary sequence comparisons or to develop phylogenetic trees to determine relationships between individual isolates.

The phylogenetic trees will be described in more detail to help explain how they are developed and how to interpret them. Phylogenetic trees are designed to show relationships between different virus isolates. To compare different viruses in a phylogenetic tree it is assumed that all the viruses in the tree had a common ancestor at one time. The viruses may have diverged greatly over time, but they should share

regions of common sequence. The first step to develop a phylogenetic tree is to make the best sequence alignment possible using one of several computer sequence alignment programs. In general the programs try to align all the different isolates so that the minimum of mismatches occur. The time and difficulty to align sequences increases depending on the number of isolates being compared and if insertions or deletions occur in the sequence of the isolates. For example, all the influenza matrix genes that have been sequenced have the same length with no deletions or insertions, but the influenza hemagglutinin gene often varies in size due to both deletions and insertions. Therefore alignment of the matrix gene is easier than properly aligning the hemagglutinin gene. For sequence alignments with many diverse isolates or with variably sized isolates, the final alignment may be different depending on the computer program you use. The same applies to when phylogenetic trees are constructed. Different programs can give different results, although in most circumstances the overall interpretations do not change. The most commonly used phylogenetic program is PAUP which stands for phylogenetic analysis using parsimony. The parsimony method compares the sequence of all the isolates and tries to find the least number of sequence differences between isolates to make a tree. The final tree can be presented in many formats, but I feel the most informative method is the phylogram method. In the phylogram tree the horizontal lines are a direct measure of how closely two isolates are related to each other. In the example in Figure 1, isolates TK/CO/13356/91 and TK/OR/71 are about 112 units away from each other (You add all the horizontal tree lengths to determine the approximate number of sequence differences between the isolates). In this type of tree the vertical lines are present only to make the tree uniform and easier to interpret, so the actual length of the vertical lines are meaningless. Virus isolates will often cluster together in small groups. These groups if separate enough from other isolates are often referred to as a clade, although I rarely feel that influenza viruses meet the clade definition.

Influenza is a segmented virus that commonly reassorts its gene segments. This means that one gene may belong to one group of viruses, but that another gene may belong to a completely separate group of viruses. For example a recent swine virus from several states in the U.S. had one viral gene of human influenza origin, another viral gene of swine influenza origin, and the remaining genes of avian influenza origin. Although this is an extreme case of reassortment, you cannot assume that just because one gene falls into a particular group that all the genes will also. The issue of reassortment will also be discussed when referring the H7N2 Northeast U.S. live bird market isolates. The practical issue when doing epidemiological analysis is that you should look at multiple genes to get an accurate picture on how the viruses might be changing in an outbreak.

MOLECULAR EPIDEMIOLOGIC ANALYSIS

During an outbreak of any infectious disease, the determination of the origin of the pathogen and if all the suspected cases of the disease are the result of infection with the same agent are critical pieces of information when trying to undertake control measures. Previously, the determination that two viruses isolated around the same time had the same hemagglutinin and neuraminidase subtypes suggested that they were related viruses. However, HA and NA subtyping only addresses 2 of the 8 viral genes, and with

the H7N2 LBM market outbreak in the NE U.S. the H7 gene was similar for all the viruses sequenced, but the nonstructural and matrix gene were from multiple lineages. Pathogenicity and host range are controlled by multiple genes, so it is difficult to characterize a viruses' pathogenicity potential if it is rapidly reassorting gene segments. Even the occurrence of the same HA and NA subtypes from viruses isolated in the same year do not assure that the viruses are from the same lineage. This situation occurred in 1993 where H5N2 viruses were isolated from multiple states in the U.S., and based on nucleotide sequencing two different lineages of HA and NA subtypes were found. This demonstrated that two different H5N2 viruses had been introduced into our poultry populations during that year.

Sequence analysis can also demonstrate relationships between outbreaks that were not previously known to be related. A good example with influenza viruses of a previously unrecognized association of outbreaks from different sources occurred with the outbreak of H5N2 virus in commercial poultry in Pennsylvania and surrounding states and the live bird markets in the Northeast United States and Florida. H5N2 virus was isolated from one or both of the live bird markets and commercial poultry in every year from 1985-1989. These H5N2 viruses from both sources appeared to be closely related based on oligonucleotide mapping and later by direct sequence analysis. The viruses also appeared related to the highly pathogenic H5N2 from Pennsylvania from 1983-84. Although the highly pathogenic avian influenza virus was successfully eradicated from the United States, a previously unknown reservoir of low pathogenic virus was present in the live bird markets in the United States, and this virus spread from the live bird markets back to many commercial poultry operations in 1985-86. The virus was eradicated from large commercial poultry operations in 1986, but this lineage of virus persisted through 1989 in the live bird market system. Molecular epidemiology techniques were able to show a relationship between viruses from seemingly unrelated outbreaks, and further highlight the role of live bird markets in the perpetuation of influenza viruses.

SEQUENCE ANALYSIS TO DETERMINE PATHOGENICITY

A major goal of many poultry disease researchers is to be able to understand how the nucleotide sequence can be directly related to pathogenicity or strain differences. For many pathogens this information is just being discovered. For avian influenza virus, it has been known for many years that the presence of multiple basic amino acids at the hemagglutinin cleavage site in H5 or H7 subtype viruses is related to the virus being highly pathogenic or not (Figure 2). The determination of sequence information has now become the standard protocol for evaluating whether H5 or H7 viruses should be considered pathogenic. The sequencing of the cleavage site is routinely done at NVSL, the reference center for avian influenza in the United States. Currently the hemagglutinin cleavage sequence information is the only factor with a known link to pathogenicity, although we believe that other influenza genes are also involved.

OTHER APPLICATIONS OF SEQUENCE INFORMATION

As more sequence information is becoming available from influenza viruses, the possibility of PCR or some other technology of diagnostic tests being used to rapidly subtype the HA, NA and nonstructural gene will be used. The differentiation by RT-PCR of viruses with the H5 and H7 HA subtypes for avian influenza viruses have already been reported. The development of RT-PCR tests for the neuraminidase gene are also in progress, because the current neuraminidase subtyping test is difficult to setup, perform, and interpret. The use of RT-PCR primers, designed from sequence information, to identify each individual subtype has the potential for providing a rapid, sensitive and economical method of subtyping influenza viruses. The difficulty with any RT-PCR test with influenza, however is that the virus mutates so rapidly that it can be difficult to develop reagents that will amplify all influenza subtypes.

CONCLUSIONS

The use of sequence analysis for poultry pathogens will only increase with time, because of the benefits of sequencing for better epidemiologic analysis, pathogenicity classification, and eventually for better diagnostic tests. Currently, the main impediment to more widespread use of this technology is the cost of the equipment, reagents, and the need to train laboratory diagnosticians to use the technology. Fortunately, the cost and methods to perform sequence analysis on clinical samples has been improving, so that it will be eventually used at most diagnostic laboratories.

Figure 1 Phylogram phylogenetic tree of the HA1 nucleotide sequence from H7 AIV

Most of the influenza viruses in this phylogenetic tree are from the live bird markets from the Northeast United States. These viruses form a distinct cluster that suggests that they all had a recent common ancestor. The vertical lines in the tree serve only as place holders to separate the isolates. The horizontal lines represent the amount of sequence differences between isolates. For example, about 112 nucleotide sequence differences exist between TK/CO/13356/91 and TK/OR/71 (10% difference in sequence). The approximate differences are determined by adding the branch lengths between the two isolates (35, 36 and 41). For most avian influenza viruses, they will fall in either the North American or the Eurasian lineage of virus, which corresponds to where they were isolated from as demonstrated in this phylogenetic tree.

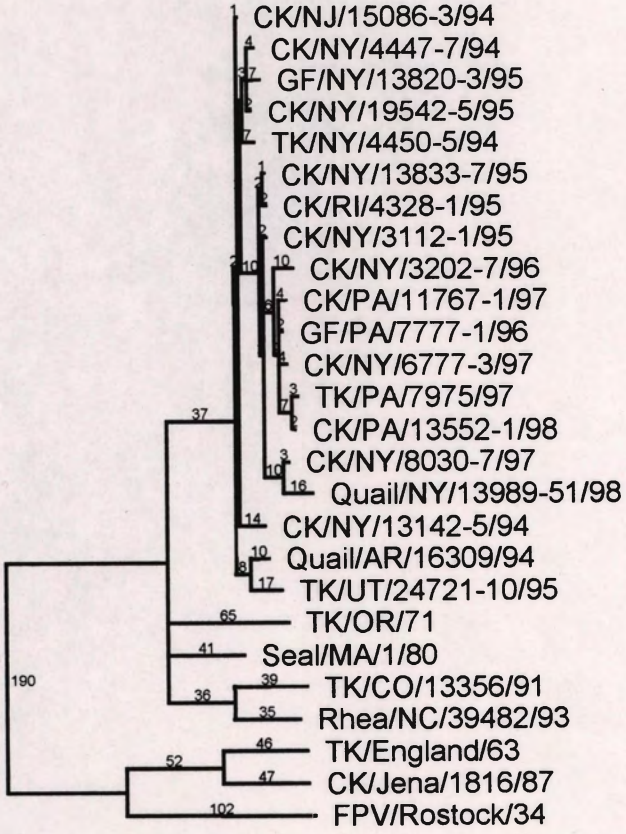

Figure 2 North American H5 Hemagglutinin Cleavage Sites

North American Consensus LP[a]	PQ**R**ET**R**/G[b]
TK/Ontario/7732/66 HP[c]	PQ**RKKR**/G
CK/PA/1370/83 HP	PQ**KKKR**/G
CK/PA/13609/93 LP	PQ**R**K**TR**/G
CK/Mexico/31381-7/93 LP	PQ**R**ET**R**/G
CK/Puebla/8624-607/94 HP	PQ**RKRKTR**/G
CK/Queretaro/7653-20/95 HP	PQ**RKRKRKTR**/G

a LP=Low pathogenic avian influenza isolate

b Basic amino acids lysine (K) and arginine (R) are in bold and the cleavage site is identified at the slash mark.

c HP=Highly pathogenic avian influenza Isolate

MOLECULAR DIAGNOSIS OF INFECTIOUS BURSAL DISEASE VIRUSES: PRACTICAL APPLICATIONS AND SIGNIFICANCE OF THE RESULTS.

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ABSTRACT

Infectious bursal disease virus (IBDV) continues to cause an immunosuppressive disease in young chickens despite the use of breeder and broiler vaccination programs. Antigenic differences among IBDV strains can be one reason for these vaccination failures. A diagnostic assay that can quickly identify and differentiate IBDV strains would aid in the control of this pathogen. A molecular diagnostic test that employs reverse transcriptase/polymerase chain reaction - restriction fragment length polymorphism (RT/PCR-RFLP) was developed for IBDV. The RFLP patterns generated are a direct result of the viral nucleotide sequences and can be used to identify differences among IBDV strains. This assay exploits the fact that antigenic changes require changes in the nucleotide sequences of the viruses. Fifty vaccine and laboratory strains of IBDV were placed into six different molecular groups using RT/PCR-RFLP. Studies on IBDV strains from field cases of the infectious bursal disease (IBD) indicated a much greater number of molecular groups. The results suggest the genetic diversity is much greater among wild-type field viruses compared to vaccine strains. The implications of these findings for the diagnosis and control of IBD are discussed.

INTRODUCTION

The reverse transcriptase/polymerase chain reaction - restriction fragment length polymorphism (RT/PCR-RFLP) assay was developed for the diagnosis of infectious bursal disease virus (IBDV) strains in 1997 (Jackwood and Sommer, 1997). Since then, this assay has been used to place 50 vaccine and laboratory strains of the virus into six molecular groups (Table 1). Examination of viruses from field outbreaks of infectious bursal disease (IBD) in the U.S. resulted in 19 additional molecular groups suggesting the genetic diversity of IBDV in the field was greater than the genetic diversity among vaccine strains (Jackwood and Sommer, 1998).

Prior to the development of the RT/PCR-RFLP assay for IBDV, the assays available for diagnosis of this virus from field outbreaks included, antigen-capture ELISA, immunofluorescence, agar-gel precipitation and virus isolation in eggs or cell culture. Each of these diagnostic assays including RT/PCR-RFLP had inherent problems that make them less than ideal. However, the RT/PCR-RFLP assay was significantly more sensitive than the other assays and was the only one designed to detect viral nucleic

acid. Diagnosis using the other assays is based on detecting viral proteins. Consequently, direct comparisons should not be made between the RT/PCR-RFLP assay results and results from diagnostic assays based on detection of viral proteins.

The RT/PCR-RFLP assay was adapted so samples preserved in phenol/chloroform could be tested from outside the United States (Jackwood, Hanes and Heins-Miller, 1996). This was an important development because for the first time IBDV strains from all over the world could be examined and compared using one standardized diagnostic assay. Consequently, the RT/PCR-RFLP assay for the diagnosis of IBDV gained world wide acceptance and currently is offered commercially by IDEXX Laboratories, Inc. (One IDEXX Drive, Westbrook, Maine 04092).

INTERPERTATION OF RESULTS

The RT/PCR-RFLP assay examines a small part of the VP2 gene that is responsible for encoding the major antigenic determinants of the virus. The same size RT/PCR product is produced for all IBDV strains so this product is cut with restriction enzymes to generate different size fragments (RFLP profile). The RFLP profile for an individual virus is used to place it into a molecular group. Although it is possible for this profile to change due to mutations, we have observed that the profiles of viruses replicating in chickens are relatively stable.

When different RFLP profiles are observed among IBDV strains, it is appropriate to conclude that the viruses are not identical. Since the assay detects genetic sequences in a section of the VP2 gene that are responsible for antigenicity, viruses with different RFLP profiles have the potential to be antigenically distinct. The *Bst*NI enzyme is used to generate an RFLP profile of IBDV strains because it targets a specific mutation that was shown to be critical in altering the specificity of a neutralizing IBDV epitope (Schnitzler et al. 1993). Specifically, the *Bst*NI enzyme identifies a single amino acid change at position 222 in the VP2 protein. When this amino acid changes from proline in classic viruses to some other amino acid (threonine in Del-E viruses), a change in the specificity of the epitope was observed (Schnitzler et al. 1993). The *Bst*NI RFLP profile identifies this change. **However, it is possible and often the case, that viruses with different RFLP profiles are antigenically similar.** This occurs because some genetic changes identified by the RFLP patterns may not affect the VP2 protein sequence and thus its antigenic properties.

When identical RFLP profiles are observed, it cannot be assumed that the viruses are identical. Because the RT/PCR-RFLP assay only examines a small part of the viral genome, it is possible that some regions of the genome could be different. Common sense should be used however. For example, if a commercial vaccine was used prior to collection of test samples, and the RFLP profile matches the vaccine strain, it can be concluded with a reasonable certainty that the virus detected was the vaccine strain.

An RFLP profile that does not match those found in molecular groups 1 through 6 indicates 1. The virus is not a vaccine strain, and **2**. It has the potential to

cause immunosuppression and clinical IBD. The current state of the art requires that identification of this potential be tested in susceptible chickens.

The RFLP profile is also important because it can be used to distinguish wild-type field virus from vaccine strains. The period when these wild-type viruses are identified is a good indication of a problem. **Identification of a virus with a non-vaccine RFLP profile while maternal antibodies to IBDV are still high might indicate that the virus is breaking through the progeny's maternal immunity.** An ELISA to detect antibodies to IBDV can be used in conjunction with the RT/PCR-RFLP assay to make this determination.

If the breeder vaccination program is adequate, we would expect to see maternal antibodies to IBDV drop during a 3 to 4 week period. During that period, IBDV should not be detected using the RT/PCR-RFLP assay, unless, a vaccine strain of the virus was used in the birds. In that case, the virus detected should have a vaccine RFLP profile. If the breeder vaccination program is not adequate, and this could be caused by several factors, we would expect to identify an IBDV strain with a non-vaccine RFLP profile during the first 2 to 3 weeks of age. It is necessary to monitor maternal anti-IBDV titers in the chicks, because, failure of a breeder vaccination program could be manifested by low titers in the chicks. In other words, the vaccine viruses used in the breeder birds may be the correct antigenic type but because the maternal antibody titers are low, the wild-type field virus may be able to infect chicks within the first 2 to 3 weeks of age.

Another factor that directly affects when a wild-type IBDV strain is detected is virulence of the virus. Virus strains that are extremely invasive and able to replicate quickly generally will break through maternal immunity sooner than viruses that are less virulent. Therefore, it is possible to detect a wild-type IBDV strain when maternal antibodies are still relatively high not because it is antigenically different, but because of its pathogenic properties. Nevertheless, detection of a wild-type IBDV strain using the RT/PCR-RFLP assay while maternal antibodies to the virus are relatively high is a sign that the breeder vaccination program is not adequate against this virus challenge.

RECOMMENDATIONS

Use the RT/PCR-RFLP assay in conjunction with regular monitoring for maternal antibodies to IBDV.

When an RFLP profile is observed that matches one of the six molecular groups, consider if a vaccine with that profile was used recently in the flock. If a vaccine with that RFLP profile was not used, it is likely that the IBDV strain is not a vaccine and thus, has the potential to cause immune suppression and/or clinical disease.

When a non-vaccine RFLP profile IBDV is identified using the RT/PCR-RFLP assay, consider the age of the bird and its maternal antibody status at the time of diagnosis. This wild-type virus may be present because the maternal immunity

in this flock is poor and not because it is antigenically different from the vaccines being used.

Test any non-vaccine IBDV strains in susceptible chicks to determine if they have the ability to cause disease.

Conformation that a non-vaccine RFLP profile virus is actually a new antigenic type is needed prior to changing a vaccine program. If a comprehensive broiler breeder vaccination program is being used, and a pathogenic virus is detected that is confirmed to be antigenically unique, chances are that a commercial vaccine does not exist for this new antigenic type. In this situation, an autogenous vaccine could be considered.

Use results from the RT/PCR-RFLP assay to keep track of the IBDV strains present on a farm over time (epidemiology). If the RFLP profiles from one flock to the next do not change and the vaccine program is keeping these viruses in check, don't make any changes. However, a change in the RFLP profile could signify a potential problem and careful consideration should then be given to the recommendations listed above.

NEW TECHNOLOGY

Current research in our laboratory has focused on identification of IBDV genes or gene segments that control virulence. During this work we identified a genetic marker (*Ngo*M IV) for the ability of the virus to replicate in cell culture. The presence of this maker in an IBDV genome, strongly suggests that the virus has been attenuated in cell culture. We have been able to adapt the detection of this marker to our standard RT/PCR-RFLP assay.

Research by Lin et al. (1993) identified a genetic marker (*Ssp*I) for very virulent IBDV (vvIBDV) strains. We have been able to incorporate the detection of this marker into our RT/PCR-RFLP assay (Jackwood and Sommer, 1999). The vvIBDV marker only identifies viruses with the very virulent phenotype. It cannot be used to determine the degree of pathogenicity among other IBDV strains.

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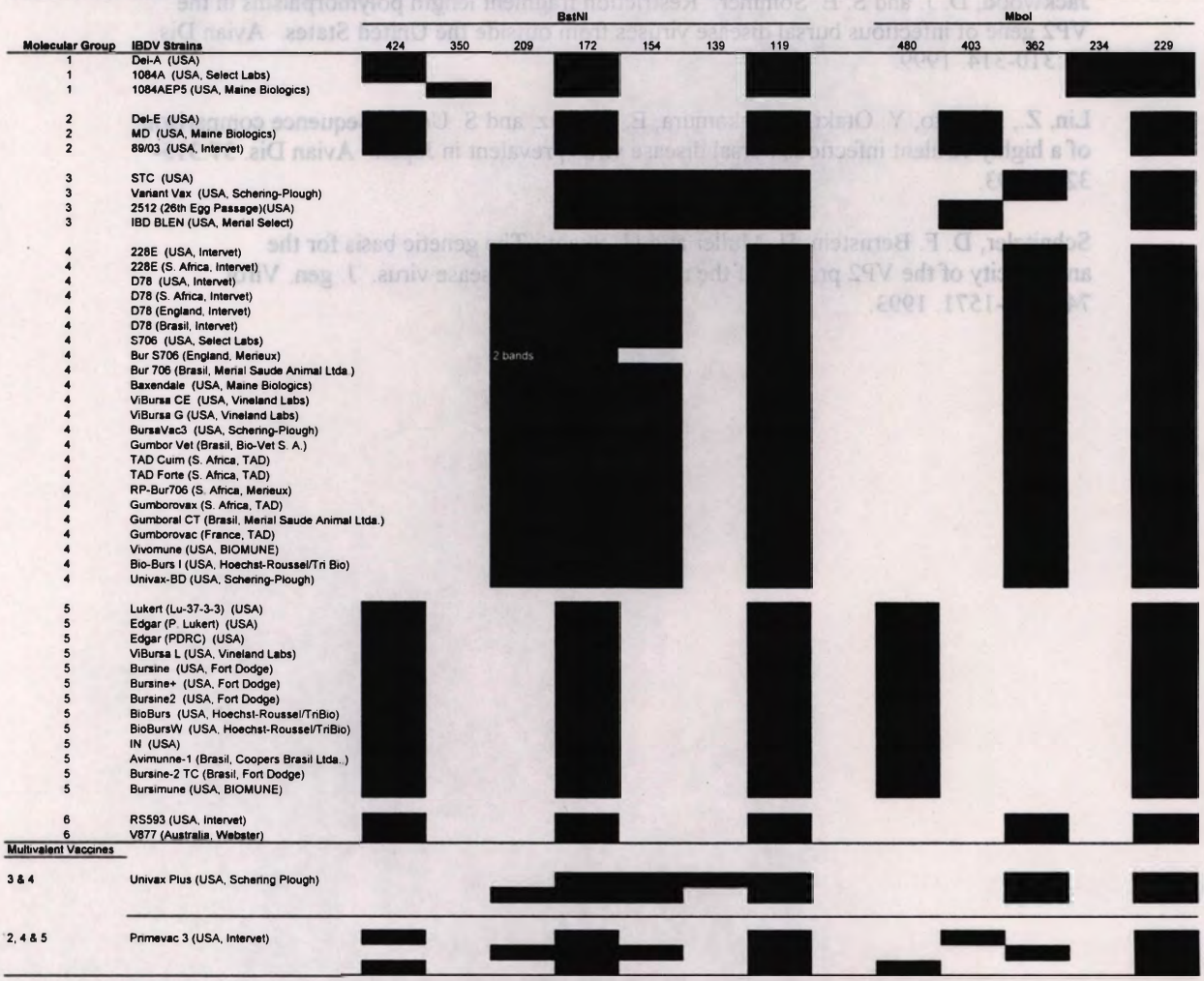
Table 1. Results of the RT/PCR-RFLP assay for vaccine and laboratory strains of IBDV.



Molecular Group	IBDV Strains
1	Del-A (USA)
1	1084A (USA, Select Labs)
1	1084AEP5 (USA, Maine Biologics)
2	Del-E (USA)
2	MD (USA, Maine Biologics)
2	89/03 (USA, Intervet)
3	STC (USA)
3	Variant Vax (USA, Schering-Plough)
3	2512 (26th Egg Passage)(USA)
3	IBD BLEN (USA, Merial Select)
4	228E (USA, Intervet)
4	228E (S. Africa, Intervet)
4	D78 (USA, Intervet)
4	D78 (S. Africa, Intervet)
4	D78 (England, Intervet)
4	D78 (Brasil, Intervet)
4	S706 (USA, Select Labs)
4	Bur S706 (England, Merieux)
4	Bur 706 (Brasil, Merial Saude Animal Ltda.)
4	Baxendale (USA, Maine Biologics)
4	ViBursa CE (USA, Vineland Labs)
4	ViBursa G (USA, Vineland Labs)
4	BursaVac3 (USA, Schering-Plough)
4	Gumbor Vet (Brasil, Bio-Vet S. A.)
4	TAD Cuim (S. Africa, TAD)
4	TAD Forte (S. Africa, TAD)
4	RP-Bur706 (S. Africa, Merieux)
4	Gumborovax (S. Africa, TAD)
4	Gumboral CT (Brasil, Merial Saude Animal Ltda.)
4	Gumborovac (France, TAD)
4	Vivomune (USA, BIOMUNE)
4	Bio-Burs I (USA, Hoechst-Roussel/Tri Bio)
4	Univax-BD (USA, Schering-Plough)
5	Lukert (Lu-37-3-3) (USA)
5	Edgar (P. Lukert) (USA)
5	Edgar (PDRC) (USA)
5	ViBursa L (USA, Vineland Labs)
5	Bursine (USA, Fort Dodge)
5	Bursine+ (USA, Fort Dodge)
5	Bursine2 (USA, Fort Dodge)
5	BioBurs (USA, Hoechst-Roussel/TriBio)
5	BioBursW (USA, Hoechst-Roussel/TriBio)
5	IN (USA)
5	Avimunne-1 (Brasil, Coopers Brasil Ltda..)
5	Bursine-2 TC (Brasil, Fort Dodge)
5	Bursimune (USA, BIOMUNE)
6	RS593 (USA, Intervet)
6	V877 (Australia, Webster)
Multivalent Vaccines	
3 & 4	Univax Plus (USA, Schering Plough)
2, 4 & 5	Primevac 3 (USA, Intervet)

Values are the length in base pairs of the restriction fragments. Colored boxes designate the presence of a restriction fragment following digestion with BstNI or MboI.
Viruses within molecular groups are antigenically similar and some viruses in different molecular groups may be antigenically related.

Industry Perspective

COMMERCIAL APPLICATIONS OF MOLECULAR DIAGNOSTIC TECHNIQUES IN VETERINARY MEDICINE: A DECADE OF EXPERIENCE AT IDEXX LABORATORIES.

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Laboratory techniques based on molecular biology have evolved rapidly over the past two decades. The power and utility of these methods are only beginning to find broader acceptance within commercial diagnostic facilities, particularly in veterinary medicine. The purpose of this presentation is to share some of the experiences gained from our introduction of the first commercial PCR based diagnostic assays into this environment. Issues associated with the product's design & development, licensing, and manufacturing will be addressed as well as technical aspects associated with these methods including considerations of the laboratory facility, installation and training of laboratory personnel, & equipment. Finally, more recent experiences gained utilizing the PCR/RFLP procedure in our reference laboratory will be discussed. Initially only a tool for research, these molecular techniques are truly catalyzing a revolution in routine diagnostic pathology (from forensic to veterinary) in many ways similar to the introduction of immunoassay techniques many years ago.

THE USE OF PCR TO MONITOR THE IBD STATUS OF BROILERS IN THE FIELD

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INTRODUCTION

In the United States, infectious bursal disease viruses (IBDVs) predominantly cause immune suppression, or subclinical Gumboro, not mortality. Since the mid-1980s, there has been a well documented emergence of several different variant strains of IBDV. Although PCR results are not a definitive answer about antigenic type, the results so far seem to reflect a continued emergence of "variant" viruses. For example, since 1998-99, we have isolated well over 40 different virus types, based on their unique PCR patterns. While it might be argued that many of these new patterns may result in only minor antigenic differences (we don't know), there are indications of at least a few novel viruses that are significantly different than our reference viruses. This means some field viruses may more readily infect broilers in the face of maternal antibodies. They may also present greater challenges for effective control through live field vaccination.

THE PCR TEST

PCR enables us to "look" at particular IBD viruses. Using Jackwood's method, a service provided by Idexx Laboratories, a 743 base pair sequence of the variable region of the VP-2 genome is amplified, then reacted with two restriction enzymes, BstN1 and Mbo1. The resulting pattern is determined by where or if the amplified molecule is cut by the enzymes, which look for specific 6-bp and 4-bp sequences, respectively. Thus, the test does not define the entire 743 bp segment, per se, only the areas where the enzymes cut. According to Jackwood, the BstN1 pattern is a good predictor of antigenic type (1). The Mbo1 enzyme helps further separate viruses that have the same BstN1 pattern (see table 1 below). Viruses within the same molecular group are more likely to be closely related to each other than to viruses having different PCR patterns. G-1 and G-2 are variant groups, G-3 and G-4 contain classic viruses, and G-5 and G-6 are not as neatly defined.

Table 1. IBDV PCR; summary chart for vaccine and laboratory strains (2, 3).

Molecular group	IBDV reference strains	BstN1 fragments	Mbo1 fragments
1	Delaware-A, SVS-510	**424, 172, 119***	234, 229
2	Delaware-E, MD, 89/03	424, 172, 119	403, 229
3	STC, Variant Vax, IBD-Blen**	172, 154, 139, 119	362, 229
4	D-78, S-706, Univax-BD, Bursavac	209, 172, 154, 119	362, 229
5	Lukert, Edgar, Bursine-2, Bursine+	424, 172, 119	480, 229
6	RS593, V877	424, 172, 119	362, 229

* This is what is referred to as the "424" pattern. For example, of the reference groups, G-1, G-2, G-5 and G-6 have the "424" pattern. They all are different on Mbo1, however.
** IBD-Blen is slightly different on Mbo1: 403, 229.

FIELD RESULTS

In table 2, results are listed from U.S. field surveys. In 1997, Jackwood first started processing a significant number of field submissions (2). In the following year, Idexx started providing this service. The 1998-99 results are all of our submissions.

Table 2. IBDV PCR; positive isolation results in the United States since 1997.

Molecular group	Jackwood 1997 (2)	(%)	FDAH 1998-99	(%)
1	2	2.9	1	0.3
2	18	25.7	67	18.4
3	0	0.0	15	4.1
4	0	0.0	2	0.5
5	2	2.9	34	9.3
6	10	14.3	84	23.1
New "424"	28	40.0	122	33.5
New "other"	10	14.3	40	11.0
Total	70		364	

In 1997 the most prevalent patterns isolated were G-2 (25.7%), which includes Del-E, followed by G-6 (14.3%). No classic G-3 or G-4 was isolated and a little G-1 (Del-A) and G-5 (Lukert) were isolated, suggesting that not much vaccine or field virus was recovered from these groups. Several different new types with the "424" pattern made up almost half of all isolations, while there were a few new types that did not have a "424" pattern (new "other"). Thus, over 85% of the field isolates had the "424" pattern on BstN1. Jackwood points out that there is an important conformational change in the G-3 and G-4 classic viruses that is not present in IBDVs which have the "424" pattern

(4). This may explain why G-3 and G-4 viruses don't tend to cross-protect as well against Del-E, for example (5).

In 1998-99, the most prevalent field patterns we isolated were again G-2 (18.4% of all positive isolations) and G-6 (23.1%), although their relative frequency flip-flopped. This suggests a rising trend of G-6 isolations. There was the rare isolation of G-1 and G-4. On the other hand, there was a rise in the incidence of G-3 (4.1%) and G-5 (9.3%) isolations. Not coincidentally, this was considered mostly due to the recovery of the stronger, more invasive vaccines IBD-Blen and Bursine-Plus, respectively. The largest category of viruses was again that consisting of the novel types sharing the "424" pattern (33.5%). Like in 1997, the "424" pattern viruses constituted about 85% of all isolations.

DESIGNING FIELD SURVEYS

Based upon past experience in the U.S., the best window to look for field virus is 21-28 days. Part of this reason is economics—the PCR test is not cheap. Unless you suspect very early field challenge, most 2 week samples will be negative. Since a positive test probably requires a fair amount of (actively replicated) virus, a high % of positive 21 day results would suggest there was at least some early IBDV infections (by 2 weeks of age). Of course, histology is still the most useful tool to pinpoint the timing of infection.

Typically, 5 bursas (from normal, healthy birds) are sampled from a given flock. Each bursa can be split into halves so histology and PCR can be run on the same samples. PCR samples should be kept cool upon harvesting and then frozen at least overnight. Samples should be packed with dry ice and shipped early in a given week via next day air. Results are usually available in 1-2 weeks.

Surveys can be conducted in many different ways, including: 1) a random sampling of farms, 2) by different regions, 3) historically good performing farms vs. poor performing farms (that should be better), 4) vaccinated vs. non-vaccinated, either historically or for that cycle, and 5) comparing different vaccination programs. Things to look for are differences in either the profile of field virus type or percentage of positives at 21 days (most flocks are positive by 28 days), and the possibility of recovering vaccine virus.

Intermediate vaccine virus is not usually recovered, while the intermediate-plus and strong vaccines are recovered with better success (see Table 2). This doesn't necessarily mean that intermediate vaccines are not working, but that they are not usually going to replicate at a high enough level—<u>on a flock basis</u>, in the face of high maternal antibody levels on one side and strong field challenge on the other—to yield a positive result. It is important to note that most flocks are vaccinated pretty early—by 8-12 days—and we sample only 5 birds/flock.

WHAT DO WE MAKE OF THE RESULTS?

Sometimes there is a correlation between a particular virus type or pattern and flock performance. This would be an indication to try to optimize the IBD vaccination program. If there is a lot of early challenge, perhaps there are opportunities to improve the breeder program—live boost and/or killed (technique or vaccine). Of couse, the effects of these changes will take time. If either early or late IBD is having an impact on performance, there may be opportunities to improve the broiler program by either the type of vaccine selected or by adjusting the timing of vaccination.

Ideally, the IBD vaccine selected would be in the same molecular group as the field virus. This vaccine would likely be the best antigenic match and have the potential to produce the most appropriate immune response. However, the vaccine's potential must also be realized by inducing a sufficient "take" response in the commercial broiler flock. If the field challenge is quite strong, this can be difficult to accomplish. In this case, a vaccine from another molecular group might actually yield better overall protection than one that is a better antigenic match, if it is capable of producing a better, more complete "take" response and a quicker onset of protection.

In reality, though, our currently available vaccines are not a perfect molecular match for the vast majority of U.S. field isolates. In addition, often a broiler complex contains a number of different molecular types, in which case vaccines are often chosen based on past performance. Some IBD vaccines have a broader spectrum of protection against different variant IBDVs, while others have advantages based on their level of activity or invasiveness (strength).

OTHER USES OF PCR

--to generate a database of virus types within a company or complex
--monitoring the prevalence or spread of new virus types in the field
--monitoring for very virulent IBDV using the Ssp1 enzyme
--importation of inactivated samples from overseas for quick study
--screening field viruses for potential vaccine candidates
--testing seed lots of vaccines for purity
--run monoclonal antibody panel on same isolates to get additional antigenic information, and vice-versa.

LIMITATIONS OF PCR ANALYSIS

--cost of a test is ~ $200.00
--does not predict pathogenicity (unless vvIBDV)
--only looking at changes in a few select regions of the VP-2 genome
--are there major/minor antigenic differences within a given PCR pattern?

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ROUTINE APPLICATION OF MOLECULAR DIAGNOSTICS IN THE POULTRY DIAGNOSTIC LABORATORY

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The justification for using molecular diagnostic procedures in the poultry diagnostic laboratory should include an assessment of resources and a demonstrated need. Molecular techniques generally do not provide information that can't be obtained by standard microbiology procedures if the proper specimens are available to adequately trained laboratory personnel. In our situation, however, the advantages of molecular techniques are considerable for certain diseases. We now provide information about diseases and agents that were not adequately addressed before. Furthermore, the information is available sooner and in greater volume than would be possible using conventional procedures within our constraints of available personnel and facilities.

We use the polymerase chain reaction (PCR) and the reverse transcriptase PCR (RT-PCR) within our panel of tests for diseases of poultry and other birds. PCR is currently used for mycoplasmas, infectious bronchitis virus, chicken infectious anemia virus, infectious laryngotracheitis virus, avian leukosis virus subgroup J provirus, psittacine polyomavirus, and eastern equine encephalomyelitis (EEE) virus. We generally try to respond to our in-house needs and those of laboratory constituents. We do not use a molecular test for every possible disease. For example, infectious bursal disease virus detection and characterization are available commercially and we have not initiated molecular IBDV diagnostics. In the Alabama diagnostic system of four total laboratories, only the central laboratory in Auburn runs molecular diagnostics. The same personnel who do poultry testing also run the mammalian PCR testing for brucellosis, Johne's disease, mycoplasmas, EEE, bluetongue, and epizootic hemorrhagic disease, among others. Laboratory personnel who understand and practice quality scientific method are essential to successful implementation of molecular diagnostics. A sound understanding of the etiology and pathogenesis of infectious disease broadens the foundation. Initiative, curiosity, creativity and persistence play a supporting role. Recent graduate courses in molecular and cellular biology are a definite asset. Detailed records and adherence to protocols are required of technicians.

The laboratory space need not be large or elaborate but it should be protected from common traffic patterns. Quiet, relatively isolated workstations are desirable to avoid interruption. It is essential that the laboratory space and surfaces be clean and capable of being disinfected. Equipment needs include many standard microbiological laboratory items (refrigerator, -70F freezer, hoods, a quality centrifuge), a thermocycler,

electrophoresis equipment, UV visualization apparatus to examine gels, Polaroid camera, micropipettes, and a decent personal computer and scanner.

We contract with commercial laboratories for making primers and for our somewhat limited sequencing efforts. Many research universities offer these services today. Primer selection is key to successful diagnostics. Verification of primer sequence is important as even peer-reviewed publications may have sequence errors. In-house specificity testing is advised for candidate diagnostic primers. The primer should be reacted with a panel of poultry pathogens, closely related viruses, and vaccine agents that may be co-infecting birds submitted for examination. Testing of primers on serial dilutions of the target agent of known titer provides information about the sensitivity of detection.

Mycoplasmosis. Our first PCR experiences in the early 1990's centered on mycoplasmosis, particularly *Mycoplasma synoviae* (MS). We were confronted with broiler breeder flocks that tested positive on the rapid plate agglutination test but then gave negative or only suspicious reactions on hemagglutination inhibition (HI) tests. Culture was often confounded by *M. gallinarum* and *M. gallinaceum*, and by inconclusive results on fluorescent antibody identification of MS. In our hands monoclonal antibody-based procedures provided inconsistent results. Our sincere but inadequate efforts in conventional diagnostics were yielding insufficient information and unacceptable delays. At stake was the prompt control of mycoplasmosis in the Alabama poultry industry. The lead to PCR came through a manuscript describing a technique for identifying mycoplasma contaminants in cell culture (2). The primers were selected from highly conserved regions of RNA within the mycoplasma ribosome. Following initial success with this general technique, our search for a specific diagnostic procedure for MS was continued. The aid of a molecular biologist, Dr. Vicky Van Santen, was enlisted to search GenBank for genomic sequence information on *M. synoviae*, our first attempt to design primers (7). This PCR test showed high specificity for *M. synoviae* and we continue to use these primers today.

Since then, general and specific primers have been designed for various species of avian mycoplasmas, including *M. gallisepticum*. Mycoplasma PCR has not eliminated the need for serology or culture but is useful as an adjunct test. It is a rapid test that can be completed in one day, without the delay of waiting for rising serological titers, or the difficulties encountered in isolating and identifying the organism. Infection can be detected in specimens from birds submitted dead for examination, from which serum is not available or the agent can no longer be cultured. PCR can be run in parallel with standard biochemical and serological speciation of mycoplasma isolates. Poultry managers use the PCR test as a rapid final test on male breeders that are added to breeding flocks during midpoint of the laying cycle. Tracheal swabs from chickens with suspicious serology are tested by PCR, and results are evaluated concurrently with HI titers while cultures are incubating.

Infectious bronchitis. The IBV RT-PCR combined with restriction fragment length polymorphism analysis (RFLP) has increased the information for epidemiology and

practical management decisions about infectious bronchitis in Alabama by hundreds-fold. It will likely never replace *in vitro* neutralization testing and *in vivo* challenge testing that fundamentally characterize IBV serotypes. Once that basic information is available, however, RT-PCR is capable of producing abundant data quickly and economically for tracking the occurrence of IBV serotypes within poultry programs and geographic regions. Amplified segments of the genome that code for the antigenically variable spike proteins are cut with restriction enzymes, which when examined by gel electrophoresis, create patterns that correlate with characterized viruses within a given serotype (4,5,6). The RT-PCR does not measure or recognize antigenic differences among serotypes; it depends on competent studies to correlate the pattern with a serotype. Newly emerged viruses merit special consideration. Slight differences in RFLP pattern can underlie considerable differences in antigenic relationship between a characterized virus and a newly emerged virus. Laboratories running these tests should maintain open communication regarding standardization of procedures and identification of trends in emerging isolates. These trends can, in turn, provide new leads in revealing other influences on IB, such as the role of immunosuppression (3). The practical advantages of RT-PCR for IBV are a reduction in the number of SPF embryonated eggs for serial passage in isolation efforts, and the rapidity with which results can be obtained. In our laboratory, we typically passage once in eggs, harvesting at 72 hours PI, and proceed directly to the molecular detection and characterization of the virus. This basic approach has yielded 50% or greater annual isolation rate of IBV from clinical specimens. The future of IBV diagnostics will likely see more laboratories able to sequence portions of the genome of new viruses, as well as applications for quantitative PCR, and possibly high-throughput PCR or DNA chip technologies to profile multiple viruses within a population of chickens.

Avian leukosis virus subgroup J (ALVJ). This PCR procedure (8) identifies and amplifies segments of ALJV proviral DNA with host cells. Despite the issues surrounding interpretation of this test, it has a place in the diagnostic laboratory. The exacting culture requirements for isolation of these viruses have pushed PCR to the forefront. Used as a sole diagnostic test for ALVJ infection and/or disease, it is easily interpreted inappropriately. While the test generally gives clear positive or negative results, those results and their implications are less clearly understood. ALVJ frequently mutates which means that primer sequences may not detect emerging viruses and thus produce false negative results. False positive reactions have been attributed to endogenous avian leukosis viruses. Sampling error, specimen contamination at necropsy, and dilution of positive tissues from pooled specimens are additional concerns. The results should be interpreted in consideration of the age at infection, whether the infection occurred vertically, congenitally, or horizontally, and pathological expression of infection in the flock, such as tumors. In practice, PCR testing for ALVJ can provide critical information about an elite breeder flock in which individual birds have data records of serology and virus isolation. PCR identification of ALVJ provirus within a specific tumor can provide definitive information for a pathologist. PCR testing as a sole criterion for ALVJ status in a flock can prove quite confusing for a breeder manager trying to improve fertility and hatchability in the presence of nematode parasitism, chronic staphylococcal arthritis, and the constraints of least-cost breeder rations.

Chicken infectious anemia (CIA). The PCR that identifies this virus (9) confirmed that infection is common in broilers in Alabama, but we are still learning about the significance of the infection. While most PCR tests have been performed using fresh spleen, bursa or thymus, we have also used PCR to examine DNA extracted from tissues processed for histopathology. PCR detected CIAV DNA *in situ* using histologic sections, and in pieces of tissues removed from paraffin blocks. This same CIAV DNA has been analyzed by DNA sequencing of the hypervariable region(s) of the CIAV genome that were specifically amplified by additional, special primers. These studies were not intended to displace traditional virology, but they have provided information that was helpful in obtaining a level of understanding about disease expression that would not have been possible for us without the molecular approach (3).

Other avian pathogens. We use a PCR for laryngotracheitis (LT) virus (1) to confirm virus isolation results. In addition, intralesional LTV DNA has been detected by PCR in paraffin-embedded tracheas with characteristic histologic lesions of LT. The DNA is extracted from tissues with lesions that are removed from the melted paraffin block. All psittacines and some other companion birds submitted for necropsy are routinely tested for psittacine polyoma virus and mycoplasma infections using PCR. The polyoma virus PCR serves as an adjunct to histopathologic identification of polyomavirus inclusion bodies. Mycoplasma infections in psittacines and other birds have proven to be rather common; most are mycoplasmas other than the poultry pathogens. It would be advantageous to add Chlamydia and psittacine beak and feather syndrome (PBFS) virus to the companion bird panel. The PBFS is interesting in that we would have to design our own primers or probes for this virus as patent and licensing issues have limited the availability of the test. PCR has been useful as an adjunct test in confirming EEE in emus. The EEE PCR is used to identify isolated viruses, and to directly test hemorrhagic gut content for the causative virus.

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PRACTICAL APPLICATION OF MOLECULAR DIAGNOSTIC TECHNIQUES IN COMMERCIAL POULTRY

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The molecularly based tools commonly used in commercial poultry include PCR, RT/PCR/RFLP, molecular based AG capture, and molecular based antibody tests. These tests are in various stages of automation and some are run "in house" while others are done in appropriate "outside" laboratories.

We use these types of tests for a variety of reasons including enhanced or quicker diagnosis, epidemiologic investigations of disease or microbial presence, vaccine application or success studies, and eradication/reduction programs. Molecular techniques have been embraced for these applications primarily due to the opportunity to speed up and automate a process that was done before but was slow and tedious.

Molecular techniques used in diagnosis have been particularly useful for mycoplasma. PCR for MG and MS have been used for a quick presumptive diagnosis. This allows you (or directs you) to hold a flock until the status is further delineated using more conventional or traditional techniques. It is used commonly in both suspect flocks and testing programs, focused on spiking males into a low fertility flock.

Molecular techniques have been very useful in epidemiological studies. They have been commonly and widely used when looking at the virus field "changes" occurring with IBD and IB. In our experience with IBD, the tests allow us to follow the prevalence, or changes over time, of a particular molecular group. With this information we can ask the question, "Does the molecular shift coincide with changes in field pathogenesis and/or field performance?"

It has been useful in vaccine application studies to use molecular techniques to determine the success of a particular treatment group in field vaccinated and field raised birds. We know the molecular pattern of a vaccine prior to application and then go look for this molecular pattern post treatment. It helps you fine tune your vaccination program in the field situation.

Molecular techniques have played a very important role in eradication/reduction programs aimed at avian leukosis virus, salmonella, and campylobacter (in the near future) to name a few. PCR has allowed us to differentiate J-virus from A-virus and adjust our practices as appropriate. Using PCR for salmonella in cecal material has greatly reduced the work load by acting as a front end "screening" test that then allows you to pursue particular positive samples utilizing more labor intensive and less automated methods. This also has allowed us to follow some molecular patterns epidemiologically. Campylobacter applications have the same opportunities.

We are using molecular techniques every day in commercial poultry. We approach them enthusiastically because of the potential for automation and speed. We approach them cautiously because of our insecurity concerning their significance. Even though molecular techniques are relatively new, we have many examples documenting that not all molecular changes are significant changes.