MOLECULAR DETECTION AND CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE AND CHICKEN ANEMIA VIRUS FROM COMMERCIAL BROILERS

by

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Under the direction of Pedro Villegas

ABSTRACT

Flinders Technology Associates filter paper (FTA® card) was compared to phenol as a inactivation method for molecular detection and characterization of Infectious Bursal disease (IBD) using RT-PCR and nucleotide sequencing. After RT-PCR and nucleotide sequencing, the FTA® card proved to be a reliable alternative to phenol for identification of IBDV. Chicken anemia virus (CAV) and IBDV are two of the most common immunosuppressive viruses associated with gangrenous dermatitis. Broiler farms with reoccurring gangrenous dermatitis were compared to farms identified as not experiencing gangrenous dermatitis. Molecular analysis of IBDV and CAV revealed that molecular changes are taking place. The isolates from a specific farm identified as MM were chosen for further characterization in vivo. Specific pathogen free and broiler birds inoculated with the MM isolates showed lymphocyte depletion indicating immunosuppression, which was seen in both groups of birds, indicating that these isolates could cause immunosuppression in the presence of maternal antibodies.

Index words: Infectious Bursal disease, Chicken anemia virus, RT-PCR, Sequencing, Immunosuppression, FTA® card
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By

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DEDICATION

This Thesis is dedicated to my husband who has always encouraged me to achieve my dreams and has supported me these past 3 years while pursuing this degree. To my parents who have always encouraged me to achieve higher things, and most of all to my Lord Jesus Christ who has blessed me beyond compare and has directed my steps to where I am today.
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CHAPTER I

INTRODUCTION

Purpose of this study

Infectious bursal disease (IBD) is a highly contagious disease of young chickens. IBD is distributed worldwide and the effects have economic significance to the poultry industry. The disease may be fatal when highly virulent strains infect poultry flocks, however, the disease can also be subclinical and lead to differing degrees of immunosuppression. The later situation is what is typically found in the United States and the results are often times bird losses due to secondary infections by a weakened immune system. Chicken anemia virus (CAV) is another common cause of immunosuppression in birds less than three weeks of age. These viruses combined can lead to a severe immunosuppression and even greater economic losses in terms of mortality and compromised bird performance.

Molecular characterization of IBDV has evolved greatly over the last decade, with the advent of RT-PCR and nucleotide sequencing availability. For almost a decade, phenol inactivation of IBDV from field samples has been the standard for transporting viruses for molecular characterization. The ability to use the Flinders Technology Associates filter paper (FTA® card) for inactivation of several avian pathogens has been proven previously; however, no work has been published on its use in IBDV nucleic acid detection. One of the objectives of this study was to use the FTA® card as an alternative to phenol inactivation for the collection of IBDV from bursal tissue.

Immunosuppression and gangrenous dermatitis have been increasingly reported in poultry flocks over the last few years. A survey of farms with repeated immunosuppression
problems and farms not experiencing problems was conducted in order to determine if there were any differences in the viruses at the molecular level. After molecular analysis was performed, viruses were selected based on differences noted and *in vivo* studies were performed in both specific pathogen free (SPF) and broiler birds to evaluate the pathogenicity of those viruses.

Results from these studies are expected to help contribute to the understanding of the current IBDV and CAV field situation and to improve diagnostic techniques for molecularly identifying IBDV with the usage of the FTA® cards for transport of nucleic acid.
CHAPTER II
LITERATURE REVIEW

Immunosuppression in chickens from infectious bursal disease and chicken anemia viruses.

A. Immunosuppression

Immunosuppression in chickens is a serious health and economic problem for the commercial poultry industry. Immunosuppression has been recognized around the world as an economically important area of poultry health (14, 20). Immuno-compromized birds are often susceptible to infections that would otherwise not affect healthy birds (2). There are several viral diseases that can result in immunosuppression. Many of these viruses can lead to a mild form of immunosuppression. A number of different viruses can cause severe immunosuppression, which include but are not limited to, infectious bursal disease, chicken anemia virus, Marek’s disease virus and reovirus. Although these viruses are known to induce immunosuppression, the exact mechanism is still not fully understood. Current knowledge on infectious bursal disease and chicken anemia virus is summarized below as they are the main focus of this research.

B. Infectious Bursal Disease Virus

i) History of IBDV

Infectious Bursal Disease virus (IBDV) is the etiological agent of Gumboro Disease or infectious bursal disease (IBD). The disease was first described in 1957 by Cosgrove(7). Originally identified as “avian nephrosis”, the syndrome quickly became known as “Gumboro disease” (7). This virus is a highly contagious disease of young chickens characterized by lymphoid destruction that occurs in the bursa of Fabricius which commonly
produces a severe immunosuppression (46, 47). The clinical features of the disease include whitish or watery diarrhea, anorexia, depression, prostration, trembling and sometimes death. Upon necropsy, birds often exhibit dehydration, hemorrhages in the leg and thigh muscles, urate deposits in the kidneys and depending on the stage of the disease, an enlargement of the bursa (29, 36).

In earlier work, the disease was believed to be caused by a strain of infectious bronchitis virus (IBV) because of the presence of urates in the kidneys and the concurrent infection with IBV that was often found in the field (29). After several studies, Winterfield et al. (67, 68) succeeded in isolating the causative agent in embryonating chicken eggs and later Hitchner named the disease “infectious bursal disease” (17, 18).

In the early 1980’s, the broiler growing area in the Delmarva peninsula began experiencing a significant increase in mortality, however, the clinical syndrome appeared different than previously seen, often with respiratory signs. Bursal lesions ranged from moderate to severe, often resulting in death because of an *E. coli* infection. Using sentinel birds, Rosenberger et al. (48) isolated four IBD viruses identified as A, D, G, and E. These isolates differed from the standard IBDV strains in gross lesions, and by producing rapid bursal destruction with minimal inflammatory response that was typically seen. The available vaccines did not provide complete protection against these new isolates when challenged with the standard challenge strain (STC). These isolates were therefore designated as antigenic variants and new vaccines were developed, tested in challenge studies and proven effective against these variants. Currently, these field variant viruses can be found throughout the United States (3, 9, 14).
In the later 1980’s high mortality outbreaks of the disease were being reported in Europe by Chettle et al. and van den Berg et al. (6, 63). These outbreaks occurred where standard biosecurity and prophylactic measures had already been taken, indicating a drastic change in the field viruses (56). Although no antigenic drift changes were detected, these viruses were labeled very virulent IBDV (vvIBDV) strains because of the increase in mortality and virulence. These strains have now spread throughout Asia, Europe and Latin America (3, 41, 44).

ii) Etiology, viral genome and organization

IBDV is a member of the Birnaviridae family, which includes three genera: Aquabirnavirus, Avibirnavirus and Entomobirnavirus. Among the most noted features of this virus is the bisegmented nature of the genome, as well as the double-stranded RNA. IBDV particles are non-enveloped with a diameter of approximately 70 nm. The virus is icosahedral in shape with a geometric skew of T=13 (8, 15, 16, 50). There are two known serotypes of IBDV. Both serotypes affect both chickens and turkeys. Serotype 1 is the pathogenic form of IBDV in chickens and has several antigenic variations in the field (50). Serotype 2 is considered apathogenic for chickens and does not cause lesions. However, one recent report showed that it could be pathogenic in chicken embryos (1). The genome of IBDV is bisegmented, double-stranded RNA with two segments that can be detected by polyacrylamide gel electrophoresis (38). The two segments are designated segment A and segment B. The molecular weight of the two double stranded segments is $2.2 \times 10^6$ and $1.9 \times 10^6$ Da, respectively (37). The segment lengths are 3.2 kb and 2.8 kb, respectively. Segment A contains two partially overlapping open reading frames (ORF). The larger ORF encodes a polyprotein which, through auto processing, matures into VP2, VP3 and VP4 viral proteins.
The shorter overlapping ORF encodes the non-structural polypeptide known as VP5. VP2 is the structural protein that composes the external surface of the virus. VP2 contains a hypervariable antigenic region responsible for serotype specificity and for producing conformational dependant neutralizing antibodies (5, 10, 59). Because these neutralizing antibodies bind to this region of the virion, it is susceptible to mutations and antigenic shifts and is the basis for molecular determination for serotype I viruses. Most of the changes that occur in the VP2 are located between amino acid 203 and 350 (8). VP3 is also a structural protein and is only found on the inner surfaces of virus-like particles. Recent findings indicate that VP3 may play a key role in assembly as well (8). VP4 is a viral protease involved in protein processing and it has been suggested that VP4 also plays a role in activation of the VP1. VP5 was the final viral protein identified in IBDV. This protein plays a role in viral pathogenesis but is not essential for viral replication in vitro or in vivo (39, 40).

The smaller genome, segment B, encodes VP1, the 95 kDa RNA-dependant RNA polymerase (RdRp) responsible for the replication and synthesis of mRNA. VP1 shares numerous sequence features with other RNA polymerases from various origins (31, 66).

iii) Forms of IBDV

The classic form of IBDV was described in the early 1960’s. This form generally affects birds around 3 weeks of age and has an incubation period that ranges from 2 to 4 days. It is characterized by an acute onset of depression in the birds. Often times the birds are reluctant to move, have ruffled feathers, and a decreased feed intake. Severely affected birds become so depressed they become dehydrated and die (7, 11, 33, 48).

The immunosuppressive or sub-clinical form is caused by low-pathogenicity forms of IBDV and is commonly described and seen throughout the United States (3, 4, 51, 54). These
strains are often labeled “variant” strains, such as the Delaware variants or the GLS strains (54). These viruses resist neutralization with antibodies developed against the classic forms of the disease and show molecular characteristics that are different from the classic forms. Although these strains are not considered to be highly pathogenic, they can cause severe immunosuppression which leads to vaccination failures and predisposition to opportunistic secondary infections (47).

The very virulent form of IBDV was first described in Europe and has since spread to almost every continent in the world. This viral form is caused by hypervirulent strains of IBDV and is characterized by an acute progression of the classic form leading to extremely high mortality rates on affected farms. There have been several publications on the molecular basis of vvIBDV, showing that they are distinctly different molecularly and antigenically from both the classic and the immunosuppressive forms of the disease (6, 41, 63).

iv.) Diagnosis of IBDV

Over the past 20 years, diagnostic tools have drastically improved making diagnosing of IBDV much easier than in the early years of the disease. Gross lesions and clinical diagnosis is based on the signs of lesions in the bursa of Fabricius, the target organ for IBDV. The gross lesions range from moderate to severe depending on the pathogenicity of the virus.

Histological diagnosis is commonly used because the lesion in the bursa is very characteristic of the disease and has been well characterized. This type of diagnosis is especially useful in determining the pathogenicity of the virus, as well as staging the time of infection. Because the virus depletes B cells present in the bursa, histopatological scores are commonly given to bursa according to the depletion that is observed. This scoring system ranges from 1 to 4, with 1 being a normal bursa and 4 being a totally lymphocyte depleted bursa (53). However,
there are other agents that can cause lymphocytic depletion, so a histopathological evaluation can not be the sole determinant for IBDV.

Serological diagnosis is a commonly used tool in diagnosing IBDV as well as monitoring maternal antibodies for IBDV. Current tests include serum-virus neutralization tests and the enzyme-linked immunoabsorbant assay (ELISA). ELISA is more widely used because it is commercially available, rapid and sensitive (34, 50).

Virus isolation was one of the first tools used for diagnosis of IBDV (50). The virus can be isolated and propagated in embryonated chicken eggs, cell culture or in susceptible birds. Propagation in susceptible birds is preferred to the other methods because other methods may cause mutations to the original virus (50).

Viral antigens may be detected as a mean of confirming the presence of IBDV. There are several different methods of detection, including direct or immunoflourescence, immunohistochemistry, agar gel immunodiffusion and antigen-capture ELISA (AC-ELISA) (13, 19). Monoclonal antibodies against IBDV were used for many years as a way to differentiate between strains identifying specific epitopes on the virus capsid (55, 56, 64).

Over the last few years, molecular techniques have dramatically improved the way IBDV is diagnosed (3, 4, 62). The reverse transcriptase polymerase chain reaction (RT-PCR) allows for the detection of viral RNA from infected clinical samples (28, 30). Fresh bursa tissue can be submitted directly or bursas can be inactivated by phenol or FTA cards for molecular analysis (21-23, 42). After confirmation of the presence of IBDV by RT-PCR, further differentiation of the strains is possible with the use of restriction enzymes that can used in a restriction fragment length polymorphism assay (RFLP) (24, 25, 43). DNA probes have also been used for determination of specific strains of IBDV. Most recently, nucleotide sequence analysis has been
made readily available to most diagnostic laboratories. Many studies of the deduced amino acid sequences from the hypervariable region of VP2, has allowed for quicker diagnosis of IBDV strains than with previous diagnostic techniques (26).

By analyzing the deduced amino acid sequence of the VP2 region, IBDV can be differentiated into classical, variant or vvIBDV forms of the virus. This is a huge advantage for the poultry industry, because they can obtain answers and results much faster than before when the virus had to be grown and propagated in vivo and additional assays conducted to characterize the virus.

C. Chicken Anemia Virus

i) History

The chicken anemia virus (CAV) was first described in 1979 in commercial chickens by Yuasa et al. (69, 70). Since then, CAV has been detected and isolated in practically every country worldwide in both laying and broiler birds. The first case of CAV isolation in the United States was reported in 1989 by Rosenberger and Cloud (45), however, recent reports have shown that there is serological evidence of the presence of CAV for at least 25 years (49, 61).

CAV can be transmitted horizontally and vertically, but when transmitted vertically, the virus can result in a severe immunodeficiency in the progeny. The disease is further exacerbated if it is co-infected with infectious bursal disease, which may cause a profound immunosuppression that can lead to further complications such as gangrenous dermatitis (12). Subclinical infections of commercial broilers may result in increased mortality and condemnations.

ii) Etiology
Chicken Anemia Virus belongs to the *Circoviridae* family and is the only member of the new genus, *Gyrovirus*. It is a small non-enveloped virus that is resistant to thermal inactivation, lipid solvents and many of the commonly used disinfectants. The genome is a circular single-stranded DNA, covalently linked and consists of approximately 2.3 kbs. The virus is icosahedral and approximately 25 nm in diameter (32). There is only one recognized serotype of CAV at this time; however, there have been reports for several years of different pathogenicities between CAV strains, however; they are all still recognized as one serotype (57, 58).

**iii) Viral Genome and organization**

The viral genome has three ORFs that encode for VP1, VP2, and VP3. The VP1 is the major capsid protein and shares some sequence similarities with the histone-binding proteins. It is believed that the VP2 aids VP1 in the virion encapsidation. VP2 may also play a role in intracellular signaling during viral replication. VP2 causes apoptosis in infected and non-infected cells. The VP3 protein, also called Apoptin, is associated only with infected cells and is a strong apoptosis inducer, which is associated with lymphoid depletion (27). The role of both viral proteins is still under research.

**iv) Disease**

Thymic atrophy and congestion are common symptoms and considered diagnostic when associated with other typical signs of CAV. The clinical disease of CAV causes aplastic bone marrow with a reduction in hematocrit values. Chickens are often anorexic, depressed and can have bone marrow that is pale or yellow in color. All of these lesions are exacerbated when chickens are co-infected with IBDV or other lymphocidal agents. Severely affected birds die
within 2 to 4 weeks of age and survivors are often stunted and perform poorly. Often, losses are the result of gangrenous dermatitis or other secondary infections (49).

v) Diagnosis and Laboratory tests

The virus can be propagated in a lymphoblastoid cell line derived from Marek’s disease tumor such as MDCC-MSB1 or MDCC-CU147 or passed in susceptible chickens (35). Antibodies to CAV can be detected using indirect immunofluorescence in MDCC-MSB1 cells or by the use of a commercially available ELISA kit. Commercial ELISA kits are also available to detect antibodies for flock screening (35, 60). If viral isolation is not needed, the use of PCR and nucleotide sequence analysis has also been used to identify CAV (65).

vi) Prevention and Control

Chicken anemia virus is highly resistant to inactivation and is easily transmitted to other birds, the virus is generally considered to be ubiquitous in both egg and meat-type chickens worldwide. Based on some serological evidence, most breeder or layer flocks produced in the U.S. are infected before 12 weeks of age; however, it is not uncommon to find serologically negative pullets that will become positive just prior to the onset of lay. Acquired immunity is the best method to prevent vertical transmission. It is well documented that immunosuppressive factors contribute to the enhanced susceptibility to CAV. One day of age infections with other immunosuppressive viruses increase the susceptibility to CAV by up to 100 fold (52).

There have been reports of specific pathogen free (SPF) flocks with a high percentage of antibodies against CAV. Based on this finding, it became more evident that producing CAV free flocks was nearly impossible (35). Although it is not feasible to totally prevent exposure to CAV, proper sanitation and control of other immunosuppressive diseases are important measures to
consider in controlling CAV. It is also important to control IBDV infection for optimal CAV vaccine-induced protection. It is believed that hyper immune breeders vaccinated with inactivated CAV vaccines can result in enhanced progeny performance. This improvement in performance may be a result of longer and more consistent maternal antibody-mediated protection during the early ages of susceptibility.
REFERENCES


CHAPTER III

EVALUATION OF FTA AND PHENOL FOR STORAGE, EXTRACTION AND
MOLECULAR CHARACTERIZATION OF INFECTIOUS BURSAL DISEASE VIRUS

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^{1}\text{ Reprinted from the Journal of Virological Methods, 138, L.B.Purvis, P.Villegas, and F.Perozo. Evaluation of FTA and Phenol for storage, extraction and molecular characterization of infectious bursal disease virus., pages 66-69. Copyright (2006), with permission from Elsevier.} \]
Abstract:
Infectious bursal disease virus (IBDV) is an important poultry pathogen and is distributed worldwide that can cause immune suppression and lesions of the bursa of Fabricius. The main component of the virus, VP2, is not only responsible for the bird’s immune response, but is important for the molecular identification of this virus as well. The nucleic acid of the virus must be adequately preserved to be analyzed by reverse-transcriptase PCR (RT-PCR) and sequenced for the molecular characterization of the field strain. Phenol inactivation has been the standard for IBDV tissue collection and international shipment; however, there have been some reports of interference with molecular detection capabilities when using phenol. Phenol is also a hazardous chemical and must be handled and shipped carefully. The ability to use the Flinders Technology Associates filter paper (FTA® card) for inactivation of several avian pathogens has been proven previously, however no work has been published on its use in IBDV nucleic acid detection. Bursas from experimentally infected birds was imprinted on FTA® cards, and then placed in phenol. Samples were evaluated and compared based on molecular detection capabilities between the two inactivation methods. The nucleic acid of the virus was detected in 85% of the FTA® card inactivated samples compared to 71% in the phenol inactivated samples. Sequence analysis was performed on samples inactivated by both methods and no differences were found. When comparing the RNA stability at different temperatures, euthanized IBDV infected birds were held at two different temperatures before sampling. No differences were detected for FTA® sampling; however, for tissues in phenol the nucleic acid was only detectable up to 2 hours post mortem in the tissues held at 4 C prior to sampling. These findings indicate that the FTA® card is an efficient and reliable alternative collection method for molecular detection and characterization of IBDV.
1. Introduction:

Infectious bursal disease (IBD) is an acute, highly contagious disease of young chickens generally characterized by severe bursal lesions and immunosuppression. Infectious bursal disease virus (IBDV) is a member of the Birnaviridae family in the genus Avibirnavirus. The genome consists of segmented, double stranded RNA (dsRNA). The virus is non-enveloped and icosahedral in shape with a diameter of approximately 55-60 nm. There are currently two recognized serotypes of IBDV, designated as 1 and 2, however, only serotype 1 induces disease in chickens (Rosenberger et al., 1998). IBDV identification and molecular characterization is commonly done by reverse-transcriptase polymerase chain reaction (RT-PCR) followed by either restriction fragment length polymorphism (RFLP) or sequencing a portion the VP2 hypervariable region (Jackwood et al., 1996, Banda et al., 2001). These molecular techniques are not available in all labs across outside the United States; therefore, international shipping of inactivated viruses for molecular identification has become popular for countries lacking this technology.

The United States requires that all infectious or hazardous agents shipped internationally be inactivated by a chemical such as formalin or phenol which is becoming more difficult to ship, as the Department of Transportation and International Civil Aviation Organization continue to enforce strict regulations (Snyder et al., 2002). Phenol has been proven to inactivate viruses while maintaining the nucleic acid for RNA amplification using RT-PCR (Banda et al., 2001, Jackwood et al., 1996). Although the use of phenol has been the standard for inactivating viruses and molecular characterization, there have been problems reported with chemically inactivated samples interfering with molecular reactions (Coombs et al., 1999, Masuda et al., 1999). An alternative way of transporting
inactivated microorganisms is represented by the Flinders Technology Associates filter paper (FTA® card) that is a chemically treated filter paper designed for the collection and room temperature storage of biological samples for molecular analysis (Moscoso et al., 2004; Smith et al., 2004). Several recent publications report the use of FTA® cards as an convenient, adequate, reliable and safe method for storing, transporting and extracting nucleic acids from inactivated infectious organisms, with no interference in molecular reactions (Moscoso et al., 2004; Moscoso et al., 2005; Ndunguru et al., 2005, Perozo et al., 2006). These reports have also shown the cards ability to completely inactivate various infectious organisms.

Fast and reliable detection of IBDV is important not only in diagnostic situations, but also for research purposes. New techniques and protocols should be constantly evaluated in order to insure that the most reliable and adequate procedures are being used. The objective of this study was to compare two methods of virus inactivation (FTA® cards vs. phenol) for the molecular detection of IBDV using standard extraction methods. To determine if time post mortem had an effect on the use of FTA® or phenol inactivation to detect IBDV, dead birds were kept at different temperatures and sampled at different times intervals and evaluated.

2. Materials and Methods

2.1 Experimental design: Phenol vs. FTA

To obtain fresh tissue samples from infected birds, seven 3 week old specific pathogen free (SPF) chickens (SPAFAS, inc.) were inoculated orally with 0.1 ml of the Edgar strain of IBDV with a titer of $10^3$ CID$_{50}$/ml (chicken infectious dose). The titer was calculated using the Reed and Muench method as previously described (Villegas et al.
1999). Three SPF chickens remained non-inoculated as a negative control. Birds were housed in Horsfall isolation units with feed and water provided *ad libitum*.

2.2 Sample collection:

At one week post inoculation, experimentally infected birds were humanely euthanized by cervical dislocation and bursas collected. The bursa tissue was cut open and imprinted on FTA® cards, as described elsewhere (Higgins, *et al.* 2000). The bursa was then cut in half where one half of the bursa was immersed in phenol and the other was frozen at -80°C until processing.

2.3. Experimental Design: Effects of storage on RNA detection

To obtain infected tissue samples, fourteen, 3 week old specific pathogen free (SPF) chickens were orally inoculated with $10^3$ CID$_{50}$ of the Edgar strain of IBDV, as described above. Three SPF chickens remained non-inoculated as negative control. Birds were housed in Horsfall isolation units and given feed and water provided *ad libitum*.

2.4 Sample collection:

At one week post inoculation, experimentally infected birds were humanely euthanized as described above. For the two different storage temperature groups (4°C and room temperature), seven birds were immediately placed in a 4°C refrigerator and the remaining seven birds were held at room temperature (approximately 27°C). At 0, 1, 2, 4, 6, 8, and 24 hours post euthanasia, the bursa tissue was collected and imprinted on FTA® cards and then placed in phenol for inactivation.

2.5 Frozen Tissue:

Frozen fresh bursa tissues were thawed at room temperature, and approximately 25 mg of tissue was minced and digested in 200 µl of 2% sodium dodecyl sulfate (SDS) with
10 µl of Proteinase K (10mg/ml) at 56 C for 30 minutes. After digestion, samples were centrifuged for 10 minutes at 10,000 g and the supernatant was used for further extraction with the High Pure RNA isolation Kit (Roche Applied Science, Indianapolis, IN) as previously described (Perozo et al., 2006).

2.6 Phenolized tissue:

The phenol inactivated bursa samples were washed 3 times with phosphate buffer saline (PBS) with a pH of 7.2 to remove excess traces of phenol prior to extraction. After washing, the phenol and fresh tissues were processed as described above. A known positive RNA internal control was included. This RNA was re-extracted using the same procedure as the phenolized tissue.

2.7 FTA® Card imprints:

Two, 2 millimeter punches were cut by a sterile hole puncher (Harris Micro-Punch), placed in 200 µl of FTA® purification reagent (Whatman International, Ltd. UK) and placed on ice for 20 minutes, vortexing every 5 minutes. After elution of the FTA® punches, the sample was processed as described above.

2.8 Reverse Transcriptase (RT) polymerase chain reaction (PCR):

The presence of IBDV nucleic acid in the samples was detected by the amplification of a 698-bp portion of the VP2 gene containing the hypervariable region (between nucleotides 567 and 1264 from the start codon) using the following set of primers: 5'-TCTGCAACAGCCAACATCAACG-3 as forward primer and 5'-TCAGGATTTGGGATCAGCTCGA-3 as reverse primer (Banda et al., 2004). RT amplification of the 698-bp portion of the VP2 gene was performed using the SuperScript
III one step- reverse transcriptase PCR kit (Invitrogen, Carlsbad, CA) following manufacturer’s recommendations. The PCR included 40 cycles consisting of denaturing at 94 C for 30 sec, annealing at 50 C for 30 sec and an extension at 72 C for 1 min, followed by a final extension at 72 C for 10 min.

2.9 Molecular characterization by sequencing:
Positive IBDV amplicons were gel purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) following manufacturer’s recommendations. The purified DNA was submitted for sequence analysis to determine if any changes were made due to the inactivation methods. Sequencing reactions were performed with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as recommended by the manufacturer and were run in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were analyzed with the aid of the DNAstar software (DNAstar, Inc., Madison, WI) and aligned using Cluster W algorithm.

3. Results
3.1 Molecular Detection: Phenol vs. FTA cards
All fresh tissue samples were positive for IBDV by RT-PCR, indicating that all birds were infected. In the phenol inactivated samples, four out of seven inoculated birds were detected by RT-PCR. Samples inactivated by the FTA® cards showed six out of seven positive (figure 3.1). The positive and negative controls worked appropriately, including the internal phenol control, which was positive, showing that the extraction method was done adequately.
3.2 Molecular characterization: Effects of storage temperature on RNA detection:
For the FTA® inactivated samples, all were positive up to 24 hours post mortem, regardless of which temperature the dead birds were held at prior to sample collection. The samples inactivated by phenol were positive only up to 2 hours when the birds were held at 4°C prior to tissue inactivation; however, no positive samples were obtained from the tissues stored at room temperature (figure 3.2). The positive, negative and internal phenol controls worked appropriately, showing that the extraction method was done adequately. In Figure 2B, the bright band below the expected amplicon size for IBDV should not be confused with a positive result. It is often seen when samples are extracted from phenol. The expected amplicon size is noted with an arrow in the figure.

3.3 Sequence analysis:
The sequence analysis from the nucleic acid analyzed from the samples showed a 100% homology between both inactivation methods (FTA® and phenol) and there was a 99% homology between the inactivated samples, the fresh tissue samples and the Edgar strain which was used to challenge in this experiment. There were no sequence changes between the two inactivation methods.

4. Discussion:
The use of FTA® cards was investigated to characterize the VP2 region of IBDV and compared with traditional phenol inactivation method using standard extractions procedures for both methods. Due to the chemical make up of phenol, extra steps must be taken during extraction in an effort to remove the traces of phenol from the tissue to decrease PCR inhibition reactions from occurring leading to a lack of detection. This can lead to added processing time and other work has shown that this can be decreased by
using FTA® cards (Moscoso et al., 2004, Moscoso et al., 2005, Perozo et al., 2006). Samples inactivated by the FTA® card require no extra removal steps and can be applied directly to the extraction procedure, making the process faster, easier and safer.

Samples inactivated by the FTA® card have been used to identify a variety of other avian pathogens such as, Mycoplasma, infectious bronchitis and Newcastle disease (Moscoso et al. 2004, Moscoso et al. 2005, Perozo et al., 2006). In this study, FTA® cards positively detected four out of seven samples, while the samples kept in phenol detected four out of seven samples.

Also, all the FTA® card samples were all positive regardless of the storage temperature the bursa tissues were held at post mortem (either 4 C or room temperature). The phenol inactivated samples, however, were positive up to 2 hours post mortem from birds stored at 4 C, but not from birds stored at room temperature. Previous researchers have reported a molecular inhibition caused by using chemical inactivation methods, which could be an explanation for a lower percentage of positive results from the phenol inactivated samples in our experiment. (Coombs et al., 1999, Masuda et al. 1999). Perhaps the phenol preserved samples could have been further washed several times in an effort to remove excess phenol; and therefore obtain a higher number of positive samples.

Although the results indicate that FTA® cards can adequately identify IBDV from deceased birds held at room temperature, it should be noted that the deceased birds in this study were not around other live birds that can speed up or aid in the decomposition of tissue as it happens under field conditions. Therefore, it is recommended that fresh samples be collected for molecular characterization, regardless of the inactivation method used.
As reported before, there were no differences between the sequences from samples inactivated by either method, showing that neither inactivation method has a negative effect on molecular identification (Perozo et al. 2006).

It should be noted that although a small number of birds were used for these experiments; the objectives were only to compare the two inactivation methods with standard extraction methods; therefore, no statistical analysis was intended. These birds were inoculated with live virus in order to closely mimic field infections for this study. For molecular identification of infectious bursal disease virus, the FTA® card is a comparable alternative to traditional phenol. Identification of IBDV is critical for controlling field outbreaks, and by utilizing new and advanced inactivation and storage methods such as FTA® cards, the process of identifying IBDV strains can be faster and easier. However, phenol is still a good alternative for those unable to obtain FTA® cards.

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A.

Figure 3.1:

MM= Molecular Marker. 1-7 are the tissue samples and are the same in all figures. Positive (+) and Negative (-) controls are included.

1A. RT- PCR positive IBDV amplicons from fresh bursa tissue.

2B. RT- PCR positive IBDV amplicons from phenol inactivated samples.

2C. RT- PCR positive IBDV amplicons from FTA inactivated bursa imprints.
Figure 3.2.

MM= Molecular Marker. Numbers across the top represent hours post mortem. Positive (+) and Negative (-) samples included. Tissue samples are in the same order in all figures.

2A: RT-PCR electrophoresis of bursas from chickens stored at two different temperatures then imprinted on FTA cards at various time intervals.

2B: RT-PCR electrophoresis of bursas from chickens stored at two different temperatures, then inactivated in phenol, sampled at various time intervals.
References:


Masuda, N., Ohnishi, T., Kawamoto, S., Monden, M. & Okubo K. (1999). Analysis of chemical modification of RNA from formalin-fixed samples and optimization of
molecular biology applications for such samples. Nucleic Acids Research, 27, 4436-4443.


CHAPTER IV

*IN VITRO AND IN VIVO STUDIES ON INFECTIOUS BURSAL DISEASE AND CHICKEN ANEMIA VIRUS FIELD ISOLATES*¹

¹ L.B.Purvis, P.Villegas, and F. Perozo. To be submitted to Avian Diseases
Abstract:

Over the last decade, the broiler industry in the Southeastern United States has reported an increasing number of outbreaks of gangrenous dermatitis, which has been closely associated with immunosuppressive diseases such as Infectious bursal disease (IBD) and chicken anemia virus (CAV). The current increase in immunosuppression seen in the field could be the result of antigenic changes in these viruses. These changes may lead to an increase in the susceptibility or pathogenicity to secondary infections which can be presented in the form of gangrenous dermatitis. Because of the immunosuppression, the occurrence of secondary opportunistic infections, and the impairment of immune response to vaccinations, it is not uncommon to see an increase in economic losses. Since gangrenous dermatitis is a known secondary infection of immunosuppressed birds, this determinant was chosen as the indicating factor to evaluate the effect of current immunosuppressive field viruses from farms that have been documented to have reoccurring problems with gangrenous dermatitis and those that appear to be healthy and unaffected. The main purpose of this study was to characterize IBDV and CAV from farms not having gangrenous dermatitis (normal farms) and from farms where gangrenous dermatitis was a recurrent problem (problem farms). One particular farm (MM farm) showed molecular changes for both IBDV and CAV and was therefore chosen to further characterize by in vitro bird studies. The MM isolates (IBDV and CAV) showed to be very immunogenic in both SPF and broiler birds, and was able to break through maternal antibodies to cause immunosuppression as shown by bursal: body ratio and histopathology bursa scores.
Introduction:

Over the last decade, the broiler industry in the Southeastern United States has reported an increasing number of outbreaks of gangrenous dermatitis, which has been closely associated with immunosuppressive diseases (30). Infectious bursal disease (IBD) and Chicken anemia virus (CAV) are the two most commonly viral diseases associated with immunosuppression in poultry (14, 26, 28, 29, 34, 37, 38). Infectious bursal disease virus (IBDV) is the etiological agent of Gumboro disease. IBDV is an *Avibirnavirus* member of the *Birnaviridae* family (21). IBD is a highly contagious disease of young chickens, characterized by destruction of the lymphocytic cells present in the bursa of Fabricius and thymus, producing a severe immunosuppression. The clinical features of IBDV includes diuresis associated with the production of whitish or watery urine followed by anorexia, depression, trembling, severe prostration, yellowish colored livers and immunosuppression leading to secondary infections and death (32). At necropsy, the birds exhibit dehydration, hemorrhages in the leg and thigh muscles, urate deposits in kidneys, and atrophy of the bursa of Fabricius and thymus (6). The bursa of Fabricius is the primary organ for viral replication following exposure to IBDV. Since this virus is extremely lymphocidal, significant lesions in the bursa of Fabricius and other lymphoid tissues are observed (5). Chicken anemia virus (CAV) is a *Gyrovirus* and belongs to the family *Circoviridae* (25). Chicken anemia virus is an acute disease that affects chicks less than 4 weeks of age. It is also commonly known as blue wing, infectious anemia, hemorrhagic syndrome or anemia dermatitis syndrome. This disease is generally characterized by anemia, lymphoid depletion of the thymus, intramuscular hemorrhages and increased mortality (33, 37). Mortality is variable, but can get high in severely affected flocks. This virus also provides opportunities for secondary infections as with IBDV (26, 28). Chicken anemia virus is an ubiquitous virus and has been identified throughout the
world (4, 20, 23). Despite vaccination programs, this virus still causes immunosuppression problems in the broiler industry. The virus has been isolated more frequently in cases where flocks were diagnosed with other diseases, such as coccidiosis, gangrenous dermatitis, and respiratory diseases (12, 26, 27).

Several reports in the past few years indicate that this immunosuppression problem is reemerging and is associated with different or unique IBDV strains (16, 19). In our lab, we have reported several different IBDV strains that show mutations in the variable region, in particular the 9109 strain that has both standard and vvIBDV mutations (2). Jackwood et al. hypothesized that some recently isolated unique IBDV viruses are able to infect birds despite the use of vaccination (17). In all the isolates characterized, several amino acid mutations among the hypervariable peak B were observed (22). The significance of these mutations and the impact they may have on the increase in gangrenous dermatitis has yet to be determined. However, any mutations in the epitope region of the virus could cause significant changes in the vaccination protection, which in turn would lead to an increase in immunosuppression and could predispose the infected birds to gangrenous dermatitis. The current increase in immunosuppression could be the result of antigenic changes in these viruses. These changes may lead to an increase in the susceptibility to secondary infections which can be presented in the form of gangrenous dermatitis. Because of the immunosuppression, the occurrence of secondary opportunistic infections, and the impairment of immune response to vaccinations, it is not uncommon to see an increase in economic losses. Combined with the cost of treatment, dead bird losses and estimated condemnations, immunosuppression resulting in gangrenous dermatitis is economically devastating to the broiler industry. The subclinical form of IBD may result in the presence of mild to severe respiratory reactions, which can result in death if complicated with bacteria or
other diseases that can rapidly multiply in immunosuppressed birds. Secondary infections, such as gangrenous dermatitis, tend to be the main cause of death in many subclinical IBD and CAV cases.

Since gangrenous dermatitis is a known secondary infection of immunosuppressed birds, this determinant was chosen as the indicating factor to analyze current immunosuppressive field viruses. Farms that have been documented to have reoccurring problems with gangrenous dermatitis were selected to be compared with those that appear to be healthy and unaffected. The main purpose of this study was to attempt to characterize IBDV and CAV from farms not having gangrenous dermatitis (normal farms) and from farms where gangrenous dermatitis was a recurrent problem (problem farms). Once these viruses were identified, molecular analysis was performed to determine if any genetic changes are taking place in the field that could have an impact on the increase in immunosuppression. Viruses that show significant deduced amino acid sequence differences were further characterized \textit{in vivo} to evaluate the relative level of immunosuppression that could lead to the reoccurrence of gangrenous dermatitis. Previous reports describe the emergence of new IBDV variants in the field that are different from the IBDV variants isolated in the 1980’s (18). Moreover, we have recently isolated field strains of IBDV from the state of Georgia that exhibit some variation in the amino acid sequence of the VP2 gene when compared to the variant strains isolated in 1985 (3). This finding further supports recently published reports (17) with the hypothesis that new IBDV strains are emerging in the field and could be playing a role in the increased incidence of gangrenous dermatitis secondary infections. The impact of these new variants on the increased immunosuppression and presence of gangrenous dermatitis should be studied. In order to determine this, IBDV and CAV field strains from farms experiencing these problems need to be surveyed and analyzed antigenically.
Recent CAV and IBDV publications indicate that some molecular changes are occurring, however the significance of these changes has not been investigated (8).

The objectives of this study were to:

1) Identify farms with recurrent gangrenous dermatitis (problem farms) and compare them with farms that had no history of gangrenous dermatitis (normal farms).

2) Molecularly evaluate CAV and IBDV strains present in these farms.

3) Further analyze selected IBDV and CAV strains in vivo.

Materials and Methods:

In Vitro studies:

Farm Tissue Sample Collection:

Five normal farms and five problem farms were identified by a local poultry integrator. Farms were visited when the birds were between 21-28 days of age. Bursa and thymus tissues were collected from all 10 farms and stored at -80 C after being processed for molecular characterization and virus isolation.

Molecular characterization:

a) CAV

Viral DNA was extracted from thymus tissue using a QIAamp® DNA mini kit (QIAGEN, Valencia, CA) according to manufacture’s instructions.

The presence of CAV was determined by the amplification of a 450 bp portion of the VP1 gene carried out in a 50 µl reaction containing 5x first strand buffer, 50 uM MgCl, Taq DNA polymerase I and 50 µM concentration of the following CAV primers.
CAV F 5’GGGGCAGTGAATCGGCGCTTAG 3’
CAV R 5’ GTGGTGCCACCGTCCTCTTCTG 3’
Amplification was carried out in 35 cycles of denaturing at 94 C for 30 seconds, annealing at 60 C for 45 seconds, and elongation at 72 C for one minute.
Electrophoresis was performed at 65 volts for 35 minutes and amplified products visualized with ethidium bromide under UV light on a 1.5% agarose gel.

b) IBDV:
Viral RNA was extracted from bursa tissues digested with 200 ul of proteinase K (10 mg/ml) for one hour at 56 C followed by extraction using a High Pure RNA isolation kit (Roche Applied Bioscience, Indianapolis, IN) according to manufacturer’s instructions.
The presence of IBDV was detected by RT-PCR amplification of a 450-bp portion of the VP2 gene containing the hypervariable region using the following set of primers (7):
GumF- ACAGGCCCAGAGTCTACA (nt 733-750)
GumR-AYCCTGTTGCCACTCTTTC (nt 1194-1212)
Superscript III one step RT-PCR kit was used for amplification following manufacturer’s instructions (Invitrogen, Carlsbald, CA). Amplification was conducted in one cycle of 94 C for four minutes, reverse transcriptase performed at 50 C for 45 minutes, followed by 40 cycles of denaturing at 94 C for 30 sec, annealing at 50 C for 30 sec and elongation at 72 C for one minute followed by a final elongation step of 72 C for five minutes. Electrophoresis was performed at 65 volts for 35 minutes and amplified products visualized with ethidium bromide under UV light on a 1.5% agarose gel.
Sequence analysis:

Amplified products were gel purified using QUIquick® gel extraction kit (Qiagen Valencia, CA) following manufacture’s recommendations. Purified DNA products were sequenced using the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, CA) as recommended by the manufacturer. Nucleotide and deduced amino acid sequences were compared with reference strains available at GenBank and aligned using the Cluster W method included in the DNAstar software program (DNAstar, Inc., Madison, WI).

Virus Isolation:

After molecular analysis, selected tissues were processed for virus isolation. Bursa and thymus tissues were minced in tryptose phosphate buffer (TPB) with antibiotics at 10% w/v. For IBDV, the bursa homogenate was passaged two times in 3 week SPF birds via cloacal inoculation of 0.1 ml. After 5 days, bursas were collected from infected birds, minced as described above and passaged a second time, after which the bursas were minced and stored for further titration. For CAV, thymus tissue was minced in TPB at 10% w/v. The thymus homogenate mixture was inoculated intramuscularly in one day old SPF birds. After 21 days, thymus tissue was collected, homogenized in TPB as previously described and passed in two day old MSB1 cells and observed daily for cytopathic effect (CPE). Once more than 50% CPE was reached, cells were collected and passaged a second time. After a second passage, cells were collected and stored at -80 C until titration could be performed.

In Vivo studies:

Experimental Design:

Virus Strains: After molecular analysis, the IBDV and CAV strains used in this study were chosen because they were isolated from a farm (MM farm) that was continually experiencing
gangrenous dermatitis and immunosuppression in several grow outs. These viruses also showed some molecular differences for both CAV and IBDV when compared to other farms surveyed and from known isolates (Edgar, variant A and E and vaccine strains for IBDV; Cux-1, Del-Ros, and SMSC -1 for CAV). These strains will be referred to as IBDV-MM and CAV-MM.

**Viral propagation, purification and preparation of MM strains:**

**CAV-MM:**

One day old SPF birds were inoculated intramuscularly (IM) with 0.2 ml of thymus homogenate from the tissue collected from the MM farm. At 21 days of age, thymuses were collected and homogenized, treated with RNAse to remove any traces of IBDV, minced in Tryptose Phosphate Broth (TPB) with antibiotics (10 % w/v) and inoculated in MSB1 cells as previously described (38). After two passages in MSB1 cells, the supernatant was harvested and titrated in MSB1 cells. Briefly, a 96 well plate was seeded with 100 ul of 30,000 cells per well and simultaneously inoculated with the CAV-MM isolate and serially diluted from $10^{-1}$ to $10^{-8}$. Cytopathic effect was observed between 5-8 days before the end point was calculated. The titer was calculated using the Proportional Distance (PD) formula. Briefly, the $PD = 100 - 50 \times \frac{\text{distance A}}{100 - \text{endpoint}} \times \frac{\text{distance B}}{100 - \text{endpoint}}$. (Dr. Ton Schat, Cornell University, Personal communication). The titered stock was stored at -80 C until needed.

**IBDV-MM:**

Three week old SPF birds were inoculated orally with 0.1 ml of the bursa homogenate from the MM farm. Five days post inoculation, bursas were collected and treated with DNAse to remove any possible CAV. Bursa were minced in TPB with antibiotics (10 % w/v). The MM strain was titrated in 3 week SPF birds and the titer calculated using the Reed and Muench method, as previously described (35). The titered stock was stored at -80 C until needed.
**Birds and Housing:** Day old broilers from a commercial poultry company were used for the commercial group and one day old SPF chickens (Merial Select, Gainesville, GA) were used for the SPF groups. Birds were housed in floor pens with wood shavings. The experimental groups are described in Table 4.1.

**Viruses and Challenge:**

The IBDV Edgar strain used in this study has been maintained in our laboratory at the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia. The CAV CL-1 strain was obtained from SPAFAS. Birds were challenged according to the outline in Table 4.1. Two IBDV strains, Edgar, and IBDV-MM strain were inoculated at $10^3 \text{CID}_{50}/0.1 \text{ml}$. Two CAV strains the CL-1 strain from SPAFAS and CAV-MM were inoculated at $10^4 \text{TCID}_{50}/0.1 \text{ml}$, and $10^{1.5} \text{CID}_{50}/0.1 \text{ml}$, respectively.

**Study parameters:** Birds were monitored and evaluated on the following parameters for 35 days. Serological titers for IBDV and CAV by ELISA, gross clinical signs, CAV- hematocrit values (pre and post challenge), histopathology, gross lesions at necropsy, and body weights.

All serum samples were tested using the commercially available ELISA kits. (IDEXX Westbrook, Maine). Hematocrit values were analyzed using a hematocrit reader. (International Equipment Company, Needham Heights, Mass).

**Statistical analysis:**

All statistical analysis was performed using the Sigma Stat 3.0 software. Dunn’s method and SNK test were performed at $P \leq 0.05$.

**Results:**

*In vitro* results:

Samples collected from the field:
As expected, by RT-PCR and PCR IBDV and CAV were identified in all problem and normal farms sampled. All bursa and thymus samples collected from the field at 21-28 days of age were processed for molecular analysis and nucleotide sequence analysis. For the IBDV deduced amino acid sequence analysis, two amino acid changes were noticed among several of the problem farms when compared to the Delaware E variant strain. At amino acid position 254 a serine (S) to an asparagine (N) switch was observed among four out of five problem farms, and three out of five normal farms. Another amino acid change was made from glutamic acid (E) to aspartic acid (D) in the hydrophilic peak B at position 323.

For CAV nucleotide sequence analysis, several changes were noticed in two of the problem farms that also had sequence changes for IBDV. As opposed to the viruses from the other farms, the MM farm had changes in both CAV and IBDV when compared to standard IBDV and CAV sequences, which influenced the decision to use the MM strain for \textit{in vivo} studies.

\textit{In vivo} results:

\textbf{Clinical signs:}

Birds were observed daily for clinical signs of disease. Both, SPF and broiler birds inoculated with the IBDV-MM strain, Edgar, CL-1 CAV strain or CAV-MM strain did not show detectable signs of disease.

\textbf{Hematocrit values for CAV:}

All packed cell volume results are shown in table 4.2 and described here briefly. Broiler birds had a statistically lower packed cell volume (PCV) at day one (20\%) as opposed to the SPF birds (30\%). Anemia values are generally considered to have PCV’s lower than 27. At 14 days of age, both the inoculated broilers and SPF birds were statistically lower than the negative control groups. At 28 days, the SPF group challenged with CAV-MM at 7 days was significantly lower
than those challenged with CL-1 strain. In the broiler group, there were statistical differences between all the groups with CAV-MM and IBDV-MM having the lowest PCV.

**Serology:**

All serological results are shown in table 4.3 (broilers) and 4.4 (SPF), and described here briefly.

a) Broilers:

At day of age, birds showed normal maternal antibody titers to CAV; however, maternal antibody titers to IBDV were considered low, since normally broilers in the USA have an average titer of 3,000 to 5,000 at day of age against IBDV. By day 14, the negative control group of broilers showed a natural decline in maternal antibody levels for both CAV and IBDV. The IBDV ELISA titers for the groups inoculated with CAV-MM at 7 and IBDV-MM at 14 days of age were statistically different from groups inoculated only with the CAV-MM strain. All antibody levels were low at 28 days of age for IBDV except the group inoculated with IBDV-MM at 7 and at 7 and 14 days of age. Although there were no statistical differences in the CAV antibody levels, the birds inoculated with the CAV-MM strain at 7 days, showed a higher antibody level than the other groups at 28 days of age. As expected, birds at 35 days of age showed higher titers in groups inoculated with CAV when compared with the negative controls. All groups, including those inoculated only with CAV had antibody levels to IBDV, indicating a possible cross-contamination between the CAV and IBDV groups.

b) SPF:

At 14 days of age, only the IBDV-MM inoculated at 7 days, CAV-MM and IBDV-MM inoculated at 7 days followed by IBDV-MM at 14 days, and the Edgar strain given at 7 days showed an antibody response considered positive. Birds at 28 days of age challenged with the IBDV-MM strain at 7 days showed a significantly higher antibody level than the negative control
birds. All groups inoculated, including those only inoculated with CAV showed an antibody response to IBDV, except Edgar inoculated at 14 days, which was lower than the other group. At 35 days, all groups had high antibody titers for IBDV, even the groups given only CAV.

**Gross Lesions:**

Upon necropsy, birds were examined for bursal, spleen and thymic atrophy as well as any other noticeable gross lesions. Results for bursa: body weight ratios are showed in figure 4.1 for all groups of birds.

**Broilers:** All birds inoculated with IBDV-MM strain showed smaller, atrophic bursas than the controls. A few birds from these groups also showed some slight signs of dermatitis and airsaculitis. Birds challenged with the CAV-MM strain at 7 days had similar lesions to those seen with the CAV CL-1 strain, including small thymus and bursas. However, the CAV-MM group inoculated at 7 days was the only group that was statistically different from the negative controls at 35 days of age by thymus: body weight ratio. Birds challenged with the CAV-MM strain followed by IBDV-MM strain at 14 days showed no differences in gross lesions. However, birds inoculated with the CAV-MM strain at 7 days followed by IBDV-MM strain at 7 and 14 days showed slightly smaller bursas, which may indicate greater immunosuppression. The bursa: body weight ratios for all groups were statistically different than the negative controls at 35 days (figure 4.1). Birds inoculated with IBDV-MM at 7 days were statistically different from those inoculated at 14 days, and the group inoculated at 7 and 14 days with IBDV-MM was statistically different than the IBDV-MM group only inoculated at 14 days. The group inoculated at 7 days with both CAV-MM and IBDV-MM followed by another IBDV-MM inoculation at 14 days was also statistically different from the group only inoculated at 14 days with IBDV-MM.

The only statistical difference found in the spleen: body weight ratios was between the IBDV
Edgar strain inoculated at 14 days and all of the IBDV-MM and CAV-MM combination groups as well as IBDV-MM given alone at either 7 or 14 days (data not shown).

**SPF:** All birds inoculated with IBDV-MM strain, in any combination showed small, atrophic bursas compared to the negative control group, and all groups were statistically different from the negative controls in bursa: body weight ratios (figure 4.1). The group inoculated with IBDV-MM and CAV-MM at 7 days followed by IBDV-MM again at 14 days was statistically different than the IBDV Edgar strain given at 14 days. This was the only statistical difference between the groups inoculated with either of the MM isolates. Consequently, birds inoculated with CAV-MM and IBDV-MM strain at 7 days showed no gross differences than the CAV-MM strain at 7 days and IBDV-MM strain at 7 and 14 days, however, in all groups, the thymus and bursa were smaller than the negative control birds.

**Histopathology:**

Bursa, spleen and thymus tissues were collected at 14 and 35 days and were evaluated histologically. Bursa collected at 35 days were scored as follows: 1 = normal to 10% follicular atrophy; 2 = 10-30% follicular atrophy; 3 = 30-70% follicular atrophy; 4 = >70% atrophy or any evidence of acute necrosis.

**Broiler results:** At 14 days, the IBDV-MM group inoculated at 7 days had severe lymphoid depletion and smaller follicle size than the negative control group.

At 35 days, this same group had bursas that averaged a bursal score of 3 with decreased amounts of lymphocytes and a decrease in follicle size. All of the other groups had a bursal score of 4. The thymus appeared mostly affected in the group inoculated with CAV-MM at 7 days followed by IBDV-MM at 7 and 14 days. In this group, the thymus, bursa and spleen all showed moderate to severe lymphoid depletion. No differences were observed in the spleen for the other groups.
**SPF results:** At 14 days, the groups inoculated with CAV-MM at 7 days showed severe thymic atrophy and lymphocyte depletion. The bursas from the group inoculated with IBDV-MM at 7 days showed smaller follicles than the negative controls, and mild heterophil infiltration in the interfollicular epithelium, which is consistent with immunosuppressive viruses. At 35 days, the IBDV-MM group inoculated at 7 days had bursal scores of 3 and 4, showing very small follicles with little to no lymphoid content. Thymus and spleens also had lymphoid depletion and an increase in germinal centers. For the groups inoculated with IBDV-MM at 7 and 14 days, bursas were also scored to be 3 and 4 with severe bursal and thymic lymphoid atrophy was observed. In all of the CAV-MM inoculated groups, the score for the thymus appeared to be within normal limits, however, the bursal score was 4 and the spleens showed some decreased amounts of lymphoid tissue indicating an infection was present. There appeared to be minor or no differences between the groups inoculated with CAV-MM at 7 days followed by one, or two inoculations of IBDV-MM at either 7 or 14 days on the bursal atrophy taking place.

**Body Weights:** Because there were no significant differences between any of the groups or replicates in body weights when compared to the negative control birds, data is not shown. However, the biggest weight gain difference was noted in the SPF group inoculated with IBDV-MM at 7 and 14 days, there was an 82 gram difference in weight gain from the negative controls at the end of 35 days. There were only minor differences noticed in the broiler birds between groups.

**Discussion:**

Immunosuppression has emerged in the last decade to be a major problem for broiler companies in the United States. Infectious bursal disease and chicken anemia viruses are known
immunosuppressive viruses (1, 4, 10, 13, 31). The infections these viruses can cause in the field can lead to further complications with secondary infectious agents such as staphylococcus, poor immune response to vaccination and gangrenous dermatitis (5, 11, 28, 29). Molecular changes in these viruses have been reported over the last several years (2, 3, 12, 19). By collecting samples from farms with reoccurring immunosuppression problems, and analyzing them molecularly, an attempt to have a better understanding of the current IBDV and CAV viruses infecting broilers in the field. There are CAV and IBDV infections occurring simultaneously in the field, regardless of the farms current problems with gangrenous dermatitis. When comparing these strains molecularly, there are some important changes taking place from which we selected the farm designated as MM. These isolates were identified as IBDV-MM and CAV-MM and analyzed both molecularly and in vivo in both SPF and commercial birds. Molecular analysis of these isolates revealed two major amino acid changes for IBDV at positions 254 (S to N) and 323 (E to D). Previous reports have noted this amino acid change at 254 as being characteristic of the variant viruses currently circulating in the field (15, 17). Because VP2 is the epitope of IBDV that induces neutralizing antibodies, changes at the amino acid level have an impact on the antigenicity of the virus in vivo (9). The molecular analysis of CAV from the MM farm also showed several nucleotide changes. A recent report on molecular changes in CAV suggests a change to glutamine at amino acid position 394 may influence pathogenicity (24, 36). However, our analysis did not include this amino acid position.

The antibody levels to IBDV in broilers showed a natural or normal decline of maternal antibodies by 14 days of age. There was a statistical difference in the broilers between the group inoculated with IBDV-MM 7 or 14 days, compared to the group inoculated with CAV-MM followed by IBDV-MM at 14 days. The SPF groups showed no significant differences for IBDV
antibody levels, indicating that the initial maternal antibodies in the broiler birds greatly impact how the birds’ immunologically respond when faced with disease. It is important to note that even at one day of age; the broilers had a relatively low geometric mean titer for IBDV since normal day of age titers can range from three to five thousand. For CAV, the broiler groups all behaved similarly, only showing differences between the inoculated groups and the negative controls. Although there were not statistical differences between the CAV inoculated groups, the CAV-MM inoculated group had the highest immunological response in SPF birds at 28 days, indicating that this is a very immunogenic virus. It should be pointed out that the birds inoculated with only CAV also showed a response to IBDV at the end of the experimental period. One possible explanation is that these groups were housed with the groups given a combination of IBDV and CAV and the viruses spread between groups. It is feasible that more differences between these groups and those inoculated with a combination of CAV and IBDV would have been observed if housed separately.

At day of age, the broilers had a significantly lower packed cell volume (PCV) than did the SPF birds (21 % vs 31%, respectively). By 7 days of age the broiler birds had recovered and were also over 30% PCV, however, if the CAV challenge had been given at day of age instead of 7 days, the broilers might have suffered more severely from the CAV virus. We chose to inoculate the CAV at 7 days of age to decrease the possibility of the virus being neutralized by maternal antibodies to CAV. In the field, birds are exposed to CAV the day they are placed on the farm and if they have low PCV’s as represented in this study, it is quite possible that severe anemia would have occurred.

Both SPF and broiler birds challenged with the IBDV-MM strain at 7, 14 or 7 and 14 days of age showed bursal atrophy when compared to negative control birds, indicating that this
virus is immunosuppressive even when given in the presence of maternal antibodies.

Histopathological analysis of bursas from these groups showed a decrease in lymphocytes and bursal scores ranged from 2 to 4. In the broiler group inoculated with CAV-MM and IBDV-MM at 7 days followed by a subsequent IBDV-MM inoculation at 14 days, all organs analyzed histopathologically showed high levels of lymphoid depletion. In the same SPF group, the depletion of lymphocytes was not as severe but appeared to be repopulating. Perhaps the broiler birds are immunosuppressed with other factors not measured in this study such as other viruses, bacteria or stress, that they were unable to recover lymphoid follicles.

In conclusion, IBDV and CAV play a very important role in immunosuppression in the broiler industry across the United States. Although both viruses have been studied extensively, it is still unclear how these viruses interact in the field to induce immunosuppression. Our findings indicate that depletion in lymphoid cells in both the bursa and thymus appears to be greater when both viruses are given simultaneously. The viruses in the field today are molecularly changing and perhaps causing more immunosuppression even in the presence of maternal antibodies. These genotype changes may be resulting in a poor response to current vaccination procedures, which could increase the incidence of secondary infections. This study supports previous reports that the viruses in the field today are genetically changing and able to cause infection, even in the presence of maternal antibodies (17, 18). The molecular changes seen in the isolates in this study show that indeed the field situation is changing. As other researchers have suggested, IBDV is a constantly changing virus that needs to be evaluated and monitored in the field if immunosuppression is to be controlled (17, 18). Chicken anemia, although thought to be no longer a problem in broiler production, is still causing immunosuppression, especially when combined with IBDV. Several reports in the last five years have also described CAV causing
problems in the field (12, 34, 36). Further studies need to be performed to evaluate ways to improve vaccines in order to help control these viruses that seem to be breaking through maternal antibodies and continuing to cause disease.
Table 4.1: Experimental Design for *in vivo* studies with the IBDV-MM and CAV MM strains using SPF and commercial broilers. Each group contained 20 birds (2 replicates).

<table>
<thead>
<tr>
<th>Challenge (days of age)</th>
<th>Route of inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>CAV-MM + IBDV-MM</td>
<td>IM/Oral*</td>
</tr>
<tr>
<td>IBDV-MM</td>
<td></td>
</tr>
<tr>
<td>CAV-MM IBDV-MM</td>
<td>IM/Oral</td>
</tr>
<tr>
<td>IBDV-MM IBDV-MM</td>
<td>Oral</td>
</tr>
<tr>
<td>CAV-MM ---</td>
<td>IM</td>
</tr>
<tr>
<td>--- IBDV-MM</td>
<td>Oral</td>
</tr>
<tr>
<td>IBDV-MM ---</td>
<td>Oral</td>
</tr>
<tr>
<td>--- ---</td>
<td>---</td>
</tr>
<tr>
<td>IBDV-EDGAR</td>
<td>Oral</td>
</tr>
<tr>
<td>CAV-CL-1 ---</td>
<td>IM</td>
</tr>
<tr>
<td>--- IBDV-EDGAR</td>
<td>Oral</td>
</tr>
</tbody>
</table>

*IM= Intramuscular inoculation for CAV viruses, and IBDV viruses were given via the oral route.
Table 4.2: SPF and broiler hematocrit values for groups of chickens infected with CAV virus. Different superscript letters denote a statistical difference at $P \leq 0.05$ by SNK method compared with the negative controls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day of inoculation</th>
<th>SPF</th>
<th></th>
<th></th>
<th></th>
<th>Broiler</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>7</td>
<td>14</td>
<td>28</td>
<td>35</td>
<td>1</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Negative Controls</td>
<td>n/a</td>
<td>31</td>
<td>33</td>
<td>40$^A$</td>
<td>36</td>
<td>35</td>
<td>20</td>
<td>30</td>
<td>41$^A$</td>
</tr>
<tr>
<td>CL-1</td>
<td>7</td>
<td></td>
<td></td>
<td>30$^B$</td>
<td>39$^A$</td>
<td>39</td>
<td></td>
<td>30$^B$</td>
<td>30</td>
</tr>
<tr>
<td>CAV-MM</td>
<td>7</td>
<td></td>
<td></td>
<td>32$^D$</td>
<td>31$^B$</td>
<td>35</td>
<td></td>
<td>32$^C$</td>
<td>32</td>
</tr>
<tr>
<td>CAV-MM IBDV-MM</td>
<td>7</td>
<td></td>
<td></td>
<td>32$^E$</td>
<td>33</td>
<td>34</td>
<td></td>
<td>30$^D$</td>
<td>31</td>
</tr>
<tr>
<td>CAV-MM IBDV-MM</td>
<td>7 &amp; 14</td>
<td></td>
<td></td>
<td>32$^F$</td>
<td>33</td>
<td>40</td>
<td></td>
<td>32$^E$</td>
<td>37</td>
</tr>
</tbody>
</table>
Table 4.3: Broiler ELISA CAV and IBDV geometric mean titers (GMT). All groups were statistically different from the controls. Different superscript letters denote statistical differences at $P \leq 0.05$ by Dunn’s method when compared to the negative controls.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day of inoculation</th>
<th>CAV GMT (days of age)</th>
<th>IBDV GMT (days of age)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Negative Controls</td>
<td>n/a</td>
<td>2187</td>
<td>1039</td>
</tr>
<tr>
<td>IBDMM</td>
<td>7</td>
<td>1056</td>
<td>999</td>
</tr>
<tr>
<td>IBD MM</td>
<td>7 &amp; 14</td>
<td>1080</td>
<td>999</td>
</tr>
<tr>
<td>IBD MM</td>
<td>14</td>
<td>1038</td>
<td>999</td>
</tr>
<tr>
<td>Edgar</td>
<td>7</td>
<td>1108</td>
<td>999</td>
</tr>
<tr>
<td>Edgar</td>
<td>14</td>
<td>1163</td>
<td>999</td>
</tr>
<tr>
<td>CL-1</td>
<td>7d</td>
<td>1122</td>
<td>999</td>
</tr>
<tr>
<td>CAV MM</td>
<td>7d</td>
<td>1237</td>
<td>1227</td>
</tr>
<tr>
<td>CAV MM IBDV MM</td>
<td>7 &amp; 14</td>
<td>1237</td>
<td>1227</td>
</tr>
</tbody>
</table>

* A titer of 999 is considered to be negative for the CAV ELISA test.
Table 4.4: SPF ELISA CAV and IBDV geometric mean titers (GMT). All groups were statistically different from the control birds. Different superscript letters denote statistical differences between the groups at $P \leq 0.05$ by Dunn’s method.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day of inoculation n/a</th>
<th>CAV GMT (days of age)</th>
<th>IBDV GMT (days of age)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Negative Controls</td>
<td>n/a</td>
<td>999</td>
<td>1073</td>
</tr>
<tr>
<td>IBD MM</td>
<td>7</td>
<td>1016</td>
<td>1819</td>
</tr>
<tr>
<td>IBD MM</td>
<td>7 &amp; 14</td>
<td>1030</td>
<td>3134</td>
</tr>
<tr>
<td>IBD MM</td>
<td>14</td>
<td>1037</td>
<td>1016</td>
</tr>
<tr>
<td>Edgar</td>
<td>7</td>
<td>1005</td>
<td>999</td>
</tr>
<tr>
<td>Edgar</td>
<td>14</td>
<td>1084</td>
<td>999</td>
</tr>
<tr>
<td>CL-1</td>
<td>7d</td>
<td>1164</td>
<td>1724</td>
</tr>
<tr>
<td>CAV MM</td>
<td>7d</td>
<td>1064</td>
<td>5563</td>
</tr>
<tr>
<td>CAV MM</td>
<td>7</td>
<td>1099</td>
<td>2210</td>
</tr>
<tr>
<td>CAV MM</td>
<td>7 &amp; 14</td>
<td>1049</td>
<td>4459</td>
</tr>
</tbody>
</table>

* A titer of 999 is considered to be negative for the CAV ELISA test.
Figure 4.1: Bursa: body weight scores at 35 days for both SPF and Broilers. Different letters represent significant differences between the groups. All groups were statistically different from the negative controls using Dunn’s method at $P < 0.001$. 
References


CHAPTER V

CONCLUSIONS

Infectious Bursal disease virus (IBDV) and Chicken anemia virus (CAV) are two very important viruses in the broiler industry. These viruses can lead to immunosuppression and secondary infections such as gangrenous dermatitis, which can lead to great economic loss.

The use of molecular techniques has become the most valuable way of identifying viruses because of its speed and accuracy. IBDV is most commonly identified by molecular techniques such as reverse transcriptase polymerase chain reaction (RT-PCR) and sequencing. Viruses must be inactivated to be shipped internationally for molecular identification. Therefore, bursas from birds experimentally infected IBDV were imprinted on FTA® cards, cut in half and one half was then placed in phenol and the other half retained as a fresh sample for comparison. Samples were evaluated and compared based on molecular detection capabilities between the two inactivation methods. The nucleic acid of the virus was detected in 85% of the FTA® card inactivated samples compared to 71% in the phenol inactivated samples. Sequence analysis was performed on samples inactivated by both methods and no differences were found. Using the FTA® card for identification of IBDV has proven to be a reliable alternative to the previously used chemical inactivation and storage methods. Because shipping regulations have become increasingly harder for international shipments, the use of the FTA® card’s ease in shipping makes it a suitable choice for inactivation of viruses. Our findings indicate that using the FTA® card is just as reliable as phenol for molecular detection of IBDV. There were no differences in the molecular
identification or sequence abilities using FTA® cards instead of phenol for inactivation and storage of nucleic acids.

There have been several reports of an increase in immunosuppression in the United States. Gangrenous dermatitis (GD) is often an identifying factor for immunosuppression, therefore, in our study, farms experiencing reoccurring GD problems (problem) were sampled and compared to healthy farms (normal). Molecular analysis was performed to determine if any changes were taking place for IBDV and CAV strains between problem and normal farms. As expected, IBDV and CAV were identified on all farms sampled, regardless of the health status. There were molecular changes found on both type of farms samples (healthy vs. normal), however, one farm (MM) in particular had a lower percentage of similarity based on the deduced amino acid sequence when compared to known strains for CAV and IBDV, and was therefore chosen to be further analyzed in vivo.

In vivo studies on the IBDV-MM and CAV-MM isolates revealed some differences in the antibody response, even in the presence of maternal antibodies. Histopathological evaluation of bursa, thymus and spleen of birds inoculated with either CAV-MM or IBDV-MM in combination or alone revealed lymphoid and lymphocyte depletion indicating immunosuppression was occurring. It is possible that these viruses are inducing immunosuppression in the field that could be causing an increased susceptibility in secondary infections such as gangrenous dermatitis, even in the presence of maternal antibodies. The amino acid differences that the MM isolates contained could be a result of various pressures that can occur in the field causing the virus to undergo changes that can cause infection even when vaccination programs are being used. It is clear that further studies need to be performed to
monitor the changes molecularly, antigenically and immunogenically in order to better understand and control these immunosuppressive viruses.