IVAN RICARDO ALVARADO
Protection conferred by commercial infectious bronchitis (IB) vaccines against the
    Nebraska 95 (California 99) isolate and persistence of IB Arkansas and
    Massachusetts virus vaccines in broilers.
(Under the direction of Dr. Pedro Villegas)

The level o protection conferred by commercial vaccines against the new variant Nebraska 95 isolate was evaluated. Complete protection was observed when the IBV Arkansas vaccine was given and when the Massachusetts-Connecticut vaccine was given at 1 and at 1 and 10 days of age. However, partial protection was observed when the Massachusetts-Connecticut vaccine was given only at 10 days of age.

The persistence of IBV Arkansas and Massachusetts vaccine strains in broilers was evaluated. By RT-PCR assay, when given as a single vaccine, the Massachusetts vaccine persisted longer in the tracheas than the Arkansas vaccine. However, only the Arkansas vaccine persisted in cecal tonsils. When the bivalent vaccine was given, the presence of the Arkansas serotype was observed at later ages when compared with the Massachusetts serotype. By the *in situ* hybridization assay, when the bivalent vaccine was given, IBV persisted longer in the cecal tonsils than in trachea.

INDEX WORDS: IBV, Nebraska 95, RT-PCR, RFLP, *in situ* hybridization, rapid plate hemagglutination assay, LightCycler, coronavirus

PROTECTION CONFERRED BY COMMERCIAL INFECTIOUS BRONCHITIS (IB)

VACCINES AGAINST THE NEBRASKA 95 (CALIFORNIA 99) ISOLATE AND

PERSISTENCE OF IB ARKANSAS AND MASSACHUSETTS VIRUS VACCINES IN

BROILERS

by

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DEDICATION

To my parents Marco Tulio and Alicia, for their love and encouragement making possible to accomplish all my goals and for teaching me precious values to guide my life.

To my sisters, Sandra and Silvia for their support and friendship.

You are the dearest treasure in my life. I love you very much.

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CHAPTER 1

INTRODUCTION

Infectious bronchitis virus (IBV) causes an infectious respiratory disease that induces

economic loses of great magnitude within the poultry industry. In layers, the economic

loses are primarily due to drops in egg production along with poor internal and external

quality of the eggs. In broilers, economic loses are produced by a decrease in weight gain

with low feed efficiency and increased condemnations in the carcasses when infectious

bronchitis is complicated with secondary bacterial infections. Although live and

inactivated vaccines are available to control the disease, the presence of multiple

serotypes and the appearance of new serotypes or variants make the control of this

disease difficult. In the presented studies, the protection afforded by selected

commercially available vaccines against one of these IBV variants and the persistence of

both the IBV Massachusetts and Arkansas type vaccines in chickens were evaluated and

assessed.

CHAPTER 2

OBJECTIVES

The objectives of these studies were to evaluate the level of protection conferred by infectious bronchitis virus (IBV) commercial vaccines against the Nebraska 95 isolate under laboratory conditions and to evaluate the persistence of IBV Arkansas and Massachusetts type vaccines in trachea and cecal tonsils of vaccinated broilers via detection techniques such as virus isolation, rapid plate hemagglutination, reverse transcriptase-polymerase chain reaction/restriction fragment length polimorphism (RT-PCR/RFLP) and *in situ* hybridization assays.

CHAPTER 3

LITERATURE REVIEW

Infectious Bronchitis (IB) is one of the most important diseases in the poultry industry. The economic impact of this disease is due to drops in egg production and poor internal and external quality of the eggs in laying hens and broiler breeders. In broilers, IB produces respiratory reactions, poor weight gain, lower feed efficiency and high condemnations at processing, when complicated with secondary bacterial infections like E. coli (11). This acute and highly transmissible disease affects chickens at different ages.

HISTORY

Infectious bronchitis was first described by Schalk and Hawn in 1931. The disease was described in North Dakota as a new respiratory disease of chickens from 2 days to 3 weeks of age with gasping and listlessness as clinical signs (66). However, the nature of the infectious agent was not determined at that time (30). The disease was reported again in 1933 by Bushnell and Brandly (11), who established that the causative agent was a virus due to the transmision of the disease from Berkefeld-filtered material. Later, the possibility of infection by infectious laryngotracheitis virus was considered (30). In 1936, Beach and Schalm (6), proved by cross-immunity that the infectious bronchitis virus (IBV) was distinct from infectious laryngotracheitis virus (ILT) and infectious coryza produced by *Haemophilus gallinarum*. In 1937, Beaudette and Hudson (7), propagated the virus in chicken embryos by the chorioallantoic route of inoculation,

becoming more lethal after continued passages. In 1941, Delaplane and Stuart (26),

confirmed the results obtained by Beaudette and Hudson. However, after successive

passages, they observed that the virus that had become highly lethal for chicken embryos,

became noninfectious and lost its pathogenicity for chickens. In 1942, van Roeckel *et al*

(71), developed the serum neutralization test in embryos by using the lethal embryo strain

described by Beaudette and Delaplane. In 1949, Fabricant considered the presence of

stunting and curling lesions in embryos as pathognomonic lesion (30). In 1950, Hofstad

and Kenzy (34), found that chicks hatched from recovered hens could still be susceptible

to IB in the presence of maternal antibodies. In the 1940's, van Roeckel *et al* (70, 72),

based on field observations indicating that chickens infected at early ages (8 to 16 weeks)

exhibited mild respiratory symptoms with protection against the disease and egg losses,

developed the bases of actual immunization programs.

ETIOLOGY

The etiologic agent is a pleomorphic-rounded virus from the family Coronaviridae, genus

Coronavirus (14). The members of this family are classified in three different serological

groups with IBV belonging to group III (35, 55). The viruses are identified in every

serogroup by their natural host, nucleotide sequence and serological relationships (35).

Infectious bronchitis is a non-segmented, single stranded, positive sense RNA virus with

a 27 to 32 Kb genome, the largest of all RNA viruses (35, 55). IBV has a lipid bi-layer

envelope with an approximate diameter of 120 nm (35, 55). When the virus is negatively

stained, the presence of petal-shaped projections or spikes is observed. IBV has three

major specific proteins known as S, M and N. The S protein is the structural protein of

the spikes, which is responsible for the attachment of the virus to the cells, fusion of the

virus envelope with the membrane of the host cell and induction of neutralizing

antibodies (35, 42, 55, 63). This protein is highly glycosylated with a carboxyterminal

cytoplasmic domain, a transmembrane domain and two external domains known as S1

and S2 (35). In the IBV Beaudette and M41 strains, the S1 and S2 portions of the S

protein result from the cleavage of the spike precursor propolypeptide at the arg-arg-phe-

arg-arg amino acid sequence (13). The S2 portion is attached to the membrane while the

S1 portion has little or no contact with the membrane forming the major part of the

bulbous end of the S protein (12). The S1 portion is present in the N-terminal portion of

the S protein while the S2 portion is present in the C-terminal portion (55). The S1

portion has a sequence of 520 amino acids and it is responsible for the presence of

hemagglutination inhibition and VN antibodies. When the amino acid sequence of the S1

portion of Coronaviruses classified in different serogroups are compared, the presence of

hypervariable regions with deletions, mutations or recombination is observed (15, 74).

The S2 portion has a sequence of 625 amino acids and it is less variable than the S1

portion (15). The M protein (formerly called E1) is comprised of 225 to 230 amino acids

and is essential for the production of Coronavirus-like particles. The M protein has three

hydrophobic alpha-helical domains, which penetrate the lipid bilayer three times. The

amino terminal portion of the M protein (20 to 22 amino acids) is highly hydrophilic and

is exposed at the virion surface while the carboxyterminal domain extends into the inner

surface of the lipid membrane interacting with the nucleocapsid protein (15, 35). The M

protein is embedded in the virus membrane and during the replication process it adheres

to the endoplasmic reticulum of the cell. During the budding process, the envelope of the

virion is taken from the endoplasmic reticulum and not from the cellular membrane, avoiding the lysis of the cell (15). The phosphorilated N or nucleocapsid protein, which has 409 amino acid residues, is present around the single RNA strand. This protein has three structural domains with the RNA binding to the second domain (12, 35, 55). The N protein is very conserved among Coronavirus in the same group with identities greater than 94% (64). The amino acid sequence of the N protein shows a high homology among different IBV strains and may be involved in the regulation of the viral RNA synthesis (12, 16, 35, 55). The carboxyl-terminal portion of this protein has been associated with the stimulation of the cell-mediated immunity by inducing the production of cytotoxic T lymphocytes protecting the chickens from acute infection (67).

SUSCEPTIBILITY TO CHEMICAL AND PHYSICAL AGENTS.

Most IBV strains are inactivated at 56 C for 15 minutes or at 45 C after 90 minutes (61). The pH is a critical factor for the stability of the virus. A decrease in up to 5 $\log_{10}$ in the titer has been reported after pH 3 treatment at room temperature for 4 h (20, 61). In cell culture, IBV is more stable at pH 6.0 to 6.5 than pH 7.0 to pH 8.0 (3). IBV is ether-labile. However, Otsuki et al (61), observed resistance in 9 of 11 strains (except the Beaudette-42 and Connaught strains) to 20% ether for 18 h at 4 C. All the strains were completely inactivated by 50% chloroform for 10 min at room temperature and by 0.1% sodium deoxycholate for 18 h at 4 C (61). The treatment with 0.05% to 0.1% beta-propiolactone or formalin at 0.1% eliminates the infectivity of the virus. The inactivation of IBV with beta-propiolactone does not affect its hemagglutination antigen activity (19, 48).

VIRUS REPLICATION

The replication of IBV starts with the attachment of the virus to O-glycosylated receptors present in the cell membrane (35). Then, after host-cell dependent proteolytic cleavage of the S protein, the fusion of the viral envelope with the plasma membrane is observed. The entry of the viral genome is followed by the translation of the polymerase gene (gene 1) from genomic RNA by two overlapping reading frames (ORFs 1a and 1b) located in the 5' end of the genome to yield an RNA-dependent RNA polymerase. These ORF are capable of coding two large polyproteins, which are probably involved in the polymerase function (35, 69). The presence of two polymerases, one for genomic RNA synthesis and one for subgenomic RNA synthesis has been suggested (10). The 700 Kd polymerase protein seems to be continuously translated as shown by the inhibition of RNA synthesis when this protein is not produced (35). Then, the plus-strand RNA is transcribed into a minus-strand RNA, which is used as a template to produce mRNAs and genomic RNA (78). The presence of subgenomic minus-strand mRNAs with similar length to positive-strand RNA has been observed serving as templates for subgenomic mRNAs synthesis. All the genomic and subgenomic mRNAs are capped and polyadenylated. All the subgenomic mRNAs present an identical 3' end to the genomic RNA and have in their 5' end a leader sequence composed from 60 to 80 bases long. The 3' end of the leader sequence shows a high homology with the intergenic sequences observed in the IBV genome, which could explain the formation of subgenomic RNAs. The genomic RNA is transcribed by using the full-length minus-strand as template (35, 69).

Translation of Viral Proteins. The mRNA 1 contains two large ORFs, overlapping

each other by 43 to 76 nucleotides, which are translated as one polyprotein by a

ribosomal frame shifting mechanism (35). This polyprotein is co- or posttranslationally

processed into multiple proteins by viral and host proteases. The polycistronic mRNA 3

of IBV contains three overlapping ORFs, which are translated. Translation of the third

ORF is regulated by an upstream internal ribosomal entry site (IRES) sequence,

translating the third ORF independently from the cap translation mechanism. The protein

encoded by this ORF is highly hydrophobic and seems to be a viral envelope protein,

known as sM (15, 35, 55).

Assembly and budding of the IBV. Initially, the N polypeptides in the nucleocapsid

and the IBV genome are formed in the cytoplasm. However, the glycoproteins are

glycosylated and processed in the Golgi apparatus. The assembling of the virions occurs

in intracellular vesicules at the Golgi apparatus and endoplasmic reticulum where the

specific glycoproteins of the virus are acquired (27). The S proteins are incorporated in

the virions and then, they are N glycosylated in the Golgi apparatus. The intracellular

transport of the whole virions favors the efficiency of the glycosylation of the S protein.

The M protein, synthesized on membrane-bound polysomes in the rough endoplasmic

reticulum, accumulates in the Golgi apparatus. The M protein, unlike S, is not

transported to the plasma membrane. Analysis of recombinant M protein suggests that

the Golgi targeting of IBV M protein depends upon four uncharged, polar amino acids on

one face of the first alpha helical, membrane-spanning domain. The M protein is critical

for the assembly of the virions interacting with the nucleocapsid protein in the presence of IBV RNA. This interaction seems to determine the site of virus assembly (16, 35, 55).

CLINICAL DISEASE

The upper respiratory tract is the main site of IBV replication (11). It occurs in ciliated epithelial and mucus secreting cells, lungs and air sacs, producing characteristic signs as sneezing, coughing, gasping, tracheal rales and nasal discharge. Inflammation of eyes and sinuses has been observed. Young birds appear depressed with reduction in feed consumption and weight gain. In birds over 6 weeks of age, similar signs are present but nasal discharge is not common (22). After respiratory infection, viremia is observed with the dissemination of the virus to different tissues (14).

In the trachea, three stages are characteristic. A degenerative phase with decilliation and desquamation during the first 2 days, with infiltration of heterophils and lymphocytes in the lamina propria, an hyperplastic phase with newly formed epithelial cells without cilia, and the recovery phase with a reparative process of the epithelial cells occurring approximately at 8 days post infection (28).

In the reproductive organs, the virus has been demonstrated in the oviduct between 6 and 9 days post infection causing glandular hypoplasia, leading to the reduction of albumen proteins like ovomucin and lysozyme. The reduction of albumen proteins changes the structural matrix of the albumen producing watery eggs. Microscopic lesions like reduction in the height of the epithelial cells, destruction of the cilia, dilation of the glands, lymphocytic foci and cellular infiltration in lamina propia are seen. When the infection affects female chicks less than 2 weeks of age, a permanent damage in the

reproductive tract is observed. This damage is seen especially in the young cells of the isthmus and magnum and is responsible for the absence of egg production in some layers after infection (28). Some strains, like the Australian T strain, are more nephrotropict producing kidney lesions with the presence of urates in the tubules and ureters (14).

In the kidneys, virus replication has been observed in the tubular epithelial cells causing alterations in the fluid and electrolyte transport. These alterations produce an increase in water losses associated with lower urine, osmolarity and higher excretion of sodium, potassium and calcium. The kidneys infected with nephropathogenic IBV are swollen and pale with the presence of urates. Nephropathogenic strains usually produce nephrosis-nephritis in young birds and urolithiasis in layers (1, 28, 59). When the birds are affected by a nephropathogenic strain, depression, wet droppings, increase in water consumption and ruffled feathers are commonly observed.

In the intestines, IBV replication has been observed. In 1986, El Houdaff *et al* (29), classified an IBV strain (strain G) as enterotropic by virtue of its prolonged persistence in the gut compared to the trachea. In 1987, Jones and Ambali (45) observed the re-excretion of an enterotropic IBV by hens at point of lay after experimental infection at one-day of age. However, Bhattarcharjee and Jones in 1997 (4), could not demonstrate the replication of 11 IBV strains in intestinal organ cultures when duodenum, jejunum and ileum explants were inoculated with 0.2 ml of virus suspension at a concentration of $10^{6.0}$ ciliostatic doses (CD50) per ml. However, in other investigations, *in vitro* explants of several gut tissues have supported the growth of IBV (8). In 1990, Lucio and Fabricant (58), isolated four viruses (Mass 41, ECV 1, ECV 2, and ECV 3) from cecal tonsils. The intestinal replication of IBV has been described in histiocytes, lymphoid

cells in the cecal tonsils and in apical epithelial cells of the villi in ileum and rectum. The

793/B UK IBV strain was more enterotropic than pneumotropic in broilers with the

presence of diarrhea (27). IBV has been isolated from other tissues as Harderian gland,

bursa of Fabricious, spleen, feces, cloacal contents and semen (28).

IMMUNITY

Passive immunity. The presence of maternal antibodies can reduce both the severity of

vaccinal reaction and the efficacy of the vaccine if the vaccine is of the same type used in

the breeder flock immunization (14). The protection offered by maternal antibodies

against the IBV challenge varies from 1 to 2 weeks (51, 52). However, in birds with

maternal antibodies vaccinated at one-day of age, the development of active immunity in

the respiratory tract is observed (60).

Active immunity. Chickens just recovered from the natural disease are resistant to

challenge with the same virus (homologous protection). However, protection against

different serotypes (heterologous protection) is unpredictable (18, 37, 73).

The determination of immunity against IBV has been evaluated by immunological

techniques like virus neutralization test (VN) and hemagluttination inhibition test (HI), as

well as the failure in the isolation of virus from tracheas five days after challenge of SPF

chickens and the evaluation of the ciliary movement in tracheal explants (24, 76). The

evaluation of ciliary movement in tracheal rings was shown to be effective in the

assessment of immunity to challenge with homologous strain of IBV after vaccination of

chickens. The results with this method correlate well with the histopathological findings

and virus isolations (5).

SEROTYPES

Until 1956, when the Connecticut strain was isolated, only the IB Massachusetts

antigenic type virus was recognized (32, 46, 70). IBV is characterized by the presence of

multiple serotypes (14, 31, 47). In 1988, King (47), determined the presence of three

IBV field isolates from layer flocks with production problems to be serologically

different from vaccine strains Mass 41, H52, H120, Florida, JMK, Connecticut and

Arkansas. The presence of new serotypes or variants has been observed mainly in layers

due to common management practices like high densities and multi-age complexes. In

addition, the continuous introduction of pullets, the longer life span of layers compared

with broilers and the presence of different levels and perhaps specificities of immunity,

exacerbate the recycling of IBV and the spread of the disease (31). The presence of

variant serotypes has been observed in layers vaccinated with only live vaccines as well

as live and inactivated vaccines suggesting that protection offered by commonly used

vaccines is not totally effective (31). The main interest of serotyping IBV is to determine

the prevalence of IBV strains in the field and compare them with the IBV strains used in

the vaccination programs, because protection against unrelated serotypes (heterologous

protection) is unpredictable (31). Cross-protection produced by some IBV serotypes

against antigenically unrelated strains have been reported (33, 62, 75). However,

complete protection against heterologous serotypes by one strain or strains has not been

observed (31). Some vaccines offer better protection against variant serotypes than

others. The Mass-Ark vaccines offer a broader protection against variant serotypes than

the Mass (Holland) alone or Mass (L-1) in combination with the Connecticut strain (31).

IBV serotypes have been classified based on different tests like virus neutralization in

chicken embryos (23), reduction of cilia movement in chicken tracheal organ culture (18, 25, 44), plaque reduction test (36), hemagglutination-inhibition (2, 49, 56), dot-immunobloting assay using monoclonal antibodies (68), monoclonal antibody-based ELISA (40), polymerase chain reaction-restriction fragment length polymorphism (RT-PCR/RFLP) (42, 54), and sequencing of the S1 gene (50, 65). In addition to the presence of multiple serotypes, no standard classification exists. For this reason, some serotypes present in one country can correspond with similar serotypes present in other countries. The presence of multiple serotypes and the appearance of new serotypes or variants, which can break through the immunity induced by vaccines, make it difficult to establish effective vaccination programs (28, 41). Programs designed to control IBV by immunization depend on the use of vaccines that are antigenically similar to circulating field strains, so the characterization of new isolates is very important due to the unpredictable cross protection among different serotypes (47).

In the U.S, the following serotypes are recognized: Massachusetts, Connecticut, Delaware 072, Florida, Arkansas 99, JMK and Georgia, California and Delaware variants (14). In the U.K, 8 serotypes have been described: 167-84, 142-86, 128-82 and 4/91 or 793B (King, J. D. Personal communication). The 793B serotype has been associated with the presence of myopathy in broilers (17). In the Netherlands, five serotypes are present: serotype A with Mass 41 and Holland 52 and 120; serotype B with D207 and D274; serotype C with D212 and D1466; Serotype D with D3128 strain and serotype E with D3896 and D274 strains (14). In Australia, serotypes A, B, C, D, E, F, G, H, I, J, K, L, M and O are present. A new classification has been established with two groups, group

I with viruses that replicate in the trachea and kidney like the T strain or N1/62 and group

II with viruses that replicate only in the trachea (21).

VACCINES

The first vaccination attempt, which was made by van Roekel, consisted of exposing

laying flocks to IBV during the onset of egg production, being the bases of current

immunization programs (72).

Live vaccines and inactivated oil emulsion vaccines have been used in IB immunization

programs in broilers and breeders. Live viruses used as vaccines have been attenuated by

successive passages in chicken embryos. However, it has been demonstrated that

successive passages although diminishing the pathogenicity, decrease the

immunogenicity. The decrease in the immunogenicity affects adversely the protection

offered by the vaccine virus against challenge viruses in the field (14).

The immunity and serological responses to live vaccine virus depend on the

multiplication of the virus in the tissues of the host. The active and passive immunity

present in the host at the moment of vaccination may prevent virus replication, partially

blocking the primary response in the case of maternal antibodies or preventing the

secondary response in the case of active immunity (9).

The serotype variation of IBV is due to high point mutation and recombination (14, 43,

53, 57, 77). IBV mutations and recombination may be associated with some management

practices like high density, multi-age flocks with different antibody levels, different

vaccination programs and methods of application. These practices exert a strong

selective pressure that favors the mutation of the virus. These mutations are natural

mechanisms of defense of the virus, trying to avoid the immune system defenses in chickens.   These resulting "variant" strains have new neutralizing epitopes that may not be recognized by the antibodies exerted by the use of the current vaccines (31, 38). The presence of multiple serotypes has lead to the production of different vaccines in different countries, depending on the prevalent serotype, in order to obtain better protection. In the U.S, seven strains are currently used for vaccine production. They are Mass 41, Connecticut, Holland, JMK, Florida 18288, Arkansas 99 and with special license Delaware 072 (14). In Australia, serotypes A and B are commonly used in vaccines. Vac1 from serotype A and Vic S from Serotype B are the commercial vaccines available (73). In the U.K the Mass type (Mass 41, H52 and H120), D 274 and 793B live vaccine and 793B killed vaccines are used. In the Netherlands, the most common strains used in vaccines are the H52 and H120 (Massachusetts type), D1466, D274 and D1201 (14).

VIRUS DIFFERENTIATION

The observations of clinical signs and pathological lesions in IB have been commonly used to evaluate the degree of attenuation of the vaccine strains. Virus strains are attenuated by serial passages in chicken embryos or cell cultures, offering an imprecise measure of severity as function of virulence (38).

After several passages in chicken embryos, the pathogenicity of the IBV is decreased. However, it has been proven that chicken passaged virus, also known as back passage, increases the pathogenicity of the virus, which is exhibited by an increase in the presence

of airsaculitis and respiratory reaction. This is especially seen when *Mycoplasma gallisepticum* is present in the field (38, 39).

In the field, IBV seems to cycle or pass from chickens that are infected with the vaccine virus to susceptible chickens. This occurs due to different levels of maternal antibodies in the flocks. At the vaccination time, birds with high antibody levels could neutralize the vaccine virus while birds with low antibody levels permit the replication of the virus and the developing of protective immunity (38).

When birds correctly vaccinated begin to shed the virus, this virus (back passaged) will be more pathogenic, infecting the birds that initially rejected the vaccine virus, presenting serious respiratory reactions. This is known as rolling reaction, cycling vaccine or circulating vaccine virus, and basically describes or explains the continuing respiratory signs in flocks following live IBV and NDV vaccination (38).

CHAPTER 4

PROTECTION CONFERRED BY COMMERCIAL INFECTIOUS BRONCHITIS

(IB) VACCINES AGAINST THE NEBRASKA 95

(CALIFORNIA 99) ISOLATE[1]

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[1]Alvarado, I. R., P. Villegas, J. El Attrache, T. P. Brown. To be submitted to

Avian Diseases

SUMMARY. An infectious bronchitis virus (IBV) was isolated from commercial broilers from the state of Nebraska. The presence of respiratory distress, inflamed tracheas, airsaculitis and edematous lungs was observed. After reverse transcriptase-polymerase chain reaction (RT-PCR), a different restriction fragment length polymorphism (RFLP) pattern was exhibited when compared to commercial vaccines and known isolates. However, this RFLP pattern was identical to several IBV isolates obtained from California. Commercial Mass-Conn and Mass-Ark mixed vaccines were used to vaccinate commercial broiler chickens via eye drop at 1, 10 and 1 and 10 days of age. At 27 days of age the birds were challenged via eye drop with the Nebraska 95 isolate. Complete protection was measured by failure to re-isolate the challenge virus from tracheas 5 days post-challenge and complemented with the tracheal and epithelium thickness scores. When the Mass-Ark vaccine was included in the vaccination programs, complete protection against challenge with the IBV Nebraska 95 isolate was observed. The Mass-Conn vaccine gave complete protection when used at 1 and at 1 and 10 days of age. However, when used as the only vaccine at 10 days of age, partial protection was observed with the re-isolation of the challenge isolate in one of the replica from this group. The birds that received the Mass-Conn vaccine at 1, 10 or at 1 and 10 days of age exhibited the highest epithelium thickness scores in all the vaccinated groups when compared with the negative control group, exhibiting a statistically significant difference $(P < 0.0017)$. Significant differences in the tracheal score $(P < 0.0003)$ were observed between the negative control and the groups that received the Mass-Conn vaccine at 1, 10 or 1 and 10 days of age. Based on these results, complete protection against the Nebraska 95 isolate was observed when the IBV Arkansas type was included in the vaccine.

INTRODUCTION

Infectious Bronchitis (IB) is one of the most important diseases present in commercial poultry farms. IB produces severe economic loses due to drops in egg production with poor internal and external quality of the eggs in layers. In broilers, IB affects weight gain, feed efficiency, and when complicated with bacterial infections like <u>E</u>. <u>coli</u> or <u>S</u>. <u>aureus</u>, mortality and increased condemnations are observed (2, 14). The characteristic clinical signs of IB are coughing, gasping, tracheal rales and nasal discharge. However, inflammation of the eyes and sinuses has been observed (2).

IB is produced by a pleomorphic virus that belongs to the Coronaviridae family, genus Coronavirus (2,11). IBV replicates in the upper respiratory tract, mainly in the trachea disseminating via the blood stream to different tissues like intestines, ovary and kidneys, remaining in these tissues for long periods of time (2, 14). During the viral replication process, in which point mutations and recombinations can be observed, changes in the nucleotide sequence of the genome are common. As a consequence, a variation in the amino acid sequence of the expressed proteins (especially in the S1 glycoprotein) makes the already present IBV antibodies unable to recognize the new protein, which can result in the presence of the disease (4).

IBV is characterized by the presence of three major proteins: S, M and N. The S protein is cleaved in the Golgi apparatus in two portions, S1 and S2 (3, 4). The S1 subunit, which varies in its amino acid conformation among different viruses, is responsible for the induction of virus neutralization (VN) and hemagglutination inhibition (HI) antibodies inducing protective immunity (2, 4, 5). The S2 subunit seems to be very conserved among viruses within the same serotype (1). However, small sequence

differences between different serotypes are observed (1). These small changes in the

sequence can create different interactions between the S1 and the S2 subunits, which can

change the location of the antibody binding sites in the S1 subunit (1).

Vaccination programs rely on the use of vaccines with virus antigenically similar to the

virus present in the field. However, the presence of multiple serotypes and the

appearance of new serotypes or variants make the establishment of an adequate

vaccination program difficult, requiring the application of multiple vaccines (6, 9). It has

been demonstrated that cross protection among different serotypes is unpredictable (9).

For this reason, the characterization of new isolates present in the field is very important

(9). IBV has been isolated from tissues like trachea, cecal tonsils, lungs, kidneys and

oviduct (2). Processed tissues are inoculated into SPF embryonated chicken eggs. The

presence of IBV in allantoic fluid has been detected by reverse transcriptase-polymerase

chain reaction (RT-PCR), using primers specific for the S1 gene and further characterized

by restriction fragment-length polymorphism (RFLP) (7, 10).

Broiler flocks in California have shown respiratory problems with inflamed tracheas,

airsaculitis and edematous and hemorrhagic lungs. Several tracheal and cecal tonsils

samples from those birds have been submitted to the diagnostic laboratory of the Poultry

Diagnostic and Research Center (PDRC) for virus isolation. An IBV was isolated from

cecal tonsils from a five and a half weeks old broiler flock and further characterized by

RFLP as Nebraska 95. This isolate exhibited a different restriction pattern when

compared with known IBV Massachusetts, Connecticut and Arkansas patterns but similar

RFLP patterns with the IBV California 99 isolate.

The objective of this study was to evaluate the level of protection conferred by IBV commercial vaccines against the Nebraska 95 (California 99) isolate under laboratory conditions.

MATERIALS AND METHODS

Virus isolation and detection. Samples of trachea and cecal tonsils were received at the diagnostic laboratory of the Poultry Diagnostic and Research Center (PDRC) of the University of Georgia for virus isolation and characterization. Different tissue samples were processed separately as follows. The samples were frozen and thawed three times. Small pieces of the tracheas and cecal tonsils were minced in 10 ml of a solution of 500 ml of tryptose phosphate broth (TPB) with 1% penicillin (10000 IU/ml) – streptomycin (10000 μg/ml) (Sigma Chemicals Co., St Louis, MO) and 1% amphotericin B (250 μg/ml) (Sigma Chemicals Co., St Louis, MO) and centrifuged at 2,000 rpm (7,600 X g) for 10 minutes in a CRU 5000 centrifuge (Damon/IEC, Needham Heights, MA). The supernatant was filtered through a sterile 0.22 μm polyethersulfone (PES) syringe filter (Whatman Inc., Clifton, NJ) into a sterile vial.

Nine-day old specific pathogen free (SPF) embryos (SPAFAS Inc., Norwich, CT) were inoculated by the chorioallantoic sac route with 0.1 ml of the filtered supernatant. Forty-eight hours later, the allantoic fluid was harvested. The presence of hemagglutinating virus (Newcastle and influenza) was determined by direct hemagglutination of 5% red blood cells. The presence of IBV was determined via two methodologies.

Firstly, a rapid plate hemagglutination assay was performed directly from fresh allantoic

fluid treated with 1 U/ml of commercial neuraminidase (Sigma Chemicals Co., St Louis,

MO) as described by Ruano et al (13).

Secondly, RT-PCR was used to amplify the S1 gene of IBV as follows. The IBV RNA

was extracted from infected allantoic fluid using the Trizol method (Life Technologies

Inc., Grand Island, NY). Briefly, 0.25 ml of allantoic fluid was mixed with 0.75 ml of

Trizol LS in a 2 ml DNase/RNase free centrifuge tube (USA Scientific Inc., Ocala, FL),

vortexed and incubated at room temperature for 5 minutes. After the addition of 0.2 ml

of chloroform, the sample was vortexed, incubated at room temperature for 8 minutes and

centrifuged at 12,000 rpm (11,750 X g) for 15 minutes at 4 C in a Heraeus Biofuge

(Kendro Laboratory Products, Germany). Then, the upper aqueous phase was transferred

to a new DNase/RNase free microfuge tube and 0.5 ml of isopropyl alcohol and 5 μl of

polyacryl carrier (Molecular Research Center Inc., Cincinnati, OH) were added. After 10

minutes of incubation at room temperature, the sample was centrifuged at 12,000 rpm

(11,750 X g) for 10 minutes at 4 C. Then, the supernatant was removed and the pellet

was washed with 1 ml of 75% ethanol, vortexed and centrifuged at 9,000 rpm (6,610 X g)

for 5 minutes at 4 C. Finally, the pellet was resuspended in DNase/RNase free water and

stored at – 80 C.

RT-PCR. A commercial one tube RT-PCR kit (Roche Diagnostics Corp., Indianapolis,

IN) was used. All of the reagents but the primers are provided by the commercial kit.

Briefly, 25 μl of DNA-RNA free water, 2.5 μl of DTT (100 mM), 4 μl of dNTP mix (10

mM), 1 μl of (1.0 μM) forward primer (TGAAACTGAACAAAAAGAC), 1μl of (1.0

μM) reverse primer (CCATAAGTACACTAAGGRCRA), 1 μl of RNAse inhibitor

(5U/μl), 10 μl of 5X PCR Buffer, 2 μl of MgCl$_2$ (25 mM) and 1 μl of enzyme mix were

used per 2 μl of RNA sample. The RT-PCR was conducted by incubation for 1 hour at

60 C, heating at 94 C for 2 minutes and 35 cycles of denaturation at 94 C for 30 seconds,

annealing at 55 C for 30 seconds and polymerization at 68 C for 80 seconds with a final

elongation step of 7 minutes at 68 C for the last cycle with a GeneAmp PCR system 2400

thermal cycler (Perking-Elmer, Norwalk, CT). The amplification products were analyzed

in a 1.5% agarose gel.

RFLP analysis was performed following the technique described by Kwon et al (10).

The RT-PCR products were digested using the commercial restriction enzymes _Bst_YI,

_Hae_III, and _Xcm_I (New England Biolabs Inc., Beverly, MA). RFLP was observed

following electrophoresis (100V constant voltage) in a 1.5% agarose gel with ethidium

bromide.

Experimental design. Two hundred, one-day old commercial broiler chickens obtained

from a local hatchery were used. One hundred eighty commercial broilers were divided

into 9 treatment groups with 20 birds per group (Table 4.1). The birds were vaccinated

via eye drop at 1, 10 or 1 and 10 days of age (Table 4.1). Every group was subdivided in

two replicas with 10 birds per replica. The birds were kept in Horsfall units under

positive pressure during the time of the study. Two Horsfall units per group were used to

keep the replicas separated. At 27 days of age, all the birds in the groups 1 through 8

were challenged by eye drop with 0.1 ml of the Nebraska 95 isolate with a titer of $10^{6.0}$

EID$_{50}$/ ml per bird.

Vaccines. Two different vaccines were used. The commercial Massachusetts-

Connecticut vaccine (Merial-Select Laboratories Inc., Gainesville, GA) and the

one microscopic unit in the score corresponds to 11 microns of actual epithelium thickness on the slide.

Statistical analyses. The statistical analyses were performed in the GraphPad InStat Database 1. ISD for Windows using tracheal scores and epithelium thickness scores as units. The mean values of the tracheal and epithelium thickness scores were separately analyzed by the nonparametric Kruskal-Wallis Test. All the vaccinated and the positive control groups were compared with the negative control group. Significative differences in the scores were established at P values less than 0.05.

RESULTS

Virus isolation and detection. Cecal tonsil samples obtained from a five and a half week old broiler flock in California were positive for IBV after the second passage in 9-day old SPF embryos. The virus was detected by the rapid plate hemagglutination test and by RT-PCR. This isolate was further characterized by RFLP as a Nebraska 95 strain showing a different RFLP pattern when compared with known IBV patterns (Fig 4.1).

Virus titration. All commercial vaccines had very similar titers, ranging from $10^{5.1}$ EID_{50}/ ml to $10^{5.6}$ EID_{50}/ml. The Nebraska 95 IBV isolate had a titer of $10^{6.0}$ EID_{50}/ ml.

Antibody levels. IBV maternal antibody levels with a geometric mean titer (GMT) of 2397 ranging from 545 to 7636 with a 69% CV were detected by the IDEXX ELISA test. In the HI test, when the Mass 41, Conn 46 and Ark 99 antigens were used, maternal GMT's of 75, 176 and 436 were detected, respectively. A marked increase in the antibody titers, detected by the IDEXX ELISA test, was observed in all groups but the controls after challenge with the Nebraska 95 isolate, as shown in table 4.2. In the groups

vaccinated at 1, 10 and 1 and 10 days of age with the Mass-Conn vaccine, an increase in the ELISA titers from 88, 2852 and 15 to 2786, 5169 and 1971 was observed, respectively. In the groups vaccinated at 1, 10 and 1 and 10 days of age with the Mass-Ark vaccine, an increase in the ELISA titers from 68, 142 and 223 to 607, 3546 and 2817 was observed, respectively. In the group vaccinated at one-day of age with the Mass-Conn vaccine and at 10 days of age with the Mass-Ark vaccine, an increase in the ELISA titers from 771 to 3137 was observed. In contrast, the ELISA titers in the unvaccinated-challenged and unvaccinated-unchallenged control groups declined from 17 and 132 to 4 and 34, respectively. In the HI test, an increase in the antibody titers after challenge with the Nebraska 95 isolate was not detected (Table 4.3).

Virus re-isolation. The protection offered by commercial vaccines, established by the re-isolation of the virus from tracheas five days post-challenge, was complete in the Massachusetts-Arkansas vaccinated groups and in the Massachusetts-Connecticut/ Massachusetts-Arkansas vaccinated group. Complete protection was observed when the Massachusetts-Connecticut vaccine was used at 1 and 1 and 10 days of age. However, when this vaccine was used as the only vaccine at 10 days of age, partial (50%) protection was observed with the re-isolation of IBV from one of the two replicas in this group. The virus was detected by rapid plate hemagglutination and RT-PCR assays and further characterized as a Nebraska 95 strain by RFLP.

Histopathology. The lowest tracheal epithelium thickness score 4 was observed in the unvaccinated-unchallenged group, as expected (Table 4.4). In all the vaccinated groups, the lower tracheal epithelium thickness scores were observed in the groups vaccinated with the bivalent vaccines containing the Arkansas strain. The higher tracheal epithelium

thickness scores were observed in the groups vaccinated with the Massachusetts-
Connecticut vaccine, which correlates with the re-isolation of the IBV Nebraska 95 strain
from one of the replicas in the group receiving the vaccine at 10 days of age as the only
vaccine (Table 4.4). Very significant (P < 0.0017) differences in the epithelium thickness
scores were observed between the negative control group and the groups that received the
Mass-Conn vaccine at 1, 10 or 1 and 10 days of age. Extremely significant differences (P
< 0.0003) in the tracheal scores were observed between the negative control
(Unvaccinated-Unchallenged) group and the unvaccinated-challenge, the Mass-Ark at 10
days and all the Mass-Conn vaccinated groups.

DISCUSSION

The maternal antibody levels detected by the ELISA and the HI tests at one-day of age
are similar to those frequently observed in progenies derived from parents that have been
vaccinated several times with live vaccines and boosted with a killed vaccine before the
onset of egg production. By 27 days of age, the antibody titers detected by the ELISA
tests in most groups were very low or negative, which was not expected considering the
high titers of the given IBV vaccines. In the groups that received the vaccine at one day
of age, these low antibody levels could result from the partial neutralization of the
vaccine virus in the target tissues by the maternal antibodies present in the broilers at that
age, with a consequent low replication of the vaccine virus and poor stimulation of the
humoral response. However, other factors associated with the test like batch variation in
adsorption of the antigen to the solid phase material, high serum dilution and antigen
concentration should be considered. After challenge with the Nebraska 95 isolate, a

marked increased in the antibody titers was observed by the ELISA test in all groups but the controls, as expected. This increase in the antibody titers after challenge shows an antigenic stimulation in all the vaccinated groups probably due to the stimulation of memory cells. A lack of correlation between infectivity, serologic response and protection against challenge virus has been described (12). In this study, the re-isolation of the challenge virus five days after challenge from one of the replicas in the group vaccinated with the Mass-Conn vaccine at 10 days of age seems to correlate with that observation. This group exhibited the highest antibody titers pre-challenge (2852) and post-challenge (5169) when compared with the other vaccinated groups (Table 4.2). In contrast, the group that received the Mass-Ark vaccine at one-day of age exhibited very low titers pre-challenge (68) and post challenge (607) (Table 4.2). However, in this group, complete protection against the challenge virus was observed. The HI results were inconclusive. The absence of antibody titers by the HI test after challenge could be due to the short period of time between the challenge with the Nebraska 95 isolate and the last serum sampling (5 days later). No correlation between the antibody levels detected by the HI test and protection was observed. The groups vaccinated at 1, 10 or 1 and 10 days of age with the Mass-Ark vaccine exhibited very low pre-challenge and post-challenge titers. However, complete protection against the challenge virus was observed in all the groups. This could be probably due to the particular subpopulation of antibodies (hemagglutination antibodies) detected by the HI test, which are not necessarily the antibodies that neutralize the virus (8). When the protection offered by the different bivalent vaccines was compared, complete protection against the Nebraska 95 isolate was observed with the Massachusetts-Arkansas vaccine. The bivalent vaccines

Mass-Conn administered at 1 day of age and Mass-Ark at 10 days of age gave complete

protection against the Nebraska 95 challenge. When the Mass-Conn vaccine was used at

1 and 1 and 10 days of age, complete protection against the Nebraska 95 isolate was

observed. However, when it was used at 10 days of age as the only vaccine, partial

protection was observed. The lowest tracheal epithelium thickness scores were observed

in the groups in which the IBV Arkansas type was present in the bivalent vaccine. In

contrast, the groups vaccinated with the Massachusetts-Connecticut vaccine showed the

highest tracheal epithelium thickness scores of all the groups. When the statistical

analysis between the negative control and the vaccinated groups was performed, a

significant difference $(P < 0.0017)$ was found only in the groups that received the Mass-

Conn vaccine at 1, 10 or 1 and 10 days of age. When the mean tracheal scores of the

vaccinated groups were compared with the negative control group, significant differences

$(P < 0.0003)$ was observed in the Mass-Conn vaccinated groups and the group vaccinated

with the Mass-Ark vaccine at 10 days of age. Based on these observation we can say that

complete protection against the Nebraska 95 isolate was achieved when the IBV

Arkansas type was present in the bivalent vaccines. A higher tracheal epithelium

thickness score was observed in the groups receiving any of the IBV bivalent vaccines at

1 or 10 days of age when compared with the groups vaccinated at 1 and 10 days of age

(Table 4.4). This could be due to the presence of a more consistent local immunity in the

groups vaccinated twice when challenged with the Nebraska 95 isolate. From these

results, it can be concluded that virus isolation and epithelium thickness scores are useful

tools to evaluate IBV protection. The absence of histopathology lesions in the kidneys,

even in the unvaccinated-challenged group, could indicate the preference of this isolate

for the respiratory tract.

The rapid plate hemagglutination test and RT-PCR showed a solid correlation for the

IBV detection as shown by Ruano et al (9). All the positive IBV samples detected by the

RT-PCR test were detected by the rapid plate hemagglutination assay. However, even

though it has been shown that the sensitivity of the RT-PCR is higher, the use of the rapid

plate hemagglutination test for IBV detection must be considered because it is less

expensive and easier to perform.

FIGURES
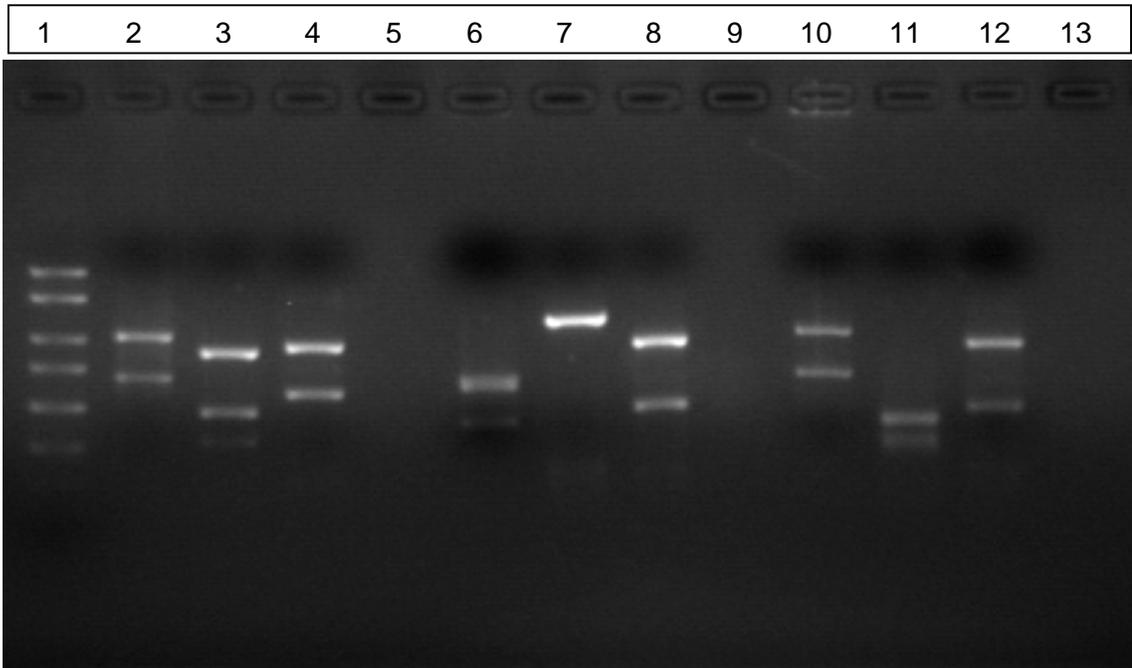
Figure 4.1. RFLP patterns of the PCR amplified S1 glycoprotein genes from IBV strains digested with the *Bst*YI, *Hae*III and *Xcm*I restriction enzymes. Lane 1 = molecular-weight marker made from a mixture of 8 defined double strand DNA markers; Lane 2, 3 and 4 = RFLP pattern of a Massachusetts IBV strain; Lane 6, 7 and 8 = RFLP pattern of an Arkansas IBV strain; Lane 10, 11 and 12 = RFLP pattern of the Nebraska 95 isolate.

TABLES

Table 4.1. Vaccines and vaccination schedules used to test the protection against challenge with
the Nebraska 95 isolate.

Group	Vaccine (1 day)	Vaccine (10 days)
1	Mass-Conn	-
2	Mass-Conn	Mass-Conn
3	-	Mass-Conn
4	Mass-Ark	-
5	Mass-Ark	Mass-Ark
6	-	Mass-Ark
7	Mass-Conn	Mass-Ark
8	Unvaccinated-Challenge	
9	Unvaccinated-Unchallenged	

Table 4.2. IBV geometric mean antibody titers detected by ELISA before and after challenge with the Nebraska 95 isolate.

Vaccines	Vaccination age (days)	Pre-Challenge (27 days)	Post-Challenge (32 days)
	1	88	2786
Mass-Conn	10	2852	5169
	1 - 10	15	1971
	1	68	607
Mass-Ark	10	142	3546
	1 - 10	223	2817
Mass-Conn/Mass-Ark	1 - 10	771	3137
Unvaccinated Challenged	-	17	4
Unvaccinated Unchallenged	-	132	34

Table 4.3. IBV geometric mean antibody titers detected by HI pre (27 days of age) and post (32 days) challenge with the Nebraska 95 isolate.

Vaccines	Age (days)	HI antigen					
		Mass 41		Conn 46		Ark 99	
		Before	After	Before	After	Before	After
	1	27	29	103	54	23	26
Mass-Conn	10	29	23	83	97	19	16
	1 - 10	15	16	123	53	16	15
	1	10	15	9	14	9	25
Mass-Ark	10	11	13	13	9	15	23
	1 - 10	12	15	10	9	11	29
Mass-Conn/Mass-Ark	1 - 10	15	11	44	47	11	18
Unvacc-Chall	-	10	4	8	9	12	22
Unvacc-Unchall	-	16	11	14	12	67	45

Table 4.4. Mean histopathology tracheal and epithelium thickness scores five days post-challenge.

Vaccines	Age (days)	Tracheal Score[A]	Epithelium thickness [B]
	1	3	10
Mass-Conn	10	3	10
	1 - 10	3	8
	1	2	7
Mass-Ark	10	3	8
	1 - 10	2	6
Mass-Conn/ Mass-Ark	1 - 10	3	7
Unvaccinated Challenged	-	3	8
Unvaccinated Unchallenged	-	2	4

[A] Tracheal scores from 1 (normal) to 4 (severe).
[B] One ephithelium thickness unit corresponds to 11 microns of thickness of the tracheal epithelium under 100X magnification.

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CHAPTER 5

PERSISTENCE OF INFECTIOUS BRONCHITIS VIRUS ARKANSAS AND MASSACHUSETTS VACCINES IN BROILERS[1]

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[1]Alvarado, I, R., P. Villegas, J. El-Attrache, M. W. Jackwood. To be submitted to Avian Diseases

SUMMARY. The persistence of infectious bronchitis virus (IBV) Arkansas and Massachusetts vaccine strains in trachea and cecal tonsils from individual commercial broilers was evaluated after vaccination by coarse spray at one-day of age with the Arkansas DPI and Massachusetts type vaccines. The persistence of IBV was determined by virus isolation, rapid plate hemagglutination assay, reverse transcriptase-polymerase chain reaction/restriction fragment length polymorphism (RT-PCR/RFLP), and *in situ* hybridization. By the RT-PCR assay, when given as a single vaccine, the Massachusetts vaccine persisted longer (up to 28 days of age) in the tracheas than the Arkansas vaccine (up to 14 days of age). However, only the Arkansas vaccine persisted in cecal tonsils (7 and 14 days of age). When the bivalent vaccine was given, the presence of the Arkansas serotype in the selected tissues was observed at later ages (up to 28 days of age) when compared with the Massachusetts serotype (up to 14 days of age). By the *in situ* hybridization assay, when given as single vaccines, the Massachusetts and Arkansas vaccines were detected in the tracheas only at 7 days of age. When the bivalent vaccine was given, the IBV persisted longer in the cecal tonsils (21 to 35 days of age) than in the trachea (7 to 21 days of age). A high correlation (97.14 %) in the detection of IBV was observed between the RT-PCR and rapid plate hemagglutination assays. However, less than 50% of the positive samples detected by these two assays were detected by the *in situ* hybridization assay. Based on this study, we can conclude that the IBV Arkansas type is more frequently observed and persists longer in the cecal tonsils than the Massachusetts type.

INTRODUCTION

Infectious bronchitis (IB) is a worldwide, acute, highly contagious disease caused by

infectious bronchitis virus, a member of the *Coronaviridae* family. Infectious bronchitis

virus (IBV) is characterized by a short incubation period, the presence of clinical signs

within 18 to 36 hours from the time of infection and high morbidity (2). The disease

usually resolves itself within two weeks. However, the presence of latent infections has

been observed in some chickens (10). In broilers, the disease is characterized by the

presence of respiratory signs with gasping, coughing, sneezing, tracheal rales and nasal

discharge. The mortality is variable depending on the virulence of the infecting serotype,

the immune status of the flock and stresses such as secondary bacterial infections and

cold (2). In layers, in addition to respiratory disease, a decrease in egg production and

egg quality is observed (6, 10). When nephropathogenic strains are involved, less clinical

respiratory disease is observed but an increase in mortality with the presence of kidney

lesions characterized by distended tubules and ureters containing uric acid crystals has

been described (3).

IBV can be isolated from different tissues such as trachea, lung, kidney, oviduct and

cecal tonsils (6). IBV can also be identified via serological techniques such as virus

neutralization, hemagglutination inhibition and identification of IBV antigens by

monoclonal antibodies. Other techniques commonly used are the rapid plate

hemagglutination assay, reverse transcriptase-polymerase chain reaction/restriction

fragment length polimorphism (RT-PCR/RFLP), and *in situ* hybridization (5, 11, 13, 16).

The existence of farms with inadequate ventilation, multiple ages and high stocking

densities can exacerbate the persistence of IB in the poultry farms. In these farms, the

horizontal transmission of the virus from flock to flock is characteristic, making it more difficult to control the disease (2). The presence of the disease can be prevented by management practices like cleaning and disinfection of the poultry houses and the use of immunization programs (2). Immunization programs are based on the use of live and inactivated vaccines. However, due to the simultaneous presence of antigenic variants in the field, the use of multiple vaccines is required (10). Live vaccines are used in broilers, layers and breeders. In layers and breeders, inactivated vaccines at point of lay are used to prevent the presentation of the disease during production and to transmit maternal antibodies from the breeders to the progeny. The presence of maternal antibodies protects the progeny against early infection of the virus and decreases the commonly observed respiratory reactions after vaccination but do not prevent the viral infection (2, 15). The presence of maternal antibodies in the trachea plays an important role in protection against IB challenge. An increase in the permeability of the blood vessels in the trachea of young chicks with the transudation of IgG antibodies from the blood as a response to the pathogenic effect of the virus has been observed (15). In chickens recovered from IB infection, other mechanisms like cytotoxic T lymphocytes, cell-mediated and antibody-mediated immunity are involved in protection (2, 4). IgG maternal antibodies have been detected in the tracheas of young chickens up to 2 weeks of age with an average half-life of 4.5 days (12). The trachea is the main target tissue for virus replication, with detection by immunohistochemestry of the viral antigen in the cytoplasm of tracheal epithelium from 16 hr to 6 days post-inoculation, with a peak of infection at 4 days post-inoculation (12).

In the field, the presence of respiratory reactions for longer periods of time after
vaccination with IBV or Newcastle disease virus (NDV) is observed probably due to the
presence of flocks with uneven levels of maternal antibodies (8, 9). These flocks come
from eggs from several breeder flocks with different ages, mixed to provide the required
number of birds to make up a broiler flock. When these birds are vaccinated at young
ages (usually during the first week of life), resistance to infection by the vaccine virus in
birds with high maternal antibody levels can be expected. Later on, when the maternal
antibody levels in these birds decline, they will become infected with the shed virus
coming from the birds that were infected with the original vaccine virus (8). Based on
these observations, continue respiratory reactions after vaccination would be favored by
the possible longer persistence of some IBV in the host.

The objective of this study was to evaluate the persistence of IBV Arkansas and
Massachusetts type vaccines in the trachea and cecal tonsils of vaccinated broilers via
detection techniques such as virus isolation, rapid plate hemagglutination assay, *in situ*
hybridization and RT-PCR/ RFLP characterization assay.

MATERIALS AND METHODS

Vaccines. Two commercial vaccines (Arkansas DPI and Massachusetts type) were
obtained from two commercial companies referred from now on as A and B (A-Ark DPI,
A-Mass, B-Ark DPI and B-Mass). These vaccines were titrated by the Reed and Müench
method (18). The vaccines were diluted in phosphate buffered saline (PBS) following the
recommendations of the companies to deliver the appropriate dose per bird. The bivalent

Mass-Ark vaccines were obtained by diluting the individual vaccines, mixing them in equal amounts before application.

Chickens. Three hundred and seventy, one-day old commercial broiler chickens obtained from a local hatchery were used. Maternal antibody levels were determined from 20 commercial broilers at one-day of age by using the hemagglutination inhibition (HI) and enzyme linked immunosorbent assay (ELISA) tests. The remaining birds were distributed in seven groups with 50 chickens per group. Every group was subdivided in two replicas with 25 birds per replica. The birds were kept in Horsfall units under positive pressure during the time of the study. Six groups were vaccinated by coarse spray at one-day of age with a commercial Preval spray gun (Precision Valve Company., Yonkers, NY). Three groups were used per company, receiving the Massachusetts, Arkansas or the bivalent Massachusetts-Arkansas vaccine. The remaining unvaccinated group was used as a negative control.

Sample processing for virus isolation. Six birds per group (three per replicate) were necropsied at 3, 7, 14, 21, 28, 35 and 42 days of age. Tracheas and cecal tonsils from every group were pulled. Half of every tissue was kept frozen at – 80 C for virus isolation and half was immersed in 10% formalin for 18 hours for virus detection by *in situ* hybridization.

The tissues were frozen and thawed three times. Small pieces of the tracheas and cecal tonsils were minced in 10 ml of a solution of 500 ml of tryptose phosphate broth (TPB) with 1% penicillin (10000 IU/ml) – streptomycin (10000 μg/ml) (Sigma Chemicals Co., St Louis, MO) and 1% amphotericin B (250 μg/ml) (Sigma Chemicals Co., St Louis, MO) and centrifuged at 2,000 rpm (7,600 g) for 10 minutes in a CRU 5000 centrifuge

(Damon/IEC, Needham Heights, MA). The supernatant was filtered through a sterile

0.22 μm polyethersulfone (PES) syringe filter (Whatman Inc., Clifton, NJ) into a sterile

vial (17).

Virus isolation and detection. Nine-day old specific pathogen free (SPF) chicken

embryos (SPAFAS Inc., Norwich, CO) were inoculated with 0.1 ml of the filtered

supernatant. Forty-eight hours after inoculation, the allantoic fluid was collected. The

presence of contaminating hemagglutinating virus such as Newcastle and Influenza was

determined by direct hemagglutination of 5% red blood cells (17). The presence of IB

vaccine virus was initially detected by a rapid plate hemagglutination assay as described

by Ruano *et al* (16), and by the amplification of the IBV S1 gene of the IBV using the

Roche Light Cycler system (Roche Diagnostics Co., Indianapolis, IN).

RT-PCR. Briefly, RNA was extracted from the allantoic fluid obtained from the

inoculated embryos following the procedures described for the Trizol LS reagent (Life

Technologies Inc., Grand Island, NY) where 250 μl of allantoic fluid was added to 750 μl

of Trizol LS reagent, vortexed and incubated for 5 min. Two hundred μl of chloroform

was added, vortexed and centrifuged at 12,000 rpm (11,750 g) for 15 min at 4 C.

Supernatant was removed and the RNA pellet was washed with ethanol. The RNA pellet

was allowed to air dry and was re-suspended in 50 μl of DNase/RNase free water and

stored at – 80 C. For the RT-PCR reaction, a commercial Roche Light Cycler-RNA

Amplification Kit SYBR Green I (Roche Diagnostics Corp., Indianapolis, IN) was used.

All the reagents except for the primers were provided with the commercial kit. Eight μl

of DNase-RNase free water, 4 μl of reaction mix (SYBR Green I, 5x), 1 μl (1.0 μM) of

forward primer (TACACTAGCCTTGCGCTAGA), 1 μl (1.0 μM) of reverse primer

(TCACTAAACACCACCAGAAC), 1.6 μl of MgCl$_2$ (25 mM) and 0.4 μl of enzyme mix were used per 4 μl of RNA sample. The final mixture was deposited in 20 μl capillaries (Roche Diagnostics Corp., Indianapolis, IN). RT was conducted for 10 min at 55 C. The PCR reaction included a denaturation period at 95 C for 30 seconds and 45 cycles of denaturation at 95 C for 0 seconds, annealing at 55 C for 10 seconds and polymerization at 72 C for 13 seconds. Melting curve analysis was performed at 65 C for 10 seconds and samples exhibiting fluorescence peaks at temperatures between 85 C and 88 C were considered positive for IBV as shown in figure 5.1. All the products obtained by using the Light Cycler were visualized in a 1.5% agarose gel with ethidium bromide to confirm the presence of the 456 base pair products (data not shown).

The presence of positive samples identified by the Light Cycler system was confirmed by amplifying the S1 gene using the Titan One Tube RT-PCR kit (Roche Diagnostics Corp., Indianapolis, IN). Further characterization of the S1 product was done using the commercial restriction enzymes *Bst*YI, *Hae*III, and *Xcm*I (New England Biolabs Inc., Beverly, MA). The different fragments from the three restriction enzymes were electrophoresed in a 1.5% agarose gel. The obtained patterns were compared with reference IBV RFLP patterns (11, 13).

In situ **hybridization.** Sections of the trachea and cecal tonsils were immersed in 10 % buffered formalin for 18 hours and embedded in paraffin. IBV RNA was extracted from commercial Massachusetts and Arkansas vaccines following the described Trizol procedure for RNA extraction (Life Technologies Inc., Grand Island, NY). For the RT-PCR reaction, a commercial Titan one tube RT-PCR kit (Roche Diagnostics Corp., Indianapolis, IN) was used. All of the reagents except the primers were provided by the

kit. The primers, designed by Falcone *et al* (5), amplified a 315 bp well-conserved region of the nucleocapsid gene. Twenty five μl of DNA-RNA free water, 2.5 μl of DTT (100 mM), 4 μl of dNTP mix (10 mM), 1 μl of (1.0 μM) forward primer (TCATGGCAAGCGGTAAGG), 1 μl of (1.0 μM) reverse primer (TTCAGGTTAGCGGCTGGTC), 1 μl of RNAse inhibitor (5 U/μl), 10 μl of 5X PCR Buffer, 2 μl of MgCl$_2$ (25 mM) and 1 μl of enzyme mix were used per 2 μl of RNA sample. RT-PCR included 1 hour of RT reaction at 60 C, heating at 94 C for 2 minutes and 35 cycles of denaturation at 94 C for 30 seconds, annealing at 55 C for 30 seconds and elongation at 68 C for 80 seconds with a final elongation step of 7 minutes at 68 C. RT-PCR products were extracted from the 1.5% agarose gel utilizing the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) following the recommendations of the manufacturer. The purified DNA products were cloned with a TA cloning kit (Invitrogen, Carlsbad, CA) following the recommendations of the manufacturer. Positive transformed colonies were selected and the DNA plasmid was purified with a QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) following the recommendations of the manufacturer. The purified pCR 2.1 plasmid containing the desired insert was cut with the *Kpn*I restriction enzyme (New England Biolabs Inc., Beverly, MA). The cut plasmid was resuspended in 50 μl of DNase/RNase free water and kept at – 20 C. For the antisense riboprobe preparation, the Dig RNA labeling (SP6/T7) kit (Roche Diagnostics Corp., Indianapolis, IN) was used. All the reagents were included in the kit. Briefly, 1 μg of cut plasmid dissolved in 13 μl of DNase/RNase free water was mixed with 2 μl of digoxigenin-labeled nucleotides, 2 μl of T7 RNA polymerase, 2 μl of transcription buffer and 1 μl of RNase inhibitor. The mixture was incubated for 2 hours at 37 C. Then, 2 μl

of RQI DNase were added to digest the DNA template and the mixture was incubated at

37 C for 20 min. To precipitate the RNA, 2 μl of 2 mM EDTA pH 8.0, 2.5 μl 4 M LiCl

and 80 μl of 100% cold ethanol were added. After incubation at 37 C for 12 hours, the

mixture was centrifuged for 20 min at 4 C. The pellet was washed with 75 μl of 70%

cold ethanol and centrifuged at 13,000 rpm (13,800 g) for 20 min at 4 C. The pellet was

dried at 30 C in a vacuum centrifuge. Finally, the pellet was resuspended in 100 μl of

water containing 1.0 μl of RNasin and incubated at 37 C for 10 min. The resuspended

antisense riboprobe was stored at – 20 C. A dot blot was done to assess the incorporation

of digoxygenin in the riboprobe using five 10-fold dilutions (10^{-1} to 10^{-5}) of a control dig-

RNA probe and our dig-labeled probe in DNase/RNase free water. A concentration of

100 pg/μl was observed.

The paraffin embedded sections were deparaffinized in Hemo-De (Fisher Scientific,

Pittsburg, PA), rehydrated in PBS with 5 mM of $MgCl_2$ and digested with 35 μg/ml of

proteinase K for 15 min at 37 C in a humid chamber. Tissues were prehybridized at 42 C

for 1 hr in a solution consisting of 50% formamide, 25% of 20X standard sodium citrate

(SSC), 5% blocking reagent (Roche Diagnostics Corp., Indianapolis, IN), 1% N-

laurylsarcosine, and 0.2% sodium dodecyl sulfate (SDS). Approximately 25 ng of

antisense riboprobe was used per slide. Sections were covered with Hybrid-Slips (Sigma

Chemicals Co., St Louis, MO) and sealed with nail hardener. Hybridization took place

overnight, in the above-mentioned prehybridization solution, at 42 C in a humid chamber.

After hybridization, samples were washed with 2X SSC with 1% SDS and then with 1X

SSC containing 0.1% SDS for 30 minutes at 50 C. Then, the samples were washed at

room temperature with decreasing concentrations of 1X and 0.1X SSC for 30 and 15

minutes respectively. Sections were blocked with 5% blocking reagent (Roche

Diagnostics Corp., Indianapolis, IN) and incubated at 37 C for 2 hours with a 1:300

dilution of anti-DIG-alkaline phosphatase (Roche Diagnostics Corp., Indianapolis, IN).

The substrate was nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate

(Roche Diagnostics Corp., Indianapolis, IN). Development progressed for 45 min.

Staining was reported as positive or negative for IBV as shown in figure 5.2. As positive

controls, infectious bronchitis virus-infected tissues were run before and at the same time

with the samples showing clearly positive reactions after 20 min.

RESULTS

Vaccine titration. Titers ranging from $10^{5.2} EID_{50}$/ml to $10^{5.8} EID_{50}$/ml were observed

in the IBV Arkansas DPI type vaccines while titers ranging from $10^{5.1} EID_{50}$/ml to $10^{5.6}$

EID_{50}/ml were observed in the IBV Massachusetts type vaccines.

Maternal antibodies. The presence of maternal antibodies at day of age was determined

by the HI and ELISA tests. By the HI test, when the Mass 41 and Ark 99 antigens were

used, geometric mean titers of 59 and 99 were detected at day of age, respectively.

However, these HI titers were highly variable ranging from 2 to 1024. By the ELISA

test, a geometric mean titer of 2844 at day of age was detected, however, a high

coefficient of variation (160.5) was observed.

Virus detection. By the RT-PCR assay, the persistence of IBV in the Massachusetts

vaccinated groups was detected from 3 to 28 days of age as shown in table 5.1. In the

trachea, IBV Massachusetts was detected up to 14 days of age in the birds that received

the vaccine from both companies. IBV Massachusetts was detected at 28 days of age in

the tracheas from the birds that received the vaccine from company A. IBV
Massachusetts was not detected in the cecal tonsils at any age. In the birds vaccinated
with the IBV Arkansas type, the persistence of the IBV was detected at 7 and 14 days of
age. In the trachea, IBV Arkansas was detected at 7 and 14 days of age in the birds that
received the vaccine from both companies. In the cecal tonsils, IBV Arkansas was
detected at 7 and 14 days of age in the birds that received the vaccine from company B
and A, respectively. In the birds vaccinated with the bivalent IBV Massachusetts-
Arkansas vaccine, the persistence of the IBV was detected from 3 to 28 days of age as
shown in table 5.2. In the trachea, IBV Massachusetts was detected at 14 days of age in
the birds that received the vaccine from both companies. In contrast, IBV Arkansas was
detected at 21 days of age in birds that received the vaccine from company A and at 7 and
21 days of age in birds that received the vaccine from company B. In cecal tonsils, IBV
Massachusetts was detected at 3, 7, and 14 days of age while IBV Arkansas was detected
at 14, 21, and 28 days of age.

In situ **hybridization.** Positive and negative controls, performed simultaneously with the
samples of interest, were constantly positive and negative, respectively. In the groups
given the single IBV Massachusetts vaccine, the presence of IBV was detected only at 7
days of age in the tracheas from the broilers that received the vaccine from company A.
In the groups given the single IBV Arkansas vaccine, the presence of IBV was detected
only at 7 days of age in tracheas from broilers that received the vaccine from both
companies. In the IBV Massachusetts-Arkansas vaccinated groups, IBV was detected in
the tracheas at 7 days of age in the birds that received the vaccine from company A and at
7, 14 and 21 days of age in the birds that received the vaccine from company B as shown

in table 5.3. In the cecal tonsils, IBV was detected at 21, 28 and 35 days of age in the

birds that received the vaccine from company B. From the 23 samples positive to IBV by

RT-PCR, only 8 samples were positive by in situ hybridization. However, in situ

hybridization was the only assay that detected the presence of IBV at 35 days of age as

shown in table 5.3.

DISCUSSION

The maternal antibody levels detected at one-day of age by the HI and ELISA tests

correlate with the antibody levels normally observed in broiler flocks in the field. The

inconsistency observed can be associated with individual variability in the transmission

of maternal antibodies from the breeders to the progeny or to the presence of commercial

broilers from different breeder flocks with different ages and different immune status.

The rapid plate hemagglutination and RT-PCR assays showed a 97.14% correlation for

the detection of the IBV. From a total of 35 positive samples detected by RT-PCR,

thirty-four (97.14%) were positive by rapid plate hemagglutination assay. These results

agree with the observations made by Ruano *et al* (16). In this study, up to three

consecutive passages in 9-day old SPF chicken embryos were necessary with a 67%,

14.7% and 17.64% of the samples detected in the first, second and third passage,

respectively.

In the Mass-Ark vaccinated groups, the presence of mixed IBV Arkansas and

Massachusetts types was expected, however, only one individual serotype was observed

after PCR/RFLP analysis. The presence of the Arkansas serotype in the selected tissues

was observed at 21 and 28 days of age when compared with the Massachusetts serotype,

which was observed at 3, 7 and 14 days of age. The longer persistence of the IBV

Arkansas type in the cecal tonsils could be due to a higher tropism for this organ when

compared with the Massachusetts type. In the cecal tonsils, the IBV Arkansas type

would avoid the presence of local immunity observe in the trachea persisting longer in

the host. The longer persistence of the Arkansas virus in cecal tonsils from birds

vaccinated with the bivalent vaccine seems to correlate with such observation.

IBV was not detected in any group by rapid plate hemagglutination or RT-PCR assays

beyond 28 days of age probably due to the development of immunity after vaccination at

one-day of age.

To increase the sensitivity of the *in situ* hybridization assay, several characteristics were

considered when selecting the riboprobe. A riboprobe complementary to a region located

in the nucleocapsid gene was selected. In addition to its very conserved nucleotide

sequence, the nucleocapsid gene sequence is located at the 3' end of the genomic and

subgenomic mRNAs with a higher presence of the sequence target (7, 14). Due to the

presence 10 to 100 fold more mRNA positive sense than negative sense during the

replication of IBV (7), a negative riboprobe was selected. In addition, its small size (315

bp) would decrease the risk of intra-strand folds or loops formation and would facilitate

the penetration into cells having better access to the target sequences (1). In spite of all

the characteristics present in the riboprobe, the sensitivity of the *in situ* hybridization

assay was lower when compared with the RT-PCR and rapid plate hemagglutination

assays. Less than 50% of the positive samples detected by RT-PCR and rapid plate

hemagglutination assays were detected by the *in situ* hybridization assay probably due to

the low presence of genomic and sub-genomic RNA in the selected tissues and to the

difficulty to work with RNA viruses due to the constant degradation of the viral RNA by RNAse A. However, the presence of IBV was detected by this technique at 35 days of age. The failure in the detection of IBV by rapid plate hemagglutination and RT-PCR assays at this age could have been due to the absence of infective virus in the tissues with the consequent lack of replication in the allantoic fluid of SPF chicken embryos. Some factors like distinction between specific and background staining, time required by the proteinase K to digest the selected tissues, and probe concentration could be re-evaluated in order to increase the sensitivity of the *in situ* hybridization assay.

FIGURES
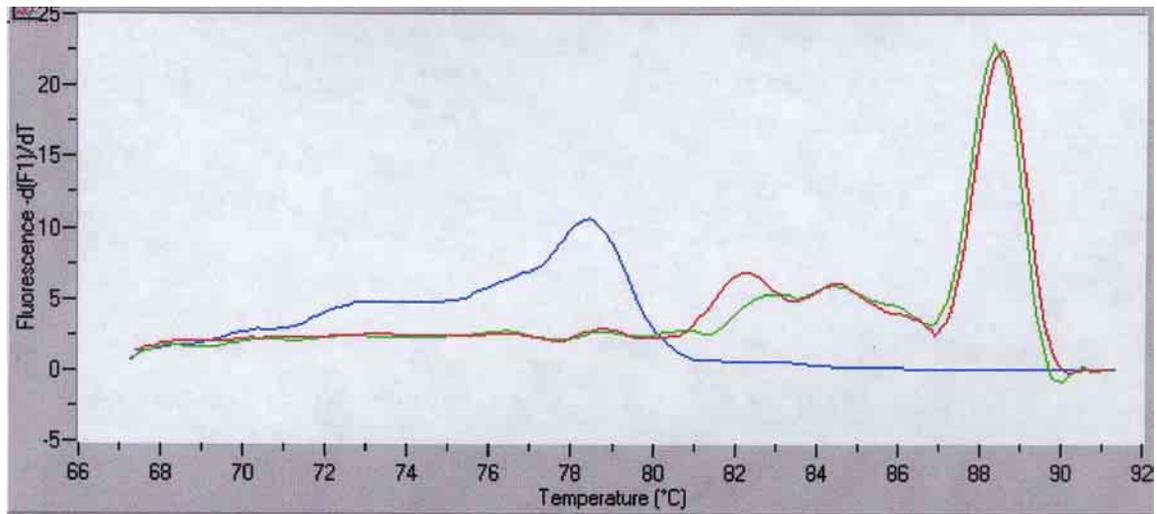
Figure 5.1. Melting curve analysis performed at 65 C for 10 seconds in one negative (blue) and two positive (red and green) IBV controls. Positive samples exhibited peaks at temperatures between 85 and 88 C.
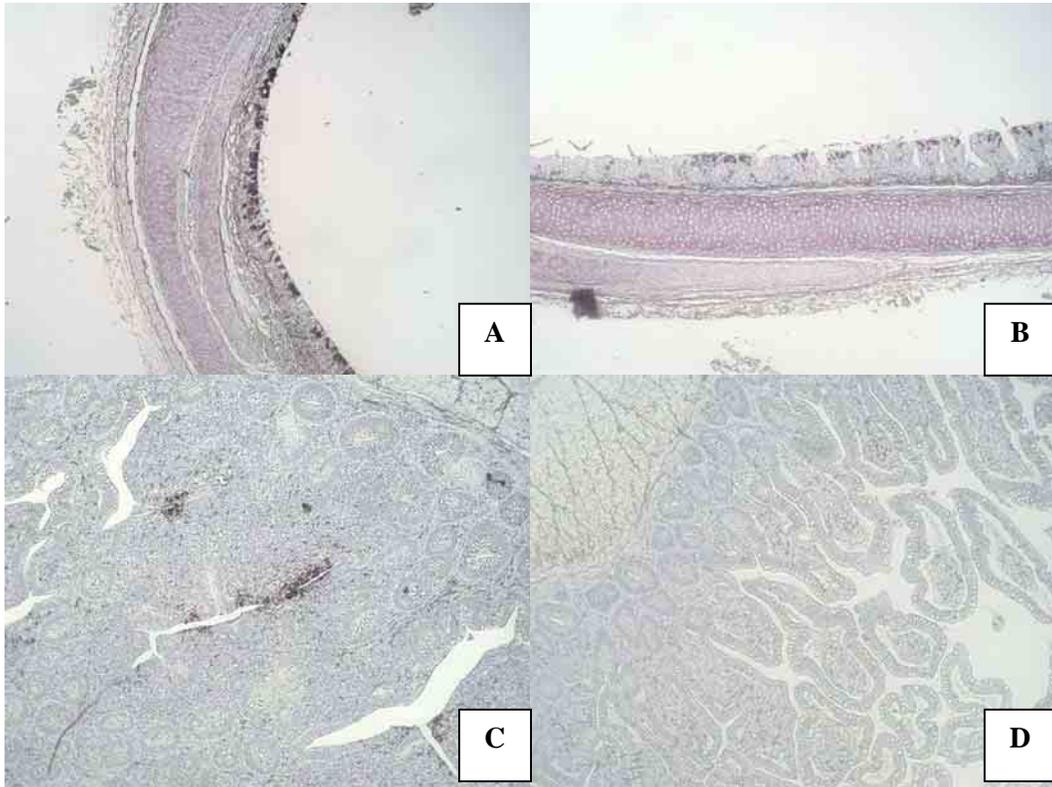
Figure 5.2. Detection of IBV by *in situ* hybridization assay in histopathology sections of trachea and cecal tonsils. The presence of IBV was detected in the epithelial cells in trachea (sections A and B) and in epithelial cells in cecal tonsils (section C). Section D corresponds to cecal tonsils from a negative control group with no presence of the IBV.

Table 5.1. Persistence of IBV detected by RT-PCR in trachea and cecal tonsils at different ages after vaccination of one-day of age commercial broilers with commercial IBV Massachusetts or IBV Arkansas vaccines.

Vaccine	Company	Tissue	3	7	14	21	28	35
Mass	A	T[1]	-	+	+	-	+	-
		C T[2]	-	-	-	-	-	-
	B	T	+	+	+	-	-	-
		C T	-	-	-	-	-	-
Ark	A	T	-	+	+	-	-	-
		C T	-	-	+	-	-	-
	B	T	-	+	+	-	-	-
		C T	-	+	-	-	-	-

Sampling age[3] (days)

[1] T = Trachea.
[2] C T = Cecal tonsils.
[3] At every sampling age, tracheal and cecal tonsils tissues from three birds were pulled.

Table 5.2. Persistence of IBV detected by RT-PCR/RFLP in trachea and cecal tonsils at different ages after vaccination of one-day old commercial broilers with the IBV Massachusetts-Arkansas vaccines and non-vaccinated controls.

Company	Tissue	Sampling age[3] (days)					
		3	7	14	21	28	35
A	T[1]	-	-	Mass	Ark	-	-
	C T[2]	Mass	Mass	Mass	-	-	-
B	T	-	Ark	Mass	Ark	-	-
	C T	-	-	Ark	Ark	Ark	-
Controls	T	-	-	-	-	-	-
	C T	-	-	-	-	-	-

[1] T = Trachea.
[2] C T = Cecal Tonsils.
[3] At every sampling age, tracheal and cecal tonsil tissues from three birds were pulled.

Table 5.3. Persistence of IBV detected by *in situ* hybridization in tracheal and cecal tonsil tissues at different sampling periods in the Mass-Ark vaccinated groups.

Company	Tissue	Age at sampling[3] (days)						
		3	7	14	21	28	35	42
A	T[1]	-	+	-	-	-	-	-
	C T[2]	-	-	-	-	-	-	-
B	T	-	+	+	+	-	-	-
	C T	-	-	-	+	+	+	-

[1] T = Trachea.
[2] C T = Cecal Tonsils.
[3] At every sampling age, tracheal and cecal tonsils tissues from three birds were pulled.

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CHAPTER 6

DISCUSSION

Protection conferred by commercial infectious bronchitis (IB) vaccines against the Nebraska 95 (California 99) isolate

In spite of the development of live and inactivated vaccines, infectious bronchitis disease (IB) continues to be a major worldwide economic disease in the poultry industry. The continual presence of IB in poultry houses is due to the constant appearance of new IBV variant strains, which differ antigenically and break through the immunity induced by vaccines. The emergence of the new IBV variants can often be associated with common management practices observed in the field in order to maximize production, while keeping the costs as low as possible. For example, IBV variant serotypes have been isolated mainly from layers probably due to the high number of layers housed in close proximity on large complexes, longer life spans and the presence of multi-age flocks with the constant introduction of pullets exhibiting different levels of antibodies with different specificities for different antigens. As a consequence, a strong selective pressure in the IBV to mutate in order to escape the immune defense system is observed.

The use of live and inactivated vaccines has been the basis of IB disease control. However, the presence of cross-protection produced by some IBV serotypes against antigenically unrelated strains (variants) is unpredictable. For this reason, a constant determination of the protection afforded by vaccine strains against challenge with field isolates must be assessed. In this study, the protection offered by currently used commercial vaccines against a new variant IBV known as Nebraska 95 (California 99) was established. This new isolate has been frequently isolated from vaccinated broiler

and layer flocks exhibiting respiratory distress. After RFLP analysis, this isolate

exhibited a different pattern when compared with Massachusetts and Arkansas IBV

patterns. The evaluation of protection offered by commercial vaccines against the IBV

Nebraska 95 isolate was determined by following the parameters established by the Code

of Federal Regulations (CFR). Complete protection was determined by the failure to re-

isolate the challenge virus from tracheas five days after challenge. Other parameters like

tracheal and epithelium thickness scores were used to complement such evaluation. The

maternal antibody titers present at one day of age were similar to those frequently

observed in commercial broiler flocks at that age. The presence of low antibody titers

before challenge (27 days of age) was not expected considering the titers of the IBV

vaccines used ($10^{5.1}$ to $10^{5.6}$ EID_{50}/ml). These low titers could be the consequence of

partial neutralization of the vaccine virus at day of age by the maternal antibodies present

in the broiler. However, other factors related with the test, like batch variation in the

adsorption of the antigen to the solid phase, high serum dilution and antigen

concentration should be considered. However, the increase in the antibody titers detected

by the ELISA test after challenge shows an antigenic stimulation of memory cells. Lack

of correlation between the antibody titers and protection against the challenge virus was

observed. This statement is based on the re-isolation of the IBV from the tracheas in the

group that received the Mass-Conn vaccine at 10 days of age, which exhibited high titers

before (2852) and after challenge (5169). No correlation between the antibody levels

detected by the HI test and protection offered by the commercial vaccines against the

IBV Nebraska 95 isolate was observed. For example, low HI titers were observed in all

the Massachusetts-Arkansas vaccinated groups before and after challenge with the

Nebraska 95 isolate. However, based on the failure to re-isolate this virus from the

tracheas 5 days after challenge, complete protection was present. These results could be

explained by the fact that the HI test detects a particular subpopulation of antibodies, the

hemagglutinating antibodies, which do not completely neutralize the virus (49). The

Massachusetts-Arkansas vaccine, when used at 1, 10 or 1 and 10 days of age, provided

complete protection against the Nebraska 95 isolate as shown by the failure to re-isolate

the Nebraska 95 isolate from tracheas five days after challenge, complemented with the

presence of lower epithelium thickness scores when compared with the unvaccinated

challenged group. The Massachusetts-Connecticut vaccine, when used at 1 day of age

followed by the Massachusetts-Arkansas vaccine, when used at 10 days of age, offered

complete protection against the Nebraska 95 isolate. These groups exhibited similar

tracheal and epithelium thickness scores as the Massachusetts-Arkansas vaccinate groups.

The Massachusetts-Connecticut vaccine, when used at 1, and 1 and 10 days of age,

conferred adequate protection against the Nebraska 95 isolate. However, when this

vaccine was used as the only vaccine at 10 days of age, partial protection was observed

with the re-isolation of the challenge virus in one of the replicas from this group. The

groups vaccinated with the Massachusetts-Connecticut vaccine exhibited the highest

tracheal and epithelium thickness scores of all groups including the unvaccinated-

challenged control group. When the statistical analysis was performed, a significant

difference in the epithelium thickness ($P < 0.0017$) and tracheal ($P < 0.003$) scores was

constantly observed only in the groups that received the Mass-Conn vaccine at 1, 10 or 1

and 10 days of age when compared with the negative control (unvaccinated-

unchallenged) group. Based on the obtained data, the presence of the Arkansas type virus

in the bivalent vaccines seems to offer a better protection against the Nebraska 95 isolate.

High correlation (100%) between the RT-PCR and rapid plate hemagglutination assays

was observed. The use of the rapid plate hemagglutination assay to diagnose the

presence of IBV must be considered due to its lower cost and easiness to perform when

compared with the RT-PCR assay. No histopathological lesions in the kidneys were

observed in any group, which could indicate the preference of this isolate for the

respiratory tract.

Persistence of IBV Arkansas and Massachusetts vaccines in broilers

Based on the results obtained in the first study, the presence of the IBV Arkansas type in

bivalent vaccines seems to offer broader protection against antigenically unrelated

serotypes (heterologous protection) and it is frequently included in vaccination programs

in the field. During the years 1999 and 2000, the most frequent isolated IBV in the state

of Georgia was the Arkansas type (66%), followed by the Massachusetts type (11%)

(Zavala, D. L. Personal communication). From all the IBV isolates from adult birds,

80% corresponded to the Arkansas type (Zavala, D. L. Personal communication).

Similar results have been observed in other states. In the state of Alabama, during the

same period of time, a higher incidence of the Arkansas type has been reported, followed

by the GA 98 and Massachusetts types (Hoerr, F. Personal communication). These

observations elicited an interesting question regarding the persistence of the Arkansas

type in the chickens for longer periods of time when compared with other serotypes. In

this study, the persistence of the IBV Arkansas and Massachusetts serotypes in trachea

and cecal tonsils from individual commercial broilers was evaluated. The persistence of

the IBV in the selected tissues was established by several approaches, like rapid plate

hemagglutination assay, RT-PCR/RFLP and *in situ* hybridization. The maternal antibody

levels detected in the commercial broilers at day of age by the HI and ELISA tests

correlated with the antibody levels normally observed in progenies from broiler breeders

exposed to several live vaccines and boosted with inactivated vaccines before the onset of

production. However, lack of uniformity in the antibody levels was observed. This lack

of uniformity could be explained by some common management practices like mixing

fertile eggs from different broiler breeder flocks with different ages to complete a broiler

flock. Even in broiler flocks coming from a single well-vaccinated breeder flock, high

variation in the maternal antibody levels is frequently observed due to the individual

variation in the transmision of the antibodies to the progeny. Due to this individual

variation, the vaccine virus can invade and replicate faster in those birds with low

maternal antibodies when compared with birds with high maternal antibodies, which

reject the vaccine virus. In this study, the presence of the vaccine virus at 3 days of age

was detected in cecal tonsils from the group that received the Massachusetts-Arkansas

vaccine from company A and in the tracheas from the group that received the

Massachusetts vaccine from company B. The low re-isolation rate of the IBV at this age

could be due to the presence of high maternal antibodies at day of age, with the

consequent neutralization and low replication of the IBV. The presence of positive

samples was more frequently observed from 7 days of age up to 28 days of age. The

presence of IBV could not be detected at 35 and 42 days of age by the rapid plate

hemagglutination and RT-PCR assays due probably due to the development of immunity

against the IBV infection. By the RT-PCR assay, the presence of the IBV Arkansas type

in the Arkansas vaccinated groups was detected only at 7 and 14 days of age. In these

groups, the presence of IBV was more consistent in tracheas than in cecal tonsils,

probably due to the presence of more infective virus in the trachea at those ages. In the

Massachusetts vaccinated groups, IBV was constantly detected up to 14 days in birds that

received the vaccine from company B and at 7, 14 and 28 days of age in birds that

received the vaccine from company A. No IBV was detected in cecal tonsils from these

groups at any age. In the Massachusetts-Arkansas vaccinated groups, the presence of

mixed populations of virus was expected with the presence of mixed restriction fragments

patterns by RFLP. However, the presence of restriction patterns from one of the two

viruses present in the bivalent vaccine was observed. In these groups, IBV

Massachusetts type was detected more consistently at early ages while the IBV Arkansas

type was detected more consistently at late ages. These results seem to correlate with the

higher isolation (80%) of the IBV Arkansas type in birds at late ages (Zavala, D. L.

Personal communication). As in the first study, a high correlation (97.14%) between the

rapid plate hemagglutination and the RT-PCR assays for IBV detection was observed.

Even though several consecutive passages in SPF chicken embryos are required for the

detection of the IBV by the rapid plate hemagglutination assay, the lower cost and lower

training capacity required to develop this assay make it an important tool for IBV

detection. In order to increase the sensitivity of the in situ hybridization assay, a

riboprobe with some specifications was designed. However, this technique exhibited

lower sensitivity when compared with RT-PCR and rapid plate hemagglutination assays

for IBV detection. This observation is based on the detection of less than 50% of the

positive samples detected by the RT-PCR and rapid plate hemagglutination assays.

However, the presence of IBV was detected by *in situ* hybridization at 35 days of age in

cecal tonsils of the group that received the Massachusetts-Arkansas vaccine from company B. The fact that this IBV could not be detected by the rapid plate hemagglutination and RT-PCR assays could be related to the absence of infective virus in the cecal tonsils at that age, with the consequent lack of replication in the allantoic fluid of the inoculated SPF chicken embryos. Non-infective virus would lose the capacity to establish in the A. fluid with a consequent degradation of its genome by the constant presence of RNases. The lower sensitivity of the *in situ* hybridization assay could be due to the presence of low genomic and sub-genomic RNA in the selected tissues and to the difficulty to work with labile RNA viruses due to the constant presence of RNAse A. The increase in the sensitivity of the *in situ* hybridization assay should be directed to standardize some variable factors like the distinction between specific and background staining, standardization of the time digestion of the selected tissue by the proteinase K, riboprobe concentration and hybridization temperature.

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