INFECTIOUS BRONCHITIS VIRUS: IN VIVO AND IN VITRO METHODS OF ATTENUATION, AND MOLECULAR CHARACTERIZATION OF FIELD STRAINS

by

IVAN R. ALVARADO

(Under the Direction of PEDRO VILLEGAS)

ABSTRACT

Infectious bronchitis (IB) is a worldwide respiratory disease of major economic importance associated with losses from production inefficiencies and mortality. Live and inactivated oil emulsion vaccines have been used in IB immunization programs. Although commercial live vaccines have been attenuated, respiratory reactions are commonly observed after vaccination. Replication of infectious bronchitis virus (IBV) in several tissues causes particular lesions. However, in the intestines, viral replication has not been associated with clinical manifestations or microscopic changes. Several IBV strains have been passaged in the intestinal tract using developed *in vivo* and *in vitro* systems to decrease not only their presence, but also their effects on the upper respiratory tract while still causing no lesions on the intestinal tract. One of the intestine passaged IBV strains, an Arkansas serotype, exhibited a lower presence and effect on the tracheal epithelium than an attenuated Arkansas vaccine. The milder lesions produced by the intestine passaged IBV strain could decrease the invasion and multiplication of secondary pathogens constantly present in chicken houses, decreasing their

negative economic impact. The use of the highly attenuated IP-Ark 1 strain in vaccination programs should be considered.

The development of vaccination programs is based on the determination of IBV strains causing the disease in the field. However, the constant presence of new IBV serotypes or variants complicates the control of the disease. In 2003, the presence of respiratory disease with high morbidity and mortality in commercial layers and broilers intensively vaccinated with the Massachusetts 41 strain was observed in Colombia. The presence of IBV in phenol-treated allantoic fluid samples obtained from broilers and commercial layers was initially determined by amplification of the N gene by reverse transcriptase-polymerase chain reaction (RT-PCR). Specific primers were designed to amplify the S1 gene of some of the Colombian IBV isolates. Further molecular characterization by RT-PCR followed by nucleotide sequencing of the HVR 1 of the S1 glycoprotein gene was performed. This study revealed for the first time the presence of four indigenous genotype clusters genetically distinct to the Mass 41 strains. This finding might indicate a low or no protection offered by the currently used Mass 41 vaccine against field isolates.

INDEX WORDS: Infectious bronchitis virus, *In vivo* intestine passages, *In vitro* intestine passages, Effect evaluation, Molecular characterization of IBV, Colombian variants, IBV hypervariable regions.

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M.S., The University of Georgia, 1994

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2004

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DEDICATION

To my parents, Alicia and Marco Tulio, and sisters, Sandra and Silvia, for their continue encouragement and love during these years that made real what once was a dream.

ACKNOWLEDGEMENTS

I want to specially extend my gratitude to my major professor, Dr. Pedro Villegas, for his priceless friendship, support, guidance and of course patience during these years.

I want to thank my committee members Drs. Charles Hofacre, Mark Jackwood, Maricarmen García and Daniel King as well as Drs. John Glisson and Hector Cervantes for their friendship and invaluable advices.

Also, I would like to express my appreciation to Drs. Carlos Estevez, Alejandro Banda and John El-Attrache for their fun-loving attitude and camaraderie along with Dr. Villegas' complicity makes our lab such a special place.

Thanks are also extended to my fellows in the Avian Medicine program for allowing me to be around during these five years without any complaint.

Finally, I must acknowledge Angela Maria for her affection and support as well as Scott Callison, Linda Purvis, Hugo Moscoso, Deborah Hilt and Silva Riblet for their authentic companionship.

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CHAPTER 1

INTRODUCTION

Infectious bronchitis (IB) is a highly contagious respiratory disease of chickens (4). Replication of the infectious bronchitis virus (IBV) causes characteristic, but not pathognomonic respiratory signs such as gasping, coughing, tracheal rales, and nasal discharge (9). Occasionally, puffy, inflamed eyes and swollen sinuses may be seen (3, 20).

The pathogenicity of IBV for different tissues has been described (9). In the respiratory tract, IBV causes decilliation and desquamation of the tracheal epithelium, resulting in a highly contagious respiratory disease (17, 21). In the kidneys, nephropathogenic strains induce clinical nephritis, gross and histological kidney lesions with mortality varying between 5% and 90% (13). In the oviduct, IBV replication has been associated with a decline in the internal and external quality of the eggs and a decrease in egg production (5, 16). Recently, IBV has been associated with a particular disease in the proventriculus (22). Infected birds appeared depressed with ruffled feathers and wet droppings with white and yellow milky feces, related with an infected alimentary tract. In the intestines, IBV replication has been observed in the tissues of the lower gut in cells resembling histiocytes and lymphoid cells in the cecal tonsils (19). The presence of the virus has also been demonstrated by immunofluorescent assay (IFA) in apical epithelial cells of the villi in ileum and rectum (1, 8). The ability of the IBV strains to survive in the presence of low pH, digestive enzymes and bile salts may also be relevant of enteric

replication (6, 18). Even though IBV has shown a wide tropism for gut tissues, no clinical manifestations or microscopic changes have been reported (9).

To control the disease, live and inactivated oil emulsion vaccines have been used. Live vaccines have been attenuated by serial passages in chicken embryos (7, 10, 14). However, live IBV vaccines have the potential to cause moderate to severe disease in susceptible chickens under certain circumstances (15). Viral damage to the respiratory tissues following vaccination may predispose young chickens to the invasion and multiplication of secondary bacterial pathogens like *E. coli*, enhancing the severity and duration of respiratory reactions, leading to death or lesions in surviving chickens (2, 9, 11). Based on the absence of clinical manifestations or histological changes after viral replication in the intestinal tissue, we hypothesized that intestine passaged IBV strains might exhibit a milder effect on the upper respiratory tract with lower respiratory reactions. Milder lesions in the tracheal epithelium would also decrease the replication of secondary bacteria and their negative economic impact. Initially, we developed an *in vivo* system to passage selected IBV strains in the intestinal tissue of specific pathogen free (SPF) chickens. After several *in vivo* passages, the effect of one of the intestine passaged IBV strains (IP Ark-1) on the upper respiratory tract was evaluated. Later, additional *in vitro* passages were performed in an intestinal organ culture system we developed. After several *in vitro* passages, the effect of two of the intestine passaged IBV strains (IP Ark-1 and IP Mass 41) on the upper respiratory tract was also evaluated.

IBV is characterized by the constant emergence or introduction of new antigenic types as the result of insertions, deletions, recombinations and point mutations. The highly transmissible nature of the disease and the presence of multiple serotypes and genotypes have complicated and increased the cost regarding its prophylaxis. The constant evolution of the virus has been

observed mainly in layers due to common management practices like high densities and multi-age complexes (12). 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 (12).

Severe respiratory conditions caused by IBV are frequently observed in many countries, including Colombia. In Colombia, Massachusetts 41 live vaccines are currently used alone or in association with inactivated vaccines in immunization programs. However, the presence of disease in vaccinated chickens has been reported. In layers, the disease has been associated with morbidity around 2% and weekly mortality of 12% while in broilers, morbidity between 10% and 80% with weekly mortality between 4.8% and 35% have been observed. In an attempt to evaluate and characterized the IBV strains present in Colombia, several IBV field strains were isolated from problem farms. By molecular characterization, the relationship between the IBV field isolates and the Massachusetts serotype and other reference strains was established.

References

1. Ambali, A. G., and R. C. Jones. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis. 34:809-817. 1990.

2. Avellaneda, G. E., P. Villegas, M. W. Jackwood, and D. J. King. In vivo evaluation of the pathogenicity of field isolates of infectious bronchitis virus. Avian Dis. 38:589-597. 1994.

3. Capua, I., R. E. Gough, M. Mancini, C. Casaccia, and C. Weiss. A 'novel' infectious bronchitis strain infecting broiler chickens in Italy. J. Vet. Med. Ser. B. 41:83-89. 1994.

4. Cavanagh, D., and S. Naqi. Infectious Bronchitis. In: Diseases of Poultry, 11th ed, Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa. pp. 101-119. 2003.

5. Cook, J., and M. B. Huggins. Newly isolated serotypes of infectious bronchitis virus: Their role in disease. Avian Path. 15:129-138. 1986.

6. Cowen, B. S., and S. B. Hitchner. PH stability studies with avian infectious bronchitis virus (Coronavirus) strains. J. Virol. 15:430-432. 1975.

7. Crinion, R. A., and M. S. Hofstad. Pathogenicity of two embryo-passage levels of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis. 16:967-973. 1972.

8. Dhinakar Raj, G., and R. C. Jones. Immunopathogenesis of infection in SPF chicks and commercial broilers of a variant infectious bronchitis virus of economic importance. Avian Path. 25:481-501. 1996.

9. Dhinakar Raj, G., and R. C. Jones. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Path. 26:677-706. 1997.

10. Gelb, J., Jr., and S. S. Cloud. Effect of serial embryo passage of an Arkansas-type avian infectious bronchitis virus isolate on clinical response, virus recovery, and immunity. Avian Dis. 27:679-687. 1983.

11. Gelb, J., Jr., R. L. Lunt, A. L. Metz, and P. A. Fries. Attenuation of avian infectious bronchitis virus by cold-adaptation. Avian Dis. 35:847-853. 1991.

12. Gelb, J., Jr., J. B. Wolff, and C. A. Moran. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Dis. 35:82-87. 1991.

13. Ignjatovic, J., D. F. Ashton, R. L. Reece, P. C. Scott, and P. Hooper. Pathogenicity of Australian strains of avian infectious bronchitis virus. J. Comp. Path. 126:115-123. 2002.

14. Larose, R. N., and H. van Roekel. The effects of rapid embryo passage upon the infectious bronchitis virus. Avian Dis. 5:157-168. 1961.

15. MacDonald, J. W., and D. A. McMartin. Observations on the effects of the H52 and H120 vaccine strains of the infectious bronchitis virus in the domestic fowl. Avian Path. 5:157-173. 1976.

16. McDougall, J. S. Infectious bronchitis in laying fowls: Its effect upon egg production and subsequent egg quality. Vet. Rec. 83:84-86. 1968.

17. Nakamura, K., J. Cook, K. Otsuki, M. B. Huggins, and J. A. Frazier. Comparative study of respiratory lesions in two chicken lines of different susceptibility, infected with infectious bronchitis virus: Histology, ultrastructure and immunochemestry. Avian Path. 20:245-261. 1991.

18. Otsuki, K., H. Yamamoto, and M. Tsubokura. Studies on avian infectious bronchitis virus (IBV). I. Resistance of IBV to chemical and physical treatments. Arch. Virol. 60:25-32. 1979.

19. Owen, R. L., B. S. Cowen, A. L. Hattel, S. Naqi, and R. A. Wilson. Detection of viral antigen following exposure of one-day old chickens to the Holland-52 strain of infectious bronchitis virus. Avian Path. 20:663-673. 1991.

20. Parsons, D., M. M. Ellis, D. Cavanagh, and J. K. Cook. Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet. Rec. 131:408-411. 1992.

21. Purcell, D. A., V. L. Tham, and P. G. Surman. The histopathology of infectious bronchitis in fowls infected with a nephrotropic "T" strain of virus. Aust. Vet. J. 52:85-91. 1976.

22. Yu, L., Y. Jiang, S. Low, Z. Wang, S. J. Nam, W. Liu, and J. Kwang. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis. 45:416-424. 2001.

CHAPTER 2

LITERATURE REVIEW

Introduction

Infectious Bronchitis virus (IBV) is a major respiratory virus of chickens (*Gallus gallus domesticus*), as it is endemic in probably all countries that raise chickens (39). Its host range is considered to be limited to the chicken (45), although genetically very similar coronaviruses cause enteric disease in turkeys (116), and respiratory and kidney disease in pheasants (180). IBV occurs globally, both in chickens kept on a large and a small scale (281). Infectious bronchitis (IB) is of major economic importance because it is a cause of poor weight gain and feed efficiency, a component of mixed infections that produce airsacculitis which may result in condemnations of broilers at processing and cause a decline in egg-production and egg-quality (45). The highly transmissible nature of the disease and the presence of multiple serotypes and genotypes have complicated and increased the cost regarding prophylaxis (39). Losses from production inefficiencies are usually of greater concern than losses from mortality, which may be directly caused by the viral infection. Mortality in broilers is economically significant with peaks between 5 to 6 weeks, usually caused by secondary bacterial infection (45).

History

IB 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 (239). However, the nature of the infectious agent was not determined at that time (94). The disease was reported again in 1933 by Bushnell and Brandly (31), who established that the causative agent was a virus due to the transmission of the disease from Berkefeld-filtered material. Later, the possibility of infection by infectious laryngotracheitis virus was considered (94). In 1936, Beach and Schalm (16), proved by cross-immunity that IBV was distinct from infectious laryngotracheitis virus (ILTV) and infectious coryza produced by *Haemophilus gallinarum*. In 1937, Beaudette and Hudson (17), propagated the virus in chicken embryos by the chorioallantoic route of inoculation, becoming more lethal after continued passages. In 1941, Delaplane and Stuart (84), 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* (264), 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 lesions (94). In 1950, Hofstad and Kenzy (125), found that chicks hatched from recovered hens could still be susceptible to IB in the presence of maternal antibodies. In the 1940s, van Roeckel *et al* (263, 265), 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 basis of actual immunization programs.

Taxonomy

Comparative analysis of selected properties have been used to organize all diversity of viruses within a hierarchical system with the order, family (subfamily), genus and species ranks. IBV belongs to the family *Coronaviridae* in the order *Nidovirales,* which also includes the morphologically different *Roniviridae* and *Arteriviridae* families (67, 92). The *Coronaviridae* family is formed by the genera *Coronavirus* and *Torovirus* (92). Using natural host, genetic and antigenic criteria, virus species in the genus *Coronavirus* have been organized into 3 groups with avian infectious bronchitis virus belonging to the third group (92). Group 1 includes porcine *Transmissible gastroenteritis virus* (TGEV), *Feline coronavirus* (FCoV), *Canine coronavirus* (CCoV), *Human coronavirus* 229E (HCoV-229E) and *Porcine epidemic diarrhea virus* (PEDV). Group 2 members are the *Murine hepatitis coronavirus* (MHV), *Bovine coronavirus* (BCoV), *Human coronavirus* OC43 (HCoV-OC43), *Porcine hemagglutinating encephalomyelitis virus* (HEV), *Rat coronavirus* (RtCoV), and *Equine coronavirus* (ECoV). Group 3 also includes the *Turkey coronavirus* (TCoV) and *Pheasant coronavirus* (109). Previous studies indicated that TCoV was closely related to Bovine coronavirus and other group 2 mammalian coronaviruses. However, antigenic and genomic similarities between TCoV and IBV, and the determination that the host range of TCoV includes chickens, led to the suggestion that TCoV might be a variant of IBV (117). Based on the sequence analysis, the extent of the differences between these two viruses are similar to differences between IBV strains. In addition, their M and N protein sequences exhibit identities greater than 90%. However, TCoV and IBV may be distinguished based on a one-way antigenic relationship (no recognition to each other by specific monoclonal antibodies), and *in vitro* growth characteristics. IBV has been propagated in allantoic sac/membranes of embryonated chicken eggs and cell cultures while the TCoV does not share

these characteristics (116). Also, TCoV is associated with enteric disease and growth in the bursa of Fabricius (14, 110, 206), while IBV is largely associated with respiratory disease and reduced egg-laying performance (45). Pheasant coronaviruses have been associated with both respiratory and kidney disease in the field (113, 180), in addition to egg production problems. Pheasant coronaviruses are located in group 3 based on the same S-3-M-5-N-3' UTR gene order, which is different than mammalian coronaviruses (92, 168).

A change in the current taxonomic classification in the family *Coronaviridae*, based on the analysis of the structural proteins N, M, E and S and two additional proteins, the RNA-dependent RNA polymerase and RNA helicase, has been suggested (109). The re-definition suggests the inclusion of the *Coronavirus* and *Torovirus* genera as two subfamilies within the *Coronaviridae* family or two families within the *Nidovirales* order with the change of the three informal *Coronavirus* groups into three genera within the *Coronaviridae* family (109).

The virus

IBV is a non-segmented, single stranded, positive sense RNA virus with an envelope of approximately 120 nm in diameter (45). The 27.6-kb genome length (mRNA1) is composed of two large overlapping reading frames (ORFs) 1a and 1b replicase genes located at the 5' unique region. It encodes a 441-kDa 1a polyprotein and a 1a/1b fusion polyprotein of 741 kDa by a frameshifting mechanism (22, 23, 27, 169). The polyproteins are subsequently processed into at least 10 nonstructural proteins by papain-like and 3C-like proteinases (179, 213). These mature and intermediate products are involved in the genomic and subgenomic RNA synthesis (212).

The four structural proteins, spike (S), envelope (E), membrane (M) and nucleocapsid (N), are encoded by subgenomic RNAs 2, 3, 4 and 6, respectively (169).

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 the induction of neutralizing antibodies (169). The S protein has been associated with *in vitro* host cell specificity (35). When the S protein gene of an infectious cDNA clone of the Beaudette strain, which replicates in mammalian Vero and BHK cells, was replaced by that of the M41 strain, which does not replicate in those cell lines, the recombinant virus was unable to replicate in them (35, 36). The recombinant did replicate in the primary chick kidney cells, as did both the Beaudette and M41 strains. The S protein is cotranslationally inserted into the rough endoplasmic reticulum (RER) and glycosylated with N glycans (169). By intramolecular disulfide bonds, the S protein forms a complex structure and is oligomerized into homotrimers (85, 216). Mature S glycoprotein is obtained during transportation to the Golgi complex by trimming of high mannose oligosaccharides and the addition of terminal sugars. Most of the S protein accumulates in the Golgi of infected cells, however, some S bloomers are transported to the plasma membrane, where it may mediate cell-cell fusion (114, 266). 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 either in the Golgi complex or extracellularly (43). 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 (37). 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 (168). 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 sequences of the S1 portion of coronaviruses classified in different serogroups are compared, the presence of hypervariable regions with deletions, mutations or recombination is observed (52,

272). The three hypervariable regions (HVRs) in the S1 subunits are located within amino acids 38-67, 91-141 and 274-387 (42, 160, 202). The S2 portion has a sequence of 625 amino acids and it is less variable than the S1 portion (52).

The M protein is by far the most abundant envelope protein of coronaviruses and has been estimated to account for approximately 40% of the mass of IBV particles (249, 254). The M protein is comprised of 225 to 230 amino acids and even though it cannot drive coronavirus budding by itself, it displays a strict requirement for a small amount of E protein for virus-like particles (VLPs) formation (15, 65). The M protein has three hydrophobic alpha-helical domains, which penetrate the lipid bilayer three times (169). 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 (52, 169). The M protein is synthesized on membrane-bound polysomes and during the replication process it adheres to the endoplasmic reticulum of the cell by an internal signal sequence (233). Unlike the S protein, the M protein is not transported to the plasma membrane (169).

The phosphorylated 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 (37, 168, 169). The N protein is very conserved among coronaviruses in the same group with identities greater than 94% and may be involved in the regulation of the viral RNA synthesis (53, 168, 235). However, amino acid identities as low as 60% to 63.3% in some IBV strains when compared with the N protein of the Australian Vic S, V5/90, N2/75, N1/62 and N9/74 IBV have been reported (235). The N protein plays a role in viral replication, assembly and immunity (169). It interacts with leader RNA sequences

facilitating viral mRNA synthesis and also binds to the viral RNA forming a helical nucleocapsid (172). 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 (242). The N protein is translated on free polysomes and rapidly phosphorylated on serine residues in the cytosol (250).

The envelope E protein plays a critical role in the budding step (64). The E protein exists as a low E protein:M protein ratio in IBV VLPs. It localizes in the Golgi complex where it interacts with the Golgi scaffold proteins. This interaction seems to result in the modulation of vesicular traffic, which might be advantageous for collecting coronavirus envelope proteins in a specific Golgi compartment for assembly (64).

Virus replication

IBV replication starts with the attachment of the virus to O-glycosylated receptors present in the cell membrane (169). Then, after host-cell dependent proteolytic cleavage of the viral S protein, the fusion of the viral envelope with the plasma membrane is observed. The entry and uncoating of the viral particle into the host cell is followed by the immediate translation of two overlapping reading frames (ORFs 1a and 1b) located in the 5' end of the genomic RNA (mRNA1). These ORFs codify the polyproteins 1a and 1a/b which yield the RNA-dependent RNA polymerase (35, 69). The presence of the two polymerases, one for genomic RNA synthesis and one for subgenomic RNA synthesis has been suggested (26). The 741 kDa 1a/b protein seems to be continuously translated by a – 1 frameshift mechanism as shown by the inhibition of RNA synthesis when this protein is not produced (169). The RNA dependent RNA polymerase, then, uses the positive sense genomic RNA as a template for the production of a full

length negative sense copy of the viral genome, which is used as a template to produce genomic and subgenomic RNAs (286). For the production of subgenomic length RNA, the viral polymerase transcribes a short leader sequence (60 to 90 nucleotides) located at the 5' end of the genome, from the negative sense, full length, RNA copy and then moves the nascent RNA strand to different intragenic regions (also known as transcript associated sequences) downstream in the genome, from which the different nested subgenomic length RNAs are finally transcribed (169). 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 the leader sequence.

Two models have been proposed for the discontinuous mechanism for the addition of coronavirus leader sequences, leader-primed transcription and discontinuous transcription during negative-strand synthesis. Both models require the presence of intergenic or transcription associated sequences (TAS) in the genomic sequence for the leader sequence acquisition during synthesis of subgenomic mRNAs (169, 250). The phenomenon of leader switching between IBV was demonstrated during the replication of the IBV Beaudette-derived defective RNA CD 61 (D-RNA CD-61) following rescue with four heterologous helper IBVs (251). The analysis of the 5' ends of the rescued D-RNAs showed that the Beaudette leader sequence, present on the initial CD-61, had been replaced with the corresponding leader sequence from the helper IBV strain but the adjacent 5' UTR sequence of the rescued D-RNAs corresponded to the original CD-61 Beaudette sequence (252). Unlike the acquisition of the leader sequence onto coronavirus subgenomic mRNAs, leader switching does not require a complete leader junction sequence (252). The authors concluded that leader switching involved a recombination event over a region of 24 nucleotides comprising the last base of the leader junction sequence and the

adjacent 23 nucleotides. Based on the replication of coronavirus D-RNAs involving the acquisition of the leader sequence from the helper virus, the authors proposed the acquisition of the leader sequence by subgenomic mRNAs via the discontinuous mechanism (252).

For the viral RNA genome replication, the presence of the first 5' 544 and last 3' 388 nucleotides located within the 5' and 3' untranslated regions (UTRs) of the IBV genome are essential (73). The first 544 nucleotides of the genome included the first 15 nucleotides of ORF 1a, however, no internal regions from the replicase 1a or 1b genes are required for viral replication (73). In the 3' UTR, a highly variable region I, adjacent to the N gene, and a much more conserved region II, proximal to the poly (A) tract are observed. Structure analysis indicated that this structure was essential for the replication of the IBV D-RNAs. The presence of three potential stem-loop structures within the 5' UTR, one of which (nt 7 to 30) would appear to be analogous to the stem-loop identified in BCoV, which is essential for the *cis*-acting replication signal associated with the leader sequence, was observed (47, 73). Only the sequences in the 5' UTR and/or region II of the 3' UTR were specifically required for packaging, similar to the findings for BCoV (47), and TGEV (135) but in contrast with other findings with MHV (98, 191, 262).

Translation of viral proteins. In spite of the polycistronic characteristic of all but the smallest mRNA, just the ORF located at the 5' end of every mRNA is translated by a cap-dependent ribosomal scanning mechanism (255). The structural proteins S, M and N are translated from separate mRNAs by this mechanism. The presence of the 5' leader sequence enhances translation of viral mRNA in virus-infected cell lysates by translational shutoff in the translation of cellular mRNA (255). The mRNA 1 contains two large ORFs, overlapping each other by 43

to 76 nucleotides, which are translated into polyproteins 1a and 1a/b by the conventional cap-dependent translation mechanism (179, 213). These polyproteins are co- or posttranslationally processed into multiple proteins by viral and host proteases (213). The polycistronic mRNA 3 of IBV contains three overlapping ORFs, which are translated. These ORFs are preceded by an internal ribosomal entry site (IRES) sequence that allows ribosomes to bypass the upstream ORF and translate the downstream ORFs by a cap-independent translation mechanism (182, 257). The protein encoded by this ORF is highly hydrophobic and seems to be the viral envelope protein, known as E (169). The mRNA 5 of IBV contains two ORFs that are both translated *in vitro* and *in vivo* by an unknown mechanism (181).

Assembly and budding. The first step in virus assembly is the binding of N protein to viral RNA forming the helical nucleocapsid (169). In MHV, an specific RNA signal that consists of a stretch of 61 nucleotides in the 3' end of gene 1b, about 20 kb from the 5' end of the genome is required for an efficient genomic RNA packaging (98, 262, 280). This nucleotide stretch is present only in the genomic-length RNA, however, packaging of small amounts of all subgenomic mRNAs into IBV virions has been observed (286). Once the nucleocapsid is formed, it interacts with the M protein in the ER or Golgi complex. The N protein cannot be packaged into virions without been associated with the viral RNA (21, 267). Although the interaction of the nucleocapsid with M protein likely triggers the packaging of the nucleocapsid into virions, the formation of VLPs do require the viral E protein (169). In fact, the formation of VLPs in the presence of the M and E proteins alone has been observed (267). The first detection of virus budding has been observed in the budding compartment, where the M protein is anchored, located between the ER and the Golgi complex (86, 159, 259, 260). Virus budding is

most likely triggered by the interaction of the E and M protein. However, due to the presence of the E protein in other sites than the ER or Golgi complex, virus budding is dictated by the M protein. Coronavirus budding never occurs on the plasma membrane probably because the M protein is never found there (169). Incorporation of the S protein occurs by interaction with M protein forming a S-M complex at the pre-Golgi complex (214, 217). After budding, virus particles may undergo further morphologic changes within the Golgi, resulting in the appearance of mature virus particles (231, 234). Viral release into extracellular space occurs by fusion of the virions with the plasma membrane (114).

IBV genetics. RNA virus replication is catalized by RNA-dependent polymerases that apparently lack a proofreading function (90). Coronaviruses are thought to mutate at a high frequency (248). However, a recent study suggests that frequent genetic changes are not inherent in all IBV genomes (140). In this study, the N-terminal region of the S1 gene of M28, a Massachusetts serotype virus from the 1940s, was compared to that of M41, a prototype Mass virus that has undergone countless number of *in vivo* and *in vitro* host passages. The two viruses differed by only 2% and 4% at the nucleotide and amino acid levels, respectively (140). In another study, the examination of two persistent viruses did not provide evidence that persistence in the case of IBV was associated with a change in virus genetic and antigenic properties (208). The presence of few mutations have been also detected in MHV genome after repeated passages of the virus in culture, however, deletion mutants have been frequently observed with deletions up to 200 amino acids in a hypervariable region of the S protein (12, 101, 222).

A unique feature of coronavirus genetics is a high frequency of RNA recombination (167). Although non segmented genomes of RNA viruses generally exhibit very low or

undetectable recombination frequencies, the recombination frequencies for the entire coronavirus genome have been calculated to be as high as 25% (13). The high frequency of RNA recombination in coronaviruses is probably the result of the unique mechanism of coronavirus RNA synthesis, which involves discontinuous transcription and polymerase jumping (167). Viral polymerase associated with the incomplete nascent RNAs dissociates from its template at a random point and switches to a homologous site on a different RNA template to complete RNA synthesis by a copy-choice mechanism (12, 167). By phylogenetic trees exhibiting different topology, the consensus intergenic sequences and the highly conserved sequence around this regions have been named as "hot spots" for RNA recombination (175). The natural evolution of coronaviruses has been closely related with the presence of the RNA recombination mechanism. New strains of IBV in poultry flocks have been the outcome of natural recombination between different field strains (38, 139, 165, 271). Studies of the S gene of the DE072 IBV strain, first isolated in 1992 in the Delmarva peninsula region of the USA (103), have demonstrated that this virus is a recombinant, in the S1 and S2 genes, with the IBV vaccine strain D1466 from the Netherlands (176). The possibility of genomic recombination among different IBV was also examined *in ovo* by coinfecting specific pathogen free embryonating chicken eggs with commonly used, embryo adapted IBV vaccine strains (93). In this study, recombination was observed between the Massachusetts and the DE072-like strains of the virus. The presence of RNA recombination in tissue culture and in experimental and natural animal infections has been demonstrated for MHV, TGEV and IBV (11, 151, 162, 271).

When a coronavirus such as IBV and MHV is passaged at a high multiplicity of infection, new RNA species smaller than the genomic RNA, known as defective interfering RNAs (DI-RNAs), can emerge (190, 191, 251, 253). DI-RNAs can replicate only in the presence of a

helper virus and may interfere with the replication of the helper virus (251, 253). All the DI-RNAs contain both the 5' and 3' ends of the viral genome, which include the *cis*-acting signals for RNA replication. Most of them also contain an ORF encoding a protein that is fused from different viral proteins (189). During serial passages of viruses in cell culture, the original DI-RNAs frequently disappear and new ones of different sizes are generated, which evolve due to recombination between the original DI-RNA and the RNA of the helper virus (100).

Reverse genetics. The ability to generate and manipulate full-length genomic cDNAs of the viral genomes with the subsequent synthesis of infectious RNA for the generation of recombinant viruses have resulted in major advances in the study of the molecular biology of RNA viruses (36). Reverse genetics systems have been achieved for a number of positive-stranded RNA viruses, with genomes ranging from 7 kb (picornaviruses) to 15 kb (arteriviruses) to 20 kb (closteroviruses) (229, 237, 238). In the *Coronaviridae* family, reverse genetic systems for the TGEV and HCoV have been developed (256, 283). A reverse genetics system for the avian infectious bronchitis virus has also been developed (36). The IBV genome cDNA was assembled, by utilizing naturally occurring restriction sites, immediately downstream of a T7 RNA polymerase promoter by *in vitro* ligation and cloned directly into the vaccinia virus genome. Infectious IBV RNA was generated *in situ* after the transfection of the restricted recombinant vaccinia virus DNA and a plasmid DNA encoding the IBV N protein into primary chicken kidney cells, previously infected with a recombinant fowlpox virus expressing T7 RNA polymerase. The primordial role of exogenous N protein (or N mRNA) for the recovery of coronavirus by stabilizing and protecting the RNA from nuclease digestion was hypothesized (36).

Susceptibility to chemical and physical agents

Most IBV strains are inactivated at 56 C for 15 minutes or at 45 C after 90 minutes (220). 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 (66, 220). In cell culture, IBV is more stable at pH 6.0 to 6.5 than pH 7.0 to pH 8.0 (4). Most of IBVs are inactivated by ether (20%) for 18 h at 4 C, chloroform (50%) for 10 min at room temperature, or sodium deoxycholate for 18 h at 4 C (220). Common disinfectants like beta-propiolactone at 0.05% to 0.1% or formalin at 0.1% inactivate the virus. The inactivation of IBV with beta-propiolactone does not affect its hemagglutination antigen activity (63, 153).

Clinical disease

The upper respiratory tract is the main site of IBV replication (45, 88, 196). The virus is primarily epitheliotropic and enters the epithelial cell by viropexis (224). The replication of IBV has been observed by immunofluorsecence, immunoperoxidase and electron microscopy in ciliated epithelial and mucus secreting cells. Infection of these cells cause the induction of characteristic signs as sneezing, coughing, gasping, tracheal rales and nasal discharge (145, 207). Occasionally, puffy, inflamed eyes and swollen sinuses may be seen (33, 223). Maximum IBV titers in the presence of clinical signs are observed in the trachea between 5 and 10 days post-infection (p.i), but occasionally virus may be present for up to 28 days (5, 8). The virus also replicates in the epithelial cells of lungs (138), and air sacs (210), with viral titers similar to those in the nose and trachea (39). IBV is not considered to cause pneumonia, even though small areas of pneumonia may be observed in the lungs of some chickens exhibiting rales with vibration emanating from the lower respiratory tract (39). From a welfare point of view, the hardest hit are

chicks of only a few days of age. Some may die directly from the viral infection but a greater number die following secondary bacterial infection (39). An overall slowing down of growth causes further economic losses. Juvenile and mature birds suffer less from IBV infection although the economic consequences of infection in egg-laying stock can be disastrous, as egg production drops precipitously and usually does not rise back to normal in the flock as a whole (39). After respiratory infection, viremia is observed with the dissemination of the virus to different internal 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 propia, a hyperplastic phase with newly formed epithelial cells without cilia, and the recovery phase with a reparative process of the epithelial cells starting by day 4 to 6 with a complete recovery by 10 to 20 days post infection (88).

In the reproductive organs, a severe decline in egg production, and later, a deterioration in shell and internal quality, accompanied by mild or no respiratory signs, is observed (58, 195, 203, 204). The presence of 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 (32). The reduction of albumen proteins changes the structural matrix of the albumen producing watery eggs. Microscopic changes like reduction in the height of the epithelial cells, reduction in number or complete absence of the cilia, dilation of the glands, lymphocytic foci and cellular infiltration in lamina propia and inter tubular stroma have been described (243). When the infection affects female chicks less than 2 weeks of age, a permanent damage in the reproductive tract is seen, resulting in ‘false layers’ that do not lay normally at sexual maturity (28, 144). This damage is seen especially in the young cells of the isthmus and

magnum, being responsible for the absence of egg production in some layers after infection (88). No evidence of oviduct pathology was observed in maternal antibody-positive chickens exposed *in ovo* at 18 days of embryonation to commercial IBV vaccines (49).

In the kidneys, virus replication has been observed in the proximal convoluted (50), distal convoluted (221), and collecting tubules (48), 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 (1, 55, 120). Negative sodium balance was a direct effect of increased output of sodium in the urine, while negative potassium balance was due to decreased intake. The kidneys infected with nephropathogenic IBV are swollen and pale with tubules and ureters distended with urates (70). The virus causes granular degeneration, vacuolation and desquamation of the tubular epithelium with massive infiltration of heterophils in the interstitium in acute stages of the disease. The changes in the chronic phase were classified as being active or inactive types of interstitial nephritis (2). Nephropathogenic strains usually produce nephrosis-nephritis in young birds and urolithiasis in layers (2, 88, 188). When the birds are affected by a nephropathogenic strain, depression, wet droppings, increase in water consumption and ruffled feathers are commonly observed. The T strain (N1/62) in Australia, the Holte and Gray in the United States, the B1648 in Belgium and the AZ23-74 in Italy are IBV strains recognized as nephropathogenic, causing histological lesions in both trachea and kidneys and variable mortality (197, 244, 279, 285). A pathogenicity evaluation of 25 IBV strains isolated in Australia between 1961 and 1994 indicated a change in the prevalent IBV strains from highly nephropathogenic (1960s to 1970s) to respiratory (1980s to early 1990s); moreover, the late 1980s saw the emergence of respiratory strains with altered tissue tropism (131). In this study, 12 strains were nephropathogenic, 10 respiratory, and 3

exhibited mixed pathogenicity. The IBV strains identified as nephropathogenic induced clinical nephritis, gross and histological kidney lesions, with variable mortality between 5% and 90% (131). According to the severity of these lesions, the nephropathogenic strains were further subdivided into high, moderate and low pathogenicity strains (131). Variable mortality has also been induced by the T strain (N1/62) (50, 245, 275). Several factors including viral dose, route of inoculation, age and strain of chicks, and environmental or nutritional factors have been shown to influence the mortality rates induced by nephropathogenic strains (2, 230).

In the intestines, IBV replication has also been observed. Several IBV strains have been isolated from cloacal swabs, feces and cecal tonsils (5, 6). In 1986, El Houdafi *et al* (91), 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 (143), observed the re-excretion of an enterotropic IBV by hens at point of lay after experimental infection at one-day of age. The *in vitro* IBV replication in small intestines has been controversial. *In vitro* explants of several gut tissues have been shown to support the growth of IBV (18). However, the same author 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 (CD_{50}) per ml (19). In 1990, Lucio and Fabricant (186), isolated four viruses (Mass 41, ECV 1, ECV 2, and ECV 3) from cecal tonsils. The intestinal replication of IBV has been described in cells resembling histiocytes, lymphoid cells in the cecal tonsils (221), and in apical epithelial cells of the villi in ileum and rectum (8, 87). The 793/B UK IBV variant strain was more enterotropic than pneumotropic and was even associated with diarrhea in broilers (87). IBV has been isolated from other tissues as Harderian gland, bursa of Fabricious,

spleen, feces, cloacal contents and semen (88). Although many alimentary tract tissues are susceptible to IBV, infection of enteric tissues usually does not manifest itself clinically (39).

Recently, three IBV strains (Q1, J2 and T3) were associated with a particular disease in the proventriculus. These IBV strains were isolated from 25 to 70 day-old H120-vaccinated chicken flocks having outbreaks of so-called "an avian disease associated with the proventriculus" at three different areas of China, between 1996 and 1998 (284). The disease was reproduced in 2, 7 and 16 wk-old SPF chickens after inoculation with the three isolates. The infected birds appeared depressed with ruffled feathers, respiratory distress and wet droppings with white and yellow milky feces associated with an infected alimentary tract. Variable mortality with persistent diarrhea was observed (284). Necropsy lesions included thickened proventriculus with milky fluid exudate while at later stage, ulcer of proventricular papilla, hemorrhagic lesions of proventricular papillary groove and cecal tonsil, and thinning of duodenum were observed (284). S1 amino acid sequences among these isolates were almost identical while only similarities between 47.3% and 82.3% were observed when compared with 47 published sequences. Based on genotyping and serology, the three isolates were described as new IBV variants (284).

Variation in virulence

The evaluation of the pathogenicity of IBV strains for the respiratory tissue has been difficult to quantify (63). The ability of the virus to cause stasis of tracheal cilia both *in vivo* and *in vitro* has been used to assess the severity of respiratory infection (88). Cubillos *et al* suggested differences in virulence of IBV strains for the trachea based on the variability of the tracheal damage in terms of ciliary activity in unvaccinated chickens (69). However, other

authors have not observed marked differences in virulence among IBV strains using this *in vitro* system (88). In 1976, Cook *et al* compared three strains of IBV on the basis of their effect on tracheal cilia, but did not find marked differences (61). In 1996, Dhinakar Raj and Jones also reported little difference among several IBV strains using measurement of ciliary activity as a criterion for damage to the tracheal epithelium (89). IBV infections usually do not occur as a single entity in the field. The presence of other respiratory diseases and secondary agents plays an important role, influencing the pathogenesis of the disease (88).

Variations among IBV strains to cause decreases in egg production and quality have also been reported (115). In 1986, Cook and Huggins found that some variant strains of IBV caused only small decreases in egg production, but had a marked effect on egg color (57). In 1992, Parson *et al* found a variant causing a substantial decline in egg production with little loss of egg color in the field (223). In 1972, Crinion and Hofstad reported differences in virulence for the immature chicken oviduct among IBV strains (68). In this study, the Massachusetts and T strains were virulent while the Connecticut and Iowa 609 strains were not. In 1984, Pradhan *et al* showed that the M41 strain caused stasis of cilia in oviduct organ cultures prepared from precociously-induced oviducts in young chicks by estrogen treatment (227). This work has been used to compare the virulence of seven strains of IBV *in vitro* using ciliostasis and a calmodulin assay to quantify the damage to oviduct epithelium (89). In this study, the isolate D207 was the most virulent while the enterotropic G variant strain was the least.

The severity of the kidney lesions produced by different nephropathogenic IBV strains has also been evaluated (2, 46). In this study, the Australian T strains induced the most rapid and severe lesions following both intra-venous and intra-ocular inoculations of susceptible chickens. Using a model to titrate kidney infectivity by intra-venous inoculation, Lambrechts *et al* did not

find differences in the infectivity among Belgian field nephropathogenic IBV isolates, but the infectivity of egg-passaged virus was highly reduced (170).

Immunity

Passive immunity. Immunoglobulin G (IgG) class antibodies are passed from vaccinated hens via the yolk to the progeny and can be detected in serum and respiratory mucus of newly hatched chicks (119, 148). 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 (157, 158). The protection offered by maternal antibodies against the IBV challenge varies from 1 to 2 weeks up to 4 weeks depending on the methods used for challenge and subsequent evaluation of protection (75, 157, 158, 198). However, in birds with maternal antibodies vaccinated at one-day of age, the development of active immunity in the respiratory tract is observed (198). In a recent study, the role of maternal antibody to infectious bronchitis virus in protection against infection and development of active immunity to vaccine was evaluated (201). In this study, at hatching, chicks with maternal antibodies (Mab+) had significant levels of antibody in serum (5.2 $\log_{10}$) and the respiratory tract (2.7 $\log_{10}$). When challenged with IBV through the intraocular route at 1 day of age, the Mab+ chicks had excellent protection (95%) at 4 days against the challenge virus. At 7 days of age, while no remarkable change was observed in serum antibody titer, that in the respiratory system had dropped to approximately half (1.5 $\log_{10}$) of the level recorded at day 1. This coincided with a drastic drop (< 30%) in protection to virus challenge at day 7. During the remaining period (10-17 days), the serum-antibody levels declined gradually, remaining at an appreciable level until day 17 (> 3.0 $\log_{10}$), whereas, respiratory antibody levels fell below 1.0 $\log_{10}$ during the same period. No

protection against IBV challenge was observed on days 14 and 17. It was concluded that the antibodies present in the respiratory tract and not in the serum provided protection against the intraocular challenge, and that vaccination of chicks at 1 day of age quickens the depletion rate of Mab in the circulation, being significant on days 14 and 21. If the serum antibody is to offer protection against spread of IBV from the respiratory tract to internal organs such as reproductive tract and kidneys via the blood stream (208), the lower presence of circulating antibody levels at these ages could be consequential with viral damage (201). The authors concluded that in Mab+ chicks, short-term protection to IBV challenge is due to respiratory antibody, vaccination at day of age hastens the rate of Mab decline, and vaccination at day of age is ineffective in the induction of an adequate primary and secondary immune response of the chicks (201). In a separate study, a negative correlation between economic losses associated with IBV infections in broilers and low or erratic maternal antibody titers was observed. The authors concluded that IBV vaccination strategies should aim at high and uniform antibody titers in the broiler breeders to obtain less variable and higher levels of maternally derived antibodies in the progeny (79).

Active immunity. Breed and strain-related genetic resistance to IBV infection has been described in chickens (29, 59, 218, 245). Innate immunity against external agents includes physical barriers provided by skin and mucous membranes, soluble factors like lysozyme, complement and acute phase proteins and cells such as granulocytes, macrophages and natural killer (NK) cells. However, the innate immunity is characterized by the lack of specificity and immunological memory (88). In IBV-infected chickens, heterophils are the most numerous early inflammatory cells in respiratory lavage fluids (99). Heterophils have no effect on virus

replication, however, they contribute to damage the tracheal epithelium (88). The role of macrophages in IBV infections is unknown while no alterations in the NK cell activity have been found after IBV infection (269). Recently, the use of *in vivo* thymulin treatments in IBV infected chickens significantly enhanced the cytolytic activity of NK cells in the lungs by up modulating the presence of IFNγ receptors (215).

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 (7, 61, 128, 268).

The determination of immunity against IBV has been evaluated by immunological techniques like virus neutralization test (VN) and hemagglutination 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 (74, 278). The evaluation of ciliary movement in tracheal rings has 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 (9). The determination of immunity in vaccinated chickens has also been evaluated by protection against mortality from a challenge with a mixture of IBV and *E. coli* (63). More vaccinal cross-protection has been found with this method than with other assessments of tracheal immunity (63). Although the S1 glycoprotein is primarily responsible for the induction of the VN and HI antibodies, the mechanism of protection against clinical disease is incomplete. The role of local antibody in preventing reinfection is also unclear. The prevention of re-infection by neutralizing antibodies in nasal secretions and the role of the Harderian gland as contributor to local immunity has been described (78, 126). Variations in IBV-specific IgG levels in serum and IgA levels in lachrymal

fluids have also been demonstrated in different chicken lines after ocular vaccination with IBV (261). A recent study describes the role of the memory T cells in protection from acute infectious bronchitis virus infection (225). In this study, T lymphocytes collected from B19/B19 chicken spleens at 2, 3, 4, and 6 weeks p.i. were transferred to six-day-old syngeneic chicks one day prior to challenging with 10^6 EID_{50}/ml of the IBV Gray strain. Memory immune T cells collected between 3 to 6 weeks p.i. provided dose responsive protection from clinical illness, while T cells collected at 2 weeks p.i. did not protect. The memory T cells subtype was determined as $CD8^+$ (225).

Serotypes

Until 1956, when the Connecticut strain was isolated, only the IB Massachusetts antigenic type virus was recognized (123, 147, 263). IBV is characterized by the presence of multiple serotypes (45, 106, 152). In 1988, King 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 (152). 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 (106). 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 (106). 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 vaccination programs, because protection against unrelated serotypes (heterologous protection) is unpredictable (106). Cross-protection produced by some IBV serotypes against antigenically unrelated strains have been reported (124, 232, 276). However, complete protection against heterologous serotypes by one strain or strains has not been observed (106). Some vaccines offer better protection against variant serotypes than others. The Massachusetts-Arkansas vaccines offered a broader protection against variant serotypes than the Massachusetts (Holland) alone or Massachusetts (L-1) in combination with the Connecticut strain (106). IBV serotypes have been classified based on different tests like virus neutralization in chicken embryos (72), reduction of cilia movement in chicken tracheal organ culture (61, 77, 142), plaque reduction test (127), hemagglutination-inhibition (3, 155, 171), dot-immunobloting assay using monoclonal antibodies (246), monoclonal antibody-based ELISA (132), polymerase chain reaction-restriction fragment length polymorphism (RT-PCR/RFLP) (137, 166), and sequencing of the S1 gene (156, 236). 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, makes it difficult to establish effective vaccination programs (88, 134). 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 (152).

In the U.S., the following serotypes are recognized: Massachusetts, Connecticut, Delaware 072, Florida, Arkansas 99, and JMK (45). In the U.K., 8 serotypes have been described including the 167-84, 142-86, 128-82 and 4/91 or 793B serotypes. The 793B serotype

has been associated with the presence of myopathy in broilers (56). 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 (45).

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 (N1/62) and group II with viruses that replicate only in the trachea (71).

Vaccination

The first vaccination attempt, made by van Roekel, consisted of exposing laying flocks to IBV during the onset of egg production, being the basis of current immunization programs (265).

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 immunogenicity adversely affects the protection offered by the vaccine virus against challenge viruses in the field (45).

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 (25).

The serotype variation of IBV is due to high point mutations and recombinations (14, 43, 53, 57, 77). IBV mutations and recombinations 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 (106, 129).

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 Massachusetts 41, Connecticut, Holland, JMK, Florida 18288, Arkansas 99 and with special license Delaware 072 (45). 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 (268). In the U.K., the Massachusetts type (Mass 41, H52 and H120), D274 and 793B vaccines are used. In the Netherlands, the most common strains used in vaccines are the H52 and H120 (Massachusetts type), D1466, D274 and D1201 (45).

Broilers are usually vaccinated at the hatchery by spray, in part for logistical/economic reasons. Also, due to the endemic characteristic of IBV in many poultry-rearing parts of the world, protection as early as possible is needed (39). Short-lived protection for the respiratory tract has been observed after single live attenuated virus vaccination with a decline in protection at 6 and 9 weeks after vaccination (76, 111). In some regions in which one vaccination gives not acceptable protection against field challenge, the birds might be revaccinated 2 to 3 weeks after

the first application with vaccines containing the same or different serotypes (39). Cook *et al* evaluated the protection of the respiratory tract provided by different live-attenuated IBV vaccines against challenge with heterologous serotypes. In this study, the administration of IB Ma5 vaccine (Massachusetts serotype) at 1 day old followed by a vaccination with the heterologous 4/91 vaccine at 2 weeks of age offered a broader protection against heterologous serotypes (62).

Homologous but not heterologous vaccines seem to prevent detectable growth of challenge virus in the trachea, as ascertained by virus isolation, with a marked reduction (84%) of IBV detection in the kidneys (226).

Inactivated oil-emulsion IBV vaccines were developed during the 1960s and 1970s to obtain long-lasting immunity to protect hens against drops in egg production (39). Single applications of inactivated virus induce little or no protection against egg loss (24, 194, 203), and no protection (193), or low protection against loss of ciliary activity in the trachea (39). Higher protections against loss of ciliary activity has been observed when birds are vaccinated at least twice with inactivated IBV (20, 133, 247). The common approach in the poultry industry is to vaccinate young females with live vaccines two or more times, followed by one dose of inactivated vaccine as the birds come into lay (24, 97, 111). However, the use of highly attenuated vaccines every 4 to 6 weeks during the entire production cycle replacing the use of inactivated vaccines has also been observed.

The induction of protection by subviral and vectored vaccines against IBV challenge has also been evaluated. Immunization with the S protein has shown to give protective immunity (41), which varies depending on the manner by which the S protein is presented to the host (39, 133). Tracheal and kidney immunity after four inoculations with 50 μg for each dose was

observed in 71% and 86% of 10 chickens (133). The S1 protein has been expressed *in situ* from fowlpox virus, adenovirus vectors and transgenic potatoes. The recombinant fowlpox virus containing the S1 gene of Massachusetts 41 strain elicited some anti-IBV protective immunity (273). Milder clinical signs, decrease titers of recovered challenge virus, and less severe histologic changes of the tracheas were observed in birds previously inoculated with the recombinant virus by the wing web and challenged with a virulent Mass-41 strain (273). A single dose of a recombinant fowl adenovirus expressing the S1 gene of the Vic S, an Australian IBV in the serotype B, gave high levels of protection against homologous (Vic S) and heterologous (N1/62) challenge, even in the face of fowl adenovirus maternal antibodies (141). The role of the IBV N protein in immunization against IBV has been evaluated (20). The authors concluded that the immunization with the N protein had induced protective immunity by activation of cytotoxic or helper T-cell responses (20). Induction of immune response in chickens by two inoculations of plasmids expressing the N protein or a fragment of the N protein against infection of IBV applied by eye-drop and intranasal inoculation has also been observed (241). The protection of immunogenic S1 glycoprotein of IBV expressed in transgenic potatoes has also been evaluated (287). In this study, specific pathogen free (SPF) leghorn chickens immunized with 5 g of the transgenic glycoprotein via oral and intramuscular routes at 1, 7 and 14 days of age were protected (100%) against challenge with 50 μl of a $10^{5.8}$ EID_{50}/ml virulent M41 strain 7 days after the third immunization (287). The protection of chickens from IBV using a DNA vaccine expressing the S1 glycoprotein has been evaluated *in ovo* after intramuscular inoculation (149). In this study, immunization of 18 day-old embryonating chicken eggs with the DNA vaccine followed by live virus vaccination completely protected bird from challenge with a dose of 10^5 EID_{50}/bird of a pathogenic IBV Ark DPI at 28 days of age.

However, a single *in ovo* application of the DNA vaccine was not sufficient for protection against IBV (149).

Laboratory systems

Most IBV isolates replicate well in chicken embryo allantoic sac, exhibiting higher titers by 1 to 2 days after inoculation (146). Coronaviruses isolated from pheasants also have been propagated in this system (113, 180). Characteristic embryo changes, observed several days after inoculation of the virus, include the presence of a dwarfed, barely moving embryo during candling. Upon opening the air cell end of the egg, the embryo is seen curled into a spherical form with feet deformed and compressed over the head and with the thickened amnion adhered to it (185). A commonly observed internal lesion of the IB-infected embryo is the presence of the mesonephros containing urates. However, this lesion seems to be associated with the stunting of the embryo and is not specific for IB infection. This lesion has also been observed after inoculation with lentogenic strains of Newcastle disease virus (NDV) (102). Microscopic lesions such as congestion with perivascular cuffing and some necrosis of the liver, pneumonia with congestion, cellular infiltration and serous exudates in the bronchial sacs, interstitial nephritis and distension of the proximal convoluted tubules with cast, have been described (185).

Cell culture systems for the propagation of IBV include chicken embryo kidney (CEK), chicken kidney (CK) and chicken embryo liver (CEL) cells (107, 187). IBV has also been propagated in chicken embryo fibroblast cultures (219), and the hepatoma (LMH) (240), African green monkey Vero (178), chicken embryo related (CER) (96), and BHK-21 (219), cell lines.

Diagnosis

The great antigenic variation exhibited by IBV strains and the availability of vaccines designed for different serotypes makes necessary the identification of the virus serotype or genotype. In general, IBV infections can be diagnosed by detection of the virus itself or the specific antibody response. The most common assays routinely used are virus isolation (VI), immunofluorescence assay (IFA), immunoperoxidase assay (IPA) and reverse transcriptase polymerase chain reaction (RT-PCR). Serologically, IBV can be detected by demonstrating a seroconversion, using paired serum sets or the demonstration of IBV-specific immunoglobulin M. The serological tests commonly used are the hemagglutination inhibition (HI) test, the agar gel precipitation test (AGPT), virus neutralization (VN), and the enzyme-linked immunosorbent assay (ELISA) (80). The level of success in the detection of IBV after a disease outbreak is influenced by a number of factors, including the time between start of infection and sampling, level of immunity in the chicken at the moment of infection, number of sampled birds, choice and quality of sampled organs, genetics of the chicken, housing system of the chicken, and possible immunosuppresion (88). All IBV strains can be isolated from the respiratory tract, with the highest concentration of IBV in the trachea during the first 3 to 5 days post-infection. After this period, the virus titer drops rapidly in the second week p.i. to below the detection level. In chronic stages, the value of examining cecal tonsils or cloacal swabs is higher than that of examining the trachea (5, 19, 60, 81, 91, 143). Depending on the history of the disease, samples from the lungs, kidneys, and oviduct should also be considered. In cases in which the isolation of IBV has been difficult, the placement of susceptible sentinel birds in problem farms for about 1 week is recommended (102). The level of immunity in the chicken at the moment of infection influences the time and amount of IBV that can be detected. Experimental IBV infections in

vaccinated and unvaccinated birds show that homologous challenge virus is detected for a much shorter period and in much lower amounts in vaccinated than in unvaccinated chickens (30, 63, 108, 277). Therefore, the period in which IBV can be isolated from the trachea after a homologous infection of vaccinated birds can be limited to a few days instead of a few weeks after infection of unvaccinated birds. Another factor that can impede the detection of an infection in well vaccinated flocks is the reduced spread of the virus between vaccinated chickens compared with unvaccinated chickens (80). In this case, it is important to sample at an early stage of infection and to use a very sensitive test (80). In the acute phase of an IBV infection of unprotected chickens, many birds will produce large amounts of IBV in the trachea. However, in chronic infections or infections in vaccinated birds, such as layers and breeders, small amounts of virus may be present in only a low percentage of the birds. Therefore, sampling of respiratory tract, kidney, cecal tonsils and cloaca of many more birds can be required for the detection of the infection (80). The quality of the samples is another important factor in the successful isolation of IBV. Samples must be chilled at 0 to 4 C as soon as possible to preserve the viability of the virus. For longer storage, the samples should be frozen at – 20 C as soon as possible. Swabs should be placed in cold tryptose phosphate broth (TPB), pH 7.0 to 7.2 with antibiotics (105). Samples can also be placed in 50% glycerin, remaining viable for many days without refrigeration (196). The classical isolation of the virus, giving a number of embryo passages until dwarfing, curling or embryo mortality occurs has been replaced by a shortened isolation procedure for multiplication of the virus and a second technique, such as IFA, antigen ELISA or RT-PCR (80). IBV can be isolated in embryonated (SPF) eggs, cell culture and chicken tracheal organ cultures (TOC) causing no specific lesions in these systems (196). Therefore, the presence of IBV antigen has to be confirmed by an IBV antigen detection method.

Embryonated SPF eggs and TOCs are more sensitive than cell cultures, especially when field strains are involved (61). IBV grows well in the developing chicken embryo, exhibiting higher titers between 48 and 60 hours post-inoculation (122, 228). However, the presence of high titers can be delayed for non-egg-adapted field strains, requiring several sequential passages to increase the amount of virus before performing the detection of the antigen (80). Direct virus isolation in conventional monolayer cell cultures has proved unsuccessful (61). Adaptation of IBV strains is necessary for fluent replication and induction of cytophatic effect (107). Tracheal organ cultures from 20 day-old SPF embryos have been very successful for isolation, titration and serotyping of IBV because no adaptation of field strains is required (56). Ciliostasis is usually observed between 3 to 4 days after inoculation, but varies between strains and inoculation dose (54).

The techniques used for detection of IBV-specific antigen using IBV-specific antibodies include detection by monoclonal antibodies (Mabs), agar-gel precipitation test (AGPT), immunofluorescent assay (IFA), immunoperoxidase assay (IPA) and antigen ELISA. Mabs react with one or small number of epitopes of the IBV antigen, providing a well-defined, reproducible and specific product (161). A disadvantage of this assay is that a mutation of only one nucleotide, resulting in a different amino acid in the epitope with which the Mab reacts, can prevent binding of the Mab to that epitope (42). The AGPT is not expensive, fast and can be easily performed in any laboratory (184), however, several antisera or an antiserum at different concentrations should be used to prevent false negatives resulting by imbalance of the antigen:antiserum ratio. The IFA is group-specific when using polyclonal anti-IBV serum or type specific by using type-specific Mabs (82). However, interpretations of the fluorescence may be complicated by non-specific reactions, especially with field samples because of

additional factors such as ammonia, dust and secondary *E. coli* infections (82). IPA allows the evaluation of antigen-bearing cells, as well as general tissue morphology. It offers some advantages over IFA such as slides evaluation in daylight with a normal microscope and easy storage due to the stability of the staining. However, it is more laborious and is sensitive to non-specific background staining by endogenous peroxidase naturally present in the sample (80). Antigen ELISA test exhibits low sensitivity for viral detection in chicken organs with a detection limit of 10^6 EID_{50} (205), 10^3 PFU (209), or 100 to 1000 median ciliostatic doses (CD_{50}) (132). Antigen ELISA has been reported as a successful confirmation test for detecting IBV antigen in allantoic fluid of inoculated eggs (282). The RT-PCR reaction does not distinguish between infectious and non-infectious virus particles. The primers for group-specific RT-PCR tests are usually annealed to segments of the IBV in the very well conserved regions of the membrane and nucleocapsid protein genes (10, 95). RT-PCR exhibits a moderate sensitivity when performed directly on tracheal swabs (137). Increased sensitivity can be achieved by performing one or two passages in embryonated eggs followed by detection of the IBV genome by RT-PCR (136). Rapid group-specific detection of avian infectious bronchitis virus by polymerase chain reaction has been performed in tissue samples by amplification of a conserved region of the N gene (95), or in embryonating eggs in association with a DNA probe (136). Nested RT-PCR has been shown to be more sensitive than conventional RT-PCR because of the addition of a second amplification step. However, nested RT-PCR is rarely used for routine diagnosis because of their extreme sensitivity and the high risk of contamination during both sampling and performing the test (44).

Several methods including sequencing, detection of genotype-specific parts of the genome by RT-PCR, or determination of the position of enzyme cleavage sites (RFLP, RNase

T1 fingerprint) have been used to group IBV strains based on genetic characterization (80). Multiplex polymerase chain reaction followed by sequence analysis (183), IBV characterization by slot blot hybridization (200), and the identification of IBV by direct automated cycle sequencing of the S-1 gene (156), have also been used to genotype IBV. A disadvantage of genotyping for use in the field is that direct translation of information about a portion of the genome of an IBV strain to biological function or antigenicity of the virus is not possible or is not without risk (51, 164). Isolates of the same serotype or protectotype can differ substantially in some genes (40), while different serotypes or protectotypes can have remarkably high similarities between their genomes (34). Therefore, for practical use in the field, exclusive use of genotyping methods is not recommended. Especially when there is suspicion in the field that the genotype of recent isolates does not provide accurate information about the true antigenic nature of these IBV isolates, conventional testing (serotyping) and especially *in vivo* studies are required (121, 139). However, some studies have shown a relationship between serotypes and genotypes. Kwon *et al* could differentiate IBV serotypes using RT-PCR and RFLP by amplifying a sequence of 1720 base pairs that contains the S1 glycoprotein gene followed by digestion with the restriction enzymes *Bst*YI, *Hae*III and *Xcm*I (166). However, the presence of mismatches at the 3' end of one or both primers can be observed enabling the amplification of some variant strains (173). Lee *et al* redesigned specific primers to amplify the DE072 variant strain of IBV due to the presence of 2-bp mismatches at the 3' en of the reverse primer (173). Other studies have demonstrated the correlation between genotyping and serotyping based on the HVR 1 sequence of the S1 gene of IBV (133, 174, 177, 211, 270).

Sequencing and subsequent comparison of the amino acid sequences is very useful to help locate conserved domains in the protein which might be essential for their structure and

function, and for epidemiological studies (80). Based on sequence data, a phylogenetic tree can be made, revealing the genomic relatedness between different strains. However, the place of a certain IBV strain in a phylogenetic tree can differ depending on the techniques used (34, 165, 288), or on which part of the genome is analyzed (163, 288). Detected differences in the sequence of two strains cannot be translated to differences in antigenicity or biological function because the secondary and tertiary structure of the protein, which are very important for its biological function and antigenicity, remain unknown. Also, the occurrence of recombination between different IBV strains during mixed reactions hampers the translation of data on a genotype to a serotype or protectotype (80). The use of RFLP after amplification of cDNA of the S1 gene has been used to genotype IBV (166). After restriction, the RFLP patterns are compared with the patterns of representatives of known serotypes (166). The correlation between RFLP pattern and serotype can be high (166), but different isolates, typed by RFLP as belonging to the same genotype can be of different serotypes or protectotypes (121).

Tests for IBV antibody detection can be grouped into group-specific and serotype-specific tests (80).

Group-specific tests include the AGPT and ELISA tests. The sensitivity of the AGPT can exhibit high variability depending on the IBV strain inoculated, application route, age at infection, presence of maternally derived antibodies or vaccinal immunity at the time of challenge and test performance. Also differences related with how the test is performed, including gel thickness, distance between the wells, diameter of wells, antigen used, incubation time and temperature used, may influence the sensitivity (80, 274). As the interpretation of AGPT results is heavily thwarted by the lack of standardization of test performance between laboratories, use of a non-validated AGPT is not recommended (80). By group-specific ELISA,

antibodies can be first detected within 1 week after vaccination or infection (119, 192, 199). Because of the short period between infection and the detection of the first antibodies by ELISA, the first paired sampling must be done at the first signs of IBV, which usually appear between 18 and 36 h after infection (45). If the first sampling is not performed in time, seroconvesion can be missed (80).

The serotype-specific tests for detection of IBV antibodies, induced primarily by the S1 spike protein, are the VN and HI test. The VNT is the gold standard test for the detection of IBV serotype-specific antibodies and is characterized by its very high sensitivity (104, 150, 192). When performing the VNT, the β-method, varying antibody concentration and constant virus dose, is preferred over the α-method, varying virus with constant antibody concentration, due to its greater sensitivity (72). Appearance of cross-reactions has been reported, especially after multiple contacts with different IBV serotypes, although low cross-reactions might also appear after repeated inoculations with the same serotype (104, 150). VNT for Ark, JMK, Florida and Massachusetts serotypes, performed on CEK cells, was highly serotype specific after one inoculation of 3 week-old SPF chickens. After a repeated inoculation at 11 weeks of age with M41 or Ark, some antibodies were induced that reacted with IBV serotypes to which the birds had not been exposed. After a second inoculation with a heterologous virus, antibody production was largely directed against the virus used for initial inoculation rather than the used for re-inoculation. In addition, the chickens that were inoculated subsequentially with heterologous IBVs tended to produce higher levels of cross-reaction antibody against IBV serotypes to which the birds had not been exposed, than birds given only homologous inoculation (104). The HI test first detect antibodies between 1 and 2 weeks p.i. (112, 258). In general, the correlation between HI and VNT when testing high titer sera is better than for low titer sera (112, 118). Although the

correlation between these test systems is high after a single inoculation, the specificity of the HI is considered lower than that of the VNT (104, 154). The serotype specificity of the HI test is much lower following re-infection with IBV, especially when the second or subsequent serotype is heterologous (104, 154). Additional inoculations with IBV can induce antibodies to react with serotypes to which the birds have not been exposed. In general, IBV antibodies can be first detected by ELISA, followed by AGPT and HI test, and last by VNT (83, 192, 199).

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 (129).

After several passages in chicken embryos, the pathogenicity of the IBV is decreased. However, it has been proven that chicken passaged virus, best known as back passage, increases the pathogenicity of the virus, which is exhibited by an increase in the presence of airsaculitis. This is especially seen when *Mycoplasma gallisepticum* is present in the field (129, 130).
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 (129). 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 (129).

References

1. Afanador, G., and J. R. Roberts. Effect of nephropathogenic infectious bronchitis viruses on renal function in young male broiler chickens. Br. Poult. Sci. 35:445-456. 1994.

2. Albassam, M. A., R. W. Winterfield, and H. L. Thacker. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis. 30:468-476. 1986.

3. Alexander, D. J., and N. J. Chettle. Procedures for the hemagglutination inhibition test for avian infectious bronchitis virus. Avian Path. 6:9-17. 1977.

4. Alexander, D. J., and M. S. Collins. Effect of pH on the growth and cytopathogenicity of avian infectious bronchitis virus in chick kidney cells. Arch. Virol. 49:339-348. 1975.

5. Alexander, D. J., and R. E. Gough. Isolation of avian infectious bronchitis virus from experimentally infected chickens. Res. Vet. Sci. 23:344-347. 1977.

6. Alexander, D. J., and R. E. Gough. A long-term study of the pathogenesis of infection of fowls with three strains of avian infectious bronchitis virus. Res. Vet. Sci. 24:228-233. 1978.

7. Alvarado, I. R., P. Villegas, J. El-Attrache, and T. P. Brown. Evaluation of the protection conferred by commercial vaccines against the California 99 isolate of infectious bronchitis virus. Avian Dis. 47:1298-1304. 2003.

8. Ambali, A. G., and R. C. Jones. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis. 34:809-817. 1990.

9. Andrade, L. F., P. Villegas, O. J. Fletcher, and R. Laudencia. Evaluation of ciliary movement in tracheal rings to assess immunity against infectious bronchitis virus. Avian Dis. 26:805-815. 1982.

10. Andreasen, J. R., Jr., M. W. Jackwood, and D. A. Hilt. Polymerase chain reaction amplification of the genome of infectious bronchitis virus. Avian Dis. 35:216-220. 1991.

11. Ballesteros, M. L., C. M. Sanchez, and L. Enjuanes. Two amino acid changes at the N-terminus of transmissible gastroenteritis coronavirus spike protein result in the loss of enteric tropism. Virology. 227:378-388. 1997.

12. Banner, L. R., J. G. Keck, and M. M. Lai. A clustering of RNA recombination sites adjacent to a hypervariable region of the peplomer gene of murine coronavirus. Virology. 175:548-555. 1990.

13. Baric, R. S., K. Fu, M. C. Schaad, and S. A. Stohlman. Establishing a genetic recombination map for murine coronavirus strain A59 complementation groups. Virology. 177:646-656. 1990.

14. Barnes, H. J., and J. S. Guy. Poult enteritis-mortality syndrome ('spiking mortality') of turkeys. In: Diseases of poultry, 10th ed, B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald and Y. M. Saif, eds. Iowa State University Press, Ames, IA. pp. 1025-1031. 1997.

15. Baudoux, P., C. Carrat, L. Besnardeau, B. Charley, and H. Laude. Coronavirus pseudoparticles formed with recombinant M and E proteins induce alpha interferon synthesis by leukocytes. J. Virol. 72:8636-8643. 1998.

16. Beach, J. R., and O. W. Schalm. A filterable virus, distinct from that of laryngotracheitis, the cause of a respiratory disease of chicks. Poult. Sci. 15:199-206. 1936.

17. Beaudette, F. R., and C. B. Hudson. Cultivation of the virus of infectious bronchitis. J. Am. Vet. Med. Ass. 90:51-58. 1937.

18. Bhattacharjee, P. S. Enterotropism and persistence of avian infectious bronchitis virus in chickens. Ph. D thesis. University of Liverpool. 1994.

19. Bhattacharjee, P. S., and R. C. Jones. Susceptibility of organ cultures from chicken tissues for strains of infectious bronchitis virus isolated from the intestine. Avian Path. 26:553-563. 1997.

20. Boots, A. M., B. J. Benaissa-Trouw, W. Hesselink, E. Rijke, C. Schrier, and E. J. Hensen. Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein. Vaccine. 10:119-124. 1992.

21. Bos, E. C., W. Luytjes, and H. van der Meulen. The production of recombinant infectious DI-particles of a murine coronavirus in the absence of helper virus. Virology. 218:52-60. 1996.

22. Boursnell, M. E., T. D. Brown, I. J. Foulds, P. F. Green, F. M. Tomley, and M. M. Binns. The complete nucleotide sequence of avian infectious bronchitis virus: analysis of the polymerase-coding region. Adv. Exp. Med. Biol. 218:15-29. 1987.

23. Boursnell, M. E., T. D. Brown, I. J. Foulds, P. F. Green, F. M. Tomley, and M. M. Binns. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J. Gen. Virol. 68:57-77. 1987.

24. Box, P. G., A. V. Beresford, and B. Roberts. Protection of laying hens against infectious bronchitis with inactivated emulsion vaccines. Vet. Rec. 106:264-268. 1980.

25. Bracewell, C. D., and D. J. Alexander. Avian infectious bronchitis. OIE Manual of Standards for diagnostic test and vaccines, 3rd ed. Chapter 3.6.6:539-548. 1996.

26. Brayton, P. R., M. M. Lai, C. D. Patton, and S. A. Stohlman. Characterization of two RNA polymerase activities induced by mouse hepatitis virus. J. Virol. 42:847-853. 1982.

27. Brierley, I., M. E. Boursnell, M. M. Binns, B. Bilimoria, V. C. Blok, T. D. Brown, and S. C. Inglis. An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. EMBO. J. 6:3779-3785. 1987.

28. Broadfoot, D. I., B. S. Pomeroy, and W. M. Smith. Effects of infectious bronchitis on egg production. J. Am. Vet. Med. Assoc. 124:128-130. 1954.

29. Bumstead, N., M. B. Huggins, and J. K. Cook. Genetic differences in susceptibility to a mixture of avian infectious bronchitis virus and Escherichia coli. Br. Poult. Sci. 30:39-48. 1989.

30. Burke, C. N., and R. E. Luginbuhl. The effect of parameter selection in evaluation of infectious bronchitis virus vaccine. I. Evaluation of the Connecticut strain by virus recovery tests. Avian Dis. 16:467-480. 1972.

31. Bushnell, L. D., and C. A. Brandly. Laryngotracheitis in chicks. Poult. Sci. 12:55-60. 1933.

32. Butler, E. J., M. J. Curtis, A. W. Pearson, and J. S. McDougall. Effect of infectious bronchitis on the structure and composition of egg albumen. J. Sci. Food Agric. 23:359-369. 1972.

33. Capua, I., R. E. Gough, M. Mancini, C. Casaccia, and C. Weiss. A 'novel' infectious bronchitis strain infecting broiler chickens in Italy. J. Vet. Med. Ser. B. 41:83-89. 1994.

34. Capua, I., K. Mawditt, D. Cavanagh, and R. E. Gough. Serological and molecular characterization of recent infectious bronchitis virus strains from Italy. Proceedings of international symposium on infectious bronchitis and pneumovirus infections in poultry. Rauischholzhausen, Germany. pp. 210-214. 1998.

35. Casais, R., B. Dove, D. Cavanagh, and P. Britton. A recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J. Virol. 77:9084-9089. 2003.

36. Casais, R., V. Thiel, S. G. Siddell, D. Cavanagh, and P. Britton. A reverse genetics system for the avian coronavirus infectious bronchitis virus. J. Virol. 75:12359-12369. 2001.

37. Cavanagh, D. Coronavirus IBV: Structural characterization of the spike protein. J. Gen. Virol. 64:2577-2583. 1983.

38. Cavanagh, D. Recent advances in avian virology. Br. Vet. J. 148:199-222. 1992.

39. Cavanagh, D. Severe acute respiratory syndrome vaccine development: Experiences of vaccination against avian infectious bronchitis coronavirus. Avian Path. 32:567-582. 2003.

40. Cavanagh, D., P. J. Davis, and J. Cook. Infectious bronchitis virus: Evidence for recombination within the Massachusetts serotype. Avian Path. 21:401-408. 1992.

41. Cavanagh, D., P. J. Davis, J. H. Darbyshire, and R. W. Peters. Coronavirus IBV: Virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J. Gen. Virol. 67:1435-1442. 1986.

42. Cavanagh, D., P. J. Davis, and A. P. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11:141-150. 1988.

43. Cavanagh, D., P. J. Davis, D. J. Pappin, M. M. Binns, M. E. Boursnell, and T. D. Brown. Coronavirus IBV: Partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor propolypeptide of IBV strains Beaudette and M41. Virus Res. 4:133-143. 1986.

44. Cavanagh, D., K. Mawditt, P. Britton, and A. D. Naylor. Longitudinal studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Path. 28:593-605. 1998.

45. Cavanagh, D., and S. Naqi. Infectious Bronchitis. In: Diseases of Poultry, 11th ed, Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa. pp. 101-119. 2003.

46. Chandra, M. Comparative nephropathogenicity of different strains of infectious bronchitis virus in chickens. Poult. Sci. 66:954-959. 1987.

47. Chang, R. Y., M. A. Hofmann, P. B. Sethna, and D. A. Brian. A cis-acting function for the coronavirus leader in defective interfering RNA replication. J. Virol. 68:8223-8231. 1994.

48. Chen, B. Y., S. Hosi, T. Nunoya, and C. Itakura. Histopathology and immunochemistry of renal lesions due to infectious bronchitis virus in chickens. Avian Path. 25:269-283. 1996.

49. Chew, P. H., P. S. Wakenell, and T. B. Farver. Pathogenicity of attenuated infectious bronchitis viruses for oviducts of chickens exposed in ovo. Avian Dis. 41:598-603. 1997.

50. Chong, K. T., and K. Apostolov. The pathogenesis of nephritis in chickens induced by infectious bronchitis virus. J. Comp. Pathol. 92:199-211. 1982.

51. Clewley, J. P., J. Morser, R. J. Avery, and B. Lomniczi. Oligonucleotide fingerprinting of ribonucleic acids of infectious bronchitis virus strains. Infect. Immun. 32:1227-1233. 1981.

52. Collisson, E. W., R. Parr, L. Wang, and A. K. Williams. An overview of the molecular characteristics of avian infectious bronchitis virus. Poult. Sci. Rev. 4:41-45. 1992.

53. Collisson, E. W., A. K. Williams, R. Vonder Haar, W. Li, and L. W. Sneed. Sequence comparisons of the 3' end of the genomes of five strains of avian infectious bronchitis virus. Adv. Exp. Med. Biol. 276:373-377. 1990.

54. Colwell, W. M., and P. D. Lukert. Effects of avian infectious bronchitis virus (IBV) on tracheal organ cultures. Avian Dis. 13:888-894. 1969.

55. Condron, R. J., and A. T. Marshall. Pathogenesis of infectious bronchitis nephritis. 1. Morphometric analysis of kidney proximal tubular epithelium in chickens. J. Comp. Path. 96:47-61. 1986.

56. Cook, J. The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. Avian Path. 13:733-741. 1984.

57. Cook, J., and J. W. Huggins. Newly isolated serotypes of infectious bronchitis virus: Their role in disease. Avian Path. 15:129-138. 1986.

58. Cook, J., and M. B. Huggins. Newly isolated serotypes of infectious bronchitis virus: Their role in disease. Avian Path. 15:129-138. 1986.

59. Cook, J., K. Otsuki, N. R. Da Silva Martins, M. M. Ellis, and M. B. Huggins. The secretory antibody response of inbred lines of chickens to avian infectious bronchitis virus infection. Avian Path. 30:233-242. 1992.

60. Cook, J. K. Duration of experimental infectious bronchitis in chickens. Res. Vet. Sci. 9:506-514. 1968.

61. Cook, J. K., J. H. Darbyshire, and R. W. Peters. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch. Virol. 50:109-118. 1976.

62. Cook, J. K., S. J. Orbell, M. A. Woods, and M. B. Huggins. Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Path. 28:477-485. 2002.

63. Cook, J. K., H. W. Smith, and M. B. Huggins. Infectious bronchitis immunity: Its study in chickens experimentally infected with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 67:1427-1434. 1986.

64. Corse, E., and C. E. Machamer. The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. Virology. 312:25-34. 2003.

65. Corse, E., and C. E. Machamer. Infectious bronchitis virus E protein is targeted to the Golgi complex and directs release of virus-like particles. J. Virol. 74:4319-4326. 2000.

66. Cowen, B. S., and S. B. Hitchner. PH stability studies with avian infectious bronchitis virus (Coronavirus) strains. J. Virol. 15:430-432. 1975.

67. Cowley, J. A., C. M. Dimmock, K. M. Spann, and P. J. Walker. Gill-associated virus of Penaeus monodon prawns: An invertebrate virus with ORF1a and ORF1b genes related to arteri and coronaviruses. J. Gen. Virol. 81:1473-1484. 2000.

68. Crinion, R. A., and M. S. Hofstad. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis. 16:351-363. 1972.

69. Cubillos, A., J. Ulloa, V. Cubillos, and J. Cook. Characterisation of strains of infectious bronchitis virus at one day-old. Avian Path. 20:85-99. 1991.

70. Cumming, R. B. Infectious avian nephrosis (uraemia) in Australia. Aust. Vet. J. 39:145-147. 1963.

71. Cumming, R. B. Studies on Australian infectious bronchitis virus. IV. Apparent farm-to-farm airborne transmission of infectious bronchitis virus. Avian Dis. 14:191-195. 1970.

72. Cunningham, C. H. Immunologic methods in avian research: Neutralization test. Avian Dis. 17:227-235. 1973.

73. Dalton, K., R. Casais, K. Shaw, K. Stirrups, S. Evans, P. Britton, K. Brown, and D. Cavanagh. Cis-acting sequences required for coronavirus infectious bronchitis virus defective-RNA replication and packaging. J. Virol. 75:125-133. 2001.

74. Darbyshire, J. H. A clearance test to assess protection in chickens vaccinated against avian infectious bronchitis virus. Avian Path. 14:497-508. 1985.

75. Darbyshire, J. H., and R. W. Peters. Humoral antibody response and assessment of protection following primary vaccination of chicks with maternally derived antibody against avian infectious bronchitis virus. Res. Vet. Sci. 38:14-21. 1985.

76. Darbyshire, J. H., and R. W. Peters. Sequential development of humoral immunity and assessment of protection in chickens following vaccination and challenge with avian infectious bronchitis virus. Res. Vet. Sci. 37:77-86. 1984.

77. Darbyshire, J. H., J. G. Rowell, J. K. Cook, and R. W. Peters. Taxonomic studies on strains of avian infectious bronchitis virus using neutralisation tests in tracheal organ cultures. Arch. Virol. 61:227-238. 1979.

78. Davelaar, F. G., and B. Kouwenhoven. Changes in the Harderian gland of the chicken following conjunctival and intranasal infection with infectious bronchitis virus in one and 20-day old chickens. Avian Path. 5:39-50. 1976.

79. De Herdt, P., R. Ducatelle, E. Uyttebroek, A. Sneep, and R. Torbeyns. Infectious bronchitis serology in broilers and broiler breeders: Correlations between antibody titers and performance in vaccinated flocks. Avian Dis. 45:612-619. 2001.

80. De Wit, J. J. Detection of infectious bronchitis. Avian Path. 29:71-93. 2000.

81. De Wit, J. J., M. C. de John, A. Pijpers, and J. H. Verheijden. Transmission of infectious bronchitis virus within vaccinated and unvaccinated groups of chickens. Avian Path. 27:464-471. 1998.

82. De Wit, J. J., G. Koch, A. Kant, and D. J. van Roozelaar. Detection by immunofluorescent assay of serotype-specific and group-specific antigens for infectious bronchitis virus in tracheas of broilers with respiratory problems. Avian Path. 24:465-474. 1995.

83. De Wit, J. J., D. R. Mekkes, D. R. Kouwenhoven, and J. H. Verheijden. Sensitivity and specificity of serological tests for detection of infectious bronchitis virus induced antibodies in broilers. Avian Path. 26:105-118. 1997.

84. Delaplane, J. P., and H. O. Stuart. The modification of infectious bronchitis virus of chickens as the result of propagation in embryonated chicken eggs. Rhode Island Agricultural Experiment Station, RI. Bull 284. 1941.

85. Delmas, B., and H. Laude. Assembly of coronavirus spike protein into trimers and its role in epitope expression. J. Virol. 64:5367-5375. 1990.

86. Denison, M. R., W. J. Spaan, and Y. van der Meer. The putative helicase of the coronavirus mouse hepatitis virus is processed from the replicase gene polyprotein and localizes in complexes that are active in viral RNA synthesis. J. Virol. 73:6862-6871. 1999.

87. Dhinakar Raj, G., and R. C. Jones. Immunopathogenesis of infection in SPF chicks and commercial broilers of a variant infectious bronchitis virus of economic importance. Avian Path. 25:481-501. 1996.

88. Dhinakar Raj, G., and R. C. Jones. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Path. 26:677-706. 1997.

89. Dhinakar Raj, G., and R. C. Jones. An in-vitro comparison of the virulence of seven strains of infectious bronchitis virus using tracheal and oviduct organ cultures. Avian Path. 25:649-662. 1996.

90. Domingo, E., C. K. Biebricher, M. Eigen, and J. J. Holland. Quasispecies and RNA virus evolution: Principles and consequences. Landes Bioscience. 2001.

91. El Houadfi, M. D., M. D. Jones, J. K. Cook, and A. G. Ambali. The isolation and characterization of six avian infectious bronchitis virus isolated in Morocco. Avian Path. 15:93-105. 1986.

92. Enjuanes, L., W. J. Spaan, E. Snijder, and D. Cavanagh. Nidovirales. In: Virus taxonomy. Classification and nomenclature of viruses, C. M. Fauquet, M. H. van Regenmortel, D. H. Bishop, E. B. Carsten, M. K. Estes, S. M. Lemon, D. J. McGeoch, J. Maniloff, M. A. Mayo, C. R. Pringle and R. B. Wickner, eds. Academic Press, San Diego, CA. pp. 827-834. 2000.

93. Estevez, C., P. Villegas, and J. El-Attrache. A recombination event, induced in ovo, between a low passage infectious bronchitis virus field isolate and a highly embryo adapted vaccine strain. Avian Dis. 47:1282-1290. 2003.

94. Fabricant, J. The early history of infectious bronchitis. Avian Dis. 42:648-650. 1998.

95. Falcone, E., E. D'Amore, L. Di Trani, A. Sili, and M. Tollis. Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction. J. Virol. Meth. 64:125-130. 1997.

96. Ferreira, H. L., D. Pilz, L. G. Mesquita, and T. Cardoso. Infectious bronchitis virus replication in the chicken embryo related cell line. Avian Path. 32:413-417. 2003.

97. Finney, P. M., P. G. Box, and H. C. Holmes. Studies with a bivalent infectious bronchitis killed virus vaccine. Avian Path. 19:435-450. 1990.

98. Fosmire, J. A., K. Hwang, and S. Makino. Identification and characterization of a coronavirus packaging signal. J. Virol. 66:3522-3530. 1992.

99. Fulton, R. M., W. M. Reed, and H. L. Thacker. Cellular response of the respiratory tract of chickens to infection with Massachusetts 41 and Australian T infectious bronchitis viruses. Avian Dis. 37:951-960. 1993.

100. Furuya, T., M. R. MacNaughton, N. La Monica, and M. M. Lai. Natural evolution of coronavirus defective-interfering RNA involves RNA recombination. Virology. 194:408-413. 1993.

101. Gallaher, T. M., S. E. Parker, and M. J. Buchmeier. Neutralization-resistant variants of a neurotropic coronavirus are generated by deletions within the amino-terminal half of the spike glycoprotein. J. Virol. 64:731-741. 1990.

102. Gelb, J., P. A. Fries, C. K. Crary, J. P. Donahoe, and D. E. Roessler. Sentinel bird approach to isolating infectious bronchitis virus. J. Am. Vet. Med. Ass. 190:1628. 1987.

103. Gelb, J., Jr., C. L. Keeler, Jr., W. A. Nix, J. K. Rosenberger, and S. S. Cloud. Antigenic and S-1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. 41:661-669. 1997.

104. Gelb, J., Jr., and S. L. Killian. Serum antibody responses of chickens following sequential inoculations with different infectious bronchitis virus serotypes. Avian Dis. 31:513-522. 1987.

105. Gelb, J., Jr., J. K. Rosenberger, P. A. Fries, S. S. Cloud, E. M. Odor, J. E. Dohms, and J. S. Jaeger. Protection afforded in infectious bronchitis virus-vaccinated sentinel chickens raised in a commercial environment. Avian Dis. 33:764-769. 1989.

106. Gelb, J., Jr., J. B. Wolff, and C. A. Moran. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Dis. 35:82-87. 1991.

107. Gillette, K. G. Plaque formation by infectious bronchitis virus in chicken embryo kidney cell cultures. Avian Dis. 17:369-378. 1973.

108. Gomez, L., and L. G. Raggi. Local immunity to avian infectious bronchitis in tracheal organ culture. Avian Dis. 18:346-368. 1974.

109. Gonzalez, J. M., P. Gomez-Puertas, D. Cavanagh, A. E. Gorbalenya, and L. Enjuanes. A comparative analysis to revise the current taxonomy of the family Coronaviridae. Arch. Virol. 148:2207-2235. 2003.

110. Goodwin, M. A., J. Brown, E. C. Player, W. L. Steffens, D. Hermes, and M. A. Dekich. Fringed membranous particles and viruses in faeces from healthy turkey poults and from poults with putative poult enteritis complex/spiking mortality. Avian Path. 24:497-505. 1995.

111. Gough, R. E., and D. J. Alexander. Comparison of duration of immunity in chickens infected with a live infectious bronchitis vaccine by three different routes. Res. Vet. Sci. 26:329-332. 1979.

112. Gough, R. E., and D. J. Alexander. Comparison of serological tests for the measurement of the primary immune response to avian infectious bronchitis vaccines. Vet. Microb. 2:289-301. 1978.

113. Gough, R. E., W. J. Cox, C. E. Winkler, M. W. Sharp, and D. Spackman. Isolation and identification of infectious bronchitis virus from pheasants. Vet. Rec. 138:208-209. 1996.

114. Griffiths, G., and P. J. Rottier. Cell biology of viruses that assemble along the biosynthetic pathway. Semin. Cell Biol. 3:367-381. 1992.

115. Guittet, M., J. P. Picault, H. Le Coq, J. Lamande, and G. Bennejean. Strategy of protection of the layer against infectious bronchitis virus. Proceedings of the 1st international symposium on infectious bronchitis. Rauischholzhausen, Germany. pp. 298-307. 1988.

116. Guy, J. S. Turkey coronavirus is more closely related to avian infectious bronchitis virus than to mammalian coronaviruses. Avian Path. 29:206-212. 2000.

117. Guy, J. S., H. J. Barnes, L. G. Smith, and J. Breslin. Antigenic characterization of a turkey coronavirus identified in poult enteritis and mortality syndrome-affected turkeys. Avian Dis. 41:583-590. 1997.

118. Hatcher, J. A., J. K. Skeeles, P. J. Blore, and J. D. Story. Comparison of the hemagglutination-inhibition test with the constant-virus diluting-serum microneutralization test for detecting antibody to avian infectious bronchitis virus. Avian Dis. 27:1157-1161. 1983.

119. Hawkes, R. A., J. H. Darbyshire, R. W. Peters, R. W. Mockett, and D. Cavanagh. Presence of viral antigen and antibody in the trachea of chickens infected with avian infectious bronchitis virus. Avian Path. 12:331-340. 1983.

120. Heath, B. C. Chemical pathology of nephrosis induced by an infectious bronchitis virus. Avian Dis. 14:95-106. 1970.

121. Hein, R. G., G. Slacum, and P. Lynch. In-vivo characterization of recent infectious bronchitis virus (IBV) variants isolated from broilers in the USA. Proceedings of international symposium on infectious bronchitis and pneumovirus infections in poultry. Rauischholzhausen, Germany. pp. 54. 1998.

122. Hitchner, S. B., and P. G. White. Growth curve studies of chick embryo-propagated infectious bronchitis virus. Poult. Sci. 34:590-594. 1955.

123. Hofstad, M. S. Antigenic differences among isolates of infectious bronchitis virus. Am. J. Vet. Res. 19:740-742. 1958.

124. Hofstad, M. S. Cross-immunity in chickens using seven isolates of avian infectious bronchitis virus. Avian Dis. 25:650-654. 1981.

125. Hofstad, M. S., and S. G. Kenzy. Susceptibility of chicks hatched from recovered hens to infectious bronchitis. Cornell Vet. 40:87-89. 1950.

126. Holmes, H. C. Neutralizing antibody in nasal secretions of chickens following administration of avian infectious bronchitis virus. Arch. Gesamte Virusforschung. 43:235-241. 1973.

127. Hopkins, S. R. Serological comparisons of strains of infectious bronchitis virus using plaque-purified isolants. Avian Dis. 18:231-239. 1974.

128. Hopkins, S. R. Typing field isolates of infectious bronchitis by the plaque-reduction test. Avian Dis. 22:71-81. 1978.

129. Hopkins, S. R., and H. W. Yoder, Jr. Increased incidence of airsacculitis in broilers infected with Mycoplasma synoviae and chicken-passaged infectious bronchitis vaccine virus. Avian Dis. 28:386-396. 1984.

130. Hopkins, S. R., and H. W. Yoder, Jr. Reversion to virulence of chicken-passaged infectious bronchitis vaccine virus. Avian Dis. 30:221-223. 1986.

131. Ignjatovic, J., D. F. Ashton, R. L. Reece, P. C. Scott, and P. Hooper. Pathogenicity of Australian strains of avian infectious bronchitis virus. J. Comp. Path. 126:115-123. 2002.

132. Ignjatovic, J., and F. Ashton. Detection and differentiation of avian infectious bronchitis viruses using a monoclonal antibody-based ELISA. Avian Path. 25:721-736. 1996.

133. Ignjatovic, J., and L. Galli. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch. Virol. 138:1-2. 1994.

134. Ignjatovic, J., and S. Sapats. Avian infectious bronchitis virus. Rev. Sci. Tech. 19:493-508. 2000.

135. Izeta, A., S. Smerdou, S. Alonso, Z. Penzes, A. Mendez, J. Plana-Duran, and L. Enjuanes. Replication and packaging of transmissible gastroenteritis coronavirus-derived synthetic minigenomes. J. Virol. 73:1535-1545. 1999.

136. Jackwood, M. W., H. M. Kwon, and D. A. Hilt. Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Dis. 36:403-409. 1992.

137. Jackwood, M. W., N. M. Yousef, and D. A. Hilt. Further development and use of a molecular serotype identification test for infectious bronchitis virus. Avian Dis. 41:105-110. 1997.

138. Janse, E. M., D. van Roozelaar, and G. Koch. Leukocyte subpopulations in kidney and trachea of chickens infected with infectious bronchitis virus. Avian Path. 23:513-523. 1994.

139. Jia, W., K. Karaca, C. R. Parrish, and S. A. Naqi. A novel variant of avian infectious bronchitis virus resulting from recombination among three different strains. Arch. Virol. 140:259-271. 1995.

140. Jia, W., S. Mondal, and S. Naqi. Genetic and antigenic diversity in avian infectious bronchitis virus isolates of the 1940s. Avian Dis. 46:437-441. 2002.

141. Johnson, M., C. Pooley, J. Ignjatovic, and S. G. Tyack. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. Vaccine. 21:2730-2736. 2003.

142. Johnson, R. B., and W. W. Marquardt. The neutralizing characteristics of strains of infectious bronchitis virus as measured by the constant-virus variable-serum method in chicken tracheal cultures. Avian Dis. 19:82-90. 1975.

143. Jones, R. C., and A. G. Ambali. Re-excretion of an enterotropic infectious bronchitis virus by hens at point of lay after experimental infection at day old. Vet. Rec. 120:617-618. 1987.

144. Jones, R. C., and F. Jordan. The exposure of day-old chicks to infectious bronchitis and subsequent development of the oviduct. Vet. Rec. 87:504-505. 1970.

145. Jones, R. C., and F. T. Jordan. Persistence of virus in the tissues and development of the oviduct in the fowl following infection at day old with infectious bronchitis virus. Res. Vet. Sci. 13:52-60. 1972.

146. Jordan, F., and T. J. Nassar. The combined influence of age of embryo and temperature and duration of incubation on the replication and yield of avian infectious bronchitis (IB) virus in the developing chick embryo. Avian Path. 2:279-294. 1973.

147. Jungherr, E. L., T. W. Chomiak, and R. E. Luginbuhl. Immunologic differences in strains of infectious bronchitis. Proceedings of the 60th annual meeting. U.S Livestock Sanit. A. pp. 203-209. 1956.

148. Jungherr, E. L., and N. L. Terrel. Naturally acquired passive immunity to infectious bronchitis in chicks. Am. J. Vet. Res. 9:201-205. 1948.

149. Kapczynski, D. R., D. A. Hilt, D. P. Shapiro, H. S. Sellers, and M. W. Jackwood. Protection of chickens from infectious bronchitis by in ovo and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. Avian Dis. 47:272-285. 2003.

150. Karaca, K., and S. Naqi. A monoclonal antibody blocking ELISA to detect serotype-specific infectious bronchitis virus antibodies. Vet. Microb. 34:249-257. 1993.

151. Keck, J. G., G. K. Matsushima, and S. Makino. In vivo RNA-RNA recombination of coronavirus in mouse brain. J. Virol. 62:1810-1813. 1988.

152. King, D. J. Identification of recent infectious bronchitis virus isolates that are serologically different from current vaccine strains. Avian Dis. 32:362-364. 1988.

153. King, D. J. Observations on the preparation and stability of infectious bronchitis virus hemagglutination antigen from virus propagated in chicken embryos and chicken kidney cell cultures. Avian Dis. 28:504-513. 1984.

154. King, D. J., and S. R. Hopkins. Evaluation of the hemagglutination-inhibition test for measuring the response of chickens to avian infectious bronchitis virus vaccination. Avian Dis. 27:100-112. 1983.

155. King, D. J., and S. R. Hopkins. Rapid serotyping of infectious bronchitis virus isolates with the hemagglutination-inhibition test. Avian Dis. 28:727-733. 1984.

156. Kingham, B. E., C. L. Keeler, Jr., W. A. Nix, B. S. Ladman, and J. Gelb, Jr. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. Avian Dis. 44:325-335. 2000.

157. Klieve, A. V., and R. B. Cumming. Immunity and cross-protection to nephritis produced by Australian infectious bronchitis viruses used as vaccines. Avian Path. 17:829-839. 1988.

158. Klieve, A. V., and R. B. Cumming. Infectious bronchitis: Safety and protection in chickens with maternal antibody. Aust. Vet. J. 65:396-397. 1988.

159. Klumperman, J., J. K. Locker, A. Meijer, M. C. Horzinek, H. J. Geuze, and P. J. Rottier. Coronavirus M proteins accumulate in the Golgi complex beyond the site of virion budding. J. Virol. 68:6523-6534. 1994.

160. Koch, G., L. Hartog, A. Kant, and D. J. van Roozelaar. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: Correlation with biological functions. J. Gen. Virol. 71:1929-1935. 1990.

161. Koch, G., L. Hartog, A. Kant, D. J. van Roozelaar, and G. G. Boer. Antigenic differentiation of avian bronchitis virus variant strains employing monoclonal antibodies. Israel J. Vet. Med. 42:89-97. 1986.

162. Kottier, S. A., D. Cavanagh, and P. Britton. Experimental evidence of recombination in coronavirus infectious bronchitis virus. Virology. 213:569-580. 1995.

163. Kusters, J. G., and E. J. Jager. Sequence evidence for RNA recombination in field isolates of avian infectious bronchitis virus. Vaccine. 8:605-608. 1990.

164. Kusters, J. G., H. G. Niesters, N. M. Bleumink-Pluym, F. G. Davelaar, M. C. Horzinek, and B. A. van der Zeijst. Molecular epidemiology of infectious bronchitis virus in the Netherlands. J. Gen. Virol. 68:343-352. 1987.

165. Kusters, J. G., H. G. Niesters, J. A. Lenstra, M. C. Horzinek, and B. A. van der Zeijst. Phylogeny of antigenic variants of avian coronavirus IBV. Virology. 169:217-221. 1989.

166. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

167. Lai, M. M. RNA recombination in animal and plat viruses. Microb. Rev. 56:61-79. 1992.

168. Lai, M. M., and D. Cavanagh. The molecular biology of coronaviruses. Adv. Virus Res. 48:1-90. 1997.

169. Lai, M. M., and K. V. Holmes. Coronaviridae: The viruses and their replication. In: Fields-Virology, 4th ed, D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman and S. E. Straus, eds. Vol. 1. Lippincott Williams & Wilkins. pp. 1163-1185. 2001.

170. Lambrechts, C., M. B. Pensaert, and R. Ducatelle. Comparative study of the infectivity of Belgian NIBV isolates for the kidney after intravenous inoculation in chickens. Proceedings of the 2nd international symposium on infectious bronchitis. Rauischholzhausen, Germany. pp. 113-119. 1991.

171. Lashgari, M. S., and J. A. Newman. Serological comparison and antigenic relationships of seven serotypes of infectious bronchitis virus using the hemagglutination-inhibition test. Avian Dis. 28:435-443. 1984.

172. Laude, H., and P. S. Masters. The Coronavirus nucleocapsid protein. In: The Coronaviridae, S. G. Sidel, eds. Plenum Press, New York. pp. 141-163. 1995.

173. Lee, C. W., D. A. Hilt, and M. W. Jackwood. Redesign of primer and application of the reverse transcriptase-polymerase chain reaction and restriction fragment length polymorphism test to the DE072 strain of infectious bronchitis virus. Avian Dis. 44:650-654. 2000.

174. Lee, C. W., D. A. Hilt, and M. W. Jackwood. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. J. Vet. Diagn. Invest. 15:344-348. 2003.

175. Lee, C. W., and M. W. Jackwood. Evidence of genetic diversity generated by recombination among avian coronavirus IBV. Arch. Virol. 145:2135-2148. 2000.

176. Lee, C. W., and M. W. Jackwood. Spike gene analysis of the DE072 strain of infectious bronchitis virus: Origin and evolution. Virus Genes. 22:85-91. 2001.

177. Lenstra, J. A., J. G. Kusters, G. Koch, and B. A. van der Zeijst. Antigenicity of the peplomer protein of infectious bronchitis virus. Mol. Immunol. 26:7-15. 1989.

178. Li, D., and D. Cavanagh. Coronavirus IBV-induced membrane fusion occurs at near-neutral pH. Arch. Virol. 122:307-316. 1992.

179. Lim, K. P., L. F. Ng, and D. X. Liu. Identification of a novel cleavage activity of the first papain-like proteinase domain encoded by open reading frame 1a of the coronavirus avian infectious bronchitis virus and characterization of the cleavage products. J. Virol. 74:1674-1685. 2000.

180. Lister, S. A., J. V. Beer, R. E. Gough, R. G. Holmes, R. G. Jones, and R. G. Orton. Outbreaks of nephritis in pheasants (Phasianus colchicus) with a possible coronavirus etiology. Vet. Rec. 117:612-613. 1985.

181. Liu, D. X., and S. C. Inglis. Identification of two new polypeptides encoded by mRNA 5 of the coronavirus infectious bronchitis virus. Virology. 186:342-347. 1992.

182. Liu, D. X., and S. C. Inglis. Internal entry of ribosomes on a tricistronic mRNA encoded by infectious bronchitis virus. J. Virol. 66:6143-6154. 1992.

183. Liu, H. J., L. H. Lee, W. L. Shih, M. Y. Lin, and M. H. Liao. Detection of infectious bronchitis virus by multiplex polymerase chain reaction and sequence analysis. J. Virol. 109:31-37. 2003.

184. Lohr, J. E. Diagnosis of infectious bronchitis (IB) by examination of tracheal mucus for IB-precipitating antigens. Avian Dis. 25:1058-1064. 1981.

185. Loomis, L. N., C. H. Cunningham, M. L. Gray, and F. J. Thorp. Pathology of the chicken embryo infected with infectious bronchitis virus. Am. J. Vet. Res. 11:245-251. 1950.

186. Lucio, B., and J. Fabricant. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. Avian Dis. 34:865-870. 1990.

187. Lukert, P. D. Comparative sensitivities of embryonating chicken eggs and primary chicken embryo kidney and liver cell cultures to infectious bronchitis virus. Avian Dis. 9:308-316. 1965.

188. MacDonald, J. W., and D. A. McMartin. Observations on the effects of the H52 and H120 vaccine strains of the infectious bronchitis virus in the domestic fowl. Avian Path. 5:157-173. 1976.

189. Makino, S., C. K. Schieh, and L. H. Soe. Primary structure and translation of a defective-interfering RNA of murine coronavirus. Virology. 166:550-560. 1988.

190. Makino, S., F. Taguchi, and K. Fujiwara. Defective interfering particles of mouse hepatitis virus. Virology. 133:9-17. 1984.

191. Makino, S., K. Yokomori, and M. M. Lai. Analysis of efficiently packaged defective interfering RNAs of murine coronavirus: Localization of a possible RNA-packaging signal. J. Virol. 64:6045-6053. 1990.

192. Marquardt, W. W., D. B. Snyder, and B. A. Schlotthober. Detection and quantification of antibodies to infectious bronchitis virus by enzyme-linked immunosorbent assay. Avian Dis. 25:713-722. 1981.

193. Martins, N. R., A. P. Mockett, A. D. Barrett, and J. K. Cook. IgM responses in chicken serum to live and inactivated infectious bronchitis virus vaccines. Avian Dis. 35:470-475. 1991.

194. McDougall, J. S. Avian infectious bronchitis: The protection afforded by an inactivated virus vaccine. Vet. Rec. 85:378-381. 1969.

195. McDougall, J. S. Infectious bronchitis in laying fowls: Its effect upon egg production and subsequent egg quality. Vet. Rec. 83:84-86. 1968.

196. McMartin, D. A. Infectious bronchitis. In: Virus infections of vertebrates. Virus infections of birds, J. B. McFerran and M. S. McNulty, eds. Vol. 4. Elsevier Science Publishers, Amsterdam, Holland. pp. 249-275. 1993.

197. Meulemans, G., M. C. Carlier, M. Gonze, P. Petit, and M. Vandenbroeck. Incidence, characterisation and prophylaxis of nephropathogenic avian infectious bronchitis viruses. Vet. Rec. 120:205-206. 1987.

198. Mockett, A., J. Cook, and M. B. Huggins. Maternally-derived antibody to infectious bronchitis virus: Its detection in chicken trachea and serum and its role in protection. Avian Path. 16:407-416. 1987.

199. Mockett, A. P., and J. H. Darbyshire. Comparative studies with an enzyme-linked immunosorbent assay (ELISA) for antibodies to avian infectious bronchitis virus. Avian Path. 10:1-10. 1981.

200. Mondal, S. P., and C. J. Cardona. Characterization of infectious bronchitis virus isolates by slot blot hybridization. Avian Dis. 47:725-730. 2003.

201. Mondal, S. P., and S. A. Naqi. Maternal antibody to infectious bronchitis virus: Its role in protection against infection and development of active immunity to vaccine. Vet. Immunol. Immunopathol. 79:31-40. 2001.

202. Moore, K. M., M. W. Jackwood, and D. A. Hilt. Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. Arch. Virol. 142:2249-2256. 1997.

203. Muneer, M. A., D. A. Halvorson, V. Sivanandan, J. A. Newman, and C. N. Coon. Effects of infectious bronchitis virus (Arkansas strain) on laying chickens. Avian Dis. 30:644-647. 1986.

204. Muneer, M. A., J. A. Newman, D. A. Halvorson, V. Sivanandan, and C. N. Coon. Effects of avian infectious bronchitis virus (Arkansas strain) on vaccinated laying chickens. Avian Dis. 31:820-828. 1987.

205. Nagano, H., M. Tsuchimoto, T. Hohdatsu, T. Yamagami, S. Ide, Y. Eiguchi, Y. Tanaka, and H. Yamagishi. Enzyme-linked immunosorbent assay for the detection of infectious bronchitis virus (IBV) antigen with monoclonal antibody. Jap. J. Vet. Sci. 52:657-659. 1990.

206. Nagaraja, K. V., and B. S. Pomeroy. Coronaviral enteritis of turkeys (bluecomb disease). In: Diseases of poultry, 10th ed, B. W. Calnek, H. J. Barnes, C. W. Beard, L. R. McDougald and Y. M. Saif, eds. Iowa State University Press, Ames, IA. pp. 686-692. 1997.

207. Nakamura, K., J. Cook, K. Otsuki, M. B. Huggins, and J. A. Frazier. Comparative study of respiratory lesions in two chicken lines of different susceptibility, infected with infectious bronchitis virus: Histology, ultrastructure and immunochemestry. Avian Path. 20:245-261. 1991.

208. Naqi, S., K. Gay, P. Patalla, S. Mondal, and R. Liu. Establishment of persistent avian infectious bronchitis virus infection in antibody-free and antibody-positive chickens. Avian Dis. 47:594-601. 2003.

209. Naqi, S., K. Karaca, and B. Bauman. A monoclonal antibody-based antigen capture enzyme-linked immunosorbent assay for identification of infectious bronchitis virus serotypes. Avian Path. 22:555-564. 1993.

210. Nauwynch, H., and M. B. Pensaert. Studies on the pathogenesis of infections with a nephropathogenic variant of infectious bronchitis virus in chickens. Proceedings of the 1st international symposium on infectious bronchitis. Rauischholzhausen, Germany. pp. 113-119. 1988.

211. Neister, H. G., N. M. Bleumink-Pluym, A. D. Osterhaus, M. C. Horzined, and B. A. van der Zeijst. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology. 118:511-519. 1987.

212. Ng, L. F., and D. X. Liu. Further characterisation of the coronavirus IBV ORF 1a products encoded by the 3C-like proteinase domain and the flanking regions. Adv. Exp. Med. Biol. 440:161-171. 1998.

213. Ng, L. F., and D. X. Liu. Further characterization of the coronavirus infectious bronchitis virus 3C-like proteinase and determination of a new cleavage site. Virology. 272:27-39. 2000.

214. Nguyen, V. P., and B. G. Hogue. Protein interactions during coronavirus assembly. J. Virol. 71:9278-9284. 1997.

215. Oliver, M. A., and J. A. Marsh. In vitro thymulin treatments enhance avian lung natural killer cell cytotoxicity in response to infectious bronchitis virus. Intern. Immunopharm. 3:107-113. 2003.

216. Opstelten, D. J., P. de Groote, and M. C. Horzinec. Disulfide bonds in folding and transport of mouse hepatitis coronavirus glycoproteins. J. Virol. 67:7394-7401. 1993.

217. Opstelten, D. J., M. J. B. Raamsman, and K. Wolfs. Envelope glycoprotein interactions in coronavirus assembly. J. Cell. Biol. 131:339-349. 1995.

218. Otsuki, K., J. W. Huggins, and J. Cook. Comparison of the susceptibility to avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens. Avian Path. 19:467-475. 1990.

219. Otsuki, K., K. Noro, H. Yamamoto, and M. Tsubokura. Studies on avian infectious bronchitis virus (IBV). II. Propagation of IBV in several cultured cells. Arch. Virol. 60:115-122. 1979.

220. Otsuki, K., H. Yamamoto, and M. Tsubokura. Studies on avian infectious bronchitis virus (IBV). I. Resistance of IBV to chemical and physical treatments. Arch. Virol. 60:25-32. 1979.

221. Owen, R. L., B. S. Cowen, A. L. Hattel, S. Naqi, and R. A. Wilson. Detection of viral antigen following exposure of one-day old chickens to the Holland-52 strain of infectious bronchitis virus. Avian Path. 20:663-673. 1991.

222. Parker, S. E., T. M. Gallagher, and M. J. Buchmeier. Sequence analysis reveals extensive polymorphism and evidence of deletions within the E2 glycoprotein gene of several strains of murine hepatitis virus. Virology. 173:664-673. 1989.

223. Parsons, D., M. M. Ellis, D. Cavanagh, and J. K. Cook. Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet. Rec. 131:408-411. 1992.

224. Patterson, S., and R. W. Bingham. Electron microscope observations on the entry of avian infectious bronchitis virus into susceptible cells. Arch. Virol. 52:191-200. 1976.

225. Pei, J., W. E. Briles, and E. W. Collisson. Memory T cells protect chicks from acute infectious bronchitis virus infection. Virology. 306:376-384. 2003.

226. Pensaert, M. B., and C. Lambrechts. Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologous and heterologous strains. Avian Path. 23:631-641. 1994.

227. Pradhan, H. K., G. C. Mohanty, and B. S. Rajya. Comparative sensitivities of oviduct and tracheal organ cultures and chicken embryo kidney cell cultures to infectious bronchitis virus. Avian Dis. 27:594-601. 1983.

228. Purchase, H. G., C. H. Cunningham, and B. R. Burmester. Genetic differences among chicken embryos in response to inoculation with an isolate of infectious bronchitis virus. Avian Dis. 10:162-172. 1966.

229. Racaniello, V. R., and D. Baltimore. Cloned poliovirus complementary DNA is infectious in mammalian cells. Science. 214:916-919. 1981.

230. Ratanasethakul, C., and R. B. Cumming. Effect of environmental temperature on the mortality in vaccinated chickens after challenge with Australian infectious bronchitis virus. Aust. Vet. J. 60:255-256. 1983.

231. Risco, C., M. Muntion, L. Enjuanes, and J. L. Carrascosa. Two types of virus-related particles are found during transmissible gastroenteritis virus morphogenesis. J. Virol. 72:4022-4031. 1998.

232. Rosenberger, J. K., R. L. Alphin, and W. C. Krauss. Cross-protection studies with a Holland strain (Nobilis H-52) of infectious bronchitis virus. Avian Dis. 20:199-201. 1976.

233. Rottier, P. J., J. Armstrong, and D. I. Meyer. Signal recognition particle-dependent insertion of coronavirus E1, an intracellular membrane glycoprotein. J. Biol. Chem. 260:4648-4652. 1985.

234. Salanueva, I. J., J. L. Carrascosa, and C. Risco. Structural maturation of the transmissible gastroenteritis coronavirus. J. Virol. 73:7952-7964. 1999.

235. Sapats, S. I., F. Ashton, P. J. Wright, and J. Ignjatovic. Novel variation in the N protein of avian infectious bronchitis virus. Virology. 226:412-417. 1996.

236. Sapats, S. I., F. Ashton, P. J. Wright, and J. Ignjatovic. Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses: Identification of a novel genotypic group in Australia. J. Gen. Virol. 77:413-418. 1996.

237. Satyanarayana, T., M. Bar-Joseph, M. Mawassi, M. R. Albiach-Marti, M. A. Ayllon, S. Gowda, M. E. Hilf, P. Moreno, S. M. Garnsey, and W. O. Dawson. Amplification of Citrus tristeza virus from a cDNA clone and infection of citrus trees. Virology. 280:87-96. 2001.

238. Satyanarayana, T., S. Gowda, V. P. Boyko, M. R. Albiach-Marti, M. Mawassi, J. Navas-Castillo, A. V. Karasev, V. Dolja, M. E. Hilf, D. J. Lewandowski, P. Moreno, M. Bar-Joseph, S. M. Garnsey, and W. O. Dawson. An engineered closterovirus RNA replicon and analysis of heterologous terminal sequences for replication. Proc. Natl. Acad. Sci. USA. 96:7433-7438. 1999.

239. Schalk, A. F., and M. C. Hawn. An apparently new respiratory disease of chicks. J. Am. Vet. Med. Ass. 78:413-422. 1931.

240. Schnitzlein, W. M., J. Radzevicius, and D. N. Tripathy. Propagation of infectious laryngotracheitis virus in an avian liver cell line. Avian Dis. 38:211-217. 1994.

241. Seo, S. H., and E. W. Collisson. Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. J. Virol. 71:5173-5177. 1997.

242. Seo, S. H., L. Wang, R. Smith, and E. W. Collisson. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. J. Virol. 71:7889-7894. 1997.

243. Sevoian, M., and P. P. Levine. Effects of infectious bronchitis on the reproductive tract, egg production and egg quality of laying chickens. Avian Dis. 1:136-164. 1957.

244. Siller, W. G., and R. B. Cumming. The histopathology of an interstitial nephritis in the fowl produced experimentally with infectious bronchitis virus. J. Path. 114:163-173. 1974.

245. Smith, H. W., J. K. Cook, and Z. E. Parsell. The experimental infection of chickens with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 66:777-786. 1985.

246. Song, C. S., J. H. Kim, Y. J. Lee, S. J. Kim, Y. Izumiya, Y. Tohya, H. K. Jang, and T. Mikami. Detection and classification of infectious bronchitis viruses isolated in Korea by dot-immunoblotting assay using monoclonal antibodies. Avian Dis. 42:92-100. 1998.

247. Song, C. S., Y. J. Lee, C. W. Lee, H. W. Sung, J. H. Kim, I. P. Mo, Y. Izumiya, H. K. Jang, and T. Mikami. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. J. Gen. Virol. 79:719-723. 1998.

248. Steinhauer, D. A., and J. J. Holland. Direct method for quantitation of extreme polymerase error frequencies at selected single base sites in viral RNA. J. Virol. 57:219-228. 1986.

249. Stern, D. F., L. Burgess, and B. M. Sefton. Structural analysis of virion proteins of the avian coronavirus infectious bronchitis virus. J. Virol. 42:208-219. 1982.

250. Stern, D. F., and B. M. Sefton. Coronavirus proteins: Biogenesis of avian infectious bronchitis virus virion proteins. J. Virol. 44:794-803. 1982.

251. Stirrups, K., K. Shaw, S. Evans, K. Dalton, R. Casais, D. Cavanagh, and P. Britton. Expression of reporter genes from the defective RNA CD-61 of the coronavirus infectious bronchitis virus. J. Gen. Virol. 81:1687-1698. 2000.

252. Stirrups, K., K. Shaw, S. Evans, K. Dalton, D. Cavanagh, and P. Britton. Leader switching occurs during the rescue of defective RNAs by heterologous strains of the coronavirus infectious bronchitis virus. J. Gen. Virol. 81:791-801. 2000.

253. Stirrups, K., K. Shaw, S. Evans, K. Dalton, D. Cavanagh, and P. Britton. Rescue of IBV D-RNA by heterologous helper virus strains. Adv. Exp. Med. Biol. 440:259-264. 1998.

254. Sturman, L. S., K. V. Holmes, and J. Behnke. Isolation of coronavirus envelope glycoproteins and interaction with the viral nucleocapsid. J. Virol. 33:449-462. 1980.

255. Tahara, S. M., T. A. Dietlin, and C. C. Bergmann. Coronavirus translational regulation: Leader affects mRNA efficiency. Virology. 202:621-630. 1994.

256. Thiel, V., J. Herold, B. Schelle, and S. G. Siddell. Infectious RNA transcribed in vitro from a cDNA copy of the human coronavirus genome cloned in vaccinia virus. J. Gen. Virol. 82:1273-1281. 2001.

257. Thiel, V., and S. G. Siddell. Internal ribosome entry in the coding region of murine hepatitis virus mRNA 5. J. Gen. Virol. 75:3041-3046. 1994.

258. Timms, L. M., C. D. Bracewell, and D. J. Alexander. Cell mediated and humoral immune response in chickens infected with infectious bronchitis. British Vet. Journal. 136:349-356. 1980.

259. Tooze, J., and S. Tooze. Infection of AtT20 murine pituitary tumour cells by mouse hepatitis virus stran A59: Virus budding is restricted to the Golgi region. Eur. J. Cell. Biol. 37:203-212. 1985.

260. Tooze, J., S. Tooze, and G. Warren. Replication of coronavirus MHV-A59 in sac- cells: Determination of the first site of budding of the progeny virions. Eur. J. Cell. Biol. 33:281-293. 1984.

261. Toro, H., E. Reyes, T. Redmann, and E. F. Kaleta. Local and systemic specific antibody response of different chicken lines after ocular vaccination against infectious bronchitis. J. Vet. Med. Ser. B. 43:449-454. 1996.

262. Van der Most, R. G., P. J. Bredenbeek, and W. J. Spaan. A domain at the 3' end of the polymerase gene is essential for encapsidation of coronavirus defective interfering RNAs. J. Virol. 65:3219-3226. 1991.

263. Van Roeckel, H., K. L. Bullis, M. K. Clarke, O. M. Oleisuk, and F. G. Sperling. Infectious bronchitis. Mass. Agr. Exp. Sta. Bull.1-47. 1950.

264. Van Roeckel, H., K. L. Bullis, O. S. Flint, and M. K. Clarke. Poultry disease control service. Massachusetts Agricultural Experiment Station, MA. Annual Report. pp. 99-102. 1942.

265. Van Roeckel, H., M. K. Clarke, K. L. Bullis, O. M. Oleisuk, and F. G. Sperling. Infectious bronchitis. Am. J. Vet. Res. 12:140-146. 1951.

266. Vennema, H., L. Heijnen, and A. J. Zijderveld. Intracellular transport of recombinant coronavirus spike proteins: Implications for virus assembly. J. Virol. 64:339-346. 1990.

267. Vennema, H., L. Heijnen, and A. J. Zijderveld. Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. EMBO. J. 15:2020-2028. 1996.

268. Wadey, C. N., and J. T. Faragher. Australian infectious bronchitis viruses: Identification of nine subtypes by a neutralisation test. Res. Vet. Sci. 30:70-74. 1981.

269. Wakenell, P. S., J. M. Sharma, and R. F. Slocombe. Embryo vaccination of chickens with infectious bronchitis virus: Histologic and ultrastructural lesion response and immunologic response to vaccination. Avian Dis. 39:752-765. 1995.

270. Wang, C. H., and Y. C. Huang. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145:291-300. 2000.

271. Wang, L., D. Junker, and E. W. Collisson. Evidence of natural recombination within the S1 genes of infectious bronchitis virus. Virology. 192:710-716. 1993.

272. Wang, L., Y. Xu, and E. W. Collisson. Experimental confirmation of recombination upstream of the S1 hypervariable region of infectious bronchitis virus. Virus Res. 49:139-145. 1997.

273. Wang, X., W. M. Schnitzlein, D. N. Tripathy, T. Girshick, and M. I. Khan. Construction and immunogenicity studies of recombinant fowl poxvirus containing the S1 gene of Massachusetts 41 strain of infectious bronchitis virus. Avian Dis. 46:831-838. 2002.

274. Wilcox, G. E., N. Nandapalan, R. L. Flower, and D. Fry-Smith. Comparison of a microneutralisation test with ELISA and precipitin tests for detection of antibodies to infectious bronchitis virus in chickens. Aust. Vet. J. 60:119-122. 1983.

275. Winterfield, R. W., and M. A. Albassam. Nephropathogenicity of infectious bronchitis virus. Poult. Sci. 63:2358-2363. 1984.

276. Winterfield, R. W., and A. M. Fadly. Potential for polyvalent infectious bronchitis vaccines. Am. J. Vet. Res. 36:524-526. 1975.

277. Winterfield, R. W., and A. M. Fadly. Some characteristics of isolates of infectious bronchitis virus from commercial vaccines. Avian Dis. 16:746-755. 1972.

278. Winterfield, R. W., A. M. Fadly, and A. A. Bickford. The immune response to infectious bronchitis virus determined by respiratory signs, virus infection, and histopathological lesions. Avian Dis. 16:260-269. 1972.

279. Winterfield, R. W., and S. B. Hitchner. Etiology of an infectious nephritis-nephrosis syndrome of chickens. Am. J. Vet. Res. 23:1273-1279. 1962.

280. Woo, K., M. Joo, and K. Narayanan. Murine coronavirus packaging signal confers packaging to nonviral RNA. J. Virol. 71:824-827. 1997.

281. Wunderwald, C., and R. K. Hoop. Serological monitoring of 40 Swiss fancy breed poultry flocks. Avian Path. 31:157-162. 2002.

282. Yagyu, K., and S. Ohta. Enzyme-linked immunosorbent assay for the detection of infectious bronchitis virus antigens. Nippon Juigaku Zasshi. 49:757-763. 1987.

283. Yount, B., K. M. Curtis, and R. S. Baric. Strategy for systematic assembly of large RNA and DNA genomes: Transmissible gastroenteritis virus model. J. Virol. 74:10600-10611. 2000.

284. Yu, L., Y. Jiang, S. Low, Z. Wang, S. J. Nam, W. Liu, and J. Kwang. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis. 45:416-424. 2001.

285. Zanella, A., R. Marchi, D. Mellano, and W. Ponti. Avian infectious bronchitis: Nephropathogenic and respiratory virus isolates and their spreading in Italy. Proceedings of the 1st international symposium on infectious bronchitis. Rauischholzhausen, West Germany. pp. 245-249. 1989.

286. Zhao, X., K. Shaw, and D. Cavanagh. Presence of subgenomic mRNAs in virions of coronavirus IBV. Virology. 196:172-178. 1993.

287. Zhou, J. Y., J. X. Wu, L. Q. Cheng, X. J. Zheng, H. Gong, S. Shang, and E. Zhou. Expression of immunogenic S1 glycoprotein of infectious bronchitis virus in transgenic potatoes. J. Virol. 77:9090-9093. 2003.

288. Zwaagstra, K. A., B. A. van der Zeijst, and J. G. Kusters. Rapid detection and identification of avian infectious bronchitis virus. J. Clin. Microb. 30:79-84. 1992.

CHAPTER 3

EFFECT OF AN IN VIVO INTESTINE PASSAGED INFECTIOUS BRONCHITIS VIRUS ON SELECTED TISSUES OF SPECIFIC PATHOGEN FREE CHICKENS [1]

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Key Words: avian infectious bronchitis virus, effect of infectious bronchitis virus, intestine passages.

Abbreviations: IB = infectious bronchitis; IBV = infectious bronchitis virus; IP = intestine-passaged; NP = Non intestine passaged; Vx = commercial vaccine; EP = epithelial proliferation; LI = lymphocytic infiltration; RT-PCR = reverse transcriptase-polymerase chain reaction; RFLP = restriction fragment length polymorphism; SNK = Student-Newman-Keuls test; SPF = specific pathogen free.

Summary. The effect of an intestine-passaged IBV Arkansas strain (IP-Ark 1, 13th passage) on the trachea and cecal tonsils of SPF chickens was evaluated following intraocular inoculation at day of age. The effect of the IP-Ark 1 was also compared with the original non-passaged Arkansas (NP-Ark 1) strain and a commercial IBV Arkansas vaccine (Vx-Ark). The presence of IBV in the allantoic fluid of SPF chicken embryos inoculated with the processed tracheal and cecal tonsil tissues was detected at several sampling ages by rapid plate hemagglutination assay and reverse transcriptase-polymerase chain reaction. Clinical signs, and tracheal lymphocytic infiltration (LI) and epithelium proliferation (EP) lesions were scored and used as parameters to determine the effect of the viruses for the upper respiratory tract. The IP-Ark 1 virus was consistently detected for a shorter period in the tracheas (up to day 6) when compared with the NP-Ark 1 (up to 12 days) and the Vx-Ark (from 6 to 12 days) viruses. Overall, no significant differences ($p<0.05$) in the LI and EP scores were observed between the IP-Ark 1 and NP-Ark 1 groups. The group inoculated with the IP-Ark 1 exhibited higher mean LI and EP scores, which were significantly higher at day 6 and 9 when compared with the Vx-Ark group. No gross or microscopic lesions were observed in the cecal tonsils at any age. Although only mild to moderate tracheal lesions and no clinical signs were observed in the IP-Ark 1 group, additional intestinal passages must be performed in an attempt to decrease the effect of this strain for the upper respiratory tract.

Introduction

Infectious bronchitis (IB) is an acute, highly contagious viral respiratory disease characterized by the presence of tracheal rales, coughing, sneezing, tracheal discharge and occasionally swollen sinuses (5). Infectious bronchitis virus (IBV) replicates in several tissues like respiratory tract, kidneys and oviduct (5). Microscopic observation of the infection in the trachea includes a degenerative phase with decilliation, desquamation, heterophilic and lymphocytic infiltration in the lamina propia, a hyperplastic phase with newly formed epithelial cells without cilia, and a recovery phase with a reparative process of the epithelial cells (7). In the kidneys, virus replication has been observed in the tubular epithelial cells causing alterations in the fluid and electrolyte transport. The kidneys infected with nephropathogenic IBV are swollen and pale with the presence of urates (7). Nephropathogenic strains usually produce nephrosis-nephritis in young birds and urolithiasis in layers (1, 7, 14). In the oviduct, glandular hypoplasia with the reduction and changes in the structural matrix of albumen proteins are associated with production of watery eggs (7). When the infection affects female chicks less than 2 weeks of age, a permanent damage in the reproductive tract with absence of egg production in some layers after infection has been observed (7).

Tropism of some IBV strains for other organs like intestines has been described (11); however, viral replication in this tissue has been controversial. El Houdafi *et al* (8), classified an IBV strain (G strain) as enterotropic by virtue of its prolonged persistence in the gut compared to the trachea. Jones and Ambali (10) 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 (4), could not demonstrate the replication of 11 IBV strains in intestinal organ cultures. 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 without the presence of macroscopic or histological lesions (3, 7).

The objectives of this study were to establish a procedure to *in vivo* passage selected IBV strains in specific pathogen free (SPF) chickens by reisolating the virus from the small intestines, and to evaluate the effect of an *in vivo* intestine-passaged IBV strain on the upper respiratory tract.

Materials and Methods

Isolation of field IBV strains. Field IBV isolates were obtained from 3 week-old SPF chickens (SPAFAS Inc., Norwich, CT) placed in commercial broiler farms showing respiratory problems in the state of Georgia. Small pieces of duodenum, jejunum, ileum and cecal tonsils from 5 sentinel birds from each problem farm were obtained and processed separately by standard procedures (2). Briefly, samples were homogenized (30% w/v) in tryptose phosphate broth (TPB), pH 7.2 with antibiotics (10,000 IU/ml penicillin, 10,000 μg/ml streptomycin) and amphotericin B (250 μg/ml). After centrifugation (20,845 X g for 20 min) at 4 C, the supernatant was removed and passed through a 0.22 μm polyethersulfone (PES) syringe filter (Whatman Inc., Clifton, NJ). The filtered supernatant fluids were inoculated into 9-11 day-old SPF chicken embryos via the allantoic sac. The presence of IBV in the allantoic fluid was evaluated 48 hours after inoculation by the rapid-plate hemagglutination assay (16), and by amplification of the S1 gene by reverse transcriptase-polymerase chain reaction (RT-PCR) (13). Further characterization of the field IBV isolates was performed by restriction fragment length

polymorphism (RFLP) of the amplified S1 gene, as described (13). Three field isolates designated as Arkansas 1, Arkansas 2 and Massachusetts 1 and two additional IBV strains, Holland 90 and Massachusetts 41 (Merial Select, Inc., Gainesville, GA) were selected to be passaged *in vivo* in one day-old SPF chickens with viral reisolation from the intestines.

Intestinal passages. *In vivo* passages of the five selected IBV strains with reisolation of the virus from the intestines were performed according to the animal use proposal guidelines. The procedure is briefly described as follows: Five groups with five, one day-old SPF chickens each were placed in positive pressure Horsfall Bauer units. Birds in each group were first inoculated by eye drop at day of age with 0.2 ml of allantoic fluid containing each of the selected IBV. Immediately after inoculation, the feed was removed for a 12-hour period. At 3 days of age, a second inoculation followed by water deprivation for 6 hours was performed. At five days of age, a third and last inoculation was performed. All the birds in each group were sacrificed at 7 days of age after a 12-hour feed withdrawal period, and the small intestines were removed, washed twice with TPB, pooled and immediately stored at – 20 C. To avoid cross contamination among the selected IBV strains, every pool of intestines from each group was processed in a separate type II A/B3 biological safety cabinet, as described (2), with the following modification: IBV present in the filtered supernatant was concentrated by centrifugation (70,000 X g for 1 hour) at 4 C in a 40% glycerol cushion gradient. The pellet was re-suspended in 4.0 ml of TPB with antibiotics and used to inoculate five 9 day-old embryos via the allantoic sac (0.2 ml per egg). The presence of IBV in the allantoic fluid was evaluated 48 hours after inoculation by rapid-plate hemagglutination assay and RT-PCR (13, 16). Further IBV characterization was performed by RFLP on the PCR amplified S1 glycoprotein gene, as described (13). Positive

IBV allantoic fluids were stored at – 20 C and used as inoculums for the next *in vivo* intestinal passage.

Viruses. The effect of the 13th intestine-passaged IBV Arkansas 1 strain (IP-Ark 1) on the trachea and cecal tonsils was evaluated and compared with the original non-passaged IBV Arkansas strain (NP-Ark 1) and a commercial IBV Arkansas DPI vaccine (Vx-Ark) (Merial Select, Inc., Gainesville, GA) used as a positive control. The commercial IBV Arkansas vaccine was diluted in PBS following the manufacturer's recommendations. The viruses were titrated by inoculating serial ten-fold dilutions of the appropriate IBV in 9 day-old SPF chicken embryos. Titers were calculated by the method of Reed and Müench (19).

Experimental design. One hundred sixty, one day-old SPF chickens were used. The birds were divided in four groups with 40 birds per group. Every group was divided in two replicates (A and B) with 20 birds per replicate (Table 2). Birds from every replicate were placed in individual positive pressure Horsfall Bauer isolation units until the end of the study. Three groups were inoculated with the NP-Ark 1, IP-Ark 1 and Vx-Ark IBV strains. The fourth group was not inoculated and used as a negative control. The birds were inoculated by eye drop at one day of age with 0.1 ml of allantoic fluid (NP-Ark 1 and IP-Ark 1) or PBS (Vx-Ark) containing the titrated viruses. Ten birds from each group (five per replicate) were randomly removed for necropsy at 3, 6, 9 and 12 days of age. Tracheas and cecal tonsils were collected. Tracheal tissues were divided in two sections. One section was kept frozen at – 80 C for virus isolation and the remaining portion was immersed in 10% formalin for 18 hours and paraffin embedded

for microscopic examination. The same procedure was performed with the cecal tonsil tissues. Virus isolation from the selected tissues was performed, as described (2).

IBV detection. Virus detection in the allantoic fluid of 9 day-old SPF chicken embryos 48 hours after inoculation with the processed tissues was performed by amplification of the S1 gene by RT-PCR (13).

Effect evaluation. Three parameters, clinical signs, lymphocytic infiltration and epithelial proliferation, were individually scored from 1 to 4, and used to evaluate the effect of the IBV strains on the upper respiratory tract. Microscopic examination of the trachea was performed under 40 X magnification. All tracheas were sectioned longitudinally and the entire length of each trachea was examined for scoring. The criteria used to score each parameter are described as follows:

a. Clinical signs. Clinical signs were examined and scored daily by the same three persons throughout the study as follows: 1 = no clinical signs; 2 = lacrimation, conjunctivitis, slight head shaking; 3 = presence of nasal exudates; 4 = same as 3 plus swollen heads.

b. Lymphocytic infiltration (LI). 1 = normal trachea without detectable or significant lymphocytic infiltration; 2 = detectable but very mild lymphocytic infiltration in the lamina propia; 3 = moderate lymphocytic infiltration in the lamina propia; 4 = severe lymphocytic infiltration in the lamina propia. A score of 4 is usually given to severe lesions (chronic severe diffuse lymphocytic infiltration) such as the one observed in chronic *Mycoplasma gallisepticum* (MG) and/or chronic, severe and complicated IBV Arkansas infection.

c. Epithelial proliferation (EP): 1 = normal trachea without significant epithelial damage of any kind; 2 = mild epithelial proliferation regardless of any other lesions: tracheas with more than one layer of epithelium; 3 = moderate epithelial proliferation characterized by the presence of two or more epithelial layers; 4 = severe epithelial proliferation characterized by gross thickening of the tracheal epithelium due to epithelial cell proliferation. Lesions with a score of 4 are frequently associated with additional lesions such as severe hyperemia, epithelial cell necrosis, severe loss of cilia, and significant infiltration with lymphocytes, plasma cells and heterophils.

Statistical analysis. An analysis of variance (ANOVA) in association with the Dunnett's multiple comparison procedure was performed utilizing the SAS system (SAS institute Inc., Cary, NC) using the mean clinical signs, LI and EP scores as units (15).

Results

Field IBV strains and intestinal passages. A total of five IBV field isolates were obtained from the intestinal tissues of 3 week–old SPF chickens used as sentinels in problem farms. Three IBV strains, two Arkansas like (Ark 1 and Ark 2), and one Connecticut (Conn 1), were isolated from cecal tonsils while two Massachusetts IBV strains (Mass 1 and Mass 2) were isolated from duodenum. Three of those five IBV field isolates (Ark 1, Ark 2 and Mass 1) and two IBV vaccine strains (Massachusetts 41 and Holland 90) (Merial Select, Inc., Gainesville, GA) were selected and passaged *in vivo* several times in the intestines of one day-old SPF chickens (Table 3.1). The IBV Mass 41, Holl 90, Ark 1, Ark 2 and Mass 1 received 8, 8, 17, 14

and 14 *in vivo* intestine passages, respectively. The presence of the virus in the allantoic fluid of SPF chicken embryos inoculated with the processed intestinal tissues was tested at each passage by rapid-plate hemagglutination assay and amplification of the S1 gene by RT-PCR (Table 3.1). Further characterization of the IBV isolates was performed by digestion of the PCR amplified S1 gene with the *Bst*YI, *Hae*III and *Xcm*I restriction enzymes (Fig. 3.1).

Virus titration. IBV titers of $10^{5.7}$, $10^{5.0}$ and $10^{4.5}$ EID_{50}/ml were obtained in the Vx- Ark, IP-Ark 1 and NP-Ark 1 IBV strains, respectively. The SPF birds in the Vx-Ark, IP-Ark 1 and NP-Ark 1 were inoculated at day of age by eye drop with $10^{4.7}$, $10^{4.0}$, and $10^{3.5}$ $EID_{50,}$ respectively.

IBV detection. IBV detection by RT-PCR in the allantoic fluid of SPF chicken embryos inoculated with the processed samples in each replicate group at different sampling ages is illustrated in figure 3.2 and summarized in table 3.2. By RT-PCR, IBV was more frequently detected (15 times) in the group inoculated with the NP-Ark 1 when compared with the groups inoculated with the IP-Ark 1 and Vx-Ark 1 (detected 9 and 7 times, respectively). IBV was detected in all the groups up to the end of the study. In the tracheas, the NP-Ark 1 virus was consistently detected (in two out of the two replicates) from 3 to 12 days, the Vx Ark virus from 6 to 12 days and the IP-Ark 1 virus at 3 and 6 days (Table 3.2). In the cecal tonsils, the NP-Ark 1 virus was consistently detected from 6 to 12 days and the IP-Ark 1 virus at 6 days. No Vx-Ark virus was detected in cecal tonsils at any age (Table 3.2). The rapid plate hemagglutination test, performed in the allantoic fluid of the only passage in SPF chicken embryos, detected the presence of IBV in 23 of the 61 (37.7%) samples positively detected by RT-PCR (Table 3.1). As

expected, no IBV was detected by RT-PCR or by rapid plate hemagglutination assay in the control groups.

Effect evaluation. No clinical signs were observed at any age in the IBV inoculated groups. All the groups received a mean daily score of 1 (normal) when examined by the same three persons up to the end of the study. No statistical differences in the scores for clinical signs were observed at any sampling age by the Dunnett's test when the IBV inoculated groups were individually compared with the non-inoculated group.

The mean LI and EP scores observed in the tracheas of SPF chickens after inoculation at day of age with the NP-Ark 1, IP-Ark 1 and Vx-Ark IBV strains at different sampling ages are presented in table 3.3. Higher mean LI and EP scores were observed in the IP-Ark 1 and NP-Ark 1 groups. The LI and EP scores in the Vx-Ark group were very similar to the scores observed in the non-inoculated group, which overall exhibited the lowest scores, as expected.

When two individual groups were compared to each other using the Dunnett's test, significant differences ($p < 0.05$) in both the LI and EP scores were observed mainly at 6 and 9 days (Table 3.4).

The IP-Ark 1 group exhibited a statistically different EP score at 6 days than the NP-Ark group and LI and EP scores at 6 and 9 days than the Vx Ark group (Table 3.4). Significant differences in the EP scores were observed in the NP-Ark 1 at 6 and 9 days when compared with the Vx-Ark group, and at 3, 6 and 9 when compared with the non-inoculated group. Significant differences between the IP-Ark 1 and non-inoculated EP scores were observed at 6 and 9 days and between their LI scores at 9 days.

Discussion

A procedure to passage *in vivo* selected IBV strains by reisolating the virus from the intestines of SPF chickens has been established. An increase in the reisolation of the virus from the intestines was observed when the birds were stressed after the IBV inoculations. Intracloacal inoculation of IBV has been described (18), and could have been use to *in vivo* passaged the selected IBV strains. However, this procedure was not used due to stronger nephrotropism and nephropathogenicity observed in the Connecticut strain after successive passages via the cloaca (18).

A positive correlation between the rapid plate hemagglutination assay and RT-PCR for the detection of IBV in the allantoic fluid of SPF chicken embryos has been described (2, 16). In this experiment, the lower IBV detection by the rapid plate hemagglutination assay might be related with a low viral concentration in the allantoic fluid after the single embryo passage. A more consistent hemagglutination activity could have been detected if additional passages had been performed. However, the embryo passage allowed us to recover and amplify the virus reisolated from the intestines.

After several passages, the effect of the *in vivo* intestine passaged IBV strains for the upper respiratory tract was evaluated. Simultaneous evaluation of all the IBV strains had been cumbersome, not only due to the number of SPF birds, chicken embryos, and isolations units involved, but also to the higher risk of cross contamination. One of the intestine passaged IBV, the IP-Ark 1 strain, was selected because it had the higher number of *in vivo* intestine passages at the time of evaluation (13 passsages) and its effect could be compared with the original field isolate. The titers obtained for the Vx-Ark, IP-Ark 1 and NP-Ark 1 strains were very close (within one log) and therefore, these viruses were used undiluted to inoculate the SPF birds at

day of age. Higher effect of the IP-Ark 1 and NP-Ark 1 on the respiratory tract might have been observed if the inoculated groups had received the same viral dose.

The lack of clinical signs in the inoculated birds without maternal antibodies could be explained by the absence of secondary bacterial infections (i.e. *Escherichia coli or Staphylococcus spp.*) or environmental factors (ammonia, dust and high densities) commonly observed in the field. Associations between these conditions and tracheal lesions produced by viral replication could induce the presence of airsaculitis, pericarditis, perihepatitis and mortality (6, 12, 17).

The absence of lesions observed in the cecal tonsils of the inoculated groups agree with previous observations implying that IBV replication in the lower gut do not cause gross lesions or microscopic changes (7).

The IP-Ark 1 was detected in the allantoic fluid of embryos inoculated with the processed tracheas in both replicates for a shorter period when compared with the NP-Ark 1 and Vx-Ark viruses. The IP-Ark 1 was also detected for a shorter period in the cecal tonsils when compared with the NP-Ark 1. 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 vaccination time, birds with high antibody levels can neutrilize the vaccine virus while birds with low antibody levels allow the replication of the virus and the developing of protective immunity. When birds correctly vaccinated begin to shed the virus, this virus (back passaged) increases its pathogenicity, infecting the birds that initially rejected the vaccine virus, presenting serious respiratory reactions (9). A shorter presence of the IP-Ark 1 virus in the tissues could result in a shorter period of virus shedding, decreasing the occurrence of the chronic respiratory reactions, known as “rolling reactions”. The consistent detection of the

Vx-Ark virus through the end of the study only in the trachea could indicate a predilection of this particular strain for the respiratory tract during the time of the study (from 3 to 12 days) with a restricted replication to this tissue.

Overall, a higher effect for the respiratory tract was observed in the IP-Ark 1 followed by the NP-Ark 1 viruses and the Vx-Ark 1 virus. Although no clinical signs were observed and the higher tracheal scores present at 6 and 9 days (EP = 2.4 and LI = 1.7) only represent a very mild lymphocytic infiltration and mild epithelial proliferation, the IP-Ark 1 virus still induced more LI and EP than the Vx-Ark virus. Additional passages must be performed to decrease the respiratory changes induced by this strain during this period. The higher effect caused by this strain could be related with the low number of *in vivo* passages or with the procedure used to perform those passages. Viral inoculation by eye drop could induce an initial viral replication in the trachea during early stages of infection, after which, some viruses would go to the intestinal tissue to continue their replication. In order to avoid the initial replication of the in *vivo* passaged IBV strains in the respiratory tract, the use of primary intestinal cell or intestine organ culture systems should be considered in an attempt to restrict the replication of the virus to the intestinal tissue. The use of *in vitro* systems would also decrease the cost and risk of cross contamination associated with the *in vivo* studies.

Table 3.1. *In vivo* passages of the selected IBV isolates in the intestinal tissues of one day-old SPF chickens. The presence of IBV in every passage was detected by rapid-plate hemagglutination assay and RT-PCR in the allantoic fluid of embryos 48 hours after inoculation with the processed intestinal tissues.

	Number of Passages																
Isolate	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Mass 41	+	+															
Holl 90	+	+	+	+													
Ark 1	+	+	+	+		+					+				+		
Ark 2	+	+			+	+			+			+					
Mass 1	+					+			+					+			

* Shaded boxes correspond to the *in vivo* intestine passages positive to IBV by RT-PCR.

+ Passages positive to IBV rapid-plate hemagglutination assay.

Figure 3.1. RFLP patterns of the PCR amplified S1 glycoprotein genes from the selected IBV strains after digestion with the *Bst*YI, *Hae*III and *Xcm*I restriction enzymes. Molecular characterization by RFLP was performed in the selected IBV strains after each *in vivo* intestine passage. Lane 1 = molecular-weight marker made from a mixture of defined double strand DNA markers exhibiting the following molecular weights; 2,000, 1,500, 1,000, 750, 500, and 300 bp; Lane 2, 3 and 4 = RFLP pattern of the Mass 41 strain; Lane 6, 7 and 8 = RFLP pattern of the Holl 90 strain; Lane 10, 11 and 12 = RFLP pattern of the Ark 1 strain; Lane 14, 15 and 16 = RFLP pattern of the Ark 2 strain; Lane 18, 19 and 20 = RFLP pattern of the Mass 1 strain.

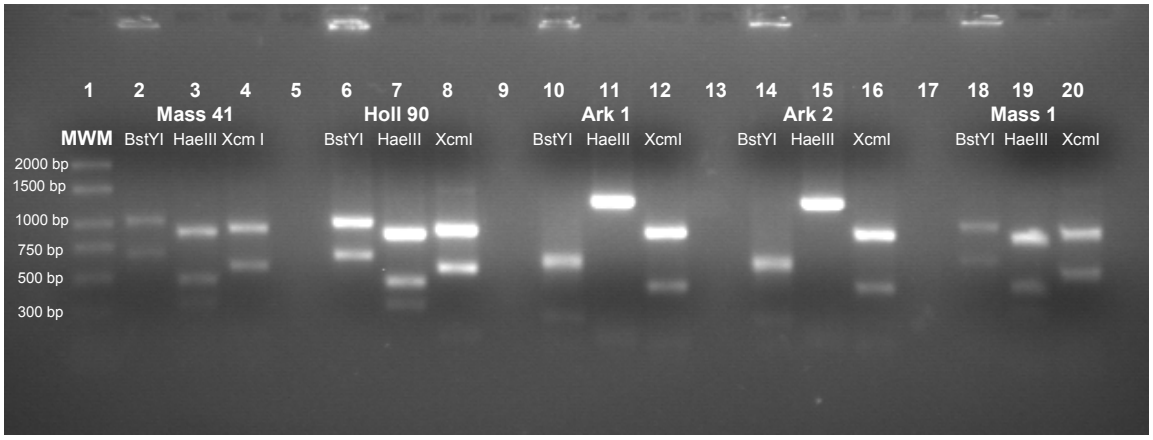
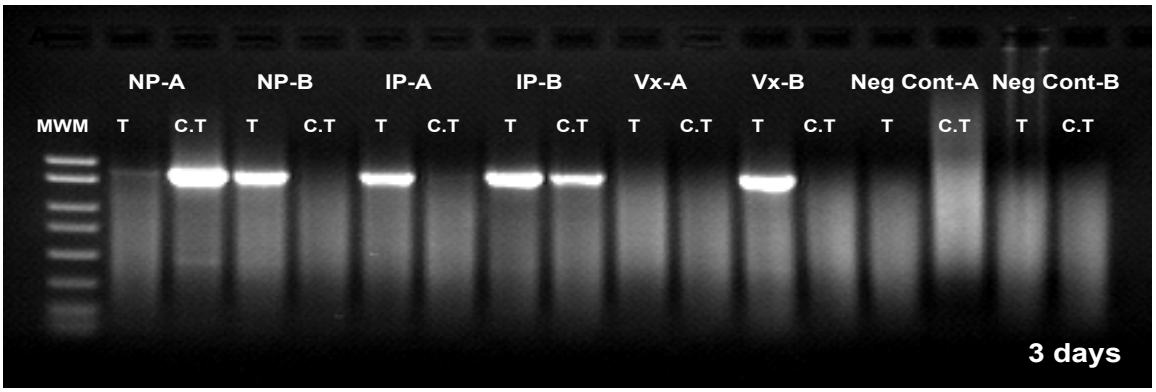
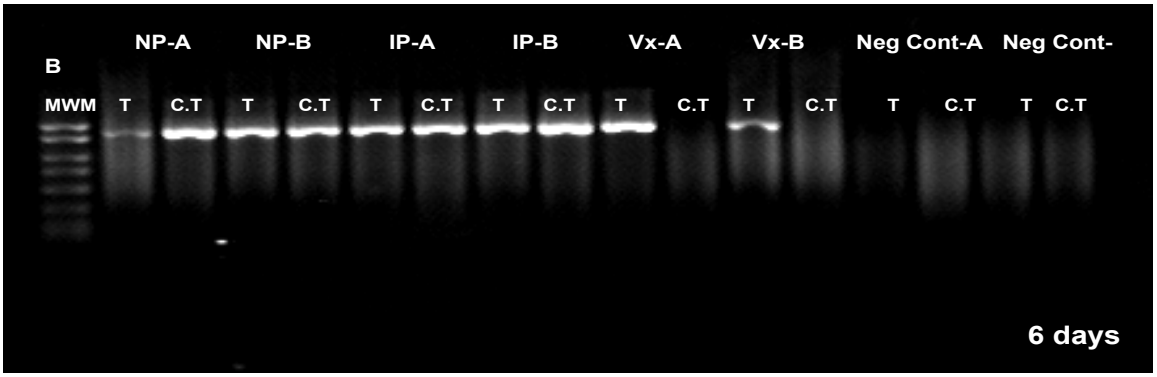
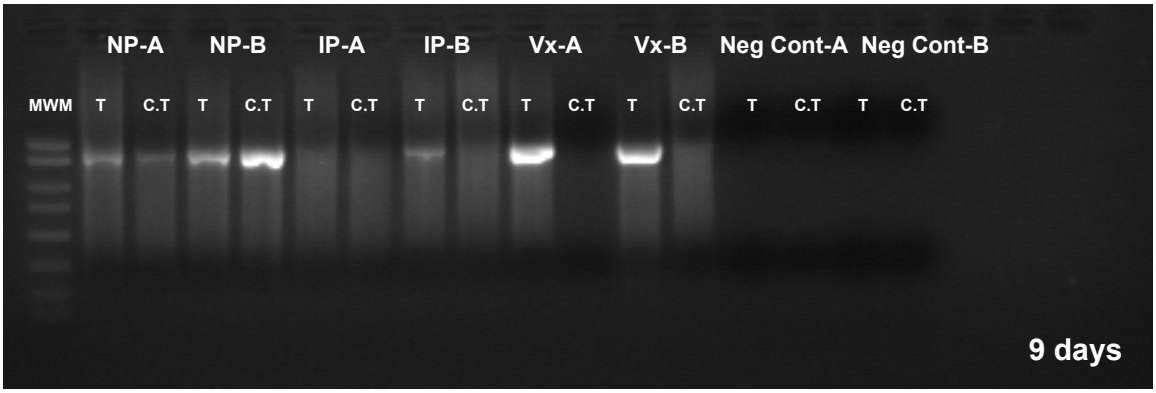

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Mass 41
Holl 90
Ark 1
Ark 2
Mass 1
MWM BstYI HaeIII Xcm I BstYI HaeIII XcmI BstYI HaeIII XcmI BstYI HaeIII XcmI BstYI HaeIII XcmI
2000 bp
1500 bp
1000 bp
750 bp
500 bp
300 bp

Figure 3.2. Positive IBV samples detected in the allantoic fluid of SPF chicken embryos 48 hours after inoculation with the processed tracheal and cecal tonsil tissues by RT-PCR in the two replicates (A and B) of the non-passaged (NP), intestine-passaged (IP), commercial vaccine (Vx) Arkansas IBV inoculated and the non inoculated groups at 3, 6, 9 and 12 days of age.


NP-A
NP-B
IP-A
IP-B
Vx-A
Vx-B
Neg Cont-A
Neg Cont-B
MWM
T
C.T
3 days


NP-A
NP-B
IP-A
IP-B
Vx-A
Vx-B
Neg Cont-A
Neg Cont-
B
MWM
T
C.T
6 days


NP-A
NP-B
IP-A
IP-B
Vx-A
Vx-B
Neg Cont-A
Neg Cont-B
MWM
T
C.T
9 days

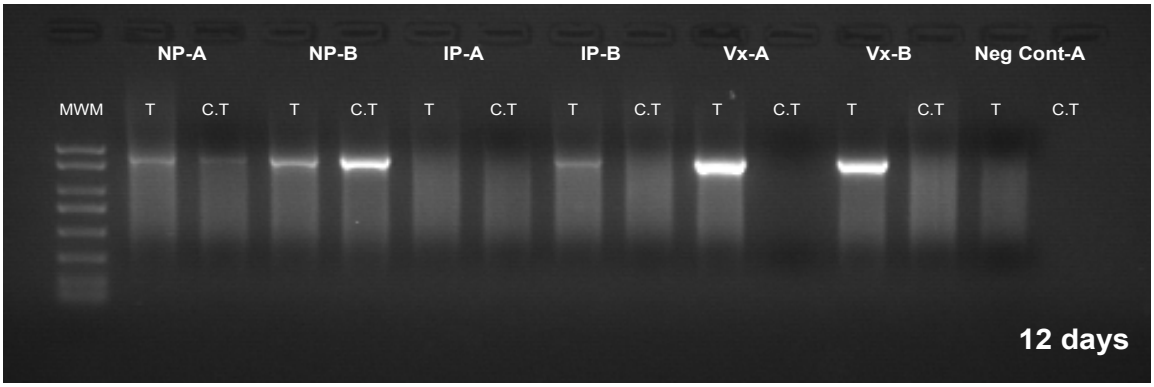

NP-A
NP-B
IP-A
IP-B
Vx-A
Vx-B
Neg Cont-A
MWM
T
C.T
12 days

Table 3.2. IBV detection by RT-PCR in the allantoic fluid of SPF chicken embryos 48 hours after inoculation with the processed tracheal and cecal tonsils tissues at several sampling ages. In each replicate, pools of five tracheas and five cecal tonsils were tested at each sampling age.

			Sampling age (days)			
Group	**Replicate**	**Tissue**	**3**	**6**	**9**	**12**
NP-Ark 1	A	Trachea	+	+	+	+
		C. tonsils	+	+	+	+
	B	Trachea	+	+	+	+
		C. tonsils	-	+	+	+
IP-Ark 1	A	Trachea	+	+	-	-
		C. tonsils	-	+	-	-
	B	Trachea	+	+	+	+
		C. tonsils	+	+	-	-
Vx-Ark	A	Trachea	-	+	+	+
		C. tonsils	-	-	-	-
	B	Trachea	+	+	+	+
		C. tonsils	-	-	-	-
Non-Inoculated	A	Trachea	-	-	-	-
		C. tonsils	-	-	-	-
	B	Trachea	-	-	-	-
		C. tonsils	-	-	-	-

Table 3.3. Mean lymphocytic infiltration (LI) and epithelial proliferation (EP) scores in the tracheas of SPF chickens inoculated at day of age by eye drop with the non-passaged (NP), intestine passaged (IP), commercial vaccine (Vx) IBV Arkansas serotype at different sampling ages.

	Sampling age							
	3		**6**		**9**		**12**	
Group	**LI [1]**	**EP [2]**	**LI**	**EP**	**LI**	**EP**	**LI**	**EP**
NP-Ark 1	1	1.7	1.4	1.9	1.3	2.0	1.62	2.12
IP-Ark 1	1	1.4	1.5	2.4	1.7	2.2	1.64	2.12
Vx-Ark	1	1.2	1	1.1	1.2	1.2	1.5	1.62
Non-inoc	1	1.1	1.3	1.1	1.1	1.2	1.38	1.5

1 = Lymphocytic infiltration scores; 1 = normal, 2 = mild, 3 = moderate, 4 = severe.

2 = Epithelial proliferation scores; 1 = normal, 2 = mild, 3 = moderate, 4 = severe.

Table 3.4. Statistical analysis of the mean lymphocytic infiltration (LI) and epithelial proliferation (EP) scores between two specific groups by the Dunnett's test at each sampling age.

	Sampling Age (days)							
	3		**6**		**9**		**12**	
Comparison	**LI**	**EP**	**LI**	**EP**	**LI**	**EP**	**LI**	**EP**
IP-Ark 1 vs. NP-Ark 1	1/1	1.4/1.7	1.5/1.4	**2.4/1.9***	1.7/1.3	2.2/2.0	1.62/1.62	2.12/2.12
IP-Ark 1 vs. Vx-Ark	1/1	1.4/1.2	**1.5/1.0**	**2.4/1.1**	**1.7/1.2**	**2.2/1.2**	1.62/1.5	2.12/1.62
NP-Ark 1 vs. Vx-Ark	1/1	1.7/1.2	1.4/1.0	**1.9/1.1**	1.3/1.2	**2.0/1.2**	1.62/1.5	2.12/1.62
NP-Ark 1 vs. Non Inoc	1/1	**1.7/1.1**	1.4/1.3	**1.9/1.1**	1.3/1.1	**2.0/1.2**	1.62/1.37	2.12/1.5
IP-Ark 1 vs. Non Inoc	1/1	1.4/1.1	1.5/1.3	**2.4/1.1**	**1.7/1.1***	**2.2/1.2**	1.62/1.37	2.12/1.5

*Bold numbers correspond to statistically different ($p<0.05$) scores.

Acknowledgements

We gratefully acknowledge Merial Select, Inc., for their economic support to develop this project.

References

1. Albassam, M. A., R. W. Winterfield, and H. L. Thacker. Comparison of the nephropathogenicity of four strains of infectious bronchitis virus. Avian Dis. 30:468-476. 1986.

2. Alvarado, I. R., P. Villegas, J. El-Attrache, and T. P. Brown. Evaluation of the protection conferred by commercial vaccines against the California 99 isolate of infectious bronchitis virus. Avian Dis. 47:1298-1304. 2003.

3. Ambali, A. G., and R. C. Jones. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis. 34:809-817. 1990.

4. Bhattacharjee, P. S., and R. C. Jones. Susceptibility of organ cultures from chicken tissues for strains of infectious bronchitis virus isolated from the intestine. Avian Path. 26:553-563. 1997.

5. Cavanagh, D., and S. Naqi. Infectious Bronchitis. In: Diseases of Poultry, 11th ed, Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa. pp. 101-119. 2003.

6. Cook, J. K., H. W. Smith, and M. B. Huggins. Infectious bronchitis immunity: Its study in chickens experimentally infected with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 67:1427-1434. 1986.

7. Dhinakar Raj, G., and R. C. Jones. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Path. 26:677-706. 1997.

8. El Houadfi, M. D., M. D. Jones, J. K. Cook, and A. G. Ambali. The isolation and characterization of six avian infectious bronchitis virus isolated in Morocco. Avian Path. 15:93-105. 1986.

9. Hopkins, S. R., and H. W. Yoder, Jr. Increased incidence of airsacculitis in broilers infected with Mycoplasma synoviae and chicken-passaged infectious bronchitis vaccine virus. Avian Dis. 28:386-396. 1984.

10. Jones, R. C., and A. G. Ambali. Re-excretion of an enterotropic infectious bronchitis virus by hens at point of lay after experimental infection at day old. Vet. Rec. 120:617-618. 1987.

11. Karaca, K., S. A. Naqi, P. Palukaitis, and B. Lucio. Serological and molecular characterization of three enteric isolates of infectious bronchitis virus of chickens. Avian Dis. 34:899-904. 1990.

12. Kleven, S. H., D. D. King, and D. P. Anderson. Airsacculitis in broilers from Mycoplasma synoviae: Effect on air-sac lesions of vaccinating with infectious bronchitis and Newcastle virus. Avian Dis. 16:915-924. 1972.

13. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

14. MacDonald, J. W., and D. A. McMartin. Observations on the effects of the H52 and H120 vaccine strains of the infectious bronchitis virus in the domestic fowl. Avian Path. 5:157-173. 1976.

15. Ott, L., and M. T. Longnecker. An introduction to statistical methods and data analysis.5th ed.:384-451. 2001.

16. Ruano, M., J. El-Attrache, and P. Villegas. A rapid-plate hemagglutination assay for the detection of infectious bronchitis virus. Avian Dis. 44:99-104. 2000.

17. Smith, H. W., J. K. Cook, and Z. E. Parsell. The experimental infection of chickens with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 66:777-786. 1985.

18. Uenaka, T., I. Kishimoto, T. Uemura, T. Ito, T. Umemura, and K. Otsuki. Cloacal inoculation with the Connecticut strain of avian infectious bronchitis virus: An attempt to produce nephropathogenic virus by in vivo passage using cloacal inoculation. J. Vet. Med. Sci. 60:495-502. 1998.

19. Villegas, P. Titration of biological suspensions. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. The American Association of Avian Pathologists, Kennett Square, PA.:248-253. 1998.

CHAPTER 4

EFFECT OF INFECTIOUS BRONCHITIS VIRUS STRAINS PASSAGED IN VITRO IN A CHICKEN INTESTINE ORGAN CULTURE ON SELECTED TISSUES OF SPECIFIC PATHOGEN FREE CHICKENS[1]

[1] I. R. Alvarado, P. Villegas and G. Zavala. To be submitted to Avian Diseases.

Key words: avian infectious bronchitis virus, *in vitro* intestinal passages, intestine organ culture, tracheal lesions.

Abbreviations: IB = infectious bronchitis; IBV = infectious bronchitis virus; IP = intestine-passaged; NP = Non intestine passaged; Vx = commercial vaccine; N = nucleocapsid gene; S = surface glycoprotein gene; ED = epithelial damage; EH = epithelial hyperplasia; LI = lymphocytic infiltration; GCP = goblet cell and compound mucus gland proliferation; RT-PCR = reverse transcriptase-polymerase chain reaction; RFLP = restriction fragment length polymorphism; SNK = Student-Newman-Keuls test.

Summary. Four selected IBV strains passaged several times *in vivo*, with reisolation of the virus from the small intestines of SPF chickens, were further passaged *in vit*ro in an intestinal organ culture system. The ability of the IBV strains to replicate in the intestinal rings after inoculation was determined by *in situ* hybridization in formalin fixed, paraffin embedded intestinal rings and virus detection by RT-PCR in the allantoic fluid of embryos inoculated with the processed intestinal rings. The effect of two of the IBV strains, the intestine passaged Arkansas 1 (IP-Ark 1) and Massachusetts 41 (IP-Mass 41) IBV strains, was evaluated in 1 day-old SPF chickens following intraocular inoculation. Overall, the IP-Ark 1 strain was detected in the tracheas of a lower percentage of birds and exhibited milder lesions than the attenuated commercial Arkansas vaccine and NP-Ark 1 strains. The IP-Mass 41 was detected in the tracheas of a higher percentage of birds than the Vx-Mass 41 at 3 days. However, no significant differences in their effect on the upper respiratory tract were observed. The IP-Mass 41 was detected in a higher percentage of birds in the cecal tonsils and duodenum than the Vx-Mass 41. Only moderate to severe diffuse and/or follicular lymphocytic infiltration without changes or lesions in the intestinal epithelium of the duodenum and cecal tonsils was observed in the inoculated groups. The intestinal passages performed on the Arkansas 1 IBV strain decreased its presence and effect for the upper respiratory tract to levels lower than those observed with currently used commercial attenuated Arkansas vaccines.

Introduction

Infectious bronchitis (IB) is a highly contagious respiratory disease of chickens distributed worldwide and characterized by tracheal rales, coughing and sneezing (5). Infectious bronchitis virus (IBV) belongs to the family *Coronaviridae*, genus *Coronavirus* and has been classified with the turkey and pheasant coronavirus in the antigenic group 3 (5). IBV can replicate in several tissues including the respiratory tract, intestinal tract, kidneys and the oviduct (8). Primary cell culture and tissue organ culture systems have been widely used for the isolation of avian viral pathogens. IBV replication in specific pathogen free (SPF) chicken embryos, chicken embryo kidney cells (CEK), chicken kidney cells (CK) and chicken embryo liver cells (CEL) has been described (5). Tracheal and oviduct organ culture systems have also been used to isolate and replicate the virus (28, 32).

In intestines, virus replication has been described in histiocytes, lymphoid cells in cecal tonsils and in apical epithelial cells of the villi in ileum and rectum without the presence of gross or histological changes (8). However, attempts to replicate IBV in the epithelial cells of chicken small intestines have not been successful (3).

Several attempts have been made to culture intestinal cells from fetal, post-natal and adult intestines in humans (4, 7, 20, 27), bovines (10), and rats (29). Recently, the development of a primary cell culture of turkey intestinal epithelial cells has been described (1). No intestinal epithelial cell lines from chickens are currently available for isolation, propagation or identification of fastidious avian enteric pathogens or other avian infectious agents.

Current production methods, including multiple ages on a farm and high-density poultry areas make the control of the disease very difficult. Prevention of production losses due to IB

are usually attempted with immunization by vaccination. Both live and inactivated virus vaccines have been widely used in IB immunization programs (5). Even though commercial live vaccines have been attenuated by serial passages in embryonated chicken eggs (18), respiratory distress is commonly observed after vaccination. The damage to tracheal epithelium caused by IBV facilitates secondary bacterial (*E. coli*) invasion and multiplication, enhancing the severity and duration of the respiratory reaction and leading to death or lesions in surviving chickens (8, 22).

The objectives of this study were to establish an *in vitro* system to passage selected IBV strains, previously passaged *in vivo* by reisolating the virus from the intestines of SPF chickens, and to determine the effect of two selected *in vitro* intestine-passaged IBV strains for the upper respiratory tract.

Materials and methods

***In vitro* intestinal passages.** An intestinal organ culture system to perform *in vitro* passages of selected IBV strains was developed. Briefly, SPF chicken embryos between 18 to 20 days of age were aseptically removed from their shells, and their small intestines were harvested, washed with phosphate buffered saline solution (PBS) and immediately cut into 1.0 mm thick rings (Figure 4.1, A). The intestinal rings were washed with PBS containing D-glucose (2% w/v), 5% fetal bovine serum (FBS), antibiotics (10,000 IU/ml penicillin, 10,000 ug/ml streptomycin) and amphotericin B (25g/ml). Intestinal rings were placed in 35 mm petri dishes, inoculated with 2 ml of IBV infected allantoic fluid and placed in a CO_2 incubator at 37 C. After one hour, the allantoic fluid was completely replaced with maintenance medium (Dulbecco's Minimal

Essential Medium (DMEM) with 10,000 IU/ml penicillin, 10,000 *u*g/ml streptomycin, D-glucose (2% w/v) and 2% FBS). IBV inoculated intestinal rings were incubated at 37 C in a 5% CO_2 atmosphere. After 60 hours of incubation, the plates were frozen and thawed to release viral particles and the medium containing the intestinal rings was transferred to a 15 ml plastic centrifuge tube. After centrifugation (1500 X g for 15 min) at 4 C, the supernatant was collected and used to inoculate new intestinal rings using 2 ml of the supernatant per plate. After 1 hour, the inoculum was also replaced by maintenance medium. After three consecutive *in vitro* blind passages, the supernatant of the third passage was used to inoculate three, 9 day-old SPF chicken embryos via the allantoic sac. The presence of IBV in the allantoic fluid was detected by RT-PCR 48 hours after inoculation, as described (16). IBV positive allantoic fluid was stored at – 80 C and used as inoculum for the first of the next three *in vitro* blind passages.

***In situ* hybridization.** The presence of IBV nucleocapsid (N) gene in the intestinal rings during the initial *in vitro* passages was detected by *in situ* hybridization with a DIG-labeled riboprobe. *In situ* hybridization was performed on formalin fixed-paraffin embedded intestinal rings 60 hours after being inoculated with IBV infected allantoic fluid following the procedure described elsewhere (17).

Construction of DIG-labeled riboprobe. Molecular cloning procedures were performed as describe elsewhere (30). A 319 bp fragment of the N gene was amplified by the Titan One Tube RT-PCR kit (Roche Diagnostics Corp., Indianapolis, IN), as described (9). The amplified fragment was cloned into the pCR 2.1 TOPO vector (Invitrogen Corp., Carlsbad, CA). The antisense DIG-labeled riboprobe was prepared by linearizing the selected plasmid with the *Kpn*I

restriction enzyme (New England Biolabs Inc., Beverly, MA) followed by phenol-chloroform-ethanol extraction and *in vitro* transcription with the Dig RNA labeling (SP6/T7) kit (Roche Diagnostics Corp., Indianapolis, IN). The riboprobe concentration was determined by dot blot comparison with a known standard DIG-labeled RNA (Roche Diagnostics Corp., Indianapolis, IN).

Evaluation of Viral Effect.

Viruses. Four IBV strains (Massachusetts 41, Holland 90, Arkansas 1 and Massachusetts 1), previously passaged *in vivo* by reisolating the virus from the small intestines of SPF chickens, were further passaged *in vitro* in an intestinal organ culture (Table 4.1). The effect of two of the four intestine passaged IBV strains on the upper respiratory and intestinal tracts was evaluated. The IP-Mass 41 (passaged 8 times *in vivo* and 38 times *in vitro* in the intestinal tissue) was evaluated and compared with the original commercial IBV Massachusetts 41 vaccine (Vx-Mass 41) (Merial Select, Inc., Gainesville, GA). The IP-Ark 1 (passaged 17 times *in vivo* and 33 times *in vitro* in the intestinal tissue) was evaluated and compared with the original non-passaged Arkansas (NP-Ark 1) and a commercial IBV Arkansas vaccine (Vx-Ark) (Merial Select, Inc., Gainesville, GA). All viruses were titrated by inoculating serial ten-fold dilutions of the appropriate IBV in 9 day-old SPF chicken embryos. Titers were calculated by the method of Reed and Müench (35).

Experimental design. One hundred eighty, one day-old SPF chickens were divided in six groups with 30 birds per group. Each group was further divided in two replicates with 15 birds

per replicate. The birds were kept in Horsfall Bauer isolation units under positive pressure during the study. Five groups were inoculated via eye drop with $10^{4.6}$, $10^{4.3}$, $10^{4.3}$, $10^{4.6}$ and $10^{4.4}$ EID_{50} of the IP-Ark 1, NP-Ark 1, Vx-Ark, IP-Mass 41 and Vx-Mass 41 IBV strains, respectively. The sixth group was not inoculated and used as a negative control. At 3, 7, 14 and 21 days of age, six birds from every group (three per replicate) were randomly removed for necropsy. Trachea, duodenum and cecal tonsils were harvested. Half of every tissue was frozen at – 80 C for virus isolation. The remaining tissues were fixed in 10% buffered formalin for 18 hours, placed in 50% ethanol and stored at 4 C until embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (HE) and used for histopathological examination.

IBV detection. The presence of IBV in the allantoic fluid of 9 day-old SPF chicken embryos inoculated with individually processed tissues was detected at every sampling age by reverse transcriptase-polymerase chain reaction (RT-PCR) (19). Further IBV characterization was performed by restriction fragment length polymorphism (RFLP) on the PCR amplified S1 glycoprotein gene, as described (19).

Evaluation of viral effect on the upper respiratory tract. Four parameters of lesions were individually scored as 1 = normal, 2 = mild, 3 = moderate, 4 = severe, and used to evaluate the effect of the IBV strains for the trachea. Tracheal scores were obtained by adding the four individual scores given in each parameter and dividing them by 4 to preserve the 1 to 4 scale. The criteria used to score each parameter are described as follows:

a. Epithelial damage. Epithelial damage included a variety of lesions like loss of cilia, which can be focal, multifocal, zonal or complete. Tracheas with multifocal to complete loss of cilia in the absence of any other lesions or changes received a score of 2. Epithelial damage also included various forms of degeneration of epithelial cells, edema, hyperemia, infiltrates into the tracheal epithelium, epithelial cell necrosis (individual necrotic cells), epithelial sloughing, etc., most of which warranted a score of 2, 3 or 4 according to severity. The degree of epithelial damage is often reflective of clinical signs and can provide insight as to the chronicity of the lesions.

b. Epithelial hyperplasia. One of the first signs of tissue healing is epithelial cell proliferation, described as hyperplasia. For most respiratory RNA viruses (infectious bronchitis virus, Newcastle disease virus, avian pneumovirus, avian influenza virus, etc.), epithelial hyperplasia usually indicates certain chronicity of the lesion (subacute to chronic).

c. Lymphocytic infiltration. Infiltrates in the lamina propia of the tracheal mucosa are non-specific and they represent mixed populations of lymphocytes, histiocytes and plasma cells. Lymphocytic infiltrates are only present after viral infection has occurred, thus representing a rather subacute to chronic response. Early infiltrates are almost invariable diffuse (non-follicular) and as they progress they may be scored between 1 and 4 arbitrarily, according to the thickness of the infiltrate in the lamina propia. As a general rule, early diffuse infiltrates are composed primarily by T cells. More chronic responses may involve B and T cells. Chronic infiltrates after viral challenge are usually composed of diffuse infiltrates containing primarily T cells (and a smaller population of B cells), and follicular infiltrates (germinal centers), containing

almost exclusively B cells. Simplistically, diffuse infiltrates with scores of 1 to 2 often represent early immune responses upon antigenic challenge. Diffuse infiltrates with scores of 3 or 4 may represent a strong chronic immunological response.

d. Goblet cell and compound mucus gland proliferation. Goblet cells are normally present in the tracheal epithelium but they will become hyperplastic and hypertrophic upon antigenic and/or environmental challenge. Goblet cell hyperplasia will result in proliferation of compound mucus glands composed of multiple hyperactive goblet cells. The significance of hypertrophy and hyperplasia of goblet cells varies according to the concomitant lesions in the trachea. For example, severe proliferation (score 4) in the absence of any other significant lesions likely reflects environmental challenge. If epithelial cell necrosis is present, viral challenge (vaccinal and/or field) is likely. In early (acute or subacute) viral infection there may be no significant goblet cell proliferation.

Statistical analysis. Statistical analysis was performed with the SAS system (SAS institute Inc., Cary, NC). Analysis of variance in association with the Dunnett's multiple comparison procedure were performed using the final mean tracheal scores as units (25).

Results

***In vitro* intestinal passages.** An intestinal organ culture system was developed to passage selected IBV strains (Fig. 4.1 A, B and C). Positive staining for the IBV N gene was observed in the apical epithelial cells of the villi in the intestinal rings 60 hours after inoculation (Fig. 4.1 D,

E and F). The previously *in vivo* intestine-passaged (IP) Massachusetts 41, Holland 90, Arkansas 1 and Massachusetts 1 IBV were further passsaged *in vitro* 38, 37, 32 and 33 times in the intestinal organ culture system, respectively (Table 4.1). Positive detection of the IBV S1 gene by RT-PCR was observed in the allantoic fluid of SPF chicken embryos inoculated with the last of the three consecutive blind passages. Appropriate RFLP patterns were also observed.

Virus titration. IBV titers of $10^{5.6}$, $10^{5.3}$, $10^{5.3}$, $10^{5.6}$ and $10^{5.4}$ EID_{50}/ml were observed in the IP-Ark 1, NP-Ark 1, Vx-Ark, IP-Mass 41 and Vx-Mass 41 IBV strains, respectively. The titrated viruses exhibited very similar titers and were used undiluted to inoculate the SPF birds in each group via eye drop with $10^{4.6}$, $10^{4.3}$, $10^{4.3}$, $10^{4.6}$ and $10^{4.4}$ EID_{50} of the IP-Ark 1, NP-Ark 1, Vx-Ark, IP-Mass 41 and Vx-Mass 41 IBV strains, respectively.

IBV detection. The percentage of birds positive to Arkansas IBV in the selected tissues at different sampling ages is shown in table 4.2-A.

In the tracheas, the IP-Ark 1 was detected in a lower percentage of birds at 7 (33% vs. 100%), 14 (0% vs. 67%) and 21 (17% vs. 50%) days than the Vx-Ark, and at 3 (67% vs. 83%), 7 (33% vs. 83%) and 14 (0% vs. 50%) days than the NP-Ark 1. The IP-Ark 1 and Vx-Ark were detected in a similar percentage of birds at 21 days (17%).

In the cecal tonsils, the IP-Ark 1 was detected in a higher percentage of birds than the Vx-Ark 1 at 3 (50% vs. 30%), 7 (100% vs. 17%), 14 (83% vs. 67%) and 21 (83% vs. 0%) days, and than the NP-Ark 1 at 7 (100% vs. 83%) and 21 (83% vs. 0%) days.

In the duodenum, the IP-Ark 1 was detected in a higher percentage of birds (100% vs. 17%) than the Vx-Ark and similarly detected (100%) than the NP-Ark 1 only at day 7.

The percentage of birds positive to Massachusetts IBV in the selected tissues at different sampling ages is shown in table 4.2 B.

In the trachea, The IP-Mass 41 was detected in a higher percentage of birds at 3 days than the Vx-Mass (83% vs. 33%) and similarly detected at 7 days (50%). In the cecal tonsils, the IP-Mass 41 was detected in a higher percentage o birds than the Vx-Mass 41 at 3 (50% vs. 33%) and 7 (33% vs. 17%) days. In the duodenum, the IP-Mass 41 was detected in a higher percentage of birds than the Vx-Mass 41 at 3 (50% vs. 17%) and 7 (33% vs. 17%) days.

No IBV Massachusetts strains were detected at 14 and 21 days of age in any of the selected tissues. As expected, no IBV was detected at any sampling age in the negative control group.

Evaluation of viral effect on the trachea. Mean tracheal scores of SPF chickens inoculated at day of age by eye drop with the Arkansas and Massachusetts IBV strains are presented in table 4.3-A and 4.3-B, respectively.

Individual comparisons of the tracheal scores between the Arkansas inoculated and negative control groups by using the Dunnett's test ($p < 0.05$) are summarized in table 4.4-A.

Although no statistically significant, the IP-Ark 1 group had lower or similar mean tracheal scores than the NP-Ark 1. The IP-Ark 1 group exhibited significantly lower mean tracheal scores at 3 (1.42 vs. 1.79) and 14 (1.41 vs. 1.83) days and higher scores at 21 days (1.66 vs. 1.33) than the Vx-Ark group. The NP-Ark 1 exhibited a significantly higher mean tracheal score than the Vx-Ark (1.75 vs. 1.33) only at 21 days. When compared with the non inoculated group, significantly higher mean tracheal scores were observed in the IP-Ark 1 at 7 days (1.83 vs. 1.25), and in the NP-Ark 1 group at 7 (1.83 vs. 1.25) and 21 (1.75 vs. 1.5) days.

Individual comparisons of the tracheal scores between the Massachusetts inoculated and negative control groups by using the Dunnett's test ($p < 0.05$) are summarized in table 4.4-B.

No significant differences in the tracheal scores were observed between the IP-Mass 41 and Vx-Mass 41 groups at 3 (1.25 vs. 1.33), 7 (1.41 vs. 1.33) and 14 (1.50 vs. 1.54) days. However, the IP-Mass 41 exhibited significantly higher tracheal scores at 21 days than the Vx-Mass 41 group (1.75 vs. 1.33). When compared with the non inoculated group, significantly lower mean tracheal scores were observed at 3 days in the IP-Mass 41 (1.25 vs. 1.66) and Vx-Mass (1.33 vs. 1.66) groups.

High tracheal scores due to high LI and GCP scores in the absence of epithelial damage and epithelial hyperplasia were observed at 21 days in the Arkansas inoculated groups (Table 4.5), and at 14 and 21 days in the Massachusetts inoculated groups (Table 4.6).

The effect of the IBV strains for the intestinal tract was evaluated by microscopic examination of the cecal tonsils and duodenum.

In the cecal tonsils, no differences among the groups were observed at any sampling age. At 3 and 7 days, all the groups exhibited very mild to non lymphocytic infiltration with no damage to the epithelial tissue. At 14 days, all the groups exhibited a moderate follicular and diffuse lymphocytic infiltration in the lamina propia with the presence of prominent germinal lymphoid centers. At 21 days, all the groups exhibited moderate to severe follicular and diffuse lymphocytic infiltration in the lamina propia with the presence of prominent lymphoid germinal centers.

In the duodenum, no lesions were observed in any of the groups at 3, 7 and 14 days. At 21 days, the cecal tonsils from the IP-Ark 1, Vx-Ark, IP-Mass 41 IBV inoculated and the negative control groups exhibited a severe follicular and non follicular lymphocytic infiltration

with at least one section of cecal tonsils with severe hyperemia. However, no damage was observed in the intestinal epithelium. At 21 days, very mild to mild lymphocytic infiltration without intralesional hemorrhages, congestion, hyperemia or intestinal epithelium damage was observed in the cecal tonsils of the Vx-Mass 41 inoculated group.

Discussion

Initial attempts to passage *in vitro* selected IBV strains, previously passaged *in vivo* in the small intestines of SPF chickens, were focused on the development of a primary intestinal cell culture system. Several approaches included the use of chicken embryo intestinal fibroblast as a co-culture system with chicken embryo intestinal epithelial cells (1), enzymatic dissociation with dispase type I and collagenase type XI (4), and non enzymatic dissociation with the cell recovery solution MatriSperse® (27). Low recoveries, fibroblast contamination and survival for a very limited time, usually 1 to 2 days, of the intestinal epithelial cells were observed. These limiting factors in the use of primary intestinal cell culture systems have been previously described (11, 26, 31). Low survival has been related with permanent cellular damage (apoptosis) occurring rapidly when the epithelial cells are separated from their extracellular matrix support using conventional dissociation methods (14, 34).

As an alternative, an intestine organ culture system to passage the selected IBV strains was developed (Fig. 4.1-A, B and C). The ability of these IBV strains to replicate in the intestinal rings was determined by the presence of the IBV N gene in the apical epithelial cells of the intestinal villi, detected by *in situ* hybridization (Fig. 4.1-D, E and F), and by the presence of

competent virus, detected by RT-PCR, in the allantoic fluid of SPF chicken embryos after each three consecutive blind passages in the intestinal rings.

The effect of two of the four *in vitro* intestine passaged IBV strains for the upper respiratory tract was evaluated. The IP-Ark1 strain was selected to compare its pathogenicity with the observed in a previous study in which this strain had been passaged several times *in vivo*. The IP-Mass 41 strain was selected based on its high number of *in vitro* passages and the possibility to compare its pathogenicity with the parental Massachusetts 41 IBV vaccine strain, commonly used in vaccination programs worldwide.

In the previous *in vivo* study (chapter 3), the *in vivo* intestine passage IBV Arkansas serotype (IP-Ark 1) exhibited a presence for a shorter period in the trachea than the NP-Ark 1 and Vx-Ark strains, and higher effect for the upper respiratory tract at 6 and 9 days than the Vx-Ark strain. In this study, the *in vitro* intestine passaged IP-Ark 1 strain was mostly detected in the trachea of a lower percentage of birds than the NP-Ark 1 and Vx Ark strains. The IP-Ark 1 also exhibited significantly milder effects on the upper respiratory tract than the Vx-Ark strain. Although no statistically significant, milder tracheal lesions were observed in the IP-Ark 1 group when compared with the NP-Ark 1 group.

The *in vitro* intestine passaged IP-Mass 41 was detected in the tracheas of a higher percentage of birds only at 3 days after infection. However, at this age, no effect of the virus on the upper respiratory tract was observed, with tracheal scores even lower than the non inoculated group. Based on the detection of the IP-Mass 41 strain in the cecal tonsils and duodenum of a higher percentage of birds than the Vx-Mass 41 strain, a further decrease in the detection of the virus in the trachea might be expected after some additional *in vitro* passages. Besides, no significant differences in their effect on the respiratory tract were observed at any age. At 21

days, higher tracheal scores were observed in the IP-Ark 1, NP-Ark 1 and IP-Mass 41 groups when compared with those observed in the Vx-Ark and Vx-Mass groups. The increased tracheal scores in these groups were produced by the higher lymphocytic infiltration and goblet cell and mucus hyperplasia observed (Table 4.5 and 4.6). However, the higher scores at this particular age in the absence of epithelial damage and epithelial hyperplasia of the trachea should not be correlated with viral induced damage and could be related with environmental challenge induced by increased levels of ammonia observed at this particular age in the isolation units. The reparative process in the IBV infected trachea begins around 4 to 6 days after infection with a complete recovery by 10 to 20 days after infection, with clinical signs disappearing within 10 to 14 days (8, 24). At early ages, around 5 to 10 days when maximum IBV titers are present, lymphocytic infiltration and goblet cell and mucus gland hyperplasia seems to correlate better with epithelial damage and epithelial hyperplasia (Tables 4.5 and 4.6). At 21 days, follicular lymphocytic infiltrates (germinal centers) were observed in the tracheas of the IP-Ark 1 group when compared with the diffuse lymphocytic infiltrates observed in the Vx-Ark inoculated group. The presence of the germinal centers, known to be mostly composed of B cells, might be indicative of a stronger production of local respiratory antibodies, which correlate better with protection than serum antibody levels (21). In commercial broilers or layers, no changes in the effect of the intestine passaged IBV strains on the upper respiratory and intestinal tracts should be expected. The virus infection in these tissues does not seem to be influenced by the presence of circulating maternal antibodies (23). However, maternally derived antibodies might block the virus from reaching the internal organs including the kidneys and reproductive tract (23).

No microscopic changes or gross lesions have been reported in the intestinal tissues after IBV infection (8). The presence of only moderate to severe lymphocytic infiltration in the

absence of changes or lesions in the intestinal epithelium of duodenum and cecal tonsils in any group at any sampling age seems to agree with this observation.

The association between IBV respiratory reactions and susceptibility to *E. coli* in chickens has been extensively studied (2, 6, 15, 33). Damage to the tracheal epithelium caused by IBV facilitates *E. coli* invasion and multiplication leading to lesions or even death, causing a major clinical and economic impact specially in broilers (12, 13, 15, 33). The intestinal passages performed on the Arkansas 1 IBV have decreased its presence and effect on the respiratory tract to levels lower than those observed with the commercial attenuated Arkansas vaccine. The ability of this intestine passaged IBV strain, when used in vaccination programs, to decrease the mortality observed in broilers during the final growing period and condemnations at processing by lowering the tracheal damage and replication of secondary pathogens (*E. coli*) should be evaluated. Additional *in vitro* passages of the IP-Mass 41, IP-Holl 90 and IP-Mass 1 should be continued to further decrease their presence and effect on the respiratory tract.

Figure 4.1. Intestinal organ culture system developed to passage selected IBV strains previously passaged *in vivo* in the small intestines of SPF chickens (A = intestinal ring, B = intestinal villi adhered to the basal matrix support, and C = individual intestinal villi separated from their basal matrix support). Positive IBV N gene detection by *in situ* hybridization was observed in the apical epithelial cells of the villi in the intestinal rings 60 hours after inoculation (D, E, and F)

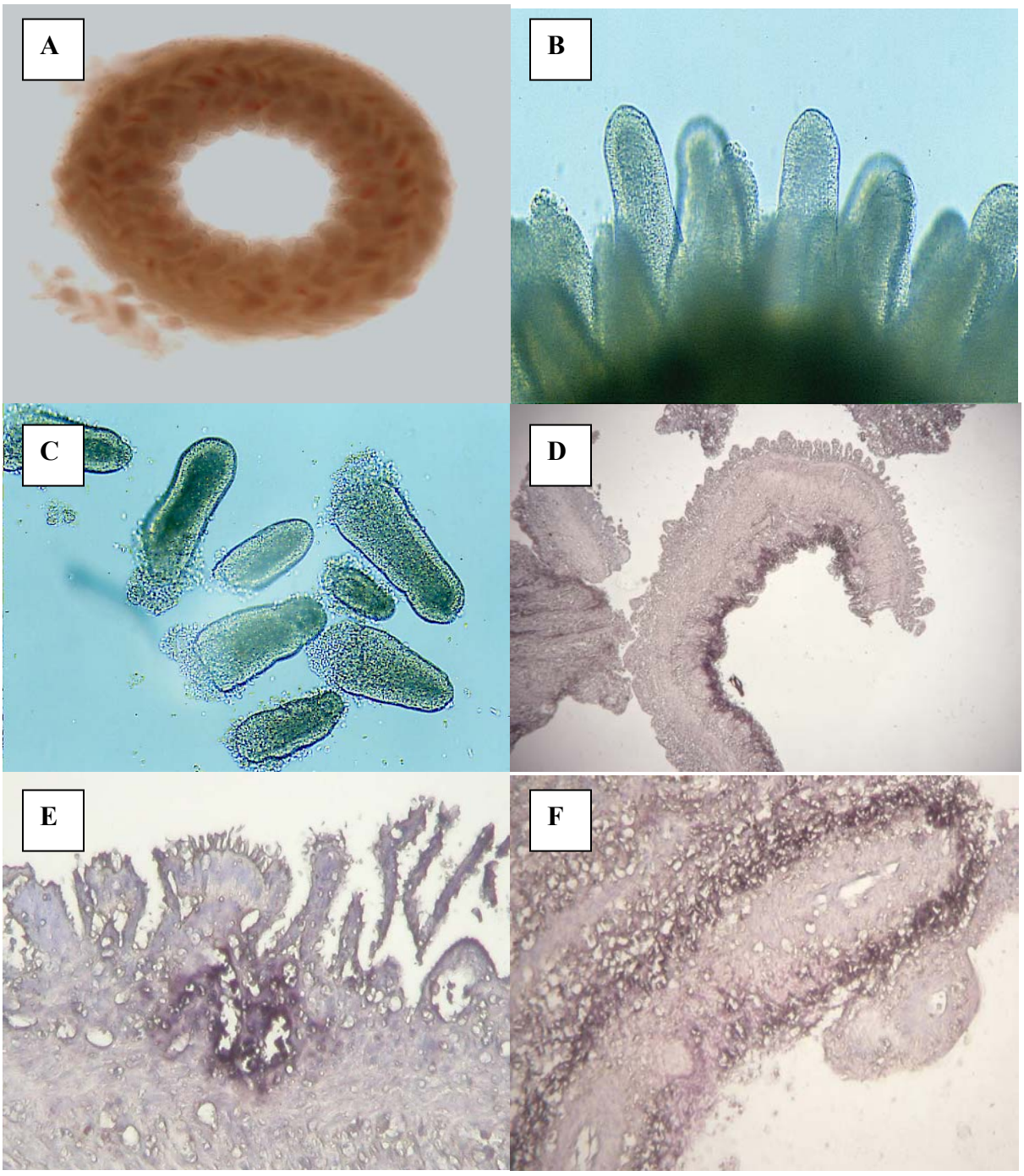

A
B
C
D
E
F

Table 4.1. *In vivo* and *in vitro* passages of the selected IBV strains performed in the intestinal tissues of one day-old SPF chickens and intestinal organ culture of 18-20 day-old SPF chicken embryos, respectively. The presence of *in vitro* intestine-passaged IBV was detected every 3 blind passages by RT-PCR in the allantoic fluid of SPF chicken embryos 48 hours after inoculation with the supernatant of each third in *vitro* intestine passage.

IBV Strain	*In Vivo* Passages	*In Vitro* Passages
IP-Mass 41*	8	38
IP-Holl 90	8	37
IP-Ark 1*	17	32
IP-Mass 1	14	33

* IBV strains used to evaluate their pathogenicity for the upper respiratory tract.

Table 4.2. Percentages of SPF birds positive to IBV after inoculation at day of age by eye drop with the IP-Ark 1, NP-Ark 1, Vx Ark IBV strains (A) or the IP-Mass 41 and Vx-Mass 41 IBV strains (B). The presence of IBV was detected by RT-PCR at every sampling age in the allantoic fluid of SPF chicken embryos 48 hours after inoculation with the processed tracheal (T), cecal tonsils (CT) and duodenal (D) tissues from 6 SPF chickens.

A	**Sampling age (days)**											
	3			**7**			**14**			**21**		
Ark Strain	**T**	**CT**	**D**	**T**	**CT**	**D**	**T**	**CT**	**D**	**T**	**CT**	**D**
IP Ark 1	67	**50**	33	33	**100**	100	0	**83**	33	17	**83**	0
NP Ark 1	83	**100**	83	83	**83**	100	50	**100**	100	17	**50**	17
Vx Ark	33	**33**	67	100	**17**	17	67	**67**	50	50	**0**	0
Neg Cont	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0

B	**Sampling age (days)**											
	3			**7**			**14**			**21**		
Mass Strain	**T**	**CT**	**D**	**T**	**CT**	**D**	**T**	**CT**	**D**	**T**	**CT**	**D**
IP Mass 41	83	**50**	50	50	**33**	33	0	**0**	0	0	**0**	0
Vx Mass 41	33	**33**	17	50	**17**	17	0	**0**	0	0	**0**	0
Neg Cont	0	**0**	0	0	**0**	0	0	**0**	0	0	**0**	0

Table 4.3. Mean tracheal scores of SPF chickens inoculated at day of age by eye drop with the IP-Ark 1, NP-Ark 1, Vx-Ark IBV strains (A) or the IP-Mass 41 and Vx-Mass 41 IBV strains (B) at different sampling ages.

A	Sampling Age (days)			
Ark Strains	**3 days**	**7 days**	**14 days**	**21 days**
IP Ark 1	1.42	1.83	1.41	1.66
NP Ark 1	1.54	1.83	1.58	1.75
Vx Ark	1.79	1.54	1.83	1.33
Neg Control	1.66	1.25	1.66	1.50 [c]

B	Sampling Age (days)			
Mass 41 Strains	**3 days**	**7 days**	**14 days**	**21 days**
IP-Mass 41	1.25	1.41	1.50	1.75
Vx-Mass 41	1.33	1.33	1.54	1.33
Neg Control	1.66	1.25	1.66	1.50

Table 4.4. Statistical analysis of the mean tracheal score between two specific groups by using the Dunnett's test in the Arkansas inoculated and negative control groups (A) and Massachusetts inoculated and negative control groups (B).

A	**Sampling Age (days)**			
Comparison	**3 days**	**7 days**	**14 days**	**21 days**
IP-Ark 1 vs. NP-Ark 1	1.42/1.54	1.83/1.83	1.41/1.58	1.66/1.75
IP-Ark 1 vs. Vx-Ark	**1.42/1.79**	1.83/1.54	**1.41/1.83**	**1.66/1.33**
NP-Ark 1 vs. Vx-Ark	1.54/1.79	1.83/1.54	1.58/1.83	**1.75/1.33**
IP-Ark 1 vs. Neg Cont	1.42/1.66	**1.83/1.25**	1.41/1.66	1.66/1.50
NP-Ark 1 vs. Neg Cont	1.54/1.66	**1.83/1.25**	1.58/1.66	**1.75/1.50**

*Bold numbers correspond to statistically different ($p<0.05$) scores.

B	**Sampling Age (days)**			
Comparison	**3 days**	**7 days**	**14 days**	**21 days**
IP-Mass 41 vs. Vx-Mass 41	1.25/1.33	1.41/1.33	1.50/1.54	**1.75/1.33**
IP-Mass 41 vs. Neg Cont	**1.25/1.66**	1.41/1.25	1.50/1.66	1.75/1.50
Vx-Mass 41 vs. Neg Cont	**1.33/1.66**	1.33/1.25	1.54/1.66	1.33/1.50

*Bold numbers correspond to statistically different ($p<0.05$) scores.

Table 4.5. Mean epithelial damage (ED), epithelial hyperplasia (EH), lymphocytic infiltration (LI) and goblet cell and compound mucus gland proliferation (GCP) tracheal scores observed in the IBV Arkansas inoculated and negative control groups at 3, 7, 14 and 21 days of age.

		IBV Strain			
Age (days)	**Parameter**	**IP-Ark1**	**NP-Ark1**	**Vx-Ark**	**Neg Cont**
3	ED	2	2	2.3	2
	EH	1	1.3	2.3	2.3
	LI	1	1.1	1	1.3
	GCP	1.6	1.6	1.5	1
7	ED	2.5	2.5	2.3	1
	EH	2.3	1.8	1.3	1
	LI	1.5	1.8	1.5	1
	GCP	1	1.1	1	2
14	ED	1.6	1.8	2.0	1
	EH	1	1.1	1.5	1.3
	LI	2	2.1	2.3	1.6
	GCP	1	1.1	1.5	2.6
21	ED	1	1	1	1
	EH	1	1	1	1
	LI	2.3	2.8	2	1.3
	GCP	2.3	2.1	1.3	2.6

*Birds inoculated with the IP-Ark 1, NP-Ark-1 and Vx-Ark received a dose of $10^{4.6}$, $10^{4.3}$ and $10^{4.3}$ EID_{50} per bird, respectively.

Table 4.6. Mean epithelial damage (ED), epithelial hyperplasia (EH), lymphocytic infiltration (LI) and goblet cell and compound mucus gland proliferation (GCP) tracheal scores observed in the IBV Massachusetts inoculated and negative control groups at 3, 7, 14 and 21 days of age.

		IBV Strain		
Age (days)	**Parameter**	**IP-Mass41**	**Vx-Mass41**	**Neg Cont**
3	ED	1.6	1	2
	EH	1	1.3	2.3
	LI	1	1	1.3
	GCP	1.3	2	1
7	ED	2	1.5	1
	EH	1.1	1.1	1
	LI	1.1	1	1
	GCP	1.3	1.6	2
14	ED	1	1	1
	EH	1	1	1.3
	LI	1.8	2	1.6
	GCP	2.1	2.1	2.6
21	ED	1.1	1	1
	EH	1.3	1	1
	LI	2	1.8	1.3
	GCP	2.5	1.5	2.6

*Birds inoculated with the IP-Mass 41 and Vx-Mass 41 received a dose of $10^{4.6}$ and $10^{4.4}$ EID_{50} per bird, respectively.

Acknowledgements

We gratefully acknowledge Merial Select, Inc. for their economic support on this project.

References

1. Ali, A., and D. L. Reynolds. Primary cell culture of turkey intestinal epithelial cells. Avian Dis. 40:103-108. 1996.

2. Avellaneda, G. E., P. Villegas, M. W. Jackwood, and D. J. King. In vivo evaluation of the pathogenicity of field isolates of infectious bronchitis virus. Avian Dis. 38:589-597. 1994.

3. Bhattacharjee, P. S., and R. C. Jones. Susceptibility of organ cultures from chicken tissues for strains of infectious bronchitis virus isolated from the intestine. Avian Path. 26:553-563. 1997.

4. Brandsch, C., P. Friedl, K. Lange, T. Richter, and T. Mothes. Primary culture and transfection of epithelial cells of human small intestine. Scand. J. Gastroenterol. 33:833-838. 1998.

5. Cavanagh, D., and S. Naqi. Infectious Bronchitis. In: Diseases of Poultry, 11th ed, Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa. pp. 101-119. 2003.

6. Cook, J., and M. B. Huggins. Newly isolated serotypes of infectious virus: Their role in disease. Avian Path. 15:129-138. 1986.

7. Deveney, C. W., L. Rand-Luby, M. J. Rutten, C. A. Luttropp, W. M. Fowler, J. Land, C. L. Meichsner, M. Farahmand, B. C. Sheppard, R. A. Crass, and K. E. Deveney. Establishment of human colonic epithelial cells in long-term culture. J. Surg. Res. 64:161-169. 1996.

8. Dhinakar Raj, G., and R. C. Jones. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Path. 26:677-706. 1997.

9. Falcone, E., E. D'Amore, L. Di Trani, A. Sili, and M. Tollis. Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction. J. Virol. Meth. 64:125-130. 1997.

10. Follmann, W., S. Weber, and S. Birkner. Primary cell cultures of bovine colon epithelium: Isolation and cell culture of colonocytes. Toxicology in Vitro. 14:435-445. 2000.

11. Fonti, R., L. G. Bises, F. Magliocca, F. Nobili, R. Caprilli, and Y. Sambuy. Human colonocytes in primary culture: A model to study epithelial growth, metabolism and differentiation. Int. J. Colorectal Dis. 9:13-22. 1994.

12. Goren, E. Observations on experimental infection of chickens with Escherichia coli. Avian Path. 7:213-224. 1978.

13. Gross, W. B. Factors affecting the development of respiratory disease complex in chickens. Avian Dis. 34:607-610. 1990.

14. Grossmann, J., C. Fiocchi, and A. D. Levine. Extracellular matrix (ECM) orchestrates death and survival of human intestinal epithelial cells. Gastroenterology. 112:A987. 1997.

15. Hopkins, S. R., and H. W. Yoder, Jr. Increased incidence of airsacculitis in broilers infected with Mycoplasma synoviae and chicken-passaged infectious bronchitis vaccine virus. Avian Dis. 28:386-396. 1984.

16. Jackwood, M. W., H. M. Kwon, and D. A. Hilt. Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and a DNA probe. Avian Dis. 36:403-409. 1992.

17. Kapczinski, D. R., H. S. Sellers, G. N. Rowland, and M. W. Jackwood. Detection of in ovo-inoculated infectious bronchitis virus by immunohistochemestry and in situ hybridization with a riboprobe in epithelial cells of the lung and cloacal bursa. Avian Diseases. 46:679-685. 2002.

18. Klieve, A. V., and R. B. Cumming. Immunity and cross-protection to nephritis produced by Australian infectious bronchitis viruses used as vaccines. Avian Path. 17:829-839. 1988.

19. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

20. McCartney, K. K., D. C. Baumgart, S. R. Carding, J. O. Brubaker, and P. A. Offit. Primary murine small intestinal epithelial cells, maintained in long-term culture, are susceptible to rotavirus infection. J. Virol. 74:5597-5603. 2000.

21. Mondal, S. P., and S. A. Naqi. Maternal antibody to infectious bronchitis virus: Its role in protection against infection and development of active immunity to vaccine. Vet. Immun. Immunopath. 79:31-40. 2001.

22. Nakamura, K., J. K. Cook, J. A. Frazier, and M. Narita. Escherichia coli multiplication and lesions in the respiratory tract of chickens inoculated with infectious bronchitis virus and/or E. coli. Avian Dis. 36:881-890. 1992.

23. Naqi, S., K. Gay, P. Patalla, S. Mondal, and R. Liu. Establishment of persistent avian infectious bronchitis virus infection in antibody-free and antibody-positive chickens. Avian Dis. 47:594-601. 2003.

24. Otsuki, K., K. Matsuo, N. Maeda, T. Sanekata, and M. Tsubokura. Selection of variants of avian infectious bronchitis virus showing tropism for different organs. Adv. Exp. Med. Biol. 276:379-384. 1990.

25. Ott, L., and M. T. Longnecker. An introduction to statistical methods and data analysis, 5th ed. 384-451. 2001.

26. Perrault, N., and F. J. Beaulieu. Use of dissociating enzyme thermolysin to generate viable human normal intestinal epithelial cell cultures. Exp. Cell. Res. 224:354-364. 1996.

27. Perreault, N., and J. F. Beaulieu. Primary cultures of fully differentiated and pure human intestinal epithelial cells. Exp. Cell. Res. 245:34-42. 1998.

28. Pradhan, H. K., G. C. Mohanty, and B. S. Rajya. Comparative sensitivities of oviduct and tracheal organ cultures and chicken embryo kidney cell cultures to infectious bronchitis virus. Avian Dis. 27:594-601. 1983.

29. Quaroni, A., J. Wands, R. L. Trelstad, and K. Isselbacher. Epithelioid cell cultures from rat small intestine. J. Cell. Biology. 80:248-265. 1979.

30. Sambrook, J., E. F. Fritsch, and T. Maniatis. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.

31. Sanderson, I. R., R. M. Ezzell, M. Kedinger, M. Erlanger, Z. X. Xu, E. Pringault, S. Leon-Robine, D. Louvard, and W. A. Walker. Human fetal enterocytes in vitro: Modulation of the phenotype by the extracellular matrix. Proc. Natl. Acad. Sci. USA. 93:7717-7722. 1996.

32. Sawaguchi, K., S. Yachida, S. Aoyama, N. Takahashi, Y. Iritani, and Y. Hayashi. Comparative use of direct organ cultures of infected chicken tracheas in isolating avian infectious bronchitis virus. Avian Dis. 29:546-551. 1985.

33. Smith, H. W., J. K. Cook, and Z. E. Parsell. The experimental infection of chickens with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 66:777-786. 1985.

34. Strater, J., U. Wedding, T. F. Barth, K. Koretz, C. Elsing, and P. Moller. Rapid onset of apoptosis in vitro follows disruption of B1-integrin/matrix interactions in human colonic crypt cells. Gastroenterology. 112:A987. 1996.

35. Villegas, P. Titration of biological suspensions. In: A laboratory manual for the isolation and identification of avian pathogens, 4th ed. D. E. Swayne, J. R. Glisson, M. W. Jackwood, J. E. Pearson, and W. M. Reed, eds. The American Association of Avian Pathologists, Kennett Square, PA.:248-253. 1998.

CHAPTER 5

MOLECULAR CHARACTERIZATION OF AVIAN INFECTIOUS BRONCHITIS VIRUS (IBV) STRAINS ISOLATED IN COLOMBIA IN 2003[1]

[1] I. R. Alvarado, P. Villegas, N. Mossos, and M. W. Jackwood. Submitted to Avian Diseases, 04/30/04.

Key Words: IBV, Colombia, variants, S1 gene, RT-PCR, cycle sequencing.

Abbreviations: IB = infectious bronchitis; IBV = infectious bronchitis virus; HVR = hypervariable region; RT-PCR = reverse transcriptase-polymerase chain reaction; RFLP = restriction fragment length polymorphism; DACS = direct automated cycle sequencing; SPF = specific pathogen free; PDRC = poultry diagnostic and research center; cDNA = complementary DNA; *Eco*RI = *Escherichia coli* RY 13.

Summary. Sixteen infectious bronchitis virus isolates were recovered from broilers and layers from five geographic regions in Colombia (Cundinamarca, Santander, Valle, Tolima and Antioquia). The viruses were isolated from tracheas, lungs and cecal tonsils of birds vaccinated with the Massachusetts strain and showing respiratory signs. Phylogenetic analysis comparing their deduced amino acid sequences in the hypervariable region 1 of the S1 gene with reference strains was conducted. Four unique genotype clusters containing isolates with indigenous genotypes were observed. Isolates within same genotype cluster were highly related at the amino acid level. Isolates in the genotype cluster A (CO8232L, CO8234CT and CO8234T) exhibited similarities between 69% and 99%. Isolates in the genotype cluster B (CO8089L and CO8091L) exhibited a 93% similarity. Isolates in the genotype cluster C (CO1657, CO1692, CO8113T and CO8110T) exhibited similarities between 91.9% and 100%. No differences at the amino acid level were observed among isolates within genotype cluster D (CO8248CT, CO8250 and CO8247). Amino acid sequences in the HVR 1 of isolates CO8232L, CO8091L, CO8110T and CO8248CT, representing the genotype clusters A, B, C and D, respectively, were compared with sequences of vaccine strains Arkansas DPI, Connecticut 46, DE 072, Holland 120 and Massachusetts 41. Isolates CO8232L and CO8091L exhibited similarities between 31% and 53% while isolates CO8110T and CO8248CT exhibited similarities between 42% and 68%. One isolate, CO8033CT, was found to be the Connecticut genotype while three isolates, CO1694, CO8043CT and CO8043T, were found to be the Massachusetts genotype.

Introduction

Infectious Bronchitis (IB) is an acute, highly contagious upper respiratory disease of chickens (16). The disease is caused by infectious bronchitis virus (IBV), a single-stranded, positive sense RNA virus member of the *Coronaviridae* family (8). Clinical signs include tracheal rales, nasal exudates, coughing, and sneezing (8). IB is an economically important disease causing a decline in egg production and egg quality in layers and poor weight gain due to reduced feed efficiency in broilers (16). The IBV genome encodes four major structural proteins, a nucleocapsid protein, a membrane protein, the envelope protein and the spike (S) glycoprotein (14). The S glycoprotein is proteolitically processed into two nonconvalently bound peptide chains known as S1 and S2 (38). In the S1 subunit, three hypervariable regions (HVR), located within amino acids 38-67, 91-141, and 274-387, are present (7, 26, 35). The S1 subunit is involved in the induction of neutralizing, serotype-specific, and hemagglutination-inhibiting antibodies (14, 38). More than 20 IBV serotypes have been identified worldwide using the virus neutralization (VN) test (9), hemagglutination-inhibition (HI) test (23), monoclonal antibodies (18, 19), and protein polymorphism (4). Other techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) with serotype specific primers (21), RNA fingerprint (27), nucleic acid probes (20, 29, 37), RT-PCR followed by restriction fragment length polymorphism (RFLP) of the amplified S1 gene (30), and direct automated cycle sequencing (DACS) of a S1 subunit with primers that amplify the HVR 1 and 2 (24), have also been used to group virus and to predict their genotype or serotype. Moreover, amino acids within the HVR 1 have been associated with major neutralizing epitopes (15, 34, 36), and have been used to genotype IBV showing correlation with serotypes (40).

Vaccination programs against IB are based on the determination of IBV strains causing the disease in the field (13). In spite of intensive vaccination, outbreaks of IB frequently occur in the field due to the continued presence of different serotypes, generated by point mutations, insertions, deletions or RNA recombination of the S1 genes (5).

In Colombia, as in many other countries, infectious bronchitis is one of the most important respiratory diseases. Vaccination programs rely on the use of the Massachusetts strain which is the only IBV vaccine strain officially approved. However, the presence of the disease in vaccinated chickens is commonly observed causing a major economic impact. The aim of this study was to obtain IBV field isolates from problem farms from different geographic regions in the country, characterize them by molecular techniques and determine their relationship with reference strains.

Materials and methods

Case history. The presence of respiratory disease in commercial layers and broilers of different age and breed was observed in several regions of the country (Fig. 5.1). Affected layer flocks were experiencing morbidity of 2% and weekly mortality of 12%, while affected broiler flocks were experiencing morbidity between 10% and 80% and weekly mortalities between 4.8% and 35%. Simultaneous infections with other respiratory pathogens as well as secondary bacterial infections were suspected in many of the flocks.

Virus isolation and inactivation. Three week-old specific pathogen free (SPF) chickens were located in selected problem farms. Tissue samples from 5 to 10 SPF sentinel birds, and

commercial layers or broilers from each problem farm were obtained in every region and sent to the main virology laboratory of the Colombian Agricultural Institute located in Bogotá. Samples, including trachea, lung and cecal tonsils, were processed by standard procedures (1). Briefly, samples were homogenized (20% w/v) in tryptose phosphate broth, pH 7.0-7.2 with antibiotics (10,000 IU/ml penicillin, 10,000 μg/ml streptomycin), centrifuged (1500 X g for 15 min), and the supernatant was passed through a sterile 0.22 μm polyethersulfone (PES) syringe filter (Whatman Inc., Clifton, NJ). The filtered supernatant fluids were inoculated into 9-11 day old SPF chicken embryos via the allantoic sac. Two serial passages were performed. Allantoic fluids from the second passage were obtained 48 h after inoculation and inactivated with an equal volume of molecular biology grade phenol, pH 4.3 (Fisher Scientific, Fair Lawn, NJ). Inactivated samples were packaged and brought to our laboratory at the Poultry Diagnostic and Research Center (PDRC) at the University of Georgia for further studies following the procedure stipulated by the United States Department of Agriculture (USDA).

RNA isolation. The phenol-treated allantoic fluids, 1.5 ml per sample, were mixed and centrifuged at 12,000 X g for 10 min. The aqueous layer was obtained and viral RNA was extracted with a High Pure RNA Isolation Kit (Roche Diagnostics Co., Indianapolis, IN) with some modifications. In steps 1 and 2 of the manufacturer's instructions, 600 ul of the aqueous layer were added to 1.2 ml of lysis/binding buffer and mixed. In steps 3 and 4, half of the mixed sample was pipetted in the High Pure filter tube, centrifuged (8000 X g for 15 sec) and the flowthrough was discarded. Remaining mixed sample was pipetted in the same filter tube, centrifuged and the flowthrough was also discarded. The remainder steps of the High Pure RNA

Isolation Kit were followed as recommended by the manufacturer. RNA was eluted in 50 μl of the provided elution buffer and stored at – 70 C.

Reverse transcriptase (RT)-polymerase chain reaction (PCR). The presence of IBV in the phenol treated samples was initially detected by the amplification of a small portion of the nucleocapsid gene as described (10), with a modification. A different reverse primer (5'-TTATCAGGAACGCCGCTA-3') was designed to obtain a 178 bp amplicon. RT amplification of the S1 gene in the nucleocapsid positive samples was performed with the SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) following manufacturer's recommendations. Complementary DNA (cDNA) was synthesized using approximately 500 ng of total RNA as template. PCR was carried out in a My Cycler thermocycler (BIO-RAD, Hercules, CA) with FailSafe PCR system and PCR 2X premix C (Epicentre, Madison, WI). For the PCR procedure, the first 3 cycles consisted of denaturing at 94 C for 30 sec, annealing at 37 C for 30 sec and an extension at 72 C for 3 min. This was followed by 32 cycles of denaturing at 94 C for 30 sec, annealing at a temperature 3 C lower than the lowest melting temperature of the primers used, and an extension at 72 C for 3 min. The final extension was performed at 72 C for 10 min. Different set of primers were used to amplify the S1 gene of the nucleocapsid positive IBV isolates (Table 5.1). Pol 4, Pol 3, S1b and S2c primers were designed according to multiple sequence alignments of the 3' end of the IBV polymerase gene and 5' end of the S2 gene of reference IBV strains on deposit in GenBank. Primers 5' S1 oligo and 3' S1 oligo, also used to amplify the S1 coding sequence, have been previously described (30).

Cloning and sequencing of S1 gene. Amplified DNA fragments containing the entire S1 gene were cloned into pCR 2.1 TOPO vector (Invitrogen Corp., Carlsbad, CA) following the manufacturer's recommendations. Clones were transformed into TOP 10 *E. coli* cells (Invitrogen Corp., Carlsbad, CA). White colonies carrying the recombinant plasmids were selected for specific *Eco*RI (New England Biolabs Inc., Beverly, MA) endonuclease restriction screening. The recombinant plasmids carrying the right size insert were purified with the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). Three sequencing forward reactions were performed per sample. Sequencing reactions, using 250 to 300 ng of template per reaction, were performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as described by the manufacturer. Sequencing reactions were run in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were edited, saved and analyzed with DNAStar software (DNAStar, Inc., Madison, WI). Analysis of the S1 nucleotide and deduced amino acid sequences were performed with the Clustal W method of DNAStar and final phylogeny (dendogram) was produced by the neighbor-joining method. Nucleotide and amino acid sequences of the HVR 1 of the S1 gene (from the start codon to nucleotide 300) were compared with several published HVR 1 S1 sequences from GenBank.

Reference nucleotide sequence accession numbers. Reference S1 sequence data obtained from GenBank and used in the phylogenetic analysis included the North American isolates GA-7994-99, AF338717; Gray, L14069; JMK, L14070; Beaudette, X02342; GA-0470-98, AF274437; GA-8077-99, AF338718; H120, J04329; M41, X04722; Connecticut, L18990; DE 072, AF274435; GA-2787-98, AF274438; GAV-92, U16157; Holte, L18988; Wolgemuth 98, AF305595; Ark 99, L10384; Ark DPI, AF094815; CU-T2, U04739 and Florida 88, AF274437,

the U.K. isolates 4/91 attenuated, AF093793; 4/91 pathogenic, AF093794; UK-7-91, Z83975, and the Australian isolates D41, AF036937; Vic S, U29519 and N1-62, U29522.

Results

The analysis of a small segment of the nucleocapsid gene detected the presence of IBV in 16 out of the 83 samples. The sixteen IBV isolates were obtained from tracheal (T), lung (L), and cecal tonsil (CT) samples of SPF chickens, and commercial layers and broilers from five geographic regions of Colombia (Table 5.2). Successful amplification of the S1 gene of the field isolates CO8250, CO8247, CO8248CT, CO8232L, CO8234T, CO8234CT and CO8033CT was achieved with the 5' and 3' S1 oligo primers (Table 5.2). Amplification of the remaining isolates was achieved with the newly designed primers Pol4, Pol3, S1b and S2c (Table 5.2). Four unique genotype clusters of IBV, identified as A, B, C, and D, were obtained on the basis of deduced amino acid sequence analysis of the HVR 1 (residues 38 to 67) of the S1 gene when compared with available sequences obtained from the GenBank database (Fig. 5.2). Isolates within the same genotype cluster were highly related at the amino acid level. The genotype cluster A, represented by the isolates CO8232L, CO8234CT and CO8234T, exhibited similarities between 69% and 99%. The genotype cluster B, represented by the isolates CO8089L and CO8091L, exhibited a 93% similarity. The genotype cluster C, represented by the isolates CO1657, CO1692, CO8113T and CO8110T, exhibited similarities between 91.9 and 100%. In the genotype cluster D, represented by the isolates CO8248CT, CO8250 and CO8247, no differences at the amino acid level were observed. Isolate CO8033CT was very closely related (98.3% at the amino acid level) with the Connecticut strain while isolates CO1694, CO8043CT and CO8043T

were very closely related (98.3% at the amino acid level) with the Massachusetts 41 strain (Fig. 5.2). Based on the high amino acid similarities observed in the isolates within each genotype cluster, one isolate from each cluster was selected. Nucleotide and amino acid sequences in the HVR 1 of isolates CO8232L, CO8091L, CO8110T and CO8248CT, representing the genotype clusters A, B, C and D, respectively, were compared with the sequences of the vaccine strains Arkansas DPI, Connecticut 46, DE 072, Holland 120 and Massachusetts 41 (Table 5.3). At the nucleotide level, isolates CO8232L and CO8091L exhibited similarities between 28.7% and 63.7% while isolates CO8110T and CO8248CT exhibited similarities between 34% and 74.3% when compared with the commercial vaccines (Table 5.3). At the amino acid level, isolates CO8232L and CO8091L exhibited similarities between 31% and 53% while isolates CO8110T and CO8248CT exhibited similarities between 42% and 68% when compared with the commercial vaccines (Table 5.3).

Accession numbers of the Colombian IBV HVR 1 sequences deposited in GenBank are: CO1657, AY604546; CO1692, AY604547; CO1694, AY604548; CO8043T, AY604549; CO8043CT, AY604550; CO8091L, AY604551; CO8250, AY604552; CO8089L, AY604553; CO8247, AY604554; CO8248CT, AY604555; CO8113T, AY604556; CO8110T, 604557; CO8232L, AY604558; CO8234T, AY604559; CO8234CT, AY604560; CO8033CT, AY604561.

Discussion

More than 20 IBV serotypes have been recognized worldwide (8, 22, 33). The constant emergence or introduction of new IBV antigenic types as the result of insertions, deletions, recombinations and point mutations has been described (8, 12, 41). Specific IBV serotypes and

neutralization epitopes have been associated with the deduced amino acid sequences present in the HVR 1 and 2 of the S1 glycoprotein (2, 17, 25, 28, 32). Furthermore, amino acids within the HVR 1 have been associated with major neutralizing epitopes (15, 34, 36), and have been used to genotype IBV showing correlation with serotypes (40).

In this study, we analyzed the presence of IBV in 83 phenol-treated allantoic fluid samples obtained from several commercial layer and broiler farms from five geographic regions of Colombia by RT-PCR followed by nucleotide sequencing of the HVR 1 of the S1 glycoprotein gene. The use of specific primers to amplify the S1 gene of IBV by RT-PCR has shown some limitations. It is known that mutations at specific primer sites might block primer binding with no amplification of the S1gene with the consequent misdiagnosis of some IB isolates (31). To overcome that possibility, we initially tested all the phenol inactivated allantoic fluid samples by RT-PCR with primers spanning a portion of the nucleocapsid gene. In addition to the highly conserved nature of the nucleocapsid gene (10), the short target amplified by the selected primers would allow us to detect positive IBV samples even in those samples with partially fragmented RNA. A total of 16 IBV isolates were recovered and amplification of the S1 gene was performed. Since earlier studies (7, 40) have demonstrated the correlation between genotyping and serotyping based on the HVR 1 sequence, we sequenced the 5' end of the S1 gene containing the HVR 1.

Analysis of the HVR 1 sequences permitted the identification of 4 different genotype clusters (A through D) which seem to be indigenous and genetically distinct to the sequences obtained from GenBank (Fig. 5.2). Isolate CO8033CT is very closely related with the Connecticut strain and seems to be a circulating vaccine virus since the Connecticut vaccine was once available and authorized to be used in Colombia. Isolates CO1694, CO8043CT and

CO8043T are very closely related with the Massachusetts strain and probably correspond to the vaccine strain Mass 41.

In Colombia, commercial live and inactivated oil emulsion vaccines are currently used in IB immunization programs in broilers and breeders. It is known that the best protection against challenge is achieved by a vaccine containing the homologous strain, while vaccines containing heterologous strains may not induce enough cross-protection (12). Initial molecular characterization of the Colombian IBV isolates resulted in the identification of new genotypes not related with the Mass 41 strain, which might indicate that current vaccines do not offer enough protection against the IBV strains present in the field. S1 amino acids of different IBV serotypes usually differ by 20 to 25% or even up to 48% (11), however, differences lower than 3% between two serotypes have been observed (3, 6). This study shows for the first time the presence of genotype variant strains of IBV in Colombia, however, *in vitro* and *in vivo* studies are needed to determine the pathogenicity of these isolates and establish the protective level offered by the government approved Mass 41 and other commercially available vaccines. *In vitro* studies including virus neutralization (VN) in SPF chicken embryos or SPF chicken kidney cell confronting the genotype variant strains against homologous and heterologous antiserums should be performed (12, 39). *In vivo* studies based on the reisolation of the challenge virus five days after challenge in vaccinated SPF chickens should also be performed as described (1). If the commercial vaccines currently available in the country offer low levels of protection, the development of new live or killed vaccines from field isolates should be considered.

Figure 5.1. Location of the five Colombian regions with vaccinated commercial layers and broiler flocks exhibiting IB respiratory disease. Regions 1 through 5 represent the states of Cundinamarca, Santander, Valle, Tolima and Antioquia, respectively.

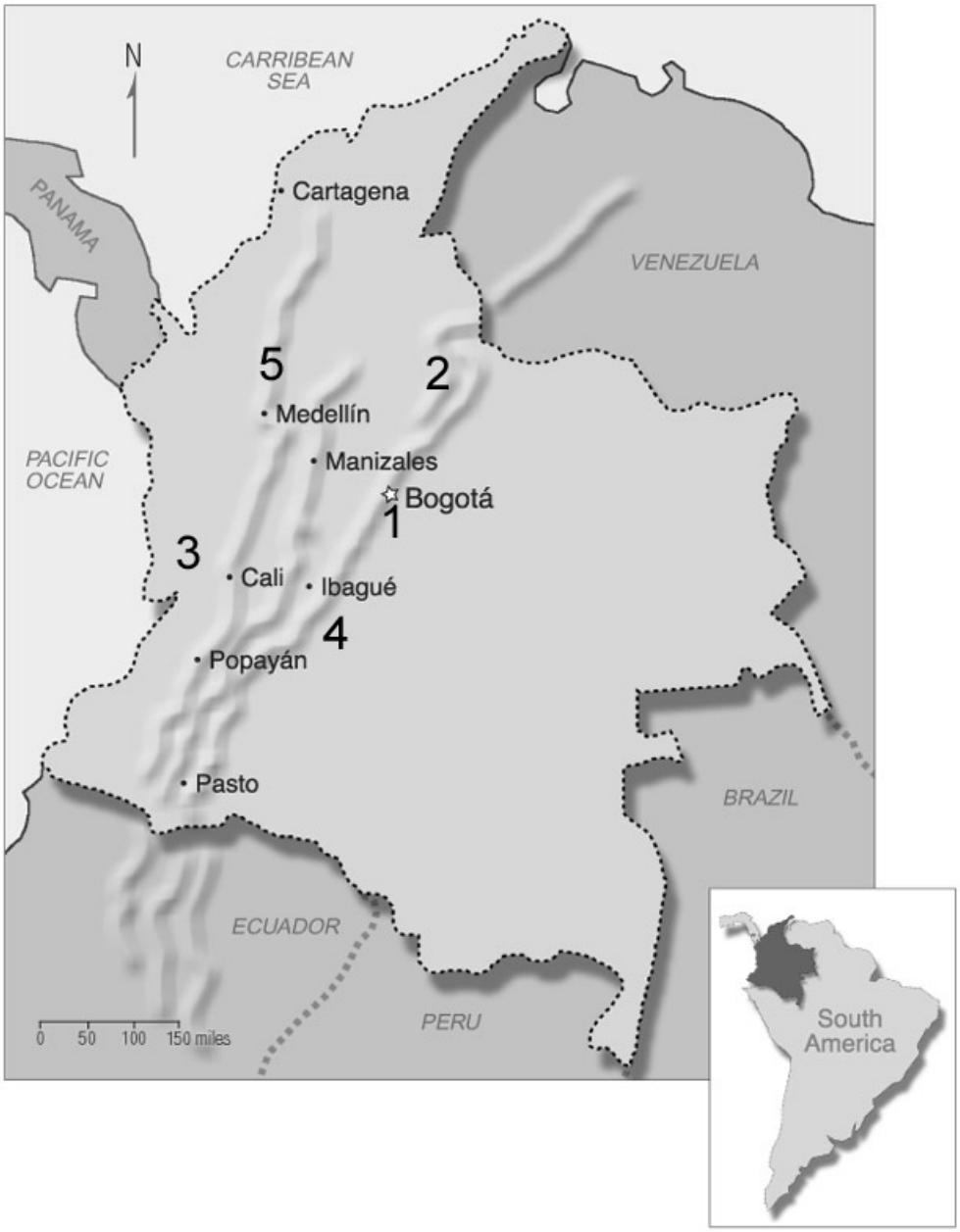

N
CARRIBEAN SEA
PANAMA
Cartagena
VENEZUELA
5
2
Medellín
Manizales
PACIFIC OCEAN
Bogotá
1
3
Cali
Ibagué
4
Popayán
Pasto
BRAZIL
ECUADOR
PERU
0 50 100 150 miles
South America

Table 5.1. Oligonucleotide primers used for polymerase chain reaction (PCR) amplification of the S1 gene of infectious bronchitis virus (IBV).

Primer	**Strand**	**Size**	**Tm**	**Location** (nucleotides)**	**5'- 3' Sequence**
Pol 4*	Forward	22	53.4	19882-19904	TGATATCTGATATGTATACAGA
Pol 3*	Forward	22	55.2	20244-20264	AGTATATTTGACGTTGCTAAGT
5' Oligo	Forward	18	50.8	20302-20320	TGAAACTGAACAAAAGAC
S1b*	Reverse	19	53.7	22144-22170	TRWACTCATCTGTYACAGT
S2c*	Reverse	17	54.8	22255-22271	ATGTTGTCGCAAACAGG
3' Oligo	Reverse	21	56.7	22002-22022	CCATAAGTAACATAAGGRCRA

* Primers designed according to multiple sequence alignment of the 3' end of the polymerase and 5' end of S2 genes from GenBank.

** Location of the primers used to amplify the S1 gene based on the complete IBV Beaudette sequence from GenBank accession number M95169.

Figure 5.2. Phylogenetic tree constructed on the basis of the amino acid sequences of the HVR 1 (residues 38-67) of the S1 protein with the Clustal W method of DNAStar showing the relationship of the Colombian IBV isolates with reference strains available in GenBank. Colombian isolates are identified by the letters CO followed by a 4-digit number and, if known, by the tissue sample from which it was isolated (trachea, T; lung, L and cecal tonsil, CT). Shaded boxes (A through D) represent the four indigenous genotype clusters.

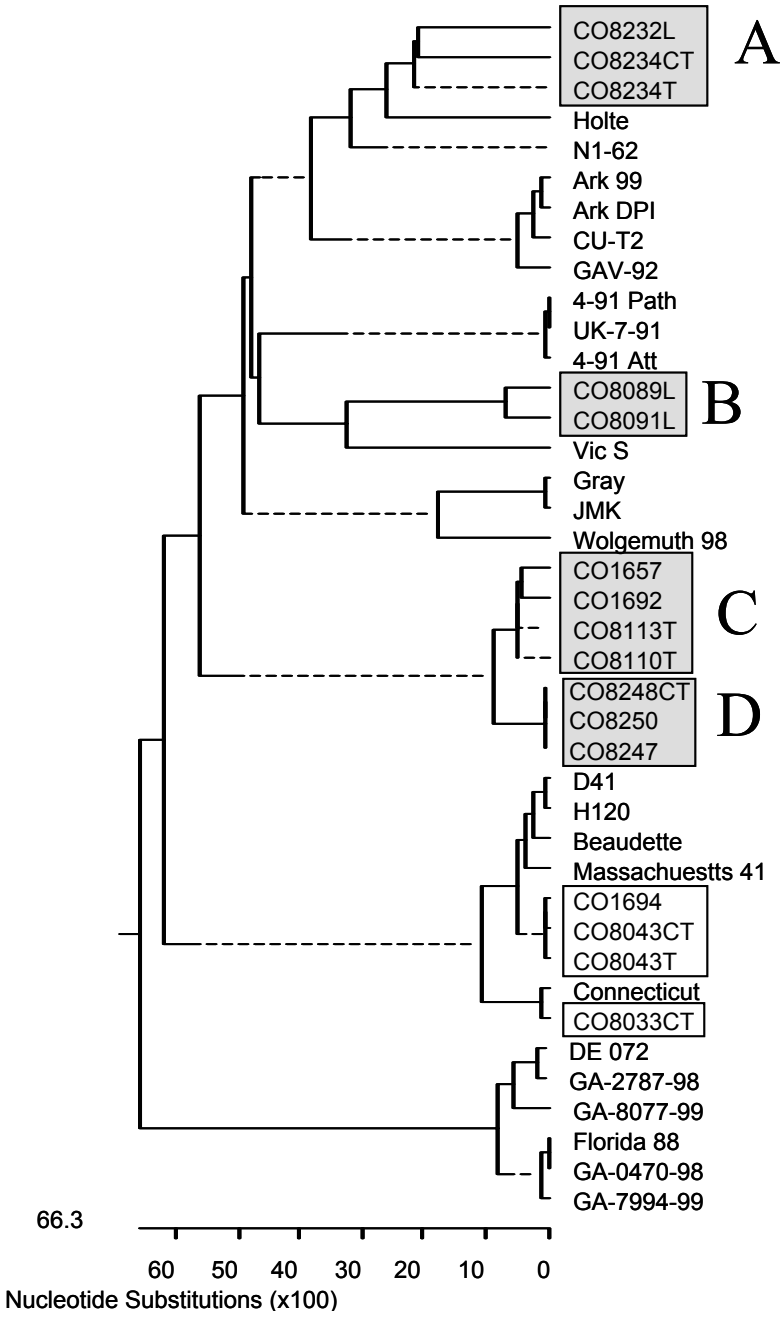

CO8232L
CO8234CT
CO8234T
A
Holte
N1-62
Ark 99
Ark DPI
CU-T2
GAV-92
4-91 Path
UK-7-91
4-91 Att
CO8089L
CO8091L
B
Vic S
Gray
JMK
Wolgemuth 98
CO1657
CO1692
CO8113T
CO8110T
C
CO8248CT
CO8250
CO8247
D
D41
H120
Beaudette
Massachuestts 41
CO1694
CO8043CT
CO8043T
Connecticut
CO8033CT
DE 072
GA-2787-98
GA-8077-99
Florida 88
GA-0470-98
GA-7994-99
66.3
60
50
40
30
20
10
0
Nucleotide Substitutions (x100)

Table 5.2. IBV isolates obtained from SPF chickens, commercial layers and broilers in Colombia in 2003 and oligonucleotide primers used for the RT reaction and PCR amplification of their S1 genes.

Region	Isolate	Bird Type	Primers (Forw/Rev)
1	CO8043T*	Broilers[A]	Pol4/S2c
	CO8043CT**	Broilers[A]	Pol 4/S2c
	CO1657	Broilers[A]	Pol4/S2c
	CO1694	Broilers[A]	Pol4/S2c
	CO1692	Broilers[A]	Pol4/S2c
2	CO8091L***	Broilers	Pol4/S1b
	CO8250	Broilers	5’ Oligo/3’ Oligo
	CO8089L	Broilers	Pol3/S2c
	CO8247	Layers	5’ Oligo/3’ Oligo
	CO8248CT	Layers	5’ Oligo/3’ Oligo
3	CO8113T	Layers	Pol4/S2c
	CO8110T	Broilers	Pol4/S1b
4	CO8232L	Layers[A]	5’ Oligo/3’ Oligo
	CO8234T	Layers[A]	5’ Oligo/3’ Oligo
	CO8234CT	Layers[A]	5’ Oligo/3’ Oligo
5	CO8033CT	Broilers[A]	5’ Oligo/3’ Oligo

* T = Trachea

** CT = Cecal tonsils

*** L = Lungs

[A] IBV strains isolated from 3 week-old SPF chickens placed in the problem farms

Table 5.3. Nucleotide (%) and amino acid similarities (%) in the HVR1 of the S1 gene of the four distinct genotype clusters typified by the isolates CO8110T, CO8248CT, CO8091L and CO8232L compared with reference vaccine strains with the Clustal W method of DNAStar.

		% Amino acid Similarity								
	Strain	**Ark DPI**	**Conn**	**DE 072**	**H 120**	**Mass 41**	**CO8232L (A)**	**CO8091L (B)**	**CO8110T (C)**	**CO8248CT (D)**
% Nucleotide Similarity	**Ark 99**		62.0	43.0	67.0	66.0	53.0	46.0	66.0	67.0
	Conn	67.0		50.0	79.0	79.0	46.0	42.0	64.0	68.0
	DE 072	36.0	66.0		48.0	47.0	31.0	33.0	42.0	43.0
	H 120	70.7	87.0	62.7		95.0	50.0	45.0	66.0	66.0
	Mass 41	70.7	88.7	61.0	96.3		47.0	43.0	64.0	64.0
	CO8232L	63.7	45.3	37.7	47.3	46.7		31.0	45.0	47.0
	CO8091L	61.0	40.3	28.7	54.0	52.7	50.0		36.0	37.0
	CO8110T	72.7	74.3	34.0	73.7	74.3	72.0	48.7		89.0
	CO8248CT	73.3	74.3	34.7	73.3	74.3	71.0	55.7	93.7	

Acknowledgments

We gratefully acknowledge Dr. Scott A. Callison, Dr. Taylor Barbosa and Deborah A. Hilt for their outstanding technical contribution to this project.

References

1. Alvarado, I. R., P. Villegas, J. El-Attrache, and T. P. Brown. Evaluation of the protection conferred by commercial vaccines against the California 99 isolate of infectious bronchitis virus. Avian Dis. 47:1298-1304. 2003.

2. Binns, M. M., M. E. Boursnell, F. M. Tomley, and D. K. Brown. Comparison of the spike precursor sequences of coronavirus IBV strains M41 and 6/82 with that of IBV Beaudette. J. Gen. Virol. 67:2825-2831. 1986.

3. Callison, S. A., M. W. Jackwood, and D. A. Hilt. Molecular characterization of infectious bronchitis virus isolates foreign to the United States and comparison with United States isolates. Avian Dis. 45:492-499. 2001.

4. Case, J. T., K. W. Sverlow, and B. J. Reynolds. A novel protein polymorphism differentiates the California serotype of infectious bronchitis from other serotypes common to California. J. Vet. Diagn. Invest. 9:149-155. 1997.

5. Cavanagh, D., P. J. Davis, and J. Cook. Infectious bronchitis virus: Evidence for recombination within the Massachusetts serotype. Avian Path. 21:401-408. 1992.

6. Cavanagh, D., P. J. Davis, J. Cook, D. Li, A. Kant, and G. Koch. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Path. 21:33-43. 1992.

7. Cavanagh, D., P. J. Davis, and A. P. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res. 11:141-150. 1988.

8. Cavanagh, D., and S. Naqi. Infectious bronchitis. in: Diseases of poultry, 11th ed, Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa. pp. 101-119. 2003.

9. Darbyshire, J. H., J. G. Rowell, J. K. Cook, and R. W. Peters. Taxonomic studies on strains of avian infectious bronchitis virus using neutralisation tests in tracheal organ cultures. Arch. Virol. 61:227-238. 1979.

10. Falcone, E., E. D'Amore, L. Di Trani, A. Sili, and M. Tollis. Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction. J. Virol. Meth. 64:125-130. 1997.

11. Gelb, J., Jr., C. L. Keeler, Jr., W. A. Nix, J. K. Rosenberger, and S. S. Cloud. Antigenic and S-1 genomic characterization of the Delaware variant serotype of infectious bronchitis virus. Avian Dis. 41:661-669. 1997.

12. Gelb, J., Jr., J. B. Wolff, and C. A. Moran. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Dis. 35:82-87. 1991.

13. Hofstad, M. S. Cross-immunity in chickens using seven isolates of avian infectious bronchitis virus. Avian Dis. 25:650-654. 1981.

14. Holmes, K. V., and M. M. Lai. Coronaviridae: The virus and their replication. in: Fundamental Virology, Fourth ed, B. N. Fields, D. M. Knipe and P. M. Howley, eds. Lippincott-Raven, Philadelphia. pp. 1163-1203. 2001.

15. Ignjatovic, J., and L. Galli. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch. Virol. 138:1-2. 1994.

16. Ignjatovic, J., and S. Sapats. Avian infectious bronchitis virus. Rev. Sci. Tech. 19:493-508. 2000.

17. Kant, A., G. Koch, D. J. van Roozelaar, J. G. Kusters, F. A. Poelwijk, and B. A. van der Zeijst. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J. Gen. Virol. 73:591-596. 1992.

18. Karaca, K., and S. Naqi. A monoclonal antibody blocking ELISA to detect serotype-specific infectious bronchitis virus antibodies. Vet. Microbiol. 34:249-257. 1993.

19. Karaca, K., S. Naqi, and J. Gelb, Jr. Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis. 36:903-915. 1992.

20. Karaca, K., P. Palukaitis, and S. Naqi. Oligonucleotide probes in infectious bronchitis virus diagnosis and strain identification. J. Virol. Methods. 42:293-300. 1993.

21. Keeler, C. L., Jr., K. L. Reed, W. A. Nix, and J. Gelb, Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis. 42:275-284. 1998.

22. King, D. J. Identification of recent infectious bronchitis virus isolates that are serologically different from current vaccine strains. Avian Dis. 32:362-364. 1988.

23. King, D. J., and S. R. Hopkins. Rapid serotyping of infectious bronchitis virus isolates with the hemagglutination-inhibition test. Avian Dis. 28:727-733. 1984.

24. Kingham, B. E., C. L. Keeler, Jr., W. A. Nix, B. S. Ladman, and J. Gelb, Jr. Identification of avian infectious bronchitis virus by direct automated cycle sequencing of the S-1 gene. Avian Dis. 44:325-335. 2000.

25. Koch, G., L. Hartog, A. Kant, and D. J. van Roozelaar. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: Correlation with biological functions. J. Gen. Virol. 71:1929-1935. 1990.

26. Kottier, S. A., D. Cavanagh, and P. Britton. Experimental evidence of recombination in coronavirus infectious bronchitis virus. Virology. 213:569-580. 1995.

27. Kusters, J. G., H. G. Niesters, N. M. Bleumink-Pluym, F. G. Davelaar, M. C. Horzinek, and B. A. Van der Zeijst. Molecular epidemiology of infectious bronchitis virus in the Netherlands. J. Gen. Virol. 68:343-352. 1987.

28. Kusters, J. G., H. G. Niesters, J. A. Lenstra, M. C. Horzinek, and B. A. van der Zeijst. Phylogeny of antigenic variants of avian coronavirus IBV. Virology. 169:217-221. 1989.

29. Kwon, H. M., M. W. Jackwood, T. P. Brown, and D. A. Hilt. Polymerase chain reaction and a biotin-labeled DNA probe for detection of infectious bronchitis virus in chickens. Avian Dis. 37:149-156. 1993.

30. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

31. Lee, C. W., D. A. Hilt, and M. W. Jackwood. Redesign of primer and application of the reverse transcriptase - polymerase chain reaction and restriction fragment length polymorphism test to the DE072 strain of infectious bronchitis virus. Avian Dis. 44:650-654. 2000.

32. Lee, C. W., D. A. Hilt, and M. W. Jackwood. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. J. Vet. Diagn. Invest. 15:344-348. 2003.

33. Lee, C. W., and M. W. Jackwood. Evidence of genetic diversity generated by recombination among avian coronavirus IBV. Arch. Virol. 145:2135-2148. 2000.

34. Lenstra, J. A., J. G. Kusters, G. Koch, and B. A. van der Zeijst. Antigenicity of the peplomer protein of infectious bronchitis virus. Mol. Immunol. 26:7-15. 1989.

35. Moore, K. M., J. D. Bennett, B. S. Seal, and M. W. Jackwood. Sequence comparison of avian infectious bronchitis virus S1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. Virus Genes. 17:63-83. 1998.

36. Neister, H. G., N. M. Bleumink-Pluym, A. D. Osterhaus, M. C. Horzined, and B. A. van der Zeijst. Epitopes on the peplomer protein of infectious bronchitis virus strain M41 as defined by monoclonal antibodies. Virology. 118:511-519. 1987.

37. Shankar, P. M., and C. J. Cardona. Characterization of infectious bronchitis virus isolates by slot blot hybridization. Avian Dis. 47:725-730. 2003.

38. Stern, D. F., and B. M. Sefton. Coronavirus proteins: Biogenesis of avian infectious bronchitis virus virion proteins. J. Virol. 44:794-803. 1982.

39. Villegas, P. Avian virus diseases. Laboratory manual. College of Veterinary Medicine, University of Georgia, Athens, GA.62. 2004.

40. Wang, C. H., and Y. C. Huang. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145:291-300. 2000.

41. Wang, L., D. Junker, and E. W. Collisson. Evidence of natural recombination within the S1 genes of infectious bronchitis virus. Virology. 192:710-716. 1993.

CHAPTER 6

DISCUSSION

IBV replication in several tissues of the respiratory tract (4, 13, 24, 29), kidneys (7, 9, 30), oviduct (3, 34) and proventriculus (38), has been associated with characteristic gross lesions and histological changes (13). IBV replication has also been observed in the intestinal tract in cells resembling histiocytes and lymphoid cells in the cecal tonsils and in apical epithelial cells of the villi in ileum and rectum (1, 13, 30). However, no histological changes or gross lesions in the intestinal tissue have been reported after IBV infection (4, 13). In spite of the wide presence of IBV strains in the intestinal tract, no studies related with the effect of intestine passaged IBV strains on the upper respiratory tract have been reported.

The first two studies involved the development of *in vivo* and *in vitro* systems to passage selected IBV strains in the intestinal tissue and the evaluation of the effect caused by these selected IBV strains, after several passages in each system, on the upper respiratory and digestive tracts. The main hypothesis supporting these studies was that intestine passages would decrease the presence and effect of IBV strains on the respiratory tract to levels even lower than those observed with commercial IBV vaccines. When used in vaccination programs, the intestine passaged IBV strains would produce milder tracheal lesions. Furthermore, milder tracheal lesions would lower the invasion and replication of secondary bacterial infections, reducing the negative effects associated with complicated respiratory reactions.

Initially, an *in vivo* system to passage selected IBV strains (Mass 41, Holland 90, Mass 1, Ark 1 and Ark 2) in the intestinal tissue of one day-old SPF chickens was developed. To date, this is the first study in which IBV strains have been consecutively passaged in chickens with reisolation of the virus from the intestines. Several passages were performed in each of the selected IBV strains. The pathogenicity of different IBV strains has been difficult to assay (2). Several models to evaluate IBV pathogenicity have been experimentally reproduced such as mixed IBV infections with *E. coli* (10, 11) or *Mycoplasma synoviae* (22), variability of ciliary activity (12), evaluation of microscopic tracheal (hyperplasia, necrosis, heterophil and lymphocytic infiltration) (16), kidney (23) or oviduct (8) lesions. After 13 *in vivo* passages, the effect of the IP-Ark 1 strain for the upper respiratory tract was evaluated and compared with the non intestine passaged (NP-Ark 1) and a commercial Arkansas vaccine (Vx-Ark) strains. Clinical signs and tracheal lymphocytic infiltration and epithelium proliferation were scored and used as parameters to evaluate the effect of the IP-Ark 1 IBV strain. No clinical signs were observed in the inoculated birds without maternal antibodies. This could be explained by the absence of secondary bacterial infections and other environmental factors commonly observed in the field. When associated with IBV infection, these factors could induce the presence of airsacculitis, pericarditis, perihepatitis and mortality (14, 18).

The IP-Ark 1 was detected in the tracheas of a lower percentage of birds when compared with the NP-Ark 1 and Vx-Ark viruses. The IP-Ark 1 also exhibited a shorter presence in the cecal tonsils when compared with the NP-Ark 1. This shorter presence in the selected tissues could be related with shedding of the virus during a shorter period, decreasing the presence of chronic respiratory reactions, known as “rolling reactions” (21).

Even though no clinical signs and only very mild lymphocytic infiltration and mild epithelial proliferation was observed at 6 and 9 days p.i., the IP-Ark 1 virus still induced higher tracheal lesions when compared with the attenuated Vx-Ark virus. The higher tracheal lesions induced by the IP-Ark 1 strain could be related with the low number of *in vivo* passages or with the procedure used to perform these passages. In the *in vivo* passages, after initial replication in the trachea, the virus might be expelled and ingested with further replication in the intestinal tissue. From this study, we concluded that primary cell or intestine organ culture system to perform additional passages would be beneficial. The *in vitro* system would not only restrict the replication of the virus to the intestinal tissue while avoiding the respiratory tract but also would further decrease their effect on the upper respiratory tract. The non enteropathogenic nature of the IP-Ark 1 strain was confirmed by the absence of gross lesions or microscopic changes in the cecal tonsils.

We initially attempted to develop a primary intestinal cell culture system to be used in the *in vitro* attenuation of selected (Mass 41, Holland 90, Mass 1 and Ark 1) IBV strains, which had been previously passaged several times *in vivo* in the intestinal tract. The approaches used to develop the intestinal primary cell culture system were unsuccessful, with low recovery, fibroblast contamination and low survival for a very limited time of the intestinal epithelial cells. These limiting factors in the use of primary intestinal cell culture systems had been previously described (15, 32, 33). An explanation of the low survival of the intestinal epithelial cells using conventional dissociation methods is the permanent cellular damage (apoptosis) occurring immediately after the epithelial cells are separated from the extracellular matrix support (20, 36).

As an alternative, an intestine organ culture system to passage the selected IBV strains was developed. The presence of the IBV N gene in the apical epithelial cells of the intestinal

villa was detected by *in situ* hybridization. Also, viral replication was demonstrated by the amplification of the S1 gene by RT-PCR in the allantoic fluid of SPF chicken embryos after each three consecutive blind passages. After several *in vitro* passages, the effect of the IP-Ark 1 and IP-Mass 41 IBV strains on the upper respiratory tract was also evaluated.

When the presence of the *in vivo* IP-Ark 1 in the trachea was evaluated, this strain was detected in a lower percentage of birds than the NP-Ark 1 and Vx-Ark strains. The IP-Ark 1 also exhibited higher lesion scores for the upper respiratory tract at 6 and 9 days p.i. than the Vx-Ark strain. After the additional *in vitro* passages, the IP-Ark 1 strain not only was detected in the tracheas of a lower percentage of birds, but also induced milder lesions in the trachea than the attenuated commercial Arkansas vaccine.

The *in vitro* IP-Mass 41 was detected in the tracheas of a higher percentage of birds at early stages of infection (3 days p.i.). However, at this age, no effect on the trachea was observed at this age with tracheal scores even lower than the observed in the non inoculated group. A tendency of the IP-Mass 41 to increase its presence in the cecal tonsils and duodenum at 3 and 7 days p.i. when compared with the attenuated Massachusetts vaccine was also observed. Further passages in the *in vitro* intestinal organ culture would further decrease the presence and effect of this IBV strain on the upper respiratory tract.

At 21 days p.i., higher tracheal scores were observed in the IP-Ark 1, NP-Ark 1 and IP-Mass 41 groups which resulted from the higher scores in the lymphocytic infiltration and goblet cell and mucus hyperplasia parameters. The higher scores observed in these parameters at this particular age in the absence of epithelium damage and epithelium hyperplasia of the trachea probably do not correlate with viral damage. The reparative process in the IBV infected trachea begins around 4 to 6 days after infection with a complete recovery by 10 to 20 days after

infection (7, 13). At early ages, around 5 to 10 days p.i., when maximum IBV titers are present, lymphocytic infiltration and goblet cell and mucus hyperplasia seemed to correlate better with epithelial damage and epithelium hyperplasia.

At 21 days p.i., the presence of germinal centers, known to be mostly composed by B cells, was observed only in the tracheas of the IP-Ark 1 inoculated birds. The presence of these germinal centers might be indicative of a stronger production of local respiratory antibodies, which correlate better with protection than serum antibody levels (28). However, further studies should be performed to confirm this hypothesis. The absence of histological changes or gross lesions in the cecal tonsils and duodenum of the inoculated groups was still observed, corroborating the non enteropathogenic nature of IBV strains after infection (4, 13).

The association between IBV respiratory reactions and susceptibility to *E. coli* in chickens has been extensively studied (17, 19, 21, 35). Damage to the tracheal epithelium caused by IBV facilitates *E. coli* invasion and multiplication leading to lesions or even death, causing a major clinical and economic impact especially in broilers (13). When used in vaccination programs, the IP-Ark 1 strain might decrease the mortality observed in broilers during the final growing period as well as decrease the condemnations at processing. Based on the milder lesions and lower presence of the IP-Ark 1 strain in the trachea, additional *in vitro* passages of the IP-Mass 41, IP-Holl 90 and IP-Mass 1 must be performed. The additional *in vitro* passages might confer the intestine passaged IBV strains some degree of attenuation to be considered as potential vaccine candidates.

IB is the major respiratory virus of the chicken, as it is endemic in probably all countries that raise chickens (5). Dozens of IBV serotypes or variants have been identified worldwide, and several vaccines designed for different serotypes are available (6, 25). Thus, diagnosis of IBV

should include identification of the serotype or genotype of the virus to select the appropriate vaccine to be used in immunization programs (6).

In Colombia, the presence of respiratory disease in vaccinated commercial layers and broilers of different age and breed was observed, causing a very negative economic impact. Live and inactivated oil emulsion vaccines are currently used in IB immunization programs. It is known that the best protection against challenge is achieved by a vaccine containing the homologous strain, while vaccines containing heterologous strains may not induce enough cross-protection (31).

We analyzed the presence of IBV in 83 phenol-treated allantoic fluid samples obtained from several commercial layer and broiler problem farms from five geographic regions. Initial detection of positive IBV samples was performed by RT-PCR amplifying a very small, well conserved region of the nucleocapsid gene. Further amplification of the S1 gene was also performed by RT-PCR using published primers (26). Amplification of the S1 gene of some of the positive samples was possible by designing new set of primers based on multiple sequence alignments of published sequences.

Since earlier studies (27, 37),had demonstrated the correlation between genotyping and serotyping based on the HVR 1 sequence, we sequenced the 5' end of the S1 gene. By analyzing the HVR 1 sequences we identified 4 different genotype clusters (A through D) which seemed to be indigenous and genetically unrelated with any reference strains present in GenBank. One isolate, the CO8033CT, was very closely related with the Connecticut strain and seems to be a circulating vaccine virus since the Connecticut vaccine was once available and authorized to be used in Colombia. Three isolates, CO1694, CO8043CT and CO8043T, were very closely related with the Massachusetts strain and probably correspond to the vaccine strain Mass 41.

Initial molecular characterization of the Colombian IBV isolates resulted in the identification of new genotypes not related with the Mass 41 strain, which might indicate that current vaccines do not offer enough protection against the IBV strains present in the field. This study shows for the first time the presence of genotype variant strains of IBV in Colombia, however, *in vitro* and *in vivo* studies are needed to determine the pathogenicity of these isolates and establish the protective level offered by the government approved Mass 41 and other commercially available vaccines. *In vitro* studies including virus neutralization (VN) in SPF chicken embryos or SPF chicken kidney cell confronting the genotype variant strains against homologous and heterologous antiserums should be performed. *In vivo* studies based on the reisolation of the challenge virus five days after challenge in vaccinated SPF chickens should also be performed. If the commercial vaccines currently available in the country offer low levels of protection, the development of new live or killed vaccines from field isolates should be considered.

References

1. Ambali, A. G., and R. C. Jones. Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Dis. 34:809-817. 1990.

2. Avellaneda, G. E., P. Villegas, M. W. Jackwood, and D. J. King. In vivo evaluation of the pathogenicity of field isolates of infectious bronchitis virus. Avian Dis. 38:589-597. 1994.

3. Butler, E. J., M. J. Curtis, A. W. Pearson, and J. S. McDougall. Effect of infectious bronchitis on the structure and composition of egg albumen. J. Sci. Food Agric. 23:359-369. 1972.

4. Cavanagh, D. Severe acute respiratory syndrome vaccine development: Experiences of vaccination against avian infectious bronchitis coronavirus. Avian Path. 32:567-582. 2003.

5. Cavanagh, D., and P. J. Davis. Evolution of avian coronavirus IBV: Sequence of the matrix glycoprotein gene and intergenic region of several serotypes. J. Gen. Virol. 69:621-629. 1988.

6. Cavanagh, D., and S. Naqi. Infectious Bronchitis. In: Diseases of Poultry, 11th ed, Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald and D. E. Swayne, eds. Iowa State University Press, Ames, Iowa. pp. 101-119. 2003.

7. Chen, B. Y., S. Hosi, T. Nunoya, and C. Itakura. Histopathology and immunochemistry of renal lesions due to infectious bronchitis virus in chickens. Avian Path. 25:269-283. 1996.

8. Chew, P. H., P. S. Wakenell, and T. B. Farver. Pathogenicity of attenuated infectious bronchitis viruses for oviducts of chickens exposed in ovo. Avian Dis. 41:598-603. 1997.

9. Chong, K. T., and K. Apostolov. The pathogenesis of nephritis in chickens induced by infectious bronchitis virus. J. Comp. Pathol. 92:199-211. 1982.

10. Cook, J., M. B. Huggins, and M. M. Ellis. Use of an infectious bronchitis virus and Escherichia coli model infection to assess the ability to vaccinate successfully against infectious bronchitis virus in the presence of maternally-derived immunity. Avian Path. 20:619-626. 1991.

11. Cook, J. K., H. W. Smith, and M. B. Huggins. Infectious bronchitis immunity: Its study in chickens experimentally infected with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 67:1427-1434. 1986.

12. Cubillos, A., J. Ulloa, V. Cubillos, and J. Cook. Characterisation of strains of infectious bronchitis virus at one day-old. Avian Path. 20:85-99. 1991.

13. Dhinakar Raj, G., and R. C. Jones. Infectious bronchitis virus: Immunopathogenesis of infection in the chicken. Avian Path. 26:677-706. 1997.

14. Fabricant, J., and P. P. Levine. Experimental production of complicated chronic respiratory disease infection ('air sac disease'). Avian Dis. 6:13-23. 1962.

15. Fonti, R., L. G. Bises, F. Magliocca, F. Nobili, R. Caprilli, and Y. Sambuy. Human colonocytes in primary culture: A model to study epithelial growth, metabolism and differentiation. Int. J. Colorectal Dis. 9:13-22. 1994.

16. Gelb, J., Jr., R. L. Lunt, A. L. Metz, and P. A. Fries. Attenuation of avian infectious bronchitis virus by cold-adaptation. Avian Dis. 35:847-853. 1991.

17. Goren, E. Observations on experimental infection of chickens with Escherichia coli. Avian Path. 7:213-224. 1978.

18. Gross, W. B. The development of "air sac disease". Avian Dis. 5:431-439. 1961.

19. Gross, W. B. Factors affecting the development of respiratory disease complex in chickens. Avian Dis. 34:607-610. 1990.

20. Grossmann, J., C. Fiocchi, and A. D. Levine. Extracellular matrix (ECM) orchestrates death and survival of human intestinal epithelial cells. Gastroenterology. 112:A987. 1997.

21. Hopkins, S. R., and H. W. Yoder, Jr. Increased incidence of airsacculitis in broilers infected with Mycoplasma synoviae and chicken-passaged infectious bronchitis vaccine virus. Avian Dis. 28:386-396. 1984.

22. Hopkins, S. R., and H. W. Yoder, Jr. Influence of infectious bronchitis strains and vaccines on the incidence of Mycoplasma synoviae airsacculitis. Avian Dis. 26:741-752. 1982.

23. Ignjatovic, J., D. F. Ashton, R. L. Reece, P. C. Scott, and P. Hooper. Pathogenicity of Australian strains of avian infectious bronchitis virus. J. Comp. Path. 126:115-123. 2002.

24. Janse, E. M., D. van Roozelaar, and G. Koch. Leukocyte subpopulations in kidney and trachea of chickens infected with infectious bronchitis virus. Avian Path. 23:513-523. 1994.

25. King, D. J. Identification of recent infectious bronchitis virus isolates that are serologically different from current vaccine strains. Avian Dis. 32:362-364. 1988.

26. Kwon, H. M., M. W. Jackwood, and J. Gelb, Jr. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis. 37:194-202. 1993.

27. Lee, C. W., D. A. Hilt, and M. W. Jackwood. Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. J. Vet. Diagn. Invest. 15:344-348. 2003.

28. Naqi, S., K. Gay, P. Patalla, S. Mondal, and R. Liu. Establishment of persistent avian infectious bronchitis virus infection in antibody-free and antibody-positive chickens. Avian Dis. 47:594-601. 2003.

29. Nauwynch, H., and M. B. Pensaert. Studies on the pathogenesis of infections with a nephropathogenic variant of infectious bronchitis virus in chickens. Proceedings of the 1st international symposium on infectious bronchitis. Rauischholzhausen, Germany. pp. 113-119. 1988.

30. Owen, R. L., B. S. Cowen, A. L. Hattel, S. Naqi, and R. A. Wilson. Detection of viral antigen following exposure of one-day old chickens to the Holland-52 strain of infectious bronchitis virus. Avian Path. 20:663-673. 1991.

31. Pensaert, M. B., and C. Lambrechts. Vaccination of chickens against a Belgian nephropathogenic strain of infectious bronchitis virus B1648 using attenuated homologous and heterologous strains. Avian Path. 23:631-641. 1994.

32. Perreault, N., and J. F. Beaulieu. Primary cultures of fully differentiated and pure human intestinal epithelial cells. Exp. Cell Res. 245:34-42. 1998.

33. Sanderson, I. R., R. M. Ezzell, M. Kedinger, M. Erlanger, Z. X. Xu, E. Pringault, S. Leon-Robine, D. Louvard, and W. A. Walker. Human fetal enterocytes in vitro: Modulation of the phenotype by the extracellular matrix. Proc. Natl. Acad. Sci. USA. 93:7717-7722. 1996.

34. Sevoian, M., and P. P. Levine. Effects of infectious bronchitis on the reproductive tract, egg production and egg quality of laying chickens. Avian Dis. 1:136-164. 1957.

35. Smith, H. W., J. K. Cook, and Z. E. Parsell. The experimental infection of chickens with mixtures of infectious bronchitis virus and Escherichia coli. J. Gen. Virol. 66:777-786. 1985.

36. Strater, J., U. Wedding, T. F. Barth, K. Koretz, C. Elsing, and P. Moller. Rapid onset of apoptosis in vitro follows disruption of B1-integrin/matrix interactions in human colonic crypt cells. Gastroenterology. 112:A987. 1996.

37. Wang, C. H., and Y. C. Huang. Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. Arch. Virol. 145:291-300. 2000.

38. Yu, L., Y. Jiang, S. Low, Z. Wang, S. J. Nam, W. Liu, and J. Kwang. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis. 45:416-424. 2001.