Avian Infectious Bronchitis Coronavirus Variation and Selection

by

Rodrigo A. Gallardo

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 12, 2011

Keywords: Infectious bronchitis virus, coronavirus evolution, chicken, genetic variation, pathology, transmission

Approved by

Haroldo Toro, Chair, Professor of Pathobiology
Vicky L. van Santen Co-chair, Professor of Pathobiology
Frederic J. Hoerr, Professor of Pathobiology
Stuart B. Price, Associate Professor of Pathobiology
Sharon R. Roberts, Associate Professor of Biological Sciences

Abstract

Infectious bronchitis virus (IBV) is a highly variable virus, its genetic variation is generated by nucleotide insertions, deletions, or point mutations made by the viral polymerase during virus replication. Another mechanism for IBV variability is RNA recombination. Selection of IBV in the host has also been previously demonstrated. Specific objectives during this three-part investigation were to assess IBV intraspatial variation in chickens, determine the IBV evolutionary pathway in viral immunodeficient hosts, and evaluate IBV variation and effects on testes of young and adult roosters.

Variation inside the chicken (Intraspatial variation) was assessed, we inoculated chickens with an Ark-type IBV commercial vaccine and characterized the sequences of the spike (S) 1 gene of IBV contained in tear fluid, trachea, and reproductive tract. The predominant IBV phenotype contained in the vaccine (prior to inoculation), became a minor or non-detectable population at all times in all tissues after replication in the chickens. Five new predominant populations designated as component (C) 1 through C5, showing distinct non-synonymous changes, were detected in the tissues or fluids of individual vaccinated chickens. Significant differences were detected in the incidence of some distinct predominant IBV populations in tissues and fluids. These results indicate for the first time that IBV undergoes intraspatial variation during the host invasion.

We evaluated the same portion of the S1 gene sequence of the dominant phenotype of an IBV Ark vaccine strain during continued passages in chickens infected

with the immunosuppressive viruses (CAV and/or IBDV) as well as in immunocompetent chickens. The dominant genotype of the vaccine strain was rapidly negatively selected in all chicken groups. Based on S1 geno/phenotype, exactly the same IBV subpopulations detected in the previous experiment emerged. During the first passage several subpopulations emerged in each group followed by establishment of one predominant population after further passages. Only subpopulation C2 successfully became established in either CAV or IBDV infected chickens. These results indicate that selection does not cease in immunodeficient chickens. Subpopulations C1 or C4 became established in immunocompetent birds but became extinct after only a few succeeding passages. A similar result was observed in chickens co-infected with CAV+IBDV. This finding constitutes further evidence for phenotypic drift occurring mainly as a result of selection.

Finally, we assessed IBV virulent strain variation and its effect on the chicken testes. Pre-puber males were inoculated with two IBV virulent strains (M41 and Ark). IBV RNA was detected in most of the testicles of both inoculated male groups. Marginal non-synonymous variation was detected in the S gene of the predominant population of IBV replicating in the testes. IBV M41 and Ark were detected in spermatogonia and Sertoli cells of testicles of infected roosters by immunofluorescence, without histopathological changes. Venereal transmission of IBV was demonstrated by artificially inseminating hens. IBV RNA was detected in the trachea of all hens inseminated with IBV-spiked semen and in 50% of hens inseminated with semen from IBV-infected males providing experimental evidence for IBV venereal transmission.

These results show high genetic variability of IBV populations resulting from mutation and selection, following the same evolutionary mechanisms originally described by Darwin for more complex species.

Acknowledgements

I would like to sincerely thank my advisor Dr. Haroldo Toro for his valuable academic and personal support. Also I would like to thank the members of my committee Drs. Vicky van Santen, Frederic Hoerr, Stuart Price, and Sharon Roberts for academic and technical guidance. I would also like to thank Cassandra Breedlove, Lisa Parsons, Stephen Gulley, Natalia Petrenko, Heather Worley, and Steven Kitchens for technical assistance during my research. Finally I would like to express gratitude to my friends and family who were crucial in this enterprise, especially my wife Ghislaine Dujovne who gave me all the support and love needed to accomplish this task.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AAALAC = association for assessment and accreditation of laboratory animal care

Ark = Arkansas

BSL = biosafety level

C = component

CAV= chicken anemia virus

CIA = chicken infectious anemia

CIAV = chicken infectious anemia virus

CID_{50} = 50% chicken infectious doses

DPI = days post inoculation

EID_{50} = 50% embryo infectious doses

FCS = fetal calf serum

FITC = fluorescein isothiocyanate

IBDV= infectious bursal disease virus

IBV= infectious bronchitis virus

IF = immunofluorescence

Mass = Massachusetts

N = Nucleocapsid

PCR = polymerase chain reaction

RT-PCR = reverse transcription-PCR

S = spike protein

S1 = S1 subunit

SARS = severe acute respiratory syndrome

SPF = specific pathogen free

I. Introduction

Avian infectious bronchitis virus (IBV) belongs to the group 3 of the Coronavirus genus along with other avian coronaviruses (30). IBV is a global poultry pathogen affecting primarily chickens and causing mainly respiratory disease and production losses. One or more serotypes can be endemic in regions with intensive poultry production (23). The IBV genome is an approximately 27.6-kb single stranded positive sense RNA molecule encoding four structural proteins, spike (S), membrane (M), envelope (E), and nucleoprotein (N). The S protein comprises two or three copies of each of its two subunits S1 (S1) and S2. However, S1 has been reported to be the most important protein in terms of coronavirus variability (103). IBV's success in the environment is due to its unique capability to evolve. Antigenic variation between strains allows the virus to escape extensive vaccination programs.

The S1 subunit of the S protein of IBV is responsible for viral attachment to cells and is a primary target for host immune responses as it induces virus neutralizing- and hemagglutination inhibition-antibodies (26-28, 36, 96-98, 122). The role of S1 in determining the species and tissue/cell tropism of several coronaviruses, including IBV, has been reported extensively (9, 10, 24, 62, 64, 74, 107, 109, 127, 136, 175). Because of the relevance of S1 for IBV's successful replication and immunological escape, the extensive variation exhibited by the S1 glycoprotein among IBV populations (102, 103)

is likely the most relevant phenotypic characteristic for this virus's "adaptation" and evolutionary success in the environment.

In evolution, the mechanisms responsible for generation of genetic variability constitute Mayr's "step one" (117), "the production of variation in every generation, that is, suitable genetic or phenotypic variants that can serve as the material of selection and this will then be exposed to the process of selection." "This first step of variation is completely independent of the actual selection process" (117). Coronavirus genetic variation is generated by nucleotide insertions, deletions, or point mutations made by the viral polymerase lacking proofreading capabilities and/or recombination events occurring during virus replication (101-103). In Mayr's second step, "the genetic endowment of the few survivors during reproduction, and thus abundant new genotypes, are then tested in the next generation. Any individual favored by that selection would contribute genotypes to the gene pool that were apt to spread in future generations and thus enhance the adaptation of the population as a whole". Based on the nucleotide sequences encoding the S1 subunit of the S protein different degrees of genetic heterogeneity have been demonstrated among four commercial Ark-DPI-derived IBV vaccines. During a single passage in chickens , subpopulations with an S gene sequence distinct from the vaccine predominant consensus were found in several organs including tears, tracheas and Harderian glands (171). These findings suggested that a distinct virus subpopulation was positively selected by the chicken upper respiratory tract. Results supporting that Ark-type IBV vaccine strains undergo selection in chickens following vaccination have also been reported (119). In chickens IBV initially replicates in the upper respiratory tract producing lesions in epithelial cells (131) of the nostrils, Harderian gland, trachea, lungs,

and air sacs (121, 141, 161). After a short viremia the virus invades distant tissues including the kidneys, the urogenital, and the gastrointestinal tract [e.g. (151)]. We hypothesized that during host invasion, the replicating IBV population is confronted with distinct selective pressures in the different tissues, leading to selection of the most fit subpopulation. To confirm this hypothesis we inoculated chickens with an Ark-type IBV via the ocular and nasal routes and analyzed the S1 gene sequences of IBV contained in different tissues and at different times post-inoculation.

Chickens in intensive production environments are exposed to multiple stressors and infectious diseases impairing innate and adaptive immunity (75). Infectious bursal disease (IBD), chicken infectious anemia (CIA), and Marek's disease (MD) are examples of endemic diseases altering the host's humoral and cell-mediated immune function. According to Kilbourne (92), "in those experiments of nature in which the immune response is effectively removed as a selective host force, viral evolution does not cease but rather appears to falter or follow different pathways". Because viral immunodeficiencies are frequent in poultry commercial settings we hypothesized that IBV passage in immunodeficient chicken populations results in distinct evolutionary pathways. To explore this hypothesis we evaluated IBV phenotypic drift during continued IBV passages in immunocompetent chickens or in chickens previously inoculated with CIA virus (CIAV) and/or IBD virus (IBDV).

Males represent 8 to 12% of birds in breeder flocks and are maintained in close contact with the breeder hens during production. The role of males on IBV dissemination in breeder flocks is largely unknown. Many poultry producers do not vaccinate males against IBV, and particularly avoid expensive inactivated vaccines. Thus, males are more

susceptible to IBV infection and can potentially act as amplifiers of IBV in breeder flocks. Another complicating factor is the practice of "spiking" male populations in older breeder flocks, i.e., replacing up to 50% of males with younger males for the purpose of increasing the fertility of the flock. This procedure interferes with the "all in-all out" biosecurity gold standard of raising chickens and increases the risk for disease transmission. Artificial insemination is a common practice in pedigree lines to control venereally transmitted diseases. IBV has been isolated from testicles (172) and semen (43) from experimentally infected birds. IBV RNA has been detected in the testicles of infected males by reverse transcriptase polymerase chain reaction (RT-PCR) (13, 66). Several authors also have associated epididymal stone formation and epididymitis with IBV live vaccination or natural infection (13, 16, 172). Because IBV is present in the testicles and in the semen it seems reasonable to hypothesize that IBV could be transmitted via the venereal route in chickens. We evaluated the effects of IBV replication in the testicles of infected males; we also investigated possible changes in the predominant IBV population replicating in the males testes using IBV Ark or Mass virulent strains. Finally, we evaluated transmission of IBV to naïve hens via artificial insemination with IBV infected semen.

II. Literature Review

History

Infectious bronchitis was first reported in 1931 in North Dakota by Schalk and Hawn (145). The new disease affected chickens from 2 days up to 3 weeks of age causing respiratory signs and high mortality. The disease was later confounded with infectious laryngotracheitis (LT) due to its similarities with disease caused by mild or low virulence type of LT virus (20). Using cross-immunity studies Beach and Schalm (11) differentiated infectious bronchitis virus (IBV) from LT virus and coryza (*Avibacterium paragallinarum*). A series of studies followed by Beaudette and Hudson, who developed replication and modification of IBV's virulence by subsequent passages in embryonated eggs (12). In 1950 Van Roeckel (169) developed the serum neutralization test for IBV in embryonated chicken eggs, which became the first established serologic method for IBV diagnosis. IBV isolation in embryonated eggs and identification by characteristic lesions in the developing embryos were described by several investigators including Cunningham and Stuart (51), Fabricant (60), and Loomis (112).

The idea of immunizing flocks against IBV emerged from field observations showing that chickens infected with IBV prior to egg lay (between 8 and 16 weeks) showed mild respiratory signs and no adverse effects, becoming immune to the disease when they reached onset of lay (61). This was the starting point for the extensive immunization programs as currently practiced. The most widely used "H" vaccines were

first developed in Germany by a Dutch graduate student in the early 1960's being its most important exponent the ubiquitous H120 (15).

In 1956 Jungherr et al. (61) reported that the Connecticut isolate of 1951 and the Massachusetts isolate of 1941 produced a similar disease but did not cross protect or cross neutralize. This was the first demonstration of the existence of antigenic variation among infectious bronchitis (IB) viruses.

Other factors such as the rapid growth of the broiler industry, the presence of endemic immunosuppressive viruses, the continuous evolution of IBV, etc. have increased the complexity of IB outbreaks and associated economic losses.

Infectious Bronchitis Virus Pathogenesis and Transmission

IB disease has an incubation period of 24 to 48 hours. Morbidity usually reaches >90% and viral spread occurs rapidly among chickens in a flock by aerosol and mechanical means. Mortality varies depending on the virulence of the infecting serotype, age, host immunity status, secondary bacterial infections, etc., varying from marginal in chickens over 6 weeks and up to ~ 25% in young chickens (under 6 weeks of age) or as high as 1% weekly in cases involving nephritis-nephrosis (37).

Respiratory signs are characterized by nasal discharge, sneezing, rales, watery eyes, and lethargy, combined with reduction in feed consumption and weight gain (37, 141). Outbreaks affecting chickens 6 weeks of age are characterized by signs similar to those in chicks, but nasal discharge does not occur as frequently. The disease may go unnoticed unless examination of the flock is done by carefully handling birds or listening to them at night when birds are quiet (37). Depression, ruffled feathers, wet droppings,

increased water intake, and mortality are the signs associated with IBV nephropathogenic strains (18, 47, 50, 177).

Economic consequences to the poultry industry comprise growth retardation and high condemnation rates in meat-type birds. In addition, decreased egg production, reduced internal and external egg quality, and reduced hatchability have been documented in layers and breeders (37).

IBV infection in chickens is known to be initiated by viral replication in the upper respiratory tract, which is usually restricted to the ciliated and mucus secreting cells, and independent of the tissue tropism of the strain (141). In addition, IBV strains also replicate in tissues along the alimentary tract (esophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, cecal tonsils, rectum, and cloaca) without significant pathological effects as well as in the kidneys, oviduct, and testes (16).

Respiratory infection damages the epithelium often predisposing young chickens to secondary infections with pathogenic bacteria. For example, meat type chickens commonly suffer from airsacculitis and systemic colibacillosis (116). Nephritis caused by viral replication in the kidneys is not uncommon during IBV outbreaks. Nephropathogenic strains produce less respiratory signs (182) and lesions (69) but can induce high mortality (44, 108, 133). Nephropathogenic IBV outbreaks can be confounded and/or exacerbated by management problems such as high dietary calcium (69).

Infection of enteric tissues does usually not manifest itself clinically but persists for long periods and results in fecal virus excretion. Outbreaks of IBV with proventriculitis have been reported frequently in China. Chinese IBV QX strains have

been associated with mortality rates of up to 80% (174). IB virulence for the reproductive tract may also differ depending on the age of the affected bird; early IBV infections induce severe damage to the oviduct in pullets (39). In layers IB shows lesion patterns varying from egg shell depigmentation to drops in egg production of up to 50% (77).

IBV infection in young pullets can result in permanent damage to the oviduct. Altered development of the oviduct results in "false layers," reduced egg production, and decreased quality eggs (16, 37). The pathology of IBV infection in the male reproductive tract has not been well documented. Although some authors describe epididymitis and stone formation in the seminiferous tubules or epididymis (13, 16, 172), others have not detected lesions associated with IBV in the testicular stroma (82). IBV has been isolated from semen 2 weeks after infection (43), but so far venereal transmission has not been demonstrated. Long term recovery of IBV from testis for up to 7 months has been reported in infected or vaccinated flocks (5, 6). Possible explanations for this phenomenon include continual cross-infections in infected or vaccinated flocks (42), continuous excretion of the virus at levels usually below the detection levels of the diagnostic tests, or reactivation in sites such as cecal tonsils or kidneys (14). Extended and intermittent shedding is evidence for the potential risk of flock to flock transmission via contamination of personnel or equipment (37).

The ability of IBV to replicate in many respiratory, enteric, and other epithelial surfaces may be related in part to the fact that attachment of IBV to host cells is dependent on N-acetylneuraminic acid (sialic acid) at the cell surface (31). This virus preferentially attaches to sialic acid when its linkage to the body of the oligosaccharide is α 2-3 (149). The affinity for α 2-3 linked sialic acid explains the pan-tropic nature of IBV

within the chicken, but it cannot be the only determinant of pathogenicity because such neuraminic acid is also present on cells that are not infected by IBV. It may be that the sialic acid binding is a primary step requiring further contacts to be made perhaps involving a more specific and less generally distributed secondary receptor (31).

The severity of the infection with IBV is influenced by the chicken genetic breed although IBV replicates to similar levels in the trachea of all breeds (128). The underlying cause of this difference is likely immunological. There is evidence for the major histocompatibility complex (MHC) haplotype of chickens influencing genetic resistance to IBV (8). Bumstead (19) demonstrated varying mortality among different breeds of chicken after inoculation with IBV alone or together with E. coli.

Infectious Bronchitis Virus Properties

Avian IBV is a single stranded, enveloped RNA virus that belongs to the group 3 of the *Coronavirus* genus along with other avian coronaviruses (30). The *Coronaviridae* family is, together with the *Arteriviridae* and *Roniviridae*, within the order *Nidovirales* (58); these viruses share the nested RNA replication strategy. The virus is round to pleomorphic in shape, possesses an envelope that is 120 nm in diameter with club shaped surface projections or spikes (37).

The IBV genome is composed by an approximately 27.6-kb single stranded positive sense RNA molecule encoding four structural proteins, spike (S), membrane (M), envelope (E), and nucleoprotein (N). The S protein comprises two or three copies of each of their two subunits S1 and S2. Neutralizing antibodies are elicited by the S1 portion. The E protein is essential for virus particle formation, the M protein is exposed

10% at the outer virus surface; the N protein surrounds the single piece of single stranded positive sense RNA genome to form the ribonucleoprotein (RNP) (37).

A replicase complex is also encoded, which carries out the unique discontinuous transcription process resulting in a set of six nested 3' co-terminal subgenomic mRNAs (156, 157). At the molecular level, coronaviruses employ a variety of unusual strategies to accomplish a complex program of replication and gene expression (115). These uncommon strategies are important factors in the generation of genetic diversity and continuous evolution. The virus replicates in the cell cytoplasm. Three to 4 hours after infection new virus particles emerge with a maximum output per cell being reached within 12 hours at 37°C (37).

IBV strains have been classified based on S antigenicity into different serotypes. In more recent years genotype classification based on the S genetic sequence has been adopted worldwide. The classification of IBV strains has assisted implementing control measures and understanding the epidemiology and evolution of the virus. Traditionally virus neutralization (VN) and hemagglutination inhibition (HI) are used to define serotypes. Some laboratories also use ELISA tests with monoclonal antibodies directed against specific epitopes of the S1 protein (37). Genotype classification is usually accomplished by RT-PCR amplification of the S1 portion of the S gene. The cDNA is further analyzed by sequencing or restriction endonuclease analysis (37). Strains of the same serotype tend to have S1 amino acid identity of ≥90% however not all isolates from the same genotype belong to the same serotype (33).

According to De Witt (56) classification of IBV is based either on functional tests investigating a biological function of the virus, or non-functional tests relying on the viral

genome. While immunotypes or protectotypes, and antigenic types are the outcomes of functional tests, genotypes are outcome of non-functional tests. Immunotypes or protectotypes provide information about the efficacy of vaccines, i.e. strains inducing protection against each other belong to the same protectotype (56).

Conventional serotyping is based on the reaction between an IBV strain and homologous and heterologous antisera produced in chickens (56). Two strains belong to the same serotype when two way heterologous neutralization titers differ less than 20 fold from the homologous titers in both directions (73). Conventionally serotyping has been performed by VN tests in various biological substrates including embryonated eggs, cell culture and tracheal organ cultures (TOCs) (23, 49, 173). Serotyping has also been conducted by HI (164). The incorporation of monoclonal antibodies to assist the classification of IBV has also been suggested (56).

IBV genotype classification is based on the genetic characterization of a segment of the viral genome. Methods used include sequencing, genotype specific sequence detection by RT-PCR, and enzyme cleavage site detection. The information obtained from these procedures has been used widely in molecular epidemiological studies (56). Unfortunately the correlation between genotype and serotype in IBV strains has been contradictory; while several authors have reported high correlation (90, 104, 110), a number of reports by other authors have detected conflicting results (22, 102). Therefore, the use of genotyping as the only tool is not recommended and conventional testing such as serotyping and *in vivo* studies should be paired with genotyping in order to corroborate the results obtained (56).

According to Han (71) genetic diversity in coronaviruses is generated by three major factors. First, the inaccuracy of the coronavirus RNA polymerase which makes one mistake every 1000 or 10,000 nucleotides (87). Second, the high frequency of homologous RNA recombination resulting from their unique random template switching during RNA replication (130). Finally, coronaviruses possess one of the largest RNA genomes providing this viral family an extra flexibility to accommodate and modify genes (115). IBV's generation of diversity results from point mutations, insertions, deletions and genome recombination (34).

Infectious Bronchitis Virus Spike Protein

The IBV S protein, a club-shaped projection on the envelope's surface of the virion, is an oligomer comprising two copies of each S1 and S2, although the possibility of a third copy of each glycopeptide cannot be ignored. The entire S protein gene has been sequenced for many strains of IBV (33, 98, 103). There is great variation among coronaviruses with respect to the size of the S polypeptide; currently the shortest S protein known is that of IBV with approximately 1160 amino acids (29). The S protein is attached to the membrane by S2, while S1 has little or no contact with the membrane and may form the major part of the bulbous end of the S protein. The association between S1 and S2 is weak, intra-peptide and not inter-peptide disulfide bonds exist in S (27). The S1 subunit of the S protein of IBV is responsible for viral attachment to cells and is a primary target for host immune responses as it induces virus neutralizing and hemagglutination inhibition antibodies (26-28, 36, 96, 122). Another common feature of the S protein is the high degree of glycosylation; both S1 and S2 are glycosylated (29).

The role of S1 in determining the species and tissue/cell tropism of several coronaviruses, including IBV, has been reported extensively (9, 10, 24, 62, 64, 74, 107, 109, 127, 136, 175). Because of the relevance of S1 for IBV's successful replication and immunological escape, the extensive variation exhibited by the S1 glycoprotein among IBV populations (100, 102) is likely the most relevant phenotypic characteristic for these virus' "adaptation" and evolutionary success in the environment.

Several reports have demonstrated that the S1 protein is highly variable. IBV serotypes commonly differ by 20% to 25% and sometimes up to 50% of the amino acids forming the S1 subunit (67, 100). This does not mean that the S protein is the only coronavirus protein that varies, but it has the most extreme variability among all coronavirus proteins (29). Indeed, most genetic changes occur in the S1 gene during adaptation to the host (38, 79). On the other hand, the S2 polypeptide differs among virus strains to an extent similar to that of other structural proteins (10 to 15%) (31). The comparison of the amino acid sequences of the S protein from IBV and other coronaviruses that infect mammals has shown that there is a far greater sequence conservation within the C terminal S2 part of the molecule than in S1 (55, 142, 148). According to Cavanagh (30) differences in the S1 protein have a selective advantage (e.g. immunization with one of the serotypes protects poorly against infection with heterologous serotypes) The S protein is the major inducer of neutralizing antibodies and protection (27, 96, 97, 122). Differences of 2 or 3% in the S1 amino acid residues can result in changes of a serotype with the subsequent lack of cross neutralization and reduction in cross protection (89, 96).

The two surface glycoproteins of IBV coronaviruses (S and M) induce the formation of VN antibodies. Although there are fewer S than M and N molecules in the virion (26), the S protein induces a good immune response following infection (29). The first and third quarter of the S1 polypeptide are known to have amino acids involved in the formation of VN epitopes (89, 96). These sites differ in closely related strains (>95% S1 amino acid identity) (63). The neutralizing activity of IBV antibodies induced by the S protein has been shown in two different ways; first by immunization with purified S protein of IBV (32), and second by immunization with vectors expressing the S gene of IBV (159).

The major VN antibody inducing sites of IBV reside in the S1 portion of the S protein. Indeed, removal of the S1 portion by urea treatment eliminates the capacity of the virus to induce neutralizing antibodies (27, 36). Additionally, VN antibodies can be induced by the monomeric form of the urea extracted S1 protein (36). Six (A-F) and two (G-H) antigenic sites have been discovered in S1 and S2 proteins respectively by competition assays (96, 97). The G site located in S2 induces weakly neutralizing antibodies (100). This S2 site is located within the first 20 residues after the N terminus of S2 (100, 138, 139). Part of this sequence is strongly conserved among isolates possessing very variable S sequences (29).

According to Wu (179) three lines of evidence suggest that S protein is more variable than the other coronavirus proteins; 1) S protein is more sensitive to mutations, 2) it has experienced more mutations in the past, and 3) has a bigger potential towards future mutations. Complete S protein gene sequencing has shown that S1 can vary several folds more than S2. Additionally, amino acid differences in S1 are not random and tend

to be concentrated in the 1 and 3 regions of the S1 subunit which is the same location of antigenic site convergence (29). Many of the differences in the 1 and 3 regions in field isolates are associated with differences in antigenicity. This evidence supports the view that changes in these regions of S1 can be advantageous to the virus avoiding, sometimes partially, the immune responses induced in chickens by prior infection or vaccination (29).

As indicated above, the S protein is a determinant of tissue tropism in coronaviruses (99) and host cell range for IB coronaviruses. Whether this protein plays a role in the pathogenicity has not been fully determined (31, 120).

Nucleotide sequencing and genetic analysis are powerful tools for monitoring phylogenetic and epidemiological evolution of IBV subtypes providing a fast and accurate method for classification and prediction of IBV serotypes (3, 35, 36, 96, 111). These techniques have been used to reveal that alterations in IBV virulence were most closely associated with differences in the S gene (65). Some authors suggest that partial S1 sequence identity values would be more strongly correlated with protection than antigenic relatedness values (105). Thus, S1 identity values could be used as a predictor of *in-vivo* protection as a more feasible alternative than laboratory challenge studies. However, some strains with high degrees of S1 identity may not cross protect against challenge. A very few amino acid differences located in major immunodominant regions may be sufficient to cause a discrepancy between the sequence and protective relatedness values correlations (34).

Although all IBV structural proteins undoubtedly play a role in immunogenicity, there is strong evidence that the S protein is the major inducer of protective immune

responses against IBV (29). Protection of 50 to 90% has been shown in mice vaccinated with an S protein expressed by an adenovirus vector and challenged with a virulent strain of murine hepatitis coronavirus (MHV) (176). A recombinant adenovirus expressing the N protein also induced protective immunity; however the protection was greater when N and S were jointly expressed compared with either protein alone (176).

Immunity Against Infectious Bronchitis Virus

Humoral and cell mediated immune responses are relevant for protection against IBV. Chickens respond to IBV infection by production of specific antibodies of all immunoglobulin (Ig) isotypes. Chickens with high levels of humoral antibodies can still be susceptible to infection suggesting that factors other than humoral immunity are involved in protection (29, 41, 150). IgM is the first antibody to be detected in serum after infection; it peaks around 8 days and levels then decline; this transient antibody is useful for detecting early infections (52, 123). At 10 to 14 days IgG is the predominant antibody, maintaining its levels for a considerable time. This antibody isotype is detected by most routinely used IBV diagnostic tests (ELISA, VN, HI, etc.). In response to a second challenge, IgG and IgM peak at the same time, but IgM declines faster (52).

Humoral immunity in response to IBV vaccination has been studied by measuring antibodies in serum using ELISA, VN, and HI (56). Serum antibody levels do not completely correlate with protection, though local antibodies are believed to play a role in the protection of the respiratory tract (140).

Local antibodies against IBV have been detected in lachrymal fluids, nasal and tracheal washings (141). Plasma cells have been retrieved from tracheal sections of

infected chickens (125). The Harderian gland is a major source for lachrymal IgA, playing an important role in the development of vaccine induced immunity (168) e.g. aerosol vaccination of day old chicks results in protective immunity, presumably because local immune mechanisms have been stimulated (53). Additionally, IBV specific IgA levels in the lachrymal fluid (46, 54), have been correlated with resistance to IBV reinfection (160). Thus increased resistance to IBV in certain chicken lines may be mediated in part by IgA. IgA's traditional role is to inhibit pathogen entry at mucosal surfaces. In addition, secretory IgA can neutralize viral particles in infected epithelial cells when on its way to be secreted (76). The lack of IgA has been correlated with increased cytokine production (25) and reduced T cell activation upon immunization (95).

Cell mediated immunity (CMI) has been demonstrated to the S1, N, and M proteins of IBV (80). Immunity in the trachea of infected birds is mediated by T cells both CD4+ and CD8+. T cells have been detected in tracheal sections of chickens infected with IBV (86). The ability to generate an effective T cell response against IBV differs among MHC haplotypes (150) demonstrating the increased susceptibility of certain chicken strains to IBV. T cell responses in chickens are important in IBV infections and are associated with initial reduction of the severity of the infection and clinical signs; this CTL activity, as previously stated, is MHC restricted and associated with CD8+ CD4- cells (41, 150).

Interferon type 1 (IFN-1) has been detected in trachea and lung and at lower levels in plasma, kidneys, liver, and spleen after IBV infection (129). IFN-1 has the capability to delay the onset of disease in chickens and its severity (31) due to its antiviral

properties. A study conducted by Guo and col., (70) characterized the specific cytokine gene expression profile of the tracheas of chickens at different time points after vaccination with IBV. After primary immunization, IFN-1 and interleukin-1 beta (IL-1β) were activated locally, triggering the antiviral state of adjacent cells and facilitating the activation and migration of T lymphocytes to local tissues modifying the local environment and dictating the type of adaptive immunity.

Viral Induced Immunodeficiency by Chicken Anemia Virus (CAV) and Infectious Bursal Disease Virus (IBDV)

Chickens in intensive production environments are exposed to multiple stressors and infectious diseases impairing innate and adaptive immunity (75). Infectious bursal disease virus (IBDV), chicken infectious anemia virus (CAV), and Marek's disease virus (MDV) are examples of viral endemic pathogens altering the host's humoral and cell-mediated immune functions. Viral induced immunosuppressions have been reported to impair the host response to infection or vaccination with numerous agents increasing the susceptibility to viral, bacterial, and parasitic diseases affecting commercial chickens (75). A considerable proportion of commercial chickens suffer from CAV and/or IBDV immunodeficiency (75). Concurrent infection with IBV is common (166). Therefore this complex of immunosuppressive diseases caused by chicken anemia virus (CAV) and infectious bursal disease virus (IBDV), coexisting with IBV is a common phenomenon in commercial flocks, adding more complexity to the disease outcomes and altering the microenvironment in which IBV replicates (166).

Chicken Anemia Virus

CAV is a small DNA virus of approximately 25 nm that belongs to the *Circoviridae* family, genus *Gyrovirus*. It possesses a single stranded covalently closed DNA genome that produces a polycystronic transcript coding for three proteins. This virus is extremely resistant to disinfectants and ubiquitous in chicken flocks. Due to the previously presented characteristics it can be present as a contaminant of viral cultures especially if propagated in embryonated chicken eggs (146).

CAV is transmitted both vertically and horizontally. Clinical disease is not frequently seen due to the existence of maternal immunity that confers protection to the chick during its first weeks of life. After 3 to 4 weeks of life, infections become subclinical but may result in significant immunosuppression (146). Virus neutralizing antibodies are crucial to restrain chicken anemia viral replication (146). When breeders are not immune, disease in the progeny (2 to 4 week-old) is characterized by generalized lymphoid atrophy with a concomitant immunosuppression, increased mortality, and severe anemia (1, 2, 40). CAV also induces thymic atrophy in older chickens (163).

CAV replication occurs in dividing cells including hemocytoblasts in the bone marrow, and T cell precursors in the thymus or dividing T cells in response to antigenic stimulation. The most important protein in the induction of immunosuppression is VP3 or apoptin, causing apoptosis of infected hemocytoblasts resulting in the decrease of erythrocytes, thrombocytes, and granulocytes (146).

In the spleen CD3+, CD8+, and TCRαβ populations constitute the main target for CAV ultimately affecting the generation of CTLs in response to vaccination or infection (1, 113). Conversely, NK cells are not affected by CAV infection (113). Peters and

colleagues (134) recently suggested that VP2 may have a role in immunosuppression down regulating MHC class 1 antigens.

Little information is available regarding CAV infection and cytokine production; quantitative RT-PCR studies 7 days post CAV infection have shown absence of changes in IFN-ϒ, IL-2, and IL-1β mRNA levels (113). Additional studies are needed in order to determine the impact of CAV infection on cytokines starting at 2-3 days post-infection (146) considering that the virus can be detected in lymphoid tissues and bone marrow as early as 3-4 days post infection (154). The thymus is extensively affected by 3 to 12 days post infection being difficult to find positive cells for viral antigens at that time. The damage of the thymus is probably related with the impairment of the cytokines needed for the T cell maturation (146).

T cell responses severely impaired by CAV are relevant in the clearance of IBV (41, 113, 150). Additionally CAV reduces mucosal immune responses to IBV (168). The deficiency of T helper cells in CAV infected chickens might adversely affect the generation of IBV specific antibodies (166).

Infectious Bursal Disease Virus

IBDV belongs to the *Birnaviridae* family characterized by a double stranded RNA genome consisting of segments A and B. Segment B codes for the RNA dependent RNA polymerase and segment A codes for two structural proteins VP2 and VP3, an auto-protease, VP4, and a small nonstructural peptide VP5. VP5 open reading frame partially overlaps with the open reading frame coding for VP234 (146).

Two serotypes have been described for IBDV, of which only serotype 1 causes immunosuppression and disease in chickens. Several pathotypes have been described within serotype 1 varying from mild to very virulent. Based on restriction analysis and sequencing IBDV strains have been subdivided in genetic groups (59). It has been recognized for a long time that serotype 1 strains can cause severe immunosuppression with impaired antibody responses and increased susceptibility to other pathogens, especially when birds are infected before 3 weeks of age (152, 153).

IBDV affects the bursa of Fabricius in chicks younger than 3 weeks of age causing B cell immunodeficiency (40, 144). The damage of the bursa is transient; follicles become repopulated with lymphocytes and the tissue architecture is restored, but primary antibody responses remain depressed until at least 7 weeks post-infection (146). In addition to replication in B cells, IBDV can also replicate in macrophages. Khatri and collaborators (91) detected viral RNA and IBDV proteins by immunohistochemistry 1 to 7 days post-infection in bursal macrophages. Additionally there was a significant decrease in the absolute number of macrophages in the bursa. The impact of the macrophage on the immune response has not been completely understood (146). Altered cytokine transcription is compatible with bursal inflammation during acute infection. However it is not clear how these changes play a role in IBDV induced immunosuppression (146).

T cells are not susceptible to IBDV infection; however they play an important role in IBDV immunopathogenesis. T cells contribute to the production of nitric oxide (NO) by the secretion of NO inducing factor, stimulating NO synthesis in macrophages and neutrophils. NO can contribute to the inflammatory lesion development but can also be

involved in down-regulation of splenic T cell responses to mitogens, which are associated with the acute phase of IBDV infection (94).

It has been previously shown that Ark type IBV persist for long periods in chickens suffering from immunodeficiency (166). IBDV infections at an early age can decrease the response to IBV vaccination, which results in increased flock's susceptibility to IBV infection (68, 132, 178). Furthermore, the levels of acquired immunity to IBV influence the period and amount of IBV that can be detected after challenge (144). Indeed, immunosuppression caused by early infection with IBDV increases the amount of IBV recovered post challenge in chickens (45).

Antibodies participate in the immune response against IBV [e.g. (97)]. Thompson (158) evaluated serum and local antibody responses after IBV vaccination in chickens infected with IBDV. The percent of chickens that developed a detectable antibody response after IBV inoculation was higher in chickens inoculated with IBV alone compared to chickens receiving both IBDV and IBV. In IBV-alone inoculated chickens the concentrations of specific IgM, IgG, IgA were evenly distributed whereas IBDV inoculated chickens produced mainly IgM antibodies and very low levels of IgA and IgG. These changes may play a role in increasing the susceptibility of IBDV infected chickens to IBV infection.

Concurrent infections with CAV and IBDV have been documented by several authors (59, 147). These combined infections have been shown to augment the severity of the immunodeficiency in chickens (40, 81, 180). Viral immunodeficiency due to CAV and IBDV seems to play a relevant role in the epidemiology and outcome of IBV infections (166).

Coronavirus Evolutionary Pathways

In evolution, the mechanisms responsible for genetic variability constitute Mayr's "step one" (117), "the production of variation in every generation, that is, suitable genetic or phenotypic variants that can serve as the material of selection and this will then be exposed to the process of selection." "This first step of variation is completely independent of the actual selection process" (117). In IBV genetic diversity is generated by mutations (nucleotide insertions, deletions, or point mutations) made by the RNA dependent RNA polymerase and recombination events [associated with concurrent infection with different IBV strains in the same host (100, 102)] occurring during virus replication. Indeed, coronaviruses including IBV have been shown to exist as mixtures of phenotypes (subpopulations) within an isolate or a vaccine (83, 126, 171, 181).

In Mayr's second step, "the genetic endowment of the few survivors during reproduction, and thus abundant new genotypes, are then tested in the next generation. Any individual favored by that selection would contribute genotypes to the gene pool that were apt to spread in future generations and thus enhance the adaptation of the population as a whole". IBV selection has been demonstrated by van Santen and Toro (171), McKinley et al., (119), and Gallardo et al., (66) using commercial Ark-DPI derived vaccines. As early as three days after ocular vaccination with an Ark-type IBV vaccine in SPF chickens, the predominant population detected in the lachrymal fluids of vaccinated birds differs from the population predominating in the vaccine prior to inoculation (119, 171). Based on S1 gene sequence, the vaccine populations selected in the chickens were more similar to the virulent ArkDPI parental strain than to the predominant population in the vaccine (171).

Since live IBV vaccines are used extensively world-wide and antigenic variants abound, IBV persists in chicken flocks (6, 42) providing ample opportunity for recombination to occur *in vivo.* McKinley et al. (119) suggested that reduced vaccine coverage (i.e. <100%) results in repeated transmission of the vaccine virus from vaccinated to unvaccinated flock mates. The infective nature of IBV combined with vaccination and re-infection of vaccine viruses in commercial chicken flocks allows the generation of diversity (119) and selection permits ultimately the emergence of new variants, including strains similar to the vaccines used in the flocks (84, 126). Continuous IBV evolution becomes apparent through the continuous identification of emergent new variants and serotypes (106). Since the first discovery of IBV antigenic variation reported by Jungherr et al, (88) in 1956, hundreds of IBV genotypes have been detected worldwide.

There is not enough evidence to suggest that any distinct protein of IBV would be of greater evolutionary relevance. Changes in the replicase proteins encoded by the ORF1ab have been associated with pathogenicity (120) and suggested by some authors to be relevant for IBV's evolutionary success. This suggestion is opposed to the general dogma that parasites inducing little harm to the host would be the most well-adapted (i.e. not killing the host permits increased replication cycles). In addition, it seems that by changing internal regulatory proteins but maintaining structural proteins which are exposed to the immune system of the host, the invading virus will still be exposed to the immune responses. Therefore, most authors attribute changes to the coronavirus spike protein (allowing escape and productive replication) as those with the greatest impact on its evolutionary fitness (38, 89, 119).

Evolution in the Immunodeficient Host

Impairment of the immune system in poultry is a frequent event which results from exposure to stress, toxins or infectious diseases. For example, a high percentage of commercial poultry is constantly affected by immunodeficiency caused by endemic CAV and IBDV (75). CAV and/or IBDV infection in chickens increases their susceptibility to numerous pathogens including IBV (75). Indeed, increased persistence of IBV has been shown in chickens inoculated with CAV and/or IBDV (166). Prolonged replication as seen in immunodeficient animals might provide increased opportunity for the emergence of IBV populations with altered behavior.

According to Kilbourne (93) "in those experiments of nature in which the immune response is effectively removed as a selective host force, viral evolution does not cease but rather appears to falter or follow different pathways." He further indicates, "The fate of viral populations in ordinarily acute infections occurring in immunocompromised hosts is one of a seemingly undirected increase in diversity." Human immunodeficiency virus (HIV) infections illustrate this thesis as increased viral heterogeneity has been observed in such patients (93). Moreover, chronic infection with human rotavirus as well as chronic natural infection with foot and mouth disease virus (FMDV), in immunodeficient hosts, have been associated with extensive genome rearrangements (4). Extensive genome variation has also been demonstrated in the VP1 protein of Sabin Type 2 poliovirus when infecting immunodeficient hosts (21). Similarly, Sabin type 3 poliovirus vaccine applied to an adult patient, evolved during the period of viral excretion (114). Additionally, Gavrilin and collaborators (Ref) demonstrated selection of wild

poliovirus and Sabrin vaccine derived isolates obtained from immunodeficient patients. Genetic and antigenic variations have also been detected in influenza viruses. Rocha et al., (143) demonstrated this in eight isolates recovered from a child with severe combined immunodeficiency syndrome infected naturally with a type A influenza virus H1N1.

Because CAV and IBDV (1, 2) drastically reduce T and B cell responses in chickens and because such responses are relevant for IBV clearance (41, 97, 150) we used these viruses as models to investigate the fate of IBV mutants/populations in immunocompetent and immunodeficient hosts.

III. Host Intraspatial Selection of Infectious Bronchitis Virus Populations

***Avian Diseases* 54: 807-813, 2010**

Rodrigo A. Gallardo, Vicky L. van Santen, Haroldo Toro[A]

Department of Pathobiology, College of Veterinary Medicine, 264 Greene Hall, Auburn University, AL 36849, USA.

SUMMARY

Ark-type infectious bronchitis virus (IBV) subpopulations with an S gene sequence distinct from the vaccine predominant consensus were previously found in the upper respiratory tract of chickens within three days after inoculation. This finding indicated that a distinct virus subpopulation was rapidly positively selected by the chicken upper respiratory tract. We hypothesized that during host invasion, the replicating IBV population further changes as it confronts the distinct environments of different tissues, leading to selection of the most fit population. We inoculated 15-day-old chickens with 10^4 50% embryo infectious doses of an Ark-type IBV commercial vaccine via the ocular and nasal routes and characterized the sequences of the S1 gene of IBV contained in tear fluid, trachea, and reproductive tract of individual chickens at different times post-inoculation. The predominant IBV phenotype contained in the vaccine (prior to inoculation), became a minor or non-detectable population at all times in all tissues after replication in the vast majority of the chickens, corroborating our previous findings. Five new predominant populations designated component (C) 1 through C5, showing distinct non-synonymous changes, i.e. nucleotide changes resulting in different amino acids encoded and thus in a phenotypic change of the predominant virus population, were detected in the tissues or fluids of individual vaccinated chickens. Due to the different biochemical properties of some amino acids that changed in the S1 glycoprotein, we anticipate that phenotypic shift occurred during the invasion process. Significant differences were detected in the incidence of some distinct IBV predominant populations in tissues and fluids; e.g. phenotype C1 showed the highest incidence in the reproductive tract of the chickens achieving a significant difference versus its incidence in the trachea

($P<0.05$). These results indicate for the first time that IBV undergoes intraspatial variation during host invasion, i.e. the dominant genotype/phenotype further changes during host invasion as the microenvironment of distinct tissues exerts selective pressure on the replicating virus population.

INTRODUCTION

Avian infectious bronchitis virus (IBV) belongs to the group 3 of the Coronavirus genus along with other avian coronaviruses (30). Coronavirus genetic variation is generated by nucleotide insertions, deletions, or point mutations made by the viral polymerase lacking proofreading capabilities and/or recombination events occurring during virus replication (101-103). In evolution, the mechanisms responsible for genetic variability constitute Mayr's "step one" (117), "the production of variation in every generation, that is, suitable genetic or phenotypic variants that can serve as the material of selection and this will then be exposed to the process of selection." "This first step of variation is completely independent of the actual selection process" (117). In Mayr's second step, "the genetic endowment of the few survivors during reproduction, and thus abundant new genotypes, are then tested in the next generation. Any individual favored by that selection would contribute genotypes to the gene pool that were apt to spread in future generations and thus enhance the adaptation of the population as a whole".

The S1 subunit (S1) of the spike protein (S) of IBV is responsible for viral attachment to cells and is a primary target for host immune responses as it induces virus neutralizing- and hemagglutination inhibition-antibodies (26-28, 36, 96, 97, 122). The role of S1 in determining the species and tissue/cell tropism of several coronaviruses, including IBV, has been reported extensively (9, 10, 24, 62, 64, 74, 107, 109, 127, 136, 175). Because of the relevance of S1 for IBV's successful replication and immunological escape, the extensive variation exhibited by the S1 glycoprotein among IBV populations

(102, 103) is likely the most relevant phenotypic characteristic for this virus's "adaptation" and evolutionary success in the environment.

We have previously reported different degrees of genetic heterogeneity among four commercial Ark-DPI-derived IBV vaccines before passage in chickens, reflected in the nucleotide sequences encoding the S1 subunit of the S protein. Within three days after inoculation into chickens, subpopulations with an S gene sequence distinct from the vaccine predominant consensus were found in several organs including tears, tracheas and Harderian glands (171). These findings suggested that a distinct virus subpopulation was positively selected by the chicken upper respiratory tract. Results supporting that Ark-type IBV vaccine strains undergo selection in chickens following vaccination have also been reported by others (119).

In the chicken host IBV initially replicates in the upper respiratory tract producing lesions in epithelial cells (131) of the nostrils, Harderian gland, trachea, lungs, and air sacs (121, 141, 161). After a short viremia the virus invades distant tissues including the kidneys, the urogenital tract, and the gastrointestinal tract [e.g. (151)]. We hypothesized that during host invasion, the replicating IBV population is confronted with distinct selective pressures in the different tissues, leading to selection of the most fit subpopulation. To confirm this hypothesis we inoculated chickens with an Ark-type IBV via the ocular and nasal routes and analyzed the S1 gene sequences of IBV contained in different tissues and at different times post-inoculation.

MATERIALS AND METHODS

Infectious bronchitis virus (IBV). A commercially available single-entity live attenuated Ark-type IBV vaccine strain was used. The lyophilized vaccine was reconstituted in sterile tryptose broth and used for inoculation of chickens.

Chickens. Seventy-two white leghorn chickens, hatched from specific pathogen free fertile eggs (Sunrise Farms, Catskill, NY) were used to evaluate IBV intraspatial variation. Hatched chickens were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an AAALAC-accredited institution.

Experimental design. All chickens were inoculated with 100 µl containing 10^4 50% embryo infective doses (EID_{50}) IBV via the ocular (25 µl each eye) and nasal (25 µl each nostril) routes at 15 days of age. Twenty four chickens were euthanized at 6, 9, and 20 days post-inoculation (DPI) and samples of trachea, oviduct or testicles, kidney, and cecal tonsil were collected from each chicken. Samples of tear fluid were obtained from each chicken prior to euthanasia as described (162).

RNA extraction. Viral RNA was prepared from tear samples of each chicken using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's

protocol. Total RNA was isolated from tracheal, kidney, cecal tonsil, oviduct, or testicle homogenates from each chicken using the Tri-Reagent RNA isolation reagent (Molecular Research Center Inc., Cincinnati, OH) following the manufacturer's protocol.

RT-PCR. cDNA for sequencing a portion of the IBV S1 gene was prepared from RNA by RT-PCR using the Qiagen one step RT-PCR kit (Qiagen, Valencia, CA) and primers NEWS1OLIGO5' and S1OLIGO3', NEWS1OLIGO5' and S1R, or S17F and S18R (Table 1.1) (85, 104, 171). For 24 of the samples, two or three separate RT-PCR reactions were carried out and products sequenced separately. The products obtained were visualized by SYBR Green staining after agarose gel electrophoresis. In cases where inadequate amounts of cDNA for sequencing were obtained, the cDNA was further amplified by PCR using the Invitrogen Platinum PCR Supermix kit (Invitrogen Corp., Carlsbad, CA) and primers S17F and S1R (for NEWS1OLIGO5'/S1R RT-PCR products) or NEWS1OLIGO5' and S1R primers (for NEWS1OLIGO5'/S1OLIGO3' RT-PCR products). A total of 12 sequences were obtained by this method.

Sequencing of cDNA generated by RT-PCR or RT-PCR plus PCR. After verification by agarose gel electrophoresis, amplified cDNA was purified using the QIAquick PCR Purification kit (Qiagen, Valencia, CA) and submitted to Massachusetts General Hospital DNA core facility for sequencing using primers S1R, S17F and/or S18R. Thus, the sequence of the first approximately 720 nt of the S1 coding sequences was obtained. Sequences were assembled and aligned using Mac Vector 10.6.0 software (Mac Vector Inc., Cary, NC). Sequences not already present in GenBank were submitted under the

accession numbers GQ484957 to GQ484963. Sequence chromatograms were examined to identify nucleotide positions with more than one nucleotide, indicating the presence of relevant levels of more than one subpopulation. For sequences showing detectable levels of more than one subpopulation, the populations were assigned to their predominant population based on the major nucleotide peaks.

Incidences of subpopulations were calculated and Chi-squared test was used to determine statistical differences between and within groups. Differences with *P* values <0.05 were considered significant.

RESULTS

IBV S1 gene amplification. As shown in Table 1.2 a portion of the S1 gene of IBV was consistently amplified from tear fluid (44 of 48 samples) and tracheal samples (32 of 48) from inoculated chickens 6 and 9 DPI, but rarely at 20 DPI. In contrast, viral S1 sequences were successfully amplified from fewer oviduct or testis samples (9 of 72) and rarely from kidneys (2 of 72) and cecal tonsils (2 of 72).

IBV S1 gene sequences in inoculated chickens. IBV-Ark found in tissues or fluids of individual inoculated chickens exhibited differences in S1 gene consensus sequences. Nucleotide and amino acid positions in which differences were observed in more than one chicken are shown in Table 1.3. Only those differences representing non-synonymous changes, i.e. resulting in different amino acids encoded and therefore in a phenotypic change of the predominant virus population, are shown in this table. (Synonymous changes were not consistently found.) Six distinct virus predominant

populations are shown, including the vaccine parent prior to inoculation. The five positively selected distinct predominant populations were designated component (C) 1 through 5, i.e. C1, C2, C3, C4, and C5. Compared to the vaccine parent, C3 exhibited nucleotide and amino acid changes in 6 positions, the most among the 5 newly selected populations. Therefore C3 showed the largest phenotypic distance to the vaccine's predominant population. In addition to the number of amino acid changes, the character of the 4 unique changes adds phenotypic distance to the predominant population of the IBV vaccine strain prior to inoculation. The hydrophobic amino acid leucine at position 76 is replaced by the bulkier hydrophobic amino acid phenylalanine in C3. At amino acid position 119, serine is replaced by proline in C3, an amino acid that has a significant effect upon the orientation of the polypeptide chain. At positions 171 and 198, amino acid substitutions in C3 involve a change from an uncharged amino acid side chain to a positively-charged one and vice versa. C1 and C4 each exhibited 3 nucleotide and amino acid changes, C2 showed 2 changes and C5 one change in this portion of the S1 sequence. The change at position 43 from hydrophobic tyrosine to positively charged histidine in virus populations C1, C2, C3, and C4 could potentially result in a different folding of the polypeptide chain and/or interaction with receptor.

Most of the samples (89%) showed a single or vastly predominant S1 nucleotide sequence. The rest (10 samples) showed more than one nucleotide peak at specific positions, indicating mixed populations. Such populations were assigned to their predominant population based on the major nucleotide peaks for the analyses to follow. Seven of these sequences were very nearly 50% each of two sequences and were assigned so that they would proportionally add to the incidence of each subpopulation. For 22 of

the 24 samples (92%) that were amplified and sequenced more than once, the same subpopulation(s) was detected each time, verifying that the amplified cDNA reliably represents the IBV population in each sample.

In addition to the six populations shown in Table 1.3, five additional variants (GenBank accession numbers GQ484959- GQ484963) were each identified as the single IBV population in one or more samples from a single chicken. Two variants each differed from the vaccine parent population in one amino acid position, one variant differed from population C1 in one position, one from population C3 in one position, and one from population C3 in two amino acid positions. For the analyses to follow, each of these variant populations was grouped with the population to which it was most similar.

Incidence of IBV subpopulations in chickens (all tissues) by day after inoculation. The incidence of the distinct IBV subpopulations, including the vaccine parent, in the chicken samples is shown in Fig. 1.1. As seen in this figure, the vaccine parent predominant population was not detected at day 6 DPI in any of the sampled chickens (n=24) demonstrating rapid negative selection of this phenotype. In contrast all newly selected phenotypes, C1 through C5, were detected at varying frequencies in the chickens. At 6 DPI, C4 was the most frequent predominant population in the birds (45.3% of the samples) followed by C1 with 26.7%. A similar pattern was observed at 9 DPI with 52.5% of the positive samples showing C4 as the predominant population followed by C1 with 15%. The vaccine parent was detected in 11.3% of the positive samples at 9 DPI. Other populations (C2, C3, and C5) showed incidences varying between 6.3% and 7.5%.

Populations by tissue or fluid (all days). Only IBV populations detected in tear fluid, trachea, and the reproductive tract were further evaluated due to the small number of S1 sequences obtained in kidneys and cecal tonsils. As seen in Fig 1.2A, C1 and C4 were the most frequently found predominant phenotypes in the tear fluid of inoculated birds. Their incidence was significantly higher ($P<0.05$) than the incidence of any of the other phenotypes. No significant differences were detected among the incidence of all other populations ($P>0.05$). In the trachea the most frequent predominant population was C4 (50%) whose incidence was significantly higher ($P<0.05$) than all other populations (Fig. 1.2B). The vaccine parent, C1, C2, and C5 showed incidences varying between 22% for C2 and 4.9% for the vaccine parent. C3 was not detected in the trachea of any chickens. The results of the reproductive tract (Fig. 1.2C) include the S1 gene sequences (n=9) detected in the oviduct of female and testis of male birds. These results were grouped because both represent a similar distance from the respiratory tract even though we are aware that the microenvironments, and thus the exerted selective pressure, may differ between these distinct tissues. As seen in Fig. 1.2C the most frequent phenotype detected in the reproductive tract was C1 (44%) followed by C4 and C5 with 22% incidence each. Neither the vaccine parent nor C2 was detected in these tissues.

Incidence of IBV subpopulations among tissues or fluids within the host. The incidences of the predominant phenotypes in samples of tissues or fluids within the chicken are compared in Fig. 1.3. The fact that some comparisons did not achieve statistical significance by Chi-squared test is likely due to the number of amplicons

sequenced. As seen in this figure, phenotype C1 showed the highest incidence in the reproductive tract of the chickens, achieving a significant difference versus its incidence in the trachea. C1 also showed a clear tendency to be more frequently found in the tear fluid than in the trachea (P=0.07). A significant difference was also detected for C3 which was found in about 10% of samples in tear fluid and in the reproductive tract but was absent in the trachea. C4 was detected with similar frequency in the tears and trachea with a clear tendency for lower frequency in the reproductive tract. However, this latter difference did not achieve statistical significance. C5's incidence was highest in the reproductive tract with a significant difference versus its incidence in the tears (P<0.05). It was interesting to notice a tendency for a decrease the incidence of the vaccine parent phenotype in the order tears (8.3%), trachea (4.9%) and reproductive tract (0%). An opposite pattern was observed with phenotype C5 which tended to increase towards the reproductive tract (22%) versus 4.2% in the tear fluids and 11% in the trachea.

Distinct IBV subpopulations in different tissues of individual chickens were found in a high proportion of animals. For example in 10/20 (50%) chickens at 6 DPI and in 6/12 (50%) chickens at 9 DPI a different predominant IBV population was found in tear versus tracheal samples.

Time-dependent kinetics of IBV subpopulations. The incidences of each IBV predominant subpopulation in the tears, trachea, and reproductive tract did not vary significantly between days 6 and 9 after inoculation. A comparison of the incidence of each IBV subpopulation in the trachea is shown in Fig. 1.4 as an example.

DISCUSSION

We have previously demonstrated genetic differences among Ark-type attenuated IBV vaccine strains. We also know both the genetic (S1 gene sequence) and phenotypic (deduced amino acids) characteristics of the S1 glycoprotein of the predominant viral subpopulation selected once this virus strain has replicated in the environment of the upper respiratory tract (171). For these reasons, and because others have also shown rapid selection of Ark-type IBV strains in chickens (119), we chose an Ark-type vaccine strain for the current experiments.

Viral RNA was consistently amplified from samples collected at 6 and 9 DPI but rarely at 20 DPI. This result can be explained by the low virulence of the IBV strain used in these experiments i.e. virulent phenotypes to the natural host have been negatively selected during multiple generations via the process of "attenuation in chicken embryos" or adaptation to the chicken embryo host. However, such an adapted virus population still shows genetic heterogeneity (171) and there is no reason to assume that the mechanisms to generate diversity could have been compromised during the artificial adaptation process.

When analyzing the incidence of the vaccine parent predominant phenotype (Fig. 1.1) in the chickens 6 days after inoculation, it becomes clear that it was negatively selected. This result corroborates our previous findings for other ArkDPI-derived vaccines as well as findings by others (119, 171).

Nucleotide alterations of the S1 coding sequences of coronaviruses have been shown to be associated with organ tropism and *in vivo* pathogenesis (99, 135). During

adaptation of IBV to Vero cells or attenuating passages in embryonated eggs, more changes accumulate in the S gene than in other genes (7, 62). In the present study, five new predominant IBV phenotypes were detected in the host tissues or fluids. The new predominant populations showed varying degrees of phenotypic distance to the predominant population of the vaccine parent. Differences varied from populations exhibiting as many as 6 non-synonymous changes to populations showing only 1 change within the region sequenced (Table 1.3). However, sequences of the entire S1 coding sequence of populations selected from this vaccine in an unrelated experiment indicate additional changes in the portion of the sequence not determined in the present study. The subpopulation showing only one non-synonymous change with the vaccine parent in the region of the S1-coding sequences determined in the present study has two additional non-synonymous differences for a total of three amino acid differences in S1 (unpublished results). In addition, some of these changes were qualitatively essential as they likely changed local charge and/or morphological properties of the S1 glycoprotein (Table 1.3). In the case of SARS coronavirus, a single amino acid change in the S protein of a palm civet isolate markedly increased its affinity for the human SARS coronavirus receptor (109). However, the impact of such amino acid changes in IBV on attachment to host cells or immunological escape requires further investigation.

During the course of a previous study, we used primers designed to specifically amplify a portion of the S1 coding sequences of ArkDPI subpopulations selected in chickens to demonstrate the presence of potentially selectable subpopulations in the vaccine used in the present study (171). The specific primers would recognize C1-C4, but would recognize neither the major vaccine population nor C5. Sequence chromatograms

of the RT-PCR products were consistent with the presence of C1-C4 in the vaccine prior to replication in chickens. A quantitative analysis of the nucleotide peak heights in the chromatograms at positions where populations C1, C3, and C4 exhibit unique nucleotides indicated that C3 was present in the highest proportion (approximately 40%), with C4 (approximately 30%) and C1 (approximately 20%) following. C2 cannot be directly estimated because it has no unique nucleotides, but might comprise the remaining approximately 10% of potentially selectable virus in the vaccine. The relative frequencies of selection of subpopulations C4, C1, and C2 in tears of the current study correspond to their previously determined relative frequencies in the vaccine. However, C3 is selected at a lower frequency in all tissues than expected based on its relative frequency in the vaccine compared to C1, C2, and C4. This suggests that C3 is not as fit in the studied environments as the other selected populations.

Even though IBV targets epithelial cells, the microenvironment of the distinct tissues harboring this type of cells varies within the host. The environment of these tissues not only differs in essential physical and biochemical characteristics (e.g. the environments of the trachea and the oviduct) but cell receptors among different tissues might vary due to for example alternative pre-mRNA splicing to produce distinct mRNAs that give rise to variant proteins (17, 124) or due to alternative glycosylation. In addition, the immune responses that clearly exert selective pressure to invading parasites also vary between tissues (e.g. different degrees of activity of mucosal or systemic immune responses). From the perspective of the virus population, it is also conceivable that the differences observed in IBV populations involve variation in affinity of cell-receptor interactions. It is also possible that the selected changes in S1 might be linked and/or

associated with changes of regulatory proteins encoded at distant sites of the viral genome that could also contribute to the fitness of the virus in distinct tissues. The current results showed that the incidence of some of the predominant phenotypes varied significantly between tissues (Fig. 1.3) of the inoculated chickens. It was also interesting to notice that the vaccine parent predominant phenotype showed a tendency to decrease its incidence towards the tissues located farther away from the site of initial inoculation. In contrast other predominant populations (e.g. C5) showed the reverse tendency. These results indicate for the first time that IBV undergoes intraspatial variation during host invasion, i.e. the dominant genotype/phenotype further changes during host invasion as the microenvironment of distinct tissues exert selective pressure on the replicating virus population.

In the present study no significant differences were detected in the incidence of predominant phenotypes within the same tissue between 6 and 9 days after inoculation. We speculate that the time span evaluated (3 days) was not long enough to allow selection. However, it is conceivable that further maturation of the specific immune responses, resulting for example in improved affinity of immune molecules, may have an impact on selection of virus subpopulations.

From an applied perspective these results strongly suggest that viruses to be used for phylogenetic studies should be obtained from the same tissues of the chickens. Otherwise, intraspatial variation may be adding variation to such data.

Evolution of viruses by selection of the fittest subpopulations can lead to the selection of virulent viruses and the emergence of new viral pathogens (57). It seems reasonable to assume that intraspatial variation is also contributing to this complex

phenomenon and to related problems, such as reversion to virulence of attenuated IBV vaccine strains after chicken passages (78).

IV. Effects of CAV and IBDV Immunodeficiency on IBV Replication and Phenotypic Drift

Avian Diseases **(in press)**

Rodrigo A. Gallardo, Vicky L. van Santen, Haroldo Toro [A]

Department of Pathobiology, College of Veterinary Medicine, 264 Greene Hall, Auburn University, AL 36849, USA.

SUMMARY

We evaluated a portion of the S1 gene sequence of the dominant phenotype of an infectious bronchitis virus (IBV) Ark vaccine strain during continued passages in chickens infected with the immunosuppressive viruses chicken anemia virus (CAV) and/or infectious bursal disease virus (IBDV) as well as in immunocompetent chickens. The IBV-Ark vaccine was applied ocularly and tears were collected from infected chickens (8 days post- infection) for subsequent ocular inoculation in passages to follow. This experiment was repeated twice. Corroborating previous results, in both trials the dominant genotype of the vaccine strain was rapidly negatively selected in all chicken groups [CAV, IBDV, CAV+IBDV, and immunocompetent]. Based on S1 geno/phenotype, exactly the same IBV subpopulations previously detected in immunocompetent chickens emerged both in immunocompetent and immunodeficient chickens during the current experiments. During the 1st passage variety of subpopulations emerged followed by establishment of one predominant population after further passages. This phenomenon can be explained by the fact that more replication cycles should result in the selection of the populations showing increased adaptedness. Only subpopulation C2 successfully became established and only in either CAV or IBDV infected chickens. These results indicate that selection indeed doesn't cease in immunodeficient chickens. The fact that C2 but no other subpopulation became established in immunodeficient hosts suggests a distinct adaptation of this phenotype to this type of environment. From an applied perspective our current results indicate that such circumstances may contribute to the emergence of novel IBV strains. Subpopulations C1 or C4 became established in immunocompetent birds but became extinct after only a few succeeding passages. A

similar result was observed in chickens co-infected with CAV+IBDV. These results suggest that the generation of genetic diversity in IBV is constrained or at least not as frequent as we would have assumed. This finding constitutes further evidence for phenotypic drift occurring mainly as a result of selection.

INTRODUCTION

Infectious bronchitis virus (IBV) is a single stranded RNA coronavirus of group 3 that shows extensive genotypic and phenotypic variability. Genetic diversity is generated during viral replication as result of the lack of proofreading activity of the RNA polymerase as well as recombination events (101-103).

The spike (S) protein is responsible for viral attachment to the host cell and is a relevant target for specific humoral and cellular host immune responses (26-28, 36, 41, 96, 97, 122). The bulb end of the S protein (S1) exhibits extensive variation among IBV populations (100, 102) providing a successful adaptation favoring immunological escape. Thus, the phenotype of S1 represents an important feature of this virus' evolutionary success.

Based on S1 gene sequencing we have previously shown that the dominant genotype/phenotype of an IBV ArkDPI vaccine strain changes during host invasion as the microenvironment of the different host's tissues exert distinct selective pressure on the replicating virus population (intraspatial variation) (66, 119, 171).

Chickens in production environments are exposed to multiple stressors and infectious diseases impairing innate and adaptive immunity (75). Infectious bursal disease (IBD), chicken infectious anemia (CIA), and Marek's disease (MD) are examples of endemic diseases altering the host's humoral and cell-mediated immune function. According to Kilbourne (92), "in those experiments of nature in which the immune response is effectively removed as a selective host force, viral evolution does not cease but rather appears to falter or follow different pathways". Because viral

immunodeficiencies are frequent in poultry commercial settings we hypothesized that IBV passage in immunodeficient chicken populations results in distinct evolutionary pathways. To explore this hypothesis we evaluated IBV phenotypic drift during continued IBV passages in immunocompetent chickens or in chickens previously inoculated with CIA virus (CIAV) and/or IBD virus (IBDV).

MATERIALS AND METHODS

Viruses. A commercially available single-entity live attenuated ArkDPI-derived IBV vaccine strain (Intervet, Millsboro, DE) was used in all experiments. The lyophilized vaccine strain was reconstituted in sterile tryptose broth containing a commercially available antibiotic-antimycotic combination (Invitrogen, Carlsbad, CA) and used in subsequent chicken passages.

CIAV strain 03-4876 previously described (170) was used to cause T lymphocyte deficiency. CIAV was propagated and tittered in MDCC-MSB1 cells as accepted (118). The efficacy of the virus batch to cause immunodeficiency was evaluated by inoculation in specific pathogen free (SPF) chickens followed by assessment of thymic lymphocyte depletion by histomorphometry as described (170).

The variant IBDV AL2 strain previously characterized (165) was used to induce B lymphocyte deficiency. The AL2 strain was propagated, tittered, and tested for efficacy at causing B lymphocyte depletion in the bursa (histomorphometric analyses) by inoculation into SPF chickens.

Chickens. White leghorn chickens which were hatched from SPF fertile eggs (Sunrise Farms, Catskill, NY) were used in all experiments. Hatched chickens were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an AAALAC accredited institution.

Experimental design.

Experiment 1. Four chicken groups (each/n=10) were hatched at 8 day intervals for serial passages of IBV vaccine. On day 7 post-hatch three chicken groups were inoculated with CAV [100µl intramuscularly (IM) containing 1.6 x 10^5 tissue culture infectious doses 50% ($TCID_{50}$] and/or IBDV [200µl subcutaneously (SC) containing 2 x 10^5 chicken infectious doses 50% (CID_{50})]. Groups were designated CAV, IBDV, and CAV+IBDV. The 4^{th} group consisted of uninoculated immunocompetent chickens. On day 15 of age all chickens were inoculated with the ArkDPI-derived IBV vaccine strain [100 µl containing 5 x $10^{5.2}$ egg infective doses 50% (EID_{50})] as follows: 25µl/each eye and 25µl/ each nostril). Tear fluids of IBV infected chickens have been shown to contain high IBV levels (168). Thus, 8 days after IBV inoculation tears were collected from all chickens of each group as described (162) for S1 gene sequencing and for subsequent passaging. Further passages were performed using pooled tears diluted in 1:2 in tryptose broth and ocularly inoculated (50µl of tear suspension) to the next generation of 15 day-old immunocompetent or immunodeficient chicken groups as previously described (168). During each passage, RNA was extracted from the collected tear samples for RT-PCR

amplification of a ~751 nucleotide portion of the IBV S1 gene to confirm the presence of IBV in the tears used for further passaging. The S1 cDNA obtained was sequenced to identify possible nucleotide changes in the S1 gene.

RT-PCR. Viral RNA was extracted from individual and pooled tear samples obtained from each chicken of all groups using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RT-PCR was performed using the Qiagen one step RT-PCR kit (Qiagen, Valencia, CA) and primers S17F and S18R previously described (66). Amplicons were visualized by gel green staining (Phoenix Research, Candler, NC) after agarose gel electrophoresis.

S1 sequencing. Amplified cDNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and submitted to the Massachusetts General Hospital DNA core facility for sequencing using primers S1R (171), S17F and/or S18R (66). Sequences were assembled and aligned using Mac Vector 10.6.0 software (Mac Vector Inc., Cary, NC). Sequence chromatograms were analyzed for the presence of subpopulation diversity. For sequences showing detectable levels of more than one population, the populations were assigned to their predominant population based on the major nucleotide peaks. The incidence of each subpopulation in IBV positive chickens was determined. Statistical differences between and within groups were analyzed by *chi* square test. Differences with $P<0.05$ were considered significant.

Experiment 2. To confirm results obtained in experiment 1, we repeated the experiment described above almost identically. The only difference was that we used a different IBV vaccine lot (same company) and chickens were inoculated with 5 x $10^{4.8}$ EID_{50}.

RESULTS

IBV RNA detection by RT-PCR during serial passages in immunocompetent or immunodeficient chickens. In experiment 1, the S1 gene of IBV was consistently amplified from tear fluid in every passage through passage 9 in all (100%) CAV immunodeficient chickens. In contrast, IBV S1 detection in immunocompetent, IBDV, and CAV+IBDV chicken groups had declined to 13% after passage 4; 20%, and 10% after passage 3 respectively. IBV RNA was undetectable in these groups by passages 4 or 5 (Fig. 2.1A).

Slightly different results were obtained in experiment 2. IBV S1 was detected by RT-PCR in the IBDV group through passage 9 with detection rates varying from 80% to 90%. On the other hand, immunocompetent, CAV, and CAV+IBDV chicken groups cleared IBV on the 5^{th}, 5^{th}, and 4^{th} passages respectively with IBV S1 detection rates during the previous passage (before clearance) of 20%, 10%, and 20% for each group respectively (Fig. 2.1B).

IBV S1 gene sequences in inoculated chickens. IBV-Ark found in tear fluids of individual inoculated chickens exhibited differences in S1 gene consensus sequences. Considering only differences representing non-synonymous changes, six distinct virus

predominant populations (including the major vaccine population prior to inoculation) were found after the 1st passage in both immunocompetent and immunodeficient birds. The emergent IBV subpopulations with nucleotide and amino acid positions in which differences were observed in more than one chicken are identical as those previously characterized and designated as component (C) 1 (C1), C2, C3, C4, and C5 (66). In brief, phenotypic differences between subpopulations encompassed both quantitative (from 1 to up to 6 changes) and qualitative (biochemically distant) amino acid substitutions (66). Corroborating previous findings, these subpopulations increased their incidence after the first passage in chickens while the vaccine predominant population was rapidly negatively selected. Interestingly, negative selection occurred both in immunocompetent as well as immunodeficient chickens. Also, a small percentage of immunocompetent and immunodeficient chickens [14.7% (5/34) in trial 1, and 18.2% (6/33) in trial 2] showed mixed IBV populations.

Frequency of subpopulations by passage. *Frequencies after passage 1.* The outcome of the first IBV passage of trials 1 and 2 consistently showed the emergence of the distinct subpopulations described above (Figs 2.2 and 2.3). For example, in immunocompetent chickens (trial 1) the most frequent subpopulation selected was C1 (67%) but other predominant populations included C4 (33%), and C5 (17%). In another example, B cell deficient chickens (IBDV infected) after passage 1 showed C2 (30%), C4 (30%), C1 (30%), and C5 (20%) without any birds showing the vaccine major. Although, the frequency of each subpopulation after passage 1 slightly differed between trials 1 and 2, C1 and C4 showed increased fitness in both experiments in immunocompetent birds

(Figs. 2.2, 2.3). In contrast to experiment 1 (shown above) in immunocompetent chickens of trial 2 the most frequently selected subpopulation was C4 (60%), followed by C1 (30%). In experiment 2, B cell deficient chickens showed C4 (80%), C2 (20%), and C1 (10%). Similarly the IBDV group of trial 1 (shown above) C4 and C2 were the fittest populations in this environment. The rank of each subpopulation by frequency in each trial is shown in table 2.1. From the data shown in this table it becomes clear that C4 seems to be the most successful population in all environments tested herein, i.e. C4 was the most frequent population in all groups in trial 2 and the 2nd most frequent in trial 1.

Frequency of subpopulations after further passages. A distinct trend was observed after the 2nd and further passages in all groups of both trials. IBV was cleared from immunocompetent chickens after the 5th passage regardless of the selected viral population predominating in the birds. Similarly, both trials showed that IBV was no longer detectable after 4 to 5 passages in chickens inoculated with IBDV+CAV. Only in those groups in which C2 was selected, IBV persisted successfully throughout the whole experimental period of 9 passages. Noticeable, in both cases where subpopulation 2 established and persisted, it was not the most prevalent during the first passage. Interestingly, persisting C2 was selected in CAV infected chickens in trial 1 and in IBDV infected chickens in trial 2. In immunocompetent chickens of both trials C1 became predominant and was cleared after the 4th passage.

Differences between the trials include that one predominant population was ultimately established in all groups in trial 1. In contrast, in trial 2 a clearly predominant population (C2) was only selected in IBDV infected chickens. In all other groups in

which C1 was more frequent, IBV was cleared from the chickens between the 4th and 5th passages.

DISCUSSION

Different portions of IBV's genome and phenotype contribute to this virus' evolutionary success. For example, the replicase proteins in ORF1ab have been shown to be associated with pathogenicity (7). However, the spike gene in IBV appears to be the most reliable measure of genetic change leading to the emergence of new viruses capable of causing disease (120). Indeed, our previous results have confirmed that population analyses based on S1 geno/phenotype allow assessment IBV drift (66).

It was fascinating to observe that based on S1 geno/phenotype, exactly the same IBV subpopulations previously detected in immunocompetent chickens (66) emerged both in immunocompetent and immunodeficient chickens during the current experiments. This result suggests that the generation of genetic diversity in IBV is constrained or at least not as frequent as we would have assumed. This assumption is enforced by the fact that the same populations arose even when different ArkDPI vaccine lots (though from the same manufacturer) were used in experiments 1 and 2. This finding constitutes further evidence for phenotypic drift occurring mainly as a result of selection.

Another interesting result involves the emergence of a variety of subpopulations during the 1st passage but a quick establishment of one predominant population after further passages. This phenomenon can be explained by the fact that more replication cycles should result in the selection of the populations showing increased adaptedness.

The genetic diversity in viral isolates of immunosuppressed individuals is attributed to shifts in population equilibrium of the replicating viral genomes in the absence of immune selection pressure (143). CAV targets lymphoblasts of the thymus cortex in young chickens causing T cell immunodeficiency (1, 2). T cell responses are relevant in the clearance of IBV (41, 150). CAV also reduces mucosal immune responses to IBV (168). On the other hand, IBDV affects the bursa of Fabricius causing B cell immunodeficiency (40, 144) and antibodies also participate in the immune response against IBV [e.g. (97)]. We previously showed that Ark-type IBV persists for longer periods of time in chicken suffering from viral immunodeficiency (166). Thus, in CAV and/or IBDV infected hosts the selective forces exerted to the replicating IBV populations are compromised. According to Kilbourne (93), "if the restricting effects of the host immune response are removed, the virus has the opportunity of emergence and testing of mutants previously absent or suppressed, antigenic changes can be seen related with altered replication properties of the virus." The current results showed that only C2 successfully became established and only in either CAV or IBDV infected chickens. These results indicate that selection indeed doesn't cease in immunodeficient chickens. The fact that C2 but no other subpopulation became established in immunodeficient hosts suggests a distinct adaptation of this phenotype to this type of environment. We would speculate that under such circumstances other selective forces, such as for example the affinity of the S protein to the cell receptors, may augment their selective preponderance.

A considerable percentage of commercial chickens suffer from CAV and/or IBDV immunodeficiency (75). Concurrent infection with IBV is common (166). From an

applied perspective our current results indicate that such circumstances may contribute to the emergence of novel IBV strains.

Immunocompetent chickens successfully cleared IBV after only a few succeeding passages. Subpopulations C1 or C4, which became established in these birds, were not fit enough to withstand this environment and became extinct. These birds were maintained in a controlled environment and the viruses did not have opportunities for recombination with other IBV strains. Thus, if generation of diversity by mutation is not as frequent as anticipated (discussed above) and IBV becomes extinct under these conditions, other mechanisms of survival (outside of the host) must play a relevant role in the fitness of this virus' family.

The CAV+IBDV infected chicken group produced intriguing results, i.e. in neither experiment an IBV population became established. It is possible that this model is inadequate to understand the drift of IBV because the incubation periods of CAV and IBDV differ. Indeed IBDV is expected to produce the most severe changes in the bursa around 3 to 5 days after inoculation (137) while CAV induces maximal changes in the thymus a few days later [around 10 days after parenteral inoculation (170)]. Thus, our experimental design may have led to the development of immune responses, particularly innate responses that provided a completely altered environment to the replicating IBV strain.

V. Infectious Bronchitis Virus in Testicles and Venereal Transmission
***Avian Diseases* 55: 255-258, 2011**

Rodrigo A. Gallardo[A], Frederic J. Hoerr[B], Wallace D. Berry[C], Vicky L. van Santen[A], Haroldo Toro[AD]

[A] Department of Pathobiology, College of Veterinary Medicine, 264 Greene Hall, Auburn University, AL 36849, USA.

[B] Thompson-Bishop-Sparks Alabama Veterinary State Diagnostic Laboratory, 890 Simms Road, Auburn, AL 36831, USA.

[C] Department of Poultry Science, College of Agriculture, 260 Lem Morrison Drive, Auburn University, AL 36849, USA.

SUMMARY

Even though males represent only 8 to 12% of the birds of a breeder flock, their role in infectious bronchitis virus (IBV) dissemination is largely unknown. We first assessed the effect of IBV replication in the chicken testes. Ten-week-old males were inoculated with Ark or Mass IBV virulent strains. Seven days post-inoculation (DPI) IBV RNA was detected in testicles of 100% of M41- and in 96% of Ark-infected males. Marginal non-synonymous variation was detected in spike (S) gene of the predominant population of IBV replicating in the testes compared to the S gene of the predominant population of viruses prior to inoculation. IBV M41 and Ark were detected in spermatogonia and Sertoli cells of testicles of infected roosters by immunofluorescence, without evident histopathological changes. We next assessed venereal transmission of IBV by artificially inseminating 54-week-old hens either with semen from IBV-infected roosters or with IBV suspended in naïve semen. IBV RNA was detected in the trachea of all hens inseminated with IBV-spiked semen and in 50% of hens inseminated with semen from IBV-infected males. The egg internal and external quality was negatively affected in hens inseminated with semen containing IBV. These results provide experimental evidence for IBV venereal transmission.

INTRODUCTION

Males represent 8 to 12% of birds in breeder flocks and are maintained in close contact with the hens during production. The role of males in infectious bronchitis virus (IBV) dissemination in breeder flocks is largely unknown. Many poultry producers do not vaccinate males against IBV, and particularly avoid expensive inactivated vaccines. Thus males are more susceptible to IBV infection and can potentially act as amplifiers of IBV in breeder flocks. Another complicating factor is the practice of "spiking" male populations in older breeder flocks, i.e., replacing up to 50% of males with younger males for the purpose of increasing the fertility of the flock. This procedure interferes with the "all in all out" gold standard of raising chickens and increases the risk for disease transmission. Artificial insemination is a common practice in pedigree lines to control venereally transmitted diseases. IBV has been isolated from testicles (172) and semen (43) from experimentally infected birds. IBV RNA has been detected in the testicles of infected males by reverse transcriptase polymerase chain reaction (RT-PCR) (13, 66). Several authors also have associated epididymal stone formation and epididymitis with IBV live vaccination or natural infection (13, 16, 172). Because IBV is present in the testicles and in the semen it seems reasonable to hypothesize that IBV could be transmitted via the venereal route in chickens.

In this study we evaluated effects of IBV replication in the testicles of infected males. Because we previously found that distinct Ark IBV vaccine subpopulations are positively selected in the reproductive tract of chickens (66), we also investigated possible changes in the predominant IBV population replicating in the males testes using

IBV Ark or Mass virulent strains. Finally we evaluated transmission of IBV to naïve hens via artificial insemination with IBV infected semen.

MATERIALS AND METHODS

Viruses. IBV Ark or M41 virulent strains were used to infect young or adult males and to spike naïve semen. These IBV virulent strains were kindly provided by Dr. Mark Jackwood (Poultry Disease Research Center, University of Georgia, Athens, GA).

Chickens. White leghorn chickens were hatched from specific pathogen free (SPF) fertile eggs (Sunrise Farms, Catskill, NY) and used in all experiments. Fifty-six 10-week-old males were used in trial 1, and 12 males and 75 females (56–week-old) were used in trial 2. All birds were maintained in biosafety level 2 (BSL 2) facilities and provided with water and food *ad libitum*. All experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an AAALAC-accredited institution.

Trial 1. IBV Ark or M41 was inoculated into groups of 10–week-old male leghorn chickens (24/group) by the ocular and nasal routes. Birds of group 1 were inoculated with 200 µl of an Ark-type virulent strain containing 1.6×10^5 50% embryo infective doses (EID_{50}). Birds of group 2 received 2×10^5 EID_{50} of a virulent M41 strain. An additional group (n=8) was used as the uninoculated control group. Seven days post-inoculation (DPI) all chickens were euthanized and samples of testicles were obtained for

histopathology, IBV RNA detection by RT-PCR followed by partial S1 gene sequencing, and for virus detection by immunofluorescence.

Trial 2. Three groups of hens (25/group) and 2 groups of males (6/group) hatched from SPF eggs and raised under BSL 2 conditions were used at 54 weeks of age. Each bird in group 1 (6 males) was inoculated with 200 µl of IBV M41 (2×10^5 EID_{50}) via the ocular and nasal routes. Semen from all males was collected 7 DPI, diluted 1:4 in semen extender E-Z MIXIN® (Animal Reproduction Systems, Chino, CA) and artificially inseminated into one group of females (70 µl per hen). Semen from uninoculated males (n=6) was collected at the same time, diluted 1:4 with semen extender, and divided into 2 aliquots. Half of this semen was spiked with IBV M41 and used to inseminate a second group of hens. Each hen of this group was inseminated with 70 µl of semen containing 5.3×10^3 EID_{50} of IBV M41. The remaining semen (IBV-negative) was used to inseminate a third group of hens (negative control). Tracheas were obtained from four hens in each group on days 7 and 15 post-insemination and tested for IBV RNA by RT-PCR. Eggs were collected from each group of hens on days 2, 3, 6, 8, and 9 after insemination and egg internal and external quality was evaluated as described (155).

Viral RNA detection. Viral RNA was prepared from the semen samples using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Total RNA was isolated from testicle samples of males and tracheal samples of females using the Tri-Reagent RNA isolation reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. cDNA for IBV detection and

sequencing of a portion of the IBV S1 gene was prepared from the extracted RNA by RT-PCR using the Qiagen one-step RT-PCR kit (Qiagen, Valencia, CA) and primers S17F and S18R as previously described (66). In addition, cDNA from a portion of the nucleocapsid (N) gene was prepared using the same Qiagen one step RT-PCR kit and IBVN(+) and IBVN(-) primers as described (72). The products obtained were visualized by gel green staining (Phoenix Research, Candler, NC) after agarose gel electrophoresis.

Sequencing of cDNA generated by RT-PCR. After verification by agarose gel electrophoresis, amplified cDNA was purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and submitted to the Massachusetts General Hospital DNA Core Facility for sequencing using S1R (171), or S18R primers. Thus, the sequence of the first 751 nucleotides of the S1 coding sequences was obtained. Sequences were assembled and aligned using Mac Vector 10.6.0 software (Mac Vector Inc., Cary, NC). The sequences of the predominant IBV populations detected in the testicles were compared to the consensus sequences of virulent M41 or Ark strains prior to inoculation.

Immunofluorescence. Cryosections (4-5 μm) were prepared from the testicles of males and blocked with 10% fetal calf serum (FCS). A commercially available monoclonal antibody against the nucleoprotein (N) of IBV Massachusetts-type strain IB95 (Hy-test, Turku, Finland) was used as the primary antibody at a 1:1000 dilution (approximately 1 μg/ml). After 2 h incubation at room temperature followed by a washing step, an anti-mouse goat IgG conjugated with FITC (Jackson ImmunoResearch, West Grove, PA) was used (1/500 in PBS containing 1% FCS) to detect the monoclonal antibody. After

appropriate washing steps, samples were mounted and evaluated in a fluorescent microscope.

Assessment of egg quality. Egg internal and external quality was evaluated essentially as described (155) on an average of 78 eggs per group of hens collected 2, 3, 6, 8, and 9 days after insemination. Egg shell texture was evaluated based on smoothness and strength of the egg shell by observation and tactile exploration. A trained technician, not directly involved in the project, blindly scored the eggs mainly as described (155) but using 5 instead of 3 scores as follows: [1] abnormal (faulty in texture, rough areas, reduced egg shell strength), [2] moderately abnormal (moderately faulty in texture and strength), [3] slightly abnormal (slightly faulty in texture), [4] normal (texture and strength, free from rough areas), and [5] exceptional (better than expected for the age of the hen). Internal egg quality was measured by the albumin height (millimeters), and egg sizes were determined by weighing (grams).

RESULTS

Trial 1. IBV RNA was detected by RT-PCR amplification of a 453-nt portion of the N gene in the chicken testicles of 96% of Ark- and 100% of M41-inoculated birds. RT-PCR amplification of a 751-nt portion of the S1 gene was less sensitive and IBV was detected in only 54% of the testicles of Ark- and 58% of M41-infected roosters. Analysis of the S1 gene sequences showed the predominant population of IBV M41 changed in the testis of only one chicken. The S1 gene of IBV in this chicken differed at nucleotide position 170 resulting in adenine being replaced by guanine. This was a non-synonymous change that

replaced the asparagine at amino acid position 57 with serine. On the other hand, synonymous mutations were identified in the S1 gene of IBV in the testicles of two individual chickens inoculated with the Ark strain of IBV.

IBV was detected in the cytoplasm of spermatogonia and Sertoli cells by immunofluorescence (IF) in the testicles of both Ark- and M41-infected 10 week-old males (Fig. 3.1). No histopathological changes were detected in the testicles of either M41- or Ark-infected chickens at the time of tissue sampling (7 DPI).

Trial 2. On day 7 after insemination IBV M41 RNA was detected (N gene RT-PCR) in 2/4 tracheal samples of hens inseminated with semen of IBV infected males, in 4/4 tracheal samples of hens inseminated with naïve semen spiked with 2 x 10^5 EID_{50} of IBV M41, and in 0/4 tracheal samples of hens inseminated with semen from uninoculated control males (Fig. 3.2). On day 15 after insemination IBV RNA was detected only in hens (4/4) inseminated with IBV spiked semen. Eggs collected from the different experimental groups showed differences in egg weight, albumin height and shell texture. External egg scores varied from 1 (rough) to 5 (smooth) (Fig. 3.3A). Eggs produced by hens inseminated with IBV-spiked semen showed a significant reduction ($P<0.05$) of shell smoothness compared to controls and hens inseminated with semen from IBV-infected males. No significant differences ($p>0.05$) in egg shell texture were detected between eggs obtained from hens inseminated with semen from IBV infected males and eggs from control hens. No significant differences ($p>0.05$) were detected among the experimental groups in the albumin height of eggs. However, a drop in albumin height started 3 days post insemination in hens inseminated with semen spiked with IBV (Fig.

3.3B). The weight of eggs laid by hens inseminated with IBV-spiked semen was significantly reduced ($P<0.05$) compared to those laid by controls and hens inseminated with semen from IBV-infected males (Fig. 3.3C).

DISCUSSION

Our detection of IBV RNA in testes corroborate findings by others that IBV replicates in the male testicles (43, 172). Ark or M41 IBV RNA was consistently detected in the testes of infected males when the N gene was targeted for amplification. In contrast, amplification of the S1 gene was less sensitive. We have also isolated IBV in SPF embryonated eggs from pools of testicles of experimentally infected males (unpublished results).

We have previously reported that distinct Ark IBV vaccine subpopulations are positively selected in the reproductive tract of chickens (66) but no information is available for Mass-type strains. Based on the deduced S1 amino acid sequence of the M41 virulent strain used herein, only one non-synonymous mutation (asparagine acid to serine) was detected in the testicles of M41 inoculated males. Because both asparagine and serine are uncharged polar amino acids, the change is unlikely to have a great effect on the structure and function of the S1 protein. Further studies are required to determine if this predominant population is the result of successful adaptation to the testicle's environment.

So far, the pathology of IBV infection in the male reproductive tract has not been well documented. While some authors describe epididymitis and stone formation in the seminiferous tubules or epididymus (13, 16, 172), others have not detected lesions

associated with IBV in the testicular stroma (82). These contradictory results may be due to the different ages of the studied males and/or with the time lapse between IBV infection and tissue sampling used by the different scientists. In agreement with some authors (13, 82), in the current study no histopathological changes were detected in the testicles of the infected chickens. However, because IBV was detected in the testes by immunofluorescence we would have anticipated also finding inflammatory cells. The fact that no immune cell infiltration was apparent may be due to the fact that samples were collected relatively shortly (7 days) after IBV respiratory inoculation. Lymphocytic infiltration is usually apparent by 3 DPI in trachea. We anticipate that the time it takes for IBV to reach testicles might delay lymphocytic infiltration in this tissue compared to trachea.

IBV infection in chickens is known to be initiated by replication in the upper respiratory tract independent of the tissue tropism of the strain (37). After viremia, IBV replicates in different tissues including the oviduct (48, 167). Insemination of hens with IBV positive semen revealed a new aspect of IBV epidemiology. Indeed, hens inseminated with semen from IBV positive males were positive for IBV RNA in the tracheas. This initial evidence for venereal transmission of IBV was corroborated by the reduction in egg quality of hens inseminated with IBV spiked semen. IBV is a highly contagious virus and mismanagement during insemination could result in accidental infection via the respiratory route. Thus, during these experiments extreme precautions were undertaken to avoid infection via a route other than the venereal, e.g., shower-in and shower-out as well as changing clothing was strictly followed by all personnel involved between semen collection steps and between insemination groups. In addition, the

insemination procedure consisted of pressing the cloaca to evert the vagina and the diluted semen was introduced using a tuberculin syringe (without the needle). When pressure was released from the cloaca, the vagina reverted placing the semen deep inside the reproductive tract, preventing exposure of the respiratory tract during self-grooming. Thus, these results provide experimental evidence that venereal transmission of IBV in chickens is feasible.

Reduced egg quality is well known to be associated with IBV infection (48). Thus, in addition to the results showing IBV RNA in the trachea of inseminated hens, the reduced internal and external quality of eggs harvested from hens inseminated with naïve semen spiked with IBV confirmed IBV infection. Hens inseminated with semen from IBV infected males did not result in reduced egg quality. It is possible that the concentration of IBV in the semen of those males was not enough to induce damage to the oviduct. Another possibility is that the experimental time was not enough to detect those changes.

These preliminary results on IBV experimental venereal transmission highlight the necessity for adequate vaccination of males against IBV in commercial settings.

VI. Conclusions

The research presented in this dissertation constitutes a three-part investigation that was conducted to potentially define avian infectious bronchitis coronavirus variation and selection. In the first study, the predominant population contained in IBV ArkDPI-derived embryo attenuated vaccines was rapidly (after 1 passage) negatively selected in the host. Minor populations with varying degrees of phenotypic distance to the vaccine predominant population became predominant at different days post IBV inoculation. It was also demonstrated that the dominant IBV genotype/phenotype changed during host invasion as the environment of distinct tissues exerted selective pressure on the replicating virus population. Thus, IBV exhibited "intraspatial variation".

In the second experiment, the predominant population contained in IBV ArkDPI-derived embryo attenuated vaccines was rapidly negatively selected, after the first passage, in the immunocompetent as well as in immunodeficient chickens. Successive passages of IBV in immunocompetent or immunodeficient chickens were performed, leading to the establishment of a distinct population in the host. The subpopulations selected in immunocompetent chickens were successfully cleared after only a few succeeding passages. The fact that such populations became extinct may indicate that the generation of genetic diversity in IBV is more constrained than assumed by numerous researchers. The immunodeficiency caused by CAV or IBDV inoculation of chickens

determined the selection of distinct IBV subpopulations corroborating that evolution doesn't cease, follows a distinct evolutionary pathway in the immunodeficient host.

The final study focused on the pathobiology of IBV infection in chicken testes and the level of variation exhibited by the virus on this tissue. We were able to detect only marginal intraspatial variation of IBV wild strains (M41 or Ark) after one passage in chickens indicating more optimal adaptedness of the virus to the host. IBV Ark and M41 virulent strains replicated in the testes of young and adult males but did not cause any apparent damage. We also demonstrated that under experimental conditions IBV is transmitted via the venereal route via IBV contaminated semen.

These results support that IBV populations show high genetic variability resulting from mutation and selection, following the same evolutionary mechanisms originally described by Darwin for more complex species.

References

1. Adair, B. M. Immunopathogenesis of chicken anemia virus infection. Dev. Comp. Immunol. 24:247-255. 2000.

2. Adair, B. M., F. McNeilly, C. D. McConnell, D. Todd, R. T. Nelson, and M. S. McNulty. Effects of chicken anemia agent on lymphokine production and lymphocyte transformation in experimentally infected chickens. Avian Dis. 35:783-792. 1991.

3. Adzhar, A., R. E. Gough, D. Haydon, K. Shaw, P. Britton, and D. Cavanagh. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Avian Pathol. 26:625-640. 1997.

4. Ahmed, R., C. S. Hahn, T. Somasundaram, L. Villarete, M. Matloubian, and J. H. Strauss. Molecular basis of organ-specific selection of viral variants during chronic infection. J. Virol. 65:4242. 1991.

5. Alexander, D. J., and R. E. Gough. Isolation of avian infectious bronchitis virus from experimentally infected chickens. Res. Vet. Sci. 23:344. 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. 1978.

7. Ammayappan, A., C. Upadhyay, J. Gelb, and V. N. Vakharia. Identification of sequence changes responsible for the attenuation of avian infectious bronchitis virus strain Arkansas DPI. Arch. Virol. 154:495-499. 2009.

8. Bacon, L. D., D. B. Hunter, H. M. Zhang, K. Brand, and R. Etches. Retrospective evidence that the MHC (B haplotype) of chickens influences genetic resistance to attenuated infectious bronchitis vaccine strains in chickens. Avian Pathol. 33:605-609. 2004.

9. 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.

10. Baric, R. S., B. Yount, L. Hensley, S. A. Peel, and W. Chen. Episodic evolution mediates interspecies transfer of a murine coronavirus. J. Virol. 71:1946. 1997.

11. 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. 1936.

12. Beaudette, F. R., and C. D. Hudson. Cultivation of the virus of infectious bronchitis J. Am. Vet. Med. Assoc 90:51-58. 1936.

13. Benyeda, Z., T. Mato, T. Suveges, E. Szabo, V. Kardi, Z. Abonyi-Toth, M. Rusvai, and V. Palya. Comparison of the pathogenicity of QX-like, M41 and 793/B infectious bronchitis strains from different pathological conditions. Avian Pathol. 38:449-456. 2009.

14. Bhattacharjee, P. S., S. D. Carter, C. E. Savage, and R. C. Jones. Re excretion of infectious bronchitis virus in chickens induced by cyclosporin. Avian Pathol. 24:435-441. 1995.

15. Bijlenga, G., J. K. A. Cook, J. Gelb Jr, and J. J. De Wit. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol. 33:550-557. 2004.

16. Boltz, D. A., M. Nakai, and J. M. Bahr. Avian infectious bronchitis virus: a possible cause of reduced fertility in the rooster. Avian Dis. 48:909-915. 2004.

17. Brimacombe, K. R., and A. N. Ladd. Cloning and embryonic expression patterns of the chicken CELF family. Develop. Dynamics 236:2216-2224. 2007.

18. Brown, T. P., J. R. Glisson, G. Rosales, P. Villegas, and R. B. Davis. Studies of avian urolithiasis associated with an infectious bronchitis virus. Avian Dis. 31:629-636. 1987.

19. 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. 1989.

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

21. Buttinelli, G., V. Donati, S. Fiore, J. Marturano, A. Plebani, P. Balestri, A. R. Soresina, R. Vivarelli, F. Delpeyroux, and J. Martin. Nucleotide variation in Sabin type 2 poliovirus from an immunodeficient patient with poliomyelitis. J. Gen. Virol. 84:1215. 2003.

22. Capua, I., K. Mawditt, D. Cavanagh, and R. E. Gough Serological and molecular characterisation of recent infectious bronchitis virus strains from Italy. In: Int. Symp. Inf. Bronchitis and Pneumovirus in Poultry. E. F. Kaleta and U. Heffels-Redmann, eds., Rauischholzhausen, Germany. pp 210-214. 1998.

23. Capua, I., Z. Minta, E. Karpinska, K. Mawditt, P. Britton, D. Cavanagh, and R. E. Gough. Co-circulation of four types of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). Avian Pathol. 28:587-592. 1999.

24. Casais, R., B. Dove, D. Cavanagh, and P. Britton. 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. 2003.

25. Cataldo, F., D. Lio, V. Marino, L. Scola, A. Crivello, and G. R. Corazza. Plasma cytokine profiles in patients with celiac disease and selective IgA deficiency. Pediatr. Allergy Immunol. 14:320-324. 2003.

26. Cavanagh, D. Structural polypeptides of coronavirus IBV. J. Gen. Virol. 53:93. 1981.

27. Cavanagh, D. Coronavirus IBV: structural characterization of the spike protein. J. Gen. Virol. 64:2577. 1983.

28. Cavanagh, D. Structural characterization of IBV glycoproteins. Adv. Exp. Med. Biol. 173:95. 1984.

29. Cavanagh, D. The coronavirus surface glycoprotein. In: The Coronaviridae. S. G. Siddel, ed. Plenum press, New York. pp 73-103. 1995.

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

31. Cavanagh, D. Coronavirus avian infectious bronchitis virus. Vet. Res. 38:281-297. 2007.

32. Cavanagh, D., J. H. Darbyshire, P. Davis, and R. W. Peters. Induction of humoral neutralising and haemagglutination inhibiting antibody by the spike protein of avian infectious bronchitis virus. Avian Pathol. 13:573-583. 1984.

33. Cavanagh, D., P. J. Davis, J. K. A. 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 Pathol. 21:33-43. 1992.

34. Cavanagh, D., P. J. Davis, and J. K. A. Cook. Infectious bronchitis virus: evidence for recombination within the Massachusetts serotype. Avian Pathol. 21:401-408. 1992.

35. 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.

36. Cavanagh, D., and P. J. Davis. Coronavirus IBV: removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. J. Gen. Virol. 67:1443. 1986.

37. Cavanagh, D., and J. Gelb Jr. Infectious Bronchitis. In: Diseases of Poultry. Y. M. Saif, ed. Blackwell Publishing, Iowa. pp 117-135. 2008.

38. Cavanagh, D., J. P. Picault, R. E. Gough, M. Hess, K. Mawditt, and P. Britton. Variation in the spike protein of the 793/B type of infectious bronchitis virus, in the field and during alternate passage in chickens and embryonated eggs. Avian Pathol. 34:20-25. 2005.

39. 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.:598-

603. 1997.

40. Cloud, S. S., H. S. Lillehoj, and J. K. Rosenberger. Immune dysfunction following infection with chicken anemia agent and infectious bursal disease virus. I. Kinetic alterations of avian lymphocyte subpopulations. Vet. Immunol. Immunop. 34:337-352. 1992.

41. Collisson, E. W., J. Pei, J. Dzielawa, and S. H. Seo. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol. 24:187-200. 2000.

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

43. Cook, J. K. A. Recovery of infectious bronchitis virus from eggs and chicks produced by experimentally inoculated hens. J. Comp. Pathol. 81:203-211. 1971.

44. Cook, J. K. A., J. Chesher, W. Baxendale, N. Greenwood, M. B. Huggins, and S. J. Orbell. Protection of chickens against renal damage caused by a nephropathogenic infectious bronchitis virus. Avian Pathol. 30:423-426. 2001.

45. Cook, J. K. A., T. F. Davison, M. B. Huggins, and P. McLaughlan. Effect of in ovo bursectomy on the course of an infectious bronchitis virus infection in line C White Leghorn chickens. Arch. Virol. 118:225-234. 1991.

46. Cook, K. A., K. Otsuki, N. R. da Silva Martins, M. M. Ellis, and M. B. Huggins. The secretory antibody response of inbred lines of chicken to avian infectious bronchitis virus infection. Avian Pathol. 21:681-692. 1992.

47. Cowen, B. S., R. F. Wideman, H. Rothenbacher, and M. O. Braune. An outbreak of avian urolithiasis on a large commercial egg farm. Avian Dis. 31:392-397. 1987.

48. Crinion, R. A. Egg quality and production following infectious bronchitis virus exposure at one day old. Poultry Sci. 51:582. 1972.

49. Csermelyi, M., R. Thijssen, F. Orthel, A. G. Burger, B. Kouwenhoven, and D. Lütticken. Serological classification of recent infectious bronchitis virus isolates by the neutralization of immunofluorescent FOCI. Avian Pathol. 17:139-148. 1988.

50. Cumming, R. B. The control of avian infectious bronchitis/nephrosis in Australia. Aus. Vet. J. 45:200-203. 1969.

51. Cunningham, C. H., and H. O. Stuart. Cultivation of the virus of infectious bronchitis of chickens in embryonated chicken eggs. Am. J. Vet. Res. 8:209-212. 1947.

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

53. Davelaar, F. G., and B. Kouwenhoven. Vaccination of 1 day old broilers against infectious bronchitis by eye drop application or coarse droplet spray and the effect of revaccination by spray. Avian Pathol. 9:499-510. 1980.

54. Davelaar, F. G., A. Noordzij, and J. A. van der Donk. A study on the synthesis and secretion of immunoglobulins by the Harderian gland of the fowl after eyedrop vaccination against infectious bronchitis at 1 day old. Avian Pathol. 11:63-79. 1982.

55. De Groot, R. J., W. Luytjes, M. C. Horzinek, B. A. M. Van der Zeijst, W. J. M. Spaan, and J. A. Lenstra. Evidence for a coiled-coil structure in the spike proteins of coronaviruses. J. Molec. Biol. 196:963-966. 1987.

56. De Wit, J. J. Detection of infectious bronchitis virus. Avian Pathol. 29:71-93. 2000.

57. Domingo, E., E. Baranowski, C. M. Ruiz-Jarabo, A. M. Martín-Hernández, J. C. Sáiz, and C. Escarmís. Quasispecies structure and persistence of RNA viruses. Emerg. Inf. Dis. 4:521. 1998.

58. Enjuanes, L., D. Brian, D. Cavanagh, K. Holmes, M. M. C. Lai, H. Laude, P. Masters, P. Rottier, S. G. Siddell, W. J. M. Spaan, F. Taguchi, and P. Talbot. Coronaviridae. In: Virus Taxonomy, Classification and Nomenclature of Viruses. M. A. Murphy, C. M. Fauquet, D. H. L. Bishop, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo and M. D. Summers, eds. Academic Press, New York. pp 835–849. 2000.

59. Eterradossi, N., and Y. M. Saif. Infectious bursal disease. In: Diseases of Poultry. Y. M. Saif, ed. Blackwell Publishing, Ames IA. pp. 185-208. 2008.

60. Fabricant, J. Studies on the diagnosis of Newcastle disease and infectious bronchitis of fowls. II. The diagnosis of infectious bronchitis by virus isolation in chick embryos. Cornell Vet. 39:414-431. 1949.

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

62. Fang, S. G., S. Shen, F. P. L. Tay, and D. X. Liu. Selection of and recombination between minor variants lead to the adaptation of an avian coronavirus to primate cells. Biochem. Bioph. Res. Comm. 336:417-423. 2005.

63. Farsang, A., C. Ros, L. H. M. Renström, C. Baule, T. Soos, and S. Belak. Molecular epizootiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain. Avian Pathol. 31:229-236. 2002.

64. Fazakerley, J. K., S. E. Parker, F. Bloom, and M. J. Buchmeier. The V5A13. 1 envelope glycoprotein deletion mutant of mouse hepatitis virus type-4 is neuroattenuated by its reduced rate of spread in the central nervous system. Virology 187:178-188. 1992.

65. Gallagher, T. M., and M. J. Buchmeier. Coronavirus spike proteins in viral entry and pathogenesis. Virology 279:371-374. 2001.

66. Gallardo, R. A., V. L. van Santen, and H. E. Toro. Host intraspatial selection of infectious bronchitis virus populations. Avian Dis. 54:807-813. 2010.

67. Gelb Jr, J., 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.

68. Giambrone, J. J., J. P. Donahoe, D. L. Dawe, and C. S. Eidson. Specific suppression of the bursa-dependent immune system of chicks with infectious bursal disease virus. Am. J. Vet. Res. 38:581. 1977.

69. Glahn, R. P., R. F. Wideman Jr, and B. S. Cowen. Order of exposure to high dietary calcium and Gray strain infectious bronchitis virus alters renal function and the incidence of urolithiasis. Poult. Sci. 68:1193. 1989.

70. Guo, X., A. J. M. Rosa, D. G. Chen, and X. Wang. Molecular mechanisms of primary and secondary mucosal immunity using avian infectious bronchitis virus as a model system. Vet. Immunol. Immunopathol. 121:332-343. 2008.

71. Han, Z., C. Sun, B. Yan, X. Zhang, Y. Wang, C. Li, Q. Zhang, Y. Ma, Y. Shao, and Q. Liu. A 15-year analysis of molecular epidemiology of avian infectious bronchitis coronavirus in China. Infect. Gen. Evol. 2010.

72. Handberg, K. J., and O. L. Nielsen Detection and strain differentiation of infectious bronchitis virus in tracheal tissues from. Avian Pathol. 28:327. 1999.

73. Hesselink, W. G. Serotyping avian infectious bronchitis virus: selection of a unified method. In: Second Int. Symp. Inf. Bronchitis. E. F. Kaleta and U. Heffels-Redmann, eds., Ruischholzhausen, Germany. pp 87–97. 1991.

74. Hingley, S. T., J. L. Gombold, E. Lavi, and S. R. Weiss. MHV-A59 fusion mutants are attenuated and display altered hepatotropism. Virology 200:1-10. 1994.

75. Hoerr, F. J. Clinical Aspects of Immunosuppression in Poultry. Avian Dis. 54:2-15. 2010.

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

77. Hopkins, S. R., and C. W. Beard. Studies on methods for determining the efficacy of oil-emulsion vaccines against infectious bronchitis virus. J. Am. Vet. Med. Assoc 187:305. 1985.

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

79. Huang, Y. P., and C. H. Wang. Sequence changes of infectious bronchitis virus isolates in the 3 7.3 kb of the genome after attenuating passage in embryonated eggs. Avian Pathol. 36:59-67. 2007.

80. Ignjatovic, J., and L. Galli. Immune responses to structural proteins of avian infectious bronchitis virus In: Avian Immunology in progress. F. Coudert, ed. INRA, Paris. pp 237-242. 1993.

81. Imai, K., M. Mase, K. Tsukamoto, H. Hihara, and N. Yuasa. Persistent infection with chicken anaemia virus and some effects of highly virulent infectious bursal disease virus infection on its persistency. Res. Vet. Sci. 67:233-238. 1999.

82. Jackson, U. H., D. A. Boltz, M. Nakai, G. Scherba, D. Bunick, and J. M. Bahr. Prepubertal exposure to the live attenuated avian infectious bronchitis virus induces epididymal stones in the rooster after puberty. Poult. Sci. 43:280-285. 2006.

83. Jackwood, M. W., D. A. Hilt, and S. A. Callison. Detection of infectious bronchitis virus by real-time reverse transcriptase-polymerase chain reaction and identification of a quasispecies in the Beaudette strain. Avian Dis. 47:718-724. 2003.

84. Jackwood, M. W., D. A. Hilt, C. W. Lee, H. M. Kwon, S. A. Callison, K. M. Moore, H. Moscoso, H. Sellers, and S. Thayer. Data from 11 years of molecular typing infectious bronchitis virus field isolates. Avian Dis. 49:614-618. 2005.

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

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

87. Jenkins, G. M., A. Rambaut, O. G. Pybus, and E. C. Holmes. Rates of molecular evolution in RNA viruses: a quantitative phylogenetic analysis. J. Molec. Evol. 54:156-165. 2002.

88. Jungherr, E. L., T. W. Chomiak, and R. E. Luginbuhl. Immunologic differences in strains of infectious bronchitis virus. In: Proc. 60th Ann. Meet. U.S. Livestk. Sanit. Ass. pp 203-209. 1956.

89. 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. 1992.

90. Keeler Jr, C. L., 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.

91. Khatri, M., J. M. Palmquist, R. M. Cha, and J. M. Sharma. Infection and activation of bursal macrophages by virulent infectious bursal disease virus. Virus Res. 113:44-50. 2005.

92. Kilbourne, E. D. Influenza, 1st ed. Plenum Medical Book Company, New York. 1987.

93. Kilbourne, E. D. Host determination of viral evolution: a variable tautology In: The evolutionary biology of viruses. S. S. Morse, ed. Raven Press Ltd., New York. pp 253-271. 1994.

94. Kim, I. J., and J. M. Sharma. IBDV-induced bursal T lymphocytes inhibit mitogenic response of normal splenocytes. Vet. Immunol. Imunopath. 74:47-57. 2000.

95. Klemola, T., E. Savilahti, A. Arato, T. Ormälä, J. Partanen, C. Eland, and S. Koskimies. Immunohistochemical findings in jejunal specimens from patients with IgA deficiency. Gut 37:519. 1995.

96. 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. 1990.

97. Koch, G., and A. Kant. Binding of antibodies that strongly neutralise infectious bronchitis virus is dependent on the glycosylation of the viral peplomer protein. Adv. Exp. Med. Biol. 1990.

98. Koch, G., and A. Kant. Nucleotide and amino acid sequence of the S1 subunit of the spike glycoprotein of avian infectious bronchitis virus, strain D3896. Nucl. Acids Res. 18:3063. 1990.

99. Kuo, L., G. J. Godeke, M. J. B. Raamsman, P. S. Masters, and P. J. M. Rottier. Retargeting of coronavirus by substitution of the spike glycoprotein ectodomain: crossing the host cell species barrier. J. Virol. 74:1393. 2000.

100. Kusters, J. G., E. J. Jager, J. A. Lenstra, G. Koch, W. P. Posthumus, R. H.

Meloen, and B. A. van der Zeijst. Analysis of an immunodominant region of infectious bronchitis virus. J. Immunol. 143:2692. 1989.

101. Kusters, J. G., E. J. Jager, H. G. M. Niesters, and B. A. M. Van Der Zeijst. Sequence evidence for RNA recombination in field isolates of avian coronavirus infectious bronchitis virus. Vaccine 8:605-608. 1990.

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

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

104. 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.

105. Ladman, B. S., A. B. Loupos, and J. Gelb. Infectious bronchitis virus S1 gene sequence comparison is a better predictor of challenge of immunity in chickens than serotyping by virus neutralization. Avian Pathol. 35:127-133. 2006.

106. Lee, C. W. Evolution of avian infectious bronchitis virus: genetic drift and recombination. Korean J. Vet. Serv. 25:97-103. 2002.

107. Leparc-Goffart, I., S. T. Hingley, M. M. Chua, X. Jiang, E. Lavi, and S. R. Weiss. Altered Pathogenesis of a Mutant of the Murine Coronavirus MHV-A59 Is Associated with a Q159L Amino Acid Substitution in the Spike Protein. Virology 239:1-10. 1997.

108. Li, H., and H. Yang. Sequence analysis of nephropathogenic infectious bronchitis virus strains of the Massachusetts genotype in Beijing. Avian Pathol. 30:535-541. 2001.

109. Li, W., C. Zhang, J. Sui, J. H. Kuhn, M. J. Moore, S. Luo, S. K. Wong, I. C. Huang, K. Xu, and N. Vasilieva. Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2. EMBO J. 24:1634-1643. 2005.

110. Lin, Z., A. Kato, Y. Kudou, and S. Ueda. A new typing method for the avian

infectious bronchitis virus using polymerase chain reaction and restriction enzyme fragment length polymorphism. Arch. Virol. 116:19-31. 1991.

111. Liu, S. W., Q. X. Zhang, J. D. Chen, Z. X. Han, X. Liu, L. Feng, Y. H. Shao, J. G. Rong, X. G. Kong, and G. Z. Tong. Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. Arch. Virol. 151:1133-1148. 2006.

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

113. Markowski Grimsrud, C. J., and K. A. Schat. Infection with chicken anaemia virus impairs the generation of pathogen specific cytotoxic T lymphocytes. Immunology 109:283-294. 2003.

114. Martin, J., G. Dunn, R. Hull, V. Patel, and P. D. Minor. Evolution of the Sabin strain of type 3 poliovirus in an immunodeficient patient during the entire 637-day period of virus excretion. J. Virol. 74:3001. 2000.

115. Masters, P. S. The molecular biology of coronaviruses. Adv. Virus Res. 66:193-292. 2006.

116. Matthijs, M. G. R., J. H. H. van Eck, J. J. De Wit, A. Bouma, and J. A. Stegeman. Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with a virulent Massachusetts-type IBV strain. Avian Dis. 49:540-545. 2005.

117. Mayr, E. Toward a new philosophy of biology: Observations of an evolutionist. Harvard University Press, Cambridge, Mass. 1988.

118. Mc Nulty, M. S., and D. Todd. Chicken anemia virus. In: Isolation, identification, and characterization of avian pathogens 5th ed. L. Dufour-Zavala, ed. The American Association of Avian Pathologists Athens, GA. pp 124-127. 2008.

119. McKinley, E. T., D. A. Hilt, and M. W. Jackwood. Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. Vaccine 26:1274-1284. 2008.

120. McKinley, E. T., M. W. Jackwood, D. A. Hilt, J. C. Kissinger, J. S. Robertson, C. Lemke, and A. H. Paterson. Attenuated live vaccine usage affects accurate measures of virus diversity and mutation rates in avian coronavirus infectious bronchitis virus. Virus Res. 158:225-234. 2011.

121. McMartin, D. A. Infectious Bronchitis. In: Virus infections of vertebrates. J. B. McFerran and M. S. McNulty, eds. Elsevier Sci. Publ., Amsterdam, the Netherlans. pp 249-275. 1993.

122. Mockett, A. P., D. Cavanagh, and T. D. K. Brown. Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41. J. Gen. Virol. 65:2281. 1984.

123. Mockett, A. P. A., and J. K. A. Cook. The detection of specific IgM to infectious bronchitis virus in chicken serum using an ELISA. Avian Pathol. 15:437-446. 1986.

124. Moore, M. J., and P. A. Silver. Global analysis of mRNA splicing. RNA 14:197. 2008.

125. Nakamura, K., J. K. A. 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 immunohistochemistry. Avian Pathol. 20:241-257. 1991.

126. Nix, W. A., D. S. Troeber, B. F. Kingham, C. L. Keeler Jr, and J. Gelb Jr. Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Dis.44:568-581. 2000.

127. Ontiveros, E., T. S. Kim, T. M. Gallagher, and S. Perlman. Enhanced virulence mediated by the murine coronavirus, mouse hepatitis virus strain JHM, is associated with a glycine at residue 310 of the spike glycoprotein. J. Virol. 77:10260. 2003.

128. Otsuki, K., M. B. Huggins, and J. K. A. Cook. Comparison of the susceptibility to avian infectious bronchitis virus infection of two inbred lines of white leghorn chickens. Avian Pathol. 19:467-475. 1990.

129. Otsuki, K., T. Nakamura, N. Kubota, Y. Kawaoka, and M. Tsubokura. Comparison of two strains of avian infectious bronchitis virus for their interferon induction, viral growth and development of virus-neutralizing antibody in

experimentally-infected chickens. Vet. Microbiol. 15:31-40. 1987.

130. Pasternak, A. O., W. J. M. Spaan, and E. J. Snijder. Nidovirus transcription: how to make sense…? J. Gen. Virol. 87:1403. 2006.

131. 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.

132. Pejkovski, C., F. G. Davelaar, and B. Kouwenhoven. Immunosuppressive effect of infectious bursal disease virus on vaccination against infectious bronchitis. Avian Pathol. 8:95-106. 1979.

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

134. Peters, M. A., B. S. Crabb, E. A. Washington, and G. F. Browning. Site-directed mutagenesis of the VP2 gene of chicken anemia virus affects virus replication, cytopathology and host-cell MHC class I expression. J. Gen. Virol. 87:823. 2006.

135. Phillips, J. J., M. M. Chua, E. Lavi, and S. R. Weiss. Pathogenesis of chimeric MHV4/MHV-A59 recombinant viruses: the murine coronavirus spike protein is a major determinant of neurovirulence. J. Virol. 73:7752. 1999.

136. Phillips, J. J., M. M. Chua, G. F. Rall, and S. R. Weiss. Murine coronavirus spike glycoprotein mediates degree of viral spread, inflammation, and virus-induced immunopathology in the central nervous system. Virology 301:109-120. 2002.

137. Pope, C. R. Lymphoid system. In: Avian Histopathology. C. Riddell, ed. AAAP, New Bolton Center. pp 17-44. 1996.

138. Posthumus, W. P., J. A. Lenstra, W. M. Schaaper, A. P. Van Nieuwstadt, L. Enjuanes, and R. H. Meloen. Analysis and simulation of a neutralizing epitope of transmissible gastroenteritis virus. J. Virol. 64:3304. 1990.

139. Posthumus, W. P., R. H. Meloen, L. Enjuanes, I. Correa, A. P. van Nieuwstadt, G. Kock, R. J. De Groot, J. G. Kusters, W. Luytjes, W. J. M. Spaan, B. A. van der Zeijst, and J. A. lenstra. Linear neutralizing epitopes on the peplomer proteins of coronavirus. .

In: Coronaviruses and their diseases. D. Cavanagh and T. D. K. Brown, eds. Plenum Press, New York. pp 181-188. 1990.

140. Raggi, L. G., and G. G. Lee. Lack of correlation between infectivity, serologic response and challenge results in immunization with an avian infectious bronchitis vaccine. J. Immunol. 94:538. 1965.

141. Raj, G. D., and R. C. Jones. Infectious bronchitis virus: immunopathogenesis of infection in the chicken. Avian Pathol. 26:677-706. 1997.

142. Rasschaert, D., and H. Laude. The predicted primary structure of the peplomer protein E2 of the porcine coronavirus transmissible gastroenteritis virus. J. Gen. Virol. 68:1883. 1987.

143. Rocha, E., N. J. Cox, R. A. Black, M. W. Harmon, C. J. Harrison, and A. P. Kendal. Antigenic and genetic variation in influenza A (H1N1) virus isolates recovered from a persistently infected immunodeficient child. J. Virol. 65:2340. 1991.

144. Rosenberger, J. K., and J. Gelb, Jr. Response to several avian respiratory viruses as affected by infectious bursal disease virus. Avian Dis. 22:95-105. 1978.

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

146. Schat, K. A., and M. A. Skinner. Avian immunosuppressive diseases. In: Avian Immunology, 1st ed. T. F. Davison, B. Kaspers and K. A. Schat, eds. Elsevier, Academic Press., San Diego CA. pp 299-322. 2008.

147. Schat, K. A., and V. L. van Santen. Chicken anemia virus and other circovirus infections. In: Diseases of Poultry. A. M. Fadly, ed. Blackwell Publ. Ames IA. pp 211-235. 2008.

148. Schmidt, I., M. Skinner, and S. Siddell. Nucleotide sequence of the gene encoding the surface projection glycoprotein of coronavirus MHV-JHM. J. Gen. virol. 68:47. 1987.

149. Schultze, B., D. Cavanagh, and G. Herrler. Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes. Virology 189:792-794. 1992.

150. 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. 1997.

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

152. Sharma, J. M., K. Karaca, and T. Pertile Virus-induced immunosuppression in chickens. Poult. Sci. 73:1082. 1994.

153. Sharma, J. M., I. J. Kim, S. Rautenschlein, and H. Y. Yeh. Infectious bursal disease virus of chickens: pathogenesis and immunosuppression. Dev. Comp. Immunol. 24:223-235. 2000.

154. Smyth, J. A., D. A. Moffett, M. S. McNulty, D. Todd, and D. P. Mackie. A sequential histopathologic and immunocytochemical study of chicken anemia virus infection at one day of age. Avian Dis. 37:324-338. 1993.

155. Stadelman, W. J. Quality identification of shell eggs. In: Egg Science and Technology, Third ed. W. J. Stadelman and O. J. Cotterill, eds. Food Products Press, New York. pp 37-63. 1990.

156. 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. 1982.

157. Stern, D. F., and S. I. Kennedy. Coronavirus multiplication strategy. II. Mapping the avian infectious bronchitis virus intracellular RNA species to the genome. J. Virol. 36:440. 1980.

158. Thompson, G., H. Mohammed, B. Bauman, and S. Naqi. Systemic and local antibody responses to infectious bronchitis virus in chickens inoculated with infectious bursal disease virus and control chickens. Avian Dis.:519-527. 1997.

159. Tomley, F. M., A. P. Mockett, M. E. Boursnell, M. M. Binns, J. K. Cook, T. D. Brown, and G. L. Smith. Expression of the infectious bronchitis virus spike protein by recombinant vaccinia virus and induction of neutralizing antibodies in vaccinated mice. J. Gen. Virol. 68:2291. 1987.

160. Toro, H., and I. Fernandez. Avian infectious bronchitis: specific lachrymal IgA level and resistance against challenge. J.Vet. Med. 41:467-472. 1994.

161. Toro, H., V. Godoy, J. Larenas, E. Reyes, and E. F. Kaleta. Avian infectious bronchitis: viral persistence in the Harderian gland and histological changes after eyedrop vaccination. Avian Dis. 40:114-120. 1996.

162. Toro, H., P. Lavaud, P. Vallejos, and A. Ferreira. Transfer of IgG from serum to lachrymal fluid in chickens. Avian Dis. 37:60-66. 1993.

163. Toro, H., A. M. Ramirez, and J. Larenas. Pathogenicity of chicken anaemia virus (isolate 10343) for young and older chickens. Avian Pathol. 26:485-499. 1997.

164. Toro, H., B. Schemera, and E. F. Kaleta. Serological differentiation of avian infectious bronchitis field isolates using an enzyme immunoassay: presence of Dutch strains in West Germany. Avian Dis.:187-192. 1987.

165. Toro, H., V. L. van Santen, F. J. Hoerr, and C. Breedlove. Effects of chicken anemia virus and infectious bursal disease virus in commercial chickens. Avian Dis. 53:94-102. 2009.

166. Toro, H., V. L. van Santen, L. Li, S. B. Lockaby, E. van Santen, and F. J. Hoerr. Epidemiological and experimental evidence for immunodeficiency affecting avian infectious bronchitis. Avian Pathol. 35:455-464. 2006.

167. van Eck, J. H. Effects of experimental infection of fowl with EDS'76 virus, infectious bronchitis virus and/or fowl adenovirus on laying performance. Vet. Quart. 5:11. 1983.

168. van Ginkel, F. W., V. L. van Santen, S. L. Gulley, and H. Toro. Infectious bronchitis virus in the chicken Harderian gland and lachrymal fluid: viral load, infectivity, immune cell responses, and effects of viral immunodeficiency. Avian Dis. 52:608-617. 2008.

169. van Roeckel, H., K. L. Bullis, O. S. Flint, and M. K. Clarke. Poultry disease control service Massachusetts Agricultural Experiment Station, MA. Annual Report. Bulletin. 388:99-103. 1942.

170. van Santen, V. L., K. S. Joiner, C. Murray, N. Petrenko, F. J. Hoerr, and H. Toro. Pathogenesis of chicken anemia virus: comparison of the oral and the intramuscular routes of infection. Avian Dis. 48:494-504. 2004.

171. van Santen, V. L., and H. Toro. Rapid selection in chickens of subpopulations within ArkDPI-derived infectious bronchitis virus vaccines. Avian Pathol. 37:293-306. 2008.

172. Villarreal, L. Y. B., P. E. Brandao, J. L. Chacon, M. S. Assayag, P. C. Maiorka, P. Raffi, A. B. S. Saidenberg, R. C. Jones, and A. J. P. Ferreira. Orchitis in roosters with reduced fertility associated with avian infectious bronchitis virus and avian metapneumovirus infections. Avian Dis. 51:900-904. 2007.

173. Wang, H. N., Q. Z. Wu, Y. Huang, and P. Liu. Isolation and identification of infectious bronchitis virus from chickens in Sichuan, China. Avian Dis. 41:279-282. 1997.

174. Wang, Y. D., Y. L. Wang, Z. C. Zhang, G. C. Fan, Y. H. Jiang, X. E. Liu, J. Ding, and S. S. Wang. Isolation and identification of glandular stomach type IBV (QX IBV) in chickens. Chin. J. Anim. Quarantine. 15:1–3. 1998.

175. Wesley, R. D., R. D. Woods, and A. K. Cheung. Genetic analysis of porcine respiratory coronavirus, an attenuated variant of transmissible gastroenteritis virus. J. Virol. 65:3369. 1991.

176. Wesseling, J. G., G. J. Godeke, V. E. C. J. Schijns, L. Prevec, F. L. Graham, M. C. Horzinek, and P. J. M. Rottier. Mouse hepatitis virus spike and nucleocapsid proteins expressed by adenovirus vectors protect mice against a lethal infection. J. Gen. Virol. 74:2061-2069. 1993.

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

178. Winterfield, R. W., F. J. Hoerr, and A. M. Fadly. Vaccination against infectious bronchitis and the immunosuppressive effects of infectious bursal disease. Poult. Sci. 57:386. 1978.

179. Wu, G., and S. Yan. Reasoning of spike glycoproteins being more vulnerable to mutations among 158 coronavirus proteins from different species. J. Molec. Mod. 11:8-16. 2005.

180. Yuasa, N., T. Taniguchi, T. Noguchi, and I. Yoshida. Effect of infectious bursal disease virus infection on incidence of anemia by chicken anemia agent. Avian Dis. 24:202-209. 1980.

181. Zhang, X., M. Hasoksuz, D. Spiro, R. Halpin, S. Wang, A. Vlasova, D. Janies, L. R. Jones, E. Ghedin, and L. J. Saif. Quasispecies of bovine enteric and respiratory coronaviruses based on complete genome sequences and genetic changes after tissue culture adaptation. Virology 363:1-10. 2007.

182. Ziegler, A. F., B. S. Ladman, P. A. Dunn, A. Schneider, S. Davison, P. G. Miller, H. Lu, D. Weinstock, M. Salem, and R. J. Eckroade. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Dis. 46:847-858. 2002.

Appendix I: Tables

Table 1.1. Primers used for amplification by RT-PCR and sequencing of a portion of the S1 gene.

Name	Sequence	Orientation	Position[A]	Ref.
NEWS1OLIGO5'	5'-TGA AAC TGA ACA AAA GAC-3'	forward	-66 - -48	(85)
S1OLIGO3'	5'-CAT AAC TAA CAT AAG GGC AA-3'	reverse	1675-1656	(104)
S1R	5'-CAT CTG AAA AAT TGC CAG-	reverse	742-725	(171)
S17F	5'-TGA AAA CTG AAC AAA AGA CCG ACT TAG-3'	forward	-66 - -40	(66)
S18R	5'-GGA TAG AAG CCA TCT GAA AAA TTG C-3'	reverse	752-728	

[A] Position in Ark vaccine S-coding sequence.

Table 1.2. S1 gene sequences obtained from tissues or tear fluids of 24 chickens on days 6, 9 and 20 after inoculation.

	Day 6	Day 9	Day 20
	Number of sequences		
Tear fluid	23	21	4
Trachea	18	14	3
Oviduct / testis	8	0	1
Cecal tonsil	2	0	0
Kidney	1	0	1

Table 1.3. Differences in amino acids encoded by S1 gene of Ark-type IBV parent strain and predominant populations detected in tissues of vaccinated chickens.

	nucleotide position	127	167	226	355	388	511	593	637	
	amino acid position	43	56	76	119	130	171	198	213	
Designation										GenBank accession number[A]
Vaccine Parent	nucleotide	T	A	C	T	A	T	A	T	EU359644
	amino acid	Tyr	Asn	Leu	Ser	Ser	Tyr	Lys	Ser	
C1[B]	nucleotide	C	A	C	T	G	T	A	G	EU359643
	amino acid	His	Asn	Leu	Ser	Gly	Tyr	Lys	Ala	
C2	nucleotide	C	A	C	T	A	T	A	G	EU359650
	amino acid	His	Asn	Leu	Ser	Ser	Tyr	Lys	Ala	
C3	nucleotide	C	A	T	C	A	C	C	G	EU359626
	amino acid	His	Asn	Phe	Pro	Ser	His	Thr	Ala	
C4	nucleotide	C	G	C	T	A	T	A	G	GQ484957
	amino acid	His	Ser	Leu	Ser	Ser	Tyr	Lys	Ala	
C5	nucleotide	T	A	C	T	A	T	A	G	GQ484958
	amino acid	Tyr	Asn	Leu	Ser	Ser	Tyr	Lys	Ala	

[A] The complete S1 or S sequences in GenBank were obtained from studies independent from the present one. The sequences in the present study match the GenBenk sequences in nucleotide positions 1-724. Sequences beyond nucleotide 724 were not determined in the present study.

[B] Selected populations were designated component (C) 1 through C5.

Table 2.1. Ranking of each subpopulation based on their incidence (high to low) in immunocompetent or immunodeficient (IBDV, CAV, CAV+IBDV infected) chickens in experiment 1 and 2.

	Experiment 1	**Experiment 2**
Immunocompetent	C1[A] > C4 > C5	C4 > C1
IBDV	C1; C4; C2 > C5	C4 > C2 > C1
CAV	C1; C4 > C2; C5	C4 > C1 > C2
CAV+IBDV	C4 > C5 > C1; Vx[B]	C4; C1 > C5 > C2

[A]**C1:** Selected subpopulations, designated component (C) 1 through C5.
[B]**Vx:** Vaccine subpopulation.

Appendix II: Figures

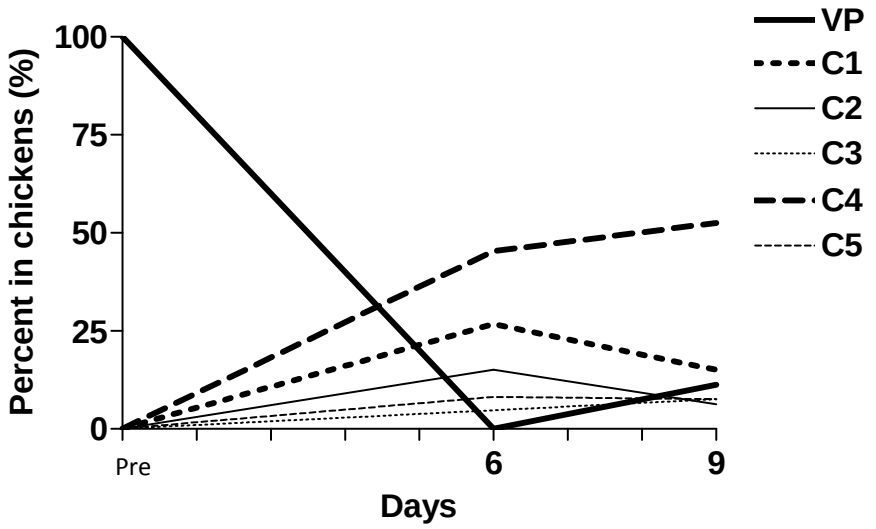

Fig. 1.1. Incidence of IBV subpopulations in chickens (all tissues) by day after inoculation. The vaccine predominant phenotype (VP) prior to inoculation (Pre) is rapidly negatively selected after replication in the host. Other subpopulations designated component (C) 1 through C5 became predominant in chicken tissues and tear fluid. C4 and C1 were the most frequent subpopulations found on days 6 and 9 after inoculation.

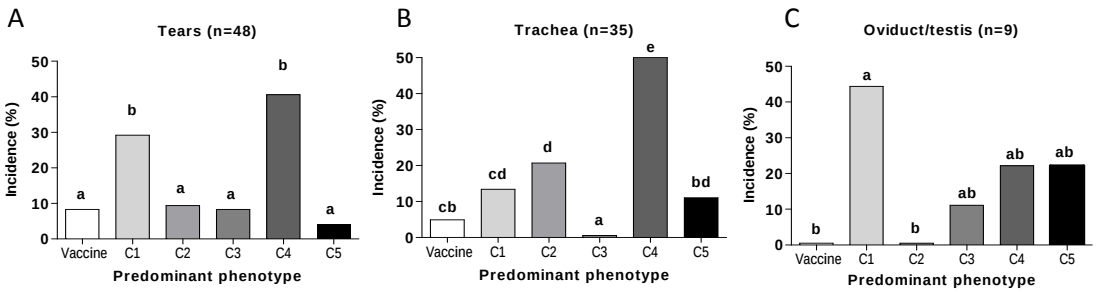

Fig. 1.2. Incidence of IBV subpopulations (bars) in tear fluid (A), trachea (B), or reproductive tract (C) in chickens after inoculation (considering all days tested) with an Ark-type vaccine strain. The number of samples containing distinct S1 gene sequences was compared by Chi-squared test. Significant differences (P<0.05) are indicated by different letters above bars.

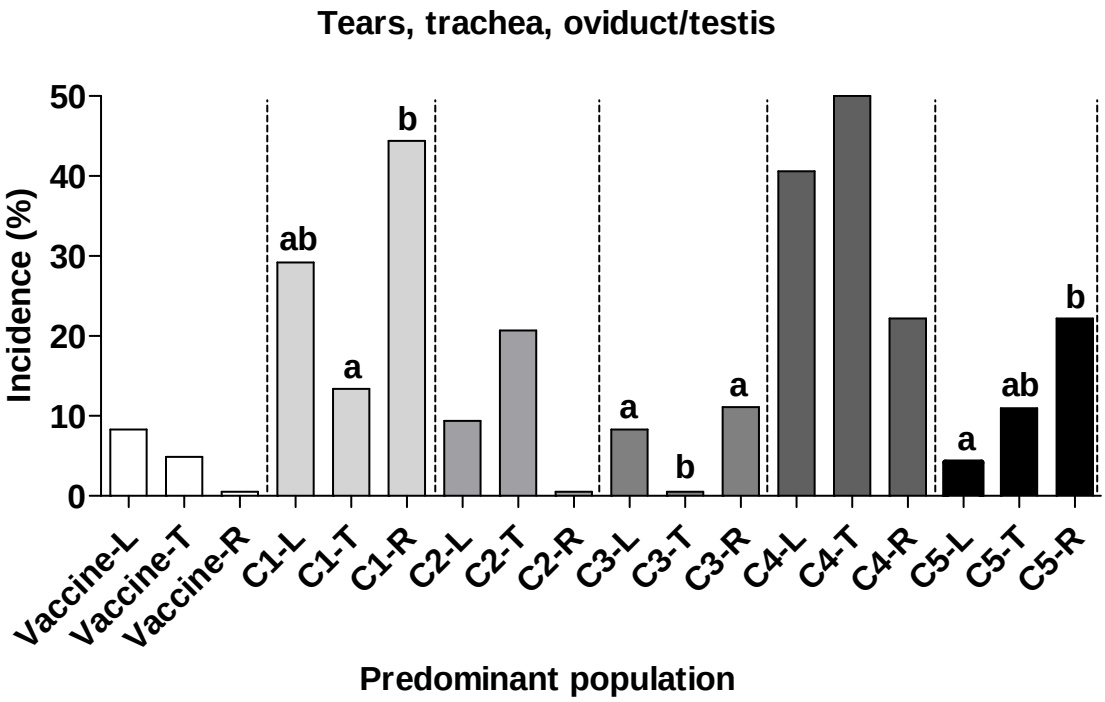

Fig. 1.3. Incidences of IBV subpopulations (bars) in tear fluid (-L), trachea (-T), or reproductive tract (-R) within chickens after inoculation (considering all days tested) with an Ark-type vaccine strain. The number of samples containing distinct S1 gene sequences was compared by Chi-squared test. Significant differences ($P<0.05$) are indicated by different letters above bars.

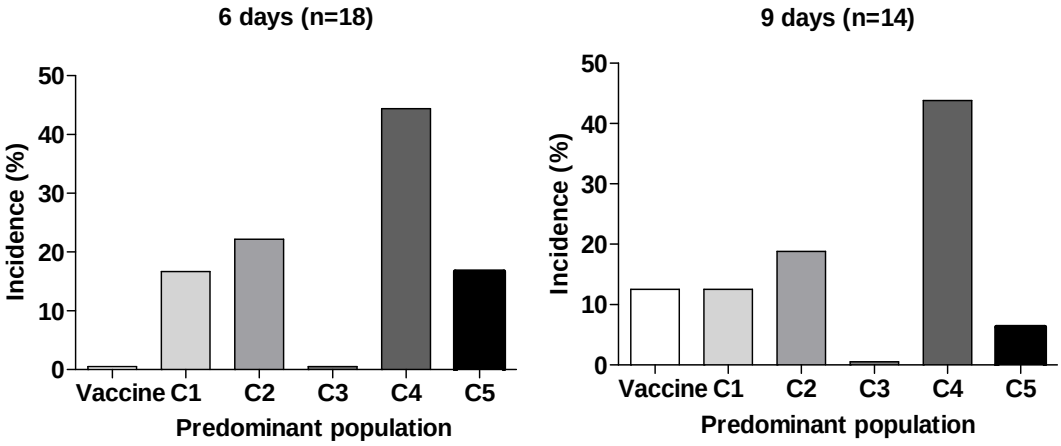

Fig. 1.4. Incidence of IBV subpopulations (bars) in the tracheas chickens at 6 and 9 days after inoculation with an Ark-type vaccine strain. Incidences of the predominant phenotypes in the tracheas on the two days were compared by Chi-squared test. No significant differences were detected between days 6 and 9.

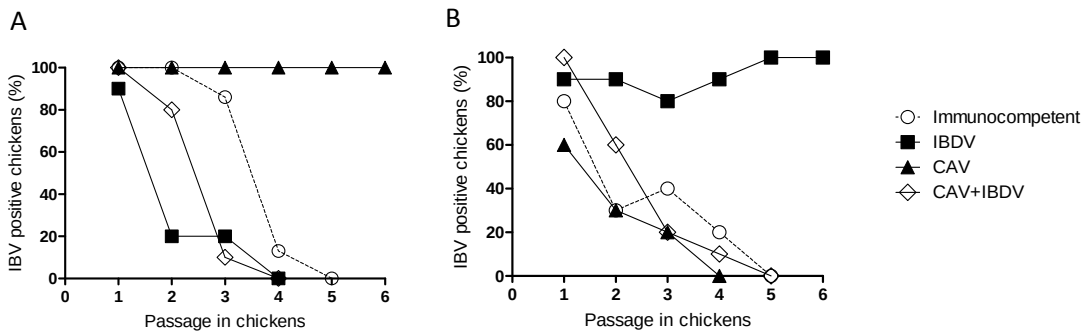

Fig. 2.1. Percent of IBV positive chickens after serial passages in immunocompetent or previously inoculated chickens (n=10/group) at 7 ds of age with CAV and/or IBDV. All chickens were inoculated with the ArkDPI-derived IBV vaccine strain on day 15 of age. 8 days after IBV inoculation tears were collected from all chickens of each group for S1 RT-PCR detection and subsequent ocular passaging. **A.** Immunocompetent, CAV, IBDV, and CAV+IBDV chicken groups in experiment 1. **B.** Immunocompetent, CAV, IBDV, and CAV+IBDV chicken groups in experiment 2.

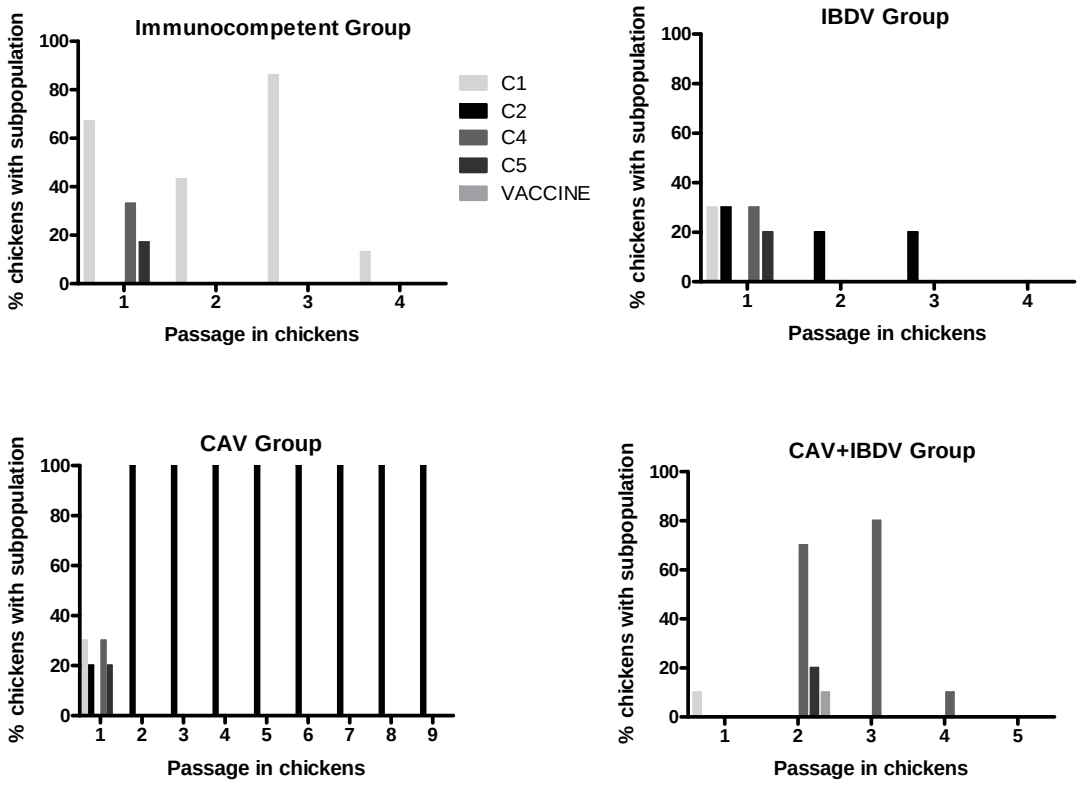

Fig. 2.2. Incidence of IBV subpopulations in immunocompetent, IBDV, CAV, and CAV+IBDV infected chicken groups after subsequent passages in experiment 1. During each passage, RNA was extracted from the collected tear samples for RT-PCR amplification of a portion of the IBV S1 gene to confirm the presence of IBV in the tears. The S1 cDNA obtained was sequenced to identify possible nucleotide changes in the S1 gene and sequence chromatograms were analyzed for the presence of subpopulation diversity.

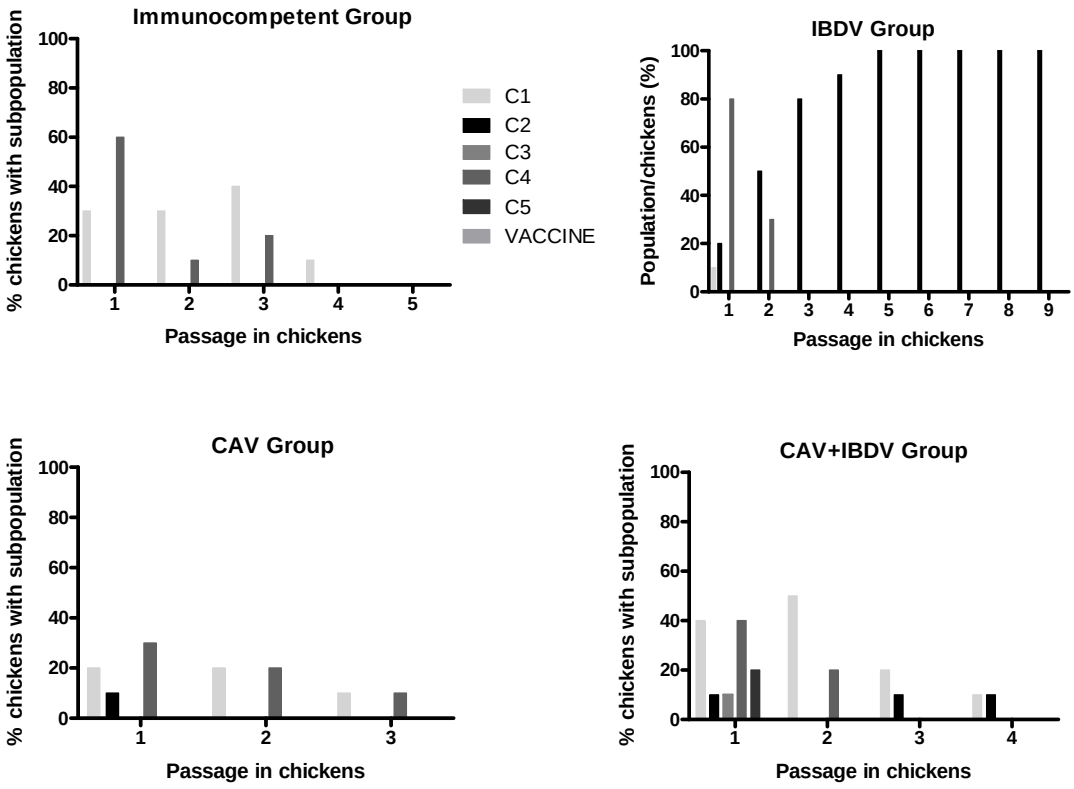

Fig. 2.3. Incidence of IBV subpopulations in immunocompetent, IBDV, CAV, and CAV+IBDV infected chicken groups after subsequent passages in experiment 2. During each passage, RNA was extracted from the collected tear samples for RT-PCR amplification of a portion of the IBV S1 gene to confirm the presence of IBV in the tears. The S1 cDNA obtained was sequenced to identify possible nucleotide changes in the S1 gene and sequence chromatograms were analyzed for the presence of subpopulation diversity.

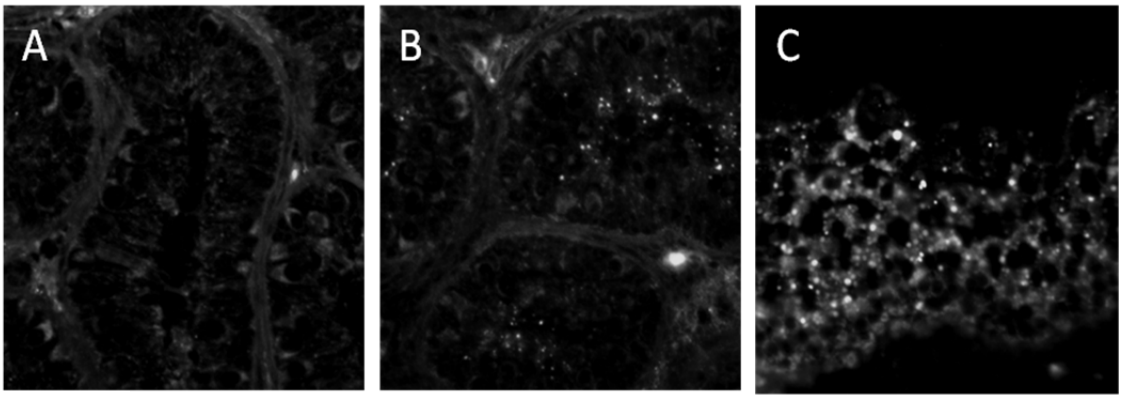

Fig. 3.1. IBV detection in the testes of 10-wk-old white leghorn males by immunofluorescence (IF) after IBV M41 or Ark inoculation via the ocular and nasal routes. (A) Seminiferous tubule of uninoculated control (40 X); (B) seminiferous tubule of males 7 days after M41 inoculation (40 X). IBV was detected in spermatogonias and Sertoli cells. (C) seminiferous tubule of males 7 days after Ark inoculation (60 X). IF staining is seen in the cell cytoplasm.

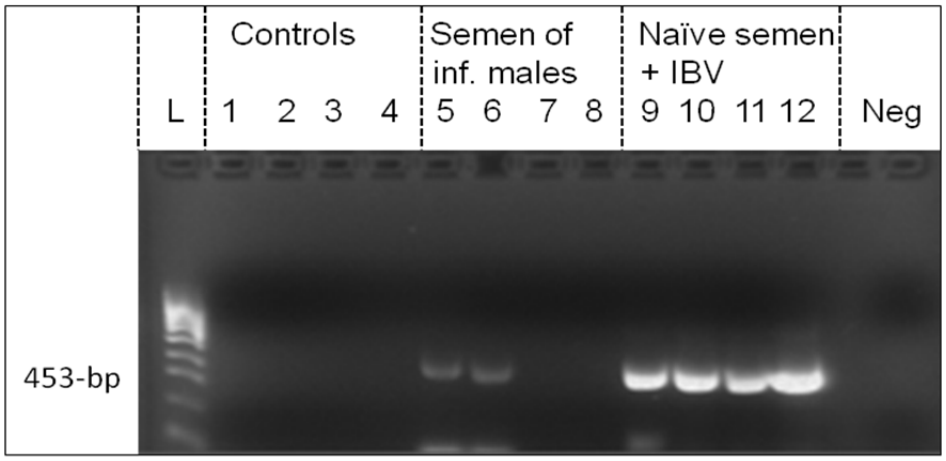

Fig. 3.2. IBV RNA detection by RT-PCR (N gene) in the trachea of hens artificially inseminated either with semen from uninfected males (lanes 1-4), IBV infected males (lanes 5-8), or naïve semen spiked with IBV M41 (lanes 9-12).

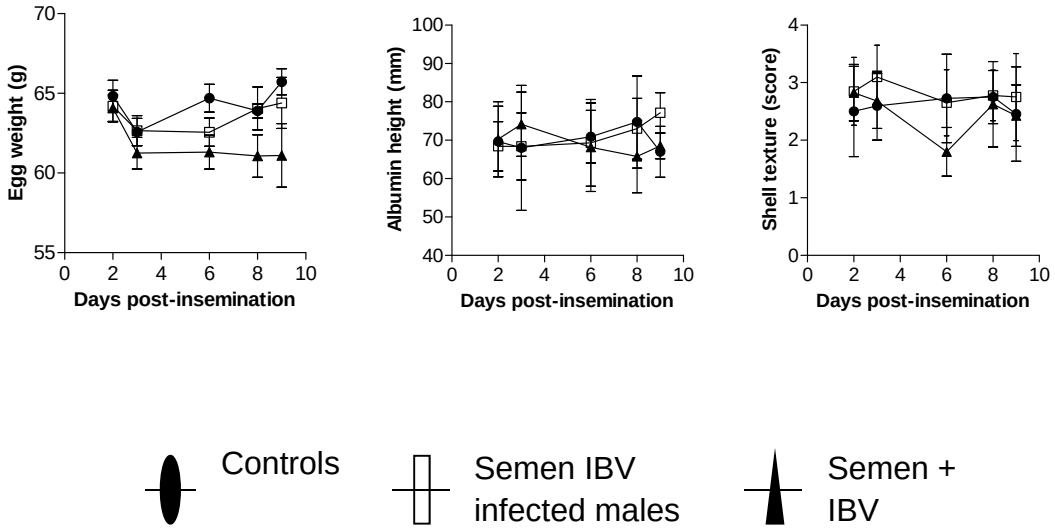

Fig. 3.3. Internal and external quality of eggs (78 per group) produced by white leghorn hens after insemination with either IBV spiked semen, semen from IBV infected males, or semen from uninfected controls. (A) Egg weight in grams (g); (B) albumin height in millimeters (mm); and (C) shell texture scoring [1 = rough through 5 = smooth]. Eggs produced by hens inseminated with IBV-spiked semen showed a significant reduction ($P<0.05$) of both shell smoothness and weight compared to controls and hens inseminated with semen from IBV-infected males.