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Workshop: Infectious Bronchitis (IB) in the Brazilian Poultry Industry

Infectious Bronchitis Virus: Dominance of ArkDPI-type Strains in the United States Broiler Industry During the Last Decade

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ABSTRACT

In the United States, more than 90% of chicken meat is produced in the southeastern states, and most egg production resides in the eastern half of the country and Texas. Several molecular epidemiological studies have indicated that most infectious bronchitis (IB) virus (IBV) isolates obtained from outbreaks of respiratory disease in these regions correspond to Ark-type IBV in spite of extensive vaccination programs which include IBV ArkDPI-derived vaccines. Accumulating evidence suggests that Ark-type strains may have a distinct capacity to circumvent preventive measures. Two strategies by which Ark-type IBV strains may maintain a high prevalence in commercial chickens are: (1) Unusually high genetic and phenotypic variability, and (2) synergism with concurrent viral immunodeficiency. Support for the first strategy includes epidemiological findings showing continued isolations of Ark-like viruses from respiratory disease affecting flocks vaccinated with serotype-specific homologous (ArkDPI-derived) vaccines, experimental data demonstrating selection of new predominant phenotypes occurring rapidly after a single passage in the host, and recent findings indicating changes of the predominant IBV population occurring within the host during the invasion process. The second strategy is supported by epidemiological data indicating increased isolations of Ark-type IBV showing minor geno-/phenotypic variation occurring in chickens simultaneously affected by immunosuppressive viruses. In addition, experimental results have shown that viral immunodeficiency leads to more severe and prolonged IB signs and lesions, delayed and reduced specific antibody responses, and increased and persistent IBV shedding. Finally, accumulating evidence confirms high genetic and phenotypic heterogeneity in commercial ArkDPI-derived vaccines. The rapid selection of new predominant phenotypes occurring in these vaccines may be facilitating the emergence of Ark-like strains. Thus, improvement of Ark-type vaccines and prevention of viral immunodeficiency's seem to be essential for an effective control of the disease.

INTRODUCTION

Infectious bronchitis (IB) virus (IBV), a member of the *Coronaviridae* family, is likely one of the most prevalent avian pathogens in the poultry industry worldwide. A mechanism to explain the prevalence was first reported in 1956 by Jungherr *et al*. (Jungherr *et al*., 1956). They demonstrated antigenic differences (sufficient to prevent cross-protection) between IBV isolates obtained in the states of Massachusetts and Connecticut. Using antibodies and different biological substrates (embryonated eggs, tracheal rings, cell cultures), numerous studies conducted into the late 1970s led to the recognition of numerous distinct IBV serotypes in the United States [e.g. (Hofstad, 1958; Hopkins, 1974;

Johnson & Marquardt, 1975)]. More recently, technologies to identify IBV such as reverse transcriptase polymerase chain reaction (RT-PCR) followed by restriction fragment length polymorphism (RFLP) (Kwon *et al*., 1993), S1 genotype-specific RT-PCR (Keeler *et al*., 1998), or nucleotide sequencing of the S1 gene (Kingham *et al*., 2000; Lee *et al*., 2003), have further demonstrated the unique capability of this virus to evolve and be maintained successfully in the environment of the poultry industry in spite of extensive control and preventive measures.

Ark-type IBV strains are highly prevalent in the U.S. poultry industry

In the United States, more than 90% of chicken meat (broiler) production occurs in the southeastern states and most of the egg production resides in the eastern half of the country and in the state of Texas. Based on reports of the Committee on Respiratory Diseases of the American Association of Avian Pathologists, most of IBV isolates obtained in 2009 from outbreaks of respiratory disease in the southeastern United States correspond to Ark-type IBV. Notably vaccination programs utilized in this region include ArkDPI (Delmarva Poultry Industry) -derived vaccines.

Surveillance studies performed at the University of Delaware concluded that most IBV isolates obtained between 1995 and 1997 in the Delmarva Peninsula were Ark-type IBV. The high number of Ark-type IBV isolates was unexpected as many integrated broiler companies used attenuated ArkDPI strains in their vaccination programs (Nix *et al*., 2000). Using RT-PCR and RFLP analysis of the IBV spike gene, Jackwood *et al*. (Jackwood *et al*., 2005) identified a total of 1,523 IBV isolates obtained between 1994 and 2004. They concluded that "by far, the Ark-DPI strain was the most frequently identified type of IBV and ranged from 23% to 65% of total isolations per year". Several Ark-like isolates also were identified every year with a tendency to increase toward 2004. Other IB viruses determined to be widespread during the 11 year period belonged to the Connecticut (Conn), DE072, and Massachusetts (Mass) serotypes. Moreover, an unusually high number of isolations in 1997 and 1999 coincided with the emergence of novel IBV variants in the State of Georgia (GAV and GA98 variant viruses),

Recently, Jackwood *et al*. (Jackwood *et al*., 2009) examined IBV vaccination in commercial broilers in the field. Different combinations of 2 to 3 IBV live vaccines were used including serotypes Mass, Conn, ArkDPI, GA98, and DE072. These authors evaluated the relative amount of IBV in tracheal swabs from birds from 10 different flocks and followed the clearance of the vaccine virus. Their findings indicated that 12% to 66% of birds were positive for IBV vaccine virus on days 21, 28 or 35 after vaccination. Most interestingly, only Ark-type viruses were recovered, indicating a unique persistence of Ark-type vaccine viruses in the chicken respiratory tract.

Collectively, these findings indicate that Ark-type IBV is currently the most relevant IBV type in the United States poultry industry. These findings also suggest that Ark-type IBV strains may have a distinct ability to circumvent preventive measures which allows them to consistently circulate in commercial poultry and cause outbreaks of disease. Finally, the increasing number of Ark-like viruses obtained from chickens vaccinated with ArkDPI-derived vaccines suggests that these vaccines might be contributing to the problem.

There is strong evidence supporting at least two important mechanisms by which Ark-type IBV strains succeed in the environment and consequently maintain a high prevalence in commercial chickens: (1) High genetic and phenotypic variability, and (2) synergistic effects of concurrent viral immunodeficiency.

High genetic and phenotypic variability of Ark-type IBV

IBV is an enveloped, single-stranded positive-sense RNA virus classified in group 3 in the *Coronavirus* genus along with other avian coronaviruses (Cavanagh, 2003). The viral genome codes for four structural proteins [spike (S), nucleocapsid, envelope, and a small membrane protein] and other regulatory proteins, including the viral RNA-dependent RNA polymerase. The S glycoprotein consists of two polypeptides, S1 and S2, which form the surface projections characterized by a globular end (S1) and a stalk (S2) that anchors S to the envelope (Lai & Holmes, 2001). The S1 subunit of the S polypeptide 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 (Cavanagh, 1981; Cavanagh, 1983; Cavanagh, 1984; Cavanagh & Davis, 1986; Koch *et al*., 1990; Koch & Kant, 1990; Mockett *et al*., 1984). The role of S1 in determining the species- and tissue/cell tropism of several coronaviruses, including IBV, has been reported extensively (Ballesteros *et al*., 1997; Baric *et al*., 1997; Casais *et al*., 2003; Fang *et al*., 2005; Fazakerley *et al*.,

1992; Hingley *et al*., 1994; Leparc-Goffart *et al*., 1997; Li *et al*., 2005; Ontiveros *et al*., 2003; Phillips *et al*., 2002; Wesley *et al*., 1991). Because of the relevance of S1 for replication and immunological escape, the extensive variation exhibited by the S1 glycoprotein among IBV populations (Kusters *et al*., 1987; Kusters *et al*., 1989) is likely the most relevant phenotypic characteristic for this virus's "adaptation" and evolutionary success. S glycoprotein variation originates from nucleotide insertions, deletions, or point mutations as a result of the viral polymerase lacking proofreading capabilities and/or from genetic recombination events occurring during viral replication (Kusters *et al*., 1990; Kusters *et al*., 1987; Kusters *et al*., 1989).

We analyzed S gene sequences of virus populations of different commercial ArkDPI-derived IBV vaccines (van Santen & Toro, 2008). Results indicated different degrees of genetic heterogeneity among ArkDPI-derived vaccines ranging from no apparent heterogeneity to heterogeneity in 20 positions in the S gene. In all except one position, nucleotide differences resulted in different amino acids encoded and therefore in a phenotypic change of the predominant virus population. The majority of amino acid differences were in the S1 subunit of the S glycoprotein.

We hypothesized that the distinct predominant phenotypes present in ArkDPI-derived vaccines from different companies (designated A, B, C, D) would show a different behavior in the host; i.e. show different pathogenicity in the upper respiratory tract of vaccinated chickens. We found significantly higher ($P<0.05$) viral loads 5 and 8 days post-vaccination (DPV) in tear fluids of chickens vaccinated with vaccines A and C vs. chickens vaccinated with vaccines B and D. Chickens vaccinated with vaccines A and C had consistently higher incidences of respiratory signs 4 through 7 DPV than chickens vaccinated with vaccine D. At 7 DPV, chickens vaccinated with vaccine C had more severe epithelial necrosis and deciliation in the trachea than chickens vaccinated with vaccine D, and more lymphocytic infiltration of the trachea than chickens vaccinated with the other three vaccines (van Santen *et al*., 2009). These results indicated that the distinct predominant IBV populations selected during the vaccine production process from the same parent virus (ArkDPI-derived vaccines) exhibit different virulence in chickens. Thus, even slight shifts of the predominant phenotype within the same IBV population are associated with changes in the behavior of these strains in the host.

Because ArkDPI viruses followed different evolutionary pathways during the process of vaccine attenuation at the commercial companies, the question arose: will these viruses further change after initial replication in the host environment? We analyzed S gene sequences of virus populations of different commercial ArkDPI-derived IBV vaccines during a single passage in specific-pathogen-free (SPF) chickens. For three of ArkDPI-derived vaccine viruses detected in the tear fluid of vaccinated chickens, a single subpopulation with an S gene sequence distinct from the vaccine major consensus at 5 to 11 codons was selected in chickens within 3 days after ocular vaccination. These findings indicated that a distinct virus subpopulation was rapidly positively selected in the environment of the chicken upper respiratory tract (van Santen & Toro, 2008). Similar results were also obtained by other researchers (McKinley *et al*., 2008).

Such adaptability of ArkDPI viruses leads to a further question: will the replicating IBV population even further change as it confronts the distinct environments of different tissues during host invasion? We inoculated 15-day-old chickens with 104 50% embryo infectious doses of an ArkDPI-derived 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 (van Santen & Toro, 2008). Five new predominant populations designated component (C) 1 through C5, showing distinct non-synonymous nucleotide changes, and thus phenotypic shift of the predominant virus population, were detected in the tissues or fluids of individual vaccinated chickens. Compared to the vaccine parent, C3 exhibited nucleotide and amino acid changes in 6 positions, the most among the 5 newly selected populations, and thereby showed the largest phenotypic distance from the vaccine's predominant population. In addition to the number of amino acid changes, the character of the 4 unique changes added to the phenotypic distance from the vaccine strain prior to inoculation. The hydrophobic amino acid leucine at position 76 was replaced by the bulkier hydrophobic amino acid phenylalanine in C3. At amino acid position 119, serine was 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 involved 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. Collectively these results indicated that phenotypic shift occurred in the Ark-type IBV population during the invasion process (Gallardo *et al*., 2009). From an applied perspective, the increased capability of ArkDPI-derived vaccines to undergo phenotypic change may be contributing to the emergence and circulation of Ark-like viruses in the poultry industry.

Significant differences also were detected in the incidence of some distinct IBV predominant populations in tissues and fluids. For example phenotype C1 had its highest incidence in the reproductive tract of the chickens, achieving a significant difference versus its incidence in the trachea ($P<0.05$). These results indicated 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 (Gallardo *et al*., 2009). From an applied perspective, these results offer an explanation for Jackwood's (Jackwood *et al*., 2009) conclusion that Ark-type vaccine viruses show increased persistence in the chicken respiratory tract. The increased persistence likely results from intraspatial variation of the replicating IBV Ark-type virus as the host immune response must follow or adapt to a continuously changing target (i.e. the antigenic characteristics of the new predominant phenotype) during viral invasion.

Collectively, the data from molecular epidemiology as well as experimental results presented above provide evidence supporting increased genetic and phenotypic variability of Ark-type IBV strains. From an epidemiological perspective, it is also interesting that Ark-type strains appear to cause problems only in areas where ArkDPI-derived vaccines are used to prevent the disease; i.e. no problems attributed to Ark strains are reported from the west coast of the United States where ArkDPI-derived vaccines have not been used by the industry. The fact that live attenuated ArkDPI-derived vaccines from different companies show genetic heterogeneity [i.e. the predominant IBV populations (based on S gene sequence) of different vaccine producers differ] is puzzling. One possible explanation might be that the ArkDPI seed virus was not adapted enough to the embryo culture at the time it was made available to vaccine companies. The existence of significant heterogeneity in the seed virus and slight differences during the process of attenuation between the different companies might have allowed different evolutionary pathways. Another possible explanation might be that this serotype has coevolved with its host for a shorter period of time and is therefore less well adapted. A third possible explanation would be the opposite option: ArkDPI originated in the Delmarva Peninsula, likely the area of the world with the most intense broiler production in terms of number of chickens per square mile. In the latter case, the extreme selective pressure resulting from extensive vaccination against IBV might have allowed immune selection of IBV ArkDPI with improved mechanisms for variability.

Synergistic effects of concurrent immunodeficiency

Outbreaks of different avian diseases [e.g. gangrenous dermatitis (Wilder *et al*., 2001)] have been associated with simultaneous immunodeficiency affecting the chicken population. In contrast, it is generally accepted that IBV can infect and induce disease in healthy-immunocompetent chickens of all ages and genetic lines (Cavanagh & Gelb, 2008). However, epidemiological data generated by Dr. F. J. Hoerr (Alabama State Diagnostic Laboratory) in which 322 respiratory broiler cases were studied, provided initial evidence for a possible association between IBV and viral immunodeficiency (Toro *et al*., 2006). IBV was isolated from broilers 13 to 54 days of age, with maximal isolation at 30 days of age. Conn, Mass, Ark, GA98, and DE072 serotypes were identified as well as a few variant profiles that did not fully match reference RFLP profiles. Differences occurred in the age of the broilers from which the viruses were isolated. Logistic regression analysis of the frequency of distinct serotypes versus broiler age revealed that Ark-type isolations, which represented nearly 60% of all isolates, followed a semicircular pattern with most isolates obtained between 27 and 43 days of age in spite of extensive ArkDPI vaccination in the region. At the same time, the histopathology of bursas and thymuses indicated that affected broilers aged 25 days or older consistently showed moderate to severe bursal and

thymic atrophy. In contrast, wild strains [Ark99, GA-98, and variant strains] tended to be isolated from older broilers towards the end of the production period, whereas increased frequency of Conn and Mass isolations occurred during the first 2 weeks of life and declined steadily with age. Evaluation of both the complete S1 nucleotide and deduced amino acid sequences of Ark-type isolates as well as virus cross-neutralization studies indicated a close similarity with the vaccine strain ArkDPI routinely used in the region. Our pathogenicity studies indicated that Ark-type IBV isolates obtained from such outbreaks of respiratory disease in Alabama broilers induced only mild respiratory disease in experimentally infected healthy and immunocompetent SPF chickens (Toro *et al*., 2006). These findings led us to hypothesize that viral immunodeficiency in chickens plays an important role in the epidemiology and outcome of IBV infection.

Atrophy of primary immune organs in chickens may be caused by different conditions or agents but immunodeficiency due to ubiquitous chicken anemia virus (CAV) and/or infectious bursal disease virus (IBDV) are the most common and relevant. CAV transiently causes generalized lymphoid atrophy with a concomitant immunodeficiency in 2-4 week old chickens (Adair, 2000; Adair *et al*., 1991; Bounous *et al*., 1995; Cloud *et al*., 1992a; McConnell *et al*., 1993), and it induces thymic atrophy in older chickens (Toro *et al*., 1997). Specific cytotoxic T lymphocytes (CTL) have been shown to be important for clearance of IBV infections (Seo & Collisson, 1997; Seo *et al*., 2000; Seo *et al*., 1997). CAV infection has been shown to abrogate CTL responses against other viruses (Markowski-Grimsrud, 2003). In addition, deficiency of T helper cells in CAV-infected chickens might adversely affect generation of IBV-specific antibodies. On the other hand, IBDV infects primarily the lymphoid tissue in the bursa of Fabricius causing a prolonged B lymphocyte immunodeficiency. Chickens exposed to IBDV have been shown to be more susceptible to various viruses including IBV (Pejkovski *et al*., 1979; Rosenberger & Gelb, 1978; Rosenberger *et al*., 1975; Sharma, 1984; Yuasa *et al*., 1980). In addition, IBV infection in bursectomized chickens is longer and more severe than in fully immunocompetent chickens (Cook *et al*., 1991), consistent with a B lymphocyte deficiency caused by IBDV infection affecting immune clearance of IBV. Combined infection with CAV and IBDV in chickens has been shown to be synergistic with a prolonged acute phase prior to recovery or mortality, significantly lower *in vitro* lymphocyte responses, and a greater reduction in protection from Newcastle disease virus challenge than in chickens infected with each agent alone (Cloud *et al*., 1992a; Cloud *et al*., 1992b; Toro *et al*., 2009; Yuasa *et al*., 1980). The epidemiological data mentioned above suggest that most outbreaks of IBV have occurred in chickens simultaneously showing bursal atrophy due to IBDV and/or thymic atrophy due to CAV infections (Toro *et al*., 2006).

To address the hypothesis that viral immunodeficiency in chickens plays an important role in the epidemiology and outcome of IBV infection, we designed experimental trials to evaluate IBV pathogenicity, viral load and persistence, and specific immune responses of chickens previously inoculated with CAV and IBDV. Our results showed that chickens inoculated with CAV+IBDV and subsequently with an Ark-type IBV isolate developed a more prolonged and more severe disease than immunocompetent chickens (Toro *et al*., 2006). While immunocompetent chickens quickly regained the same tracheal mucosal thickness as controls, immunocompromised birds still showed greater mucosal thickness than the controls at day 24 after IBV inoculation. IBV infection in the tracheal mucosa is characterized by massive infiltration of the lamina propria by lymphoid cells and the formation of a large number of germinal centers (Ficken, 1987). Our data showed that all immunocompetent chickens showed an early (9 days post inoculation) significant mononuclear cell infiltration as compared with immunocompromised chickens. Mononuclear infiltration declined linearly until achieving a plateau in the immunocompetent group. On the other hand, the immunodeficient group showed a less pronounced but more persistent mononuclear infiltration, which was most likely the result of lymphocytic depletion induced by CAV and IBDV. Consistent with the histopathological findings, CAV+IBDV infected chickens showed a delayed and reduced IBV specific local IgA response in the lachrymal fluids. Finally, IBV RNA was detected by RT-PCR in the lachrymal fluid and in the trachea through day 14 after inoculation in immunocompetent birds and through day 28 after inoculation in immunodeficient birds (Toro *et al*., 2006). The high concentrations and persistence of IBV RNA both in the trachea and in lachrymal fluids of CAV+IBDV infected chickens were likely due to reduced viral clearance as a consequence of reduced humoral and cellular immune responses (Markowski-Grimsrud, 2003; Seo & Collisson, 1997; Seo *et al*., 2000; Seo *et al*., 1997; Sharma, 1984).

To further understand the effects of viral

immunodeficiency on IBV infection we compared the immune responses to IBV in the Harderian gland and cecal tonsils of immunocompetent chickens and chickens infected with CAV and/or IBDV (van Ginkel *et al*., 2008). Flow cytometric analyses of lymphocytes in Harderian glands and cecal tonsils indicated that the relative abundance of IgM+ B cells in the Harderian glands and cecal tonsils following exposure to IBV in combination with immunosuppressive viruses was reduced compared to chickens infected with IBV alone. Infection with CAV, but not IBDV, reduced the CD4+/ CD8+ T cell ratios compared to chickens infected with IBV alone. ELISPOT assays on cells in the Harderian glands and cecal tonsils of IBV-infected chickens indicated that maximum IBV-specific IgA-secreting cell responses were reduced in chickens co-infected with CAV. IBDV co-infected chickens displayed a delayed IgA response to IBV. Thus, immunosuppressive viruses reduced B cells and T helper cells in immune secondary organs, and slowed the kinetics and/or reduced the magnitude of the mucosal immune response against IBV (van Ginkel *et al*., 2008).

The results obtained in immunodeficient chickens, i.e. more severe and prolonged clinical signs and lesions, delayed and reduced antibody responses, and increased and persisting viral shedding, support the hypothesis that viral immunodeficiency may be playing an important role in IBV epidemiology. This role may be particularly apparent in outbreaks of disease caused by IBV isolates showing only minor geno-/phenotypic variation, which would otherwise be efficiently protected against by serotype-specific homologous vaccination.

As discussed above, the Ark-type IBV has been the most relevant IBV type in the U.S. poultry industry during the last decade. The strategies for successful adaptation, which allow these strains to circulate recurrently in commercial poultry, likely include a distinct high genetic and phenotypic variability and/or synergism with concurrent immunodeficiency. Moreover ArkDPI-derived vaccine strains in use by the poultry industry may be part of the problem. Thus, improvement of such vaccine strains may be required for effective control of the disease.

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