**Infectious Bronchitis Virus Infection of Chicken: The Essential Role of Mucosal Immunity**

Kannan Ganapathy

Institute of Infection, Veterinary & Ecology Sciences, University of Liverpool,

Neston, Cheshire, CH64 7TE, United Kingdom

Corresponding author: [gana@liverpool.ac.uk](mailto:gana@liverpool.ac.uk)

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Abbreviations: List of abbreviations: CTL = Cytotoxic T cell; dpv = days post vaccination; ELISA = Enzyme-linked immunosorbent assay; HG = Harderian gland; HI = Hemagglutination inhibition; IBDV = Infectious bursal disease virus; IB = infectious bronchitis; IBV = infectious bronchitis virus; Ig = Immunoglobulin; MDA = Maternally-derived antibody; NK = Natural killer; TLR = Toll-like receptor; VN = Virus neutralisation.

**SUMMARY**

Infection with either virulent or vaccine strains of infectious bronchitis virus (IBV) elicits a complex interaction of non-specific, innate, mucosal, cellular and humoral immunity, thereby mounting an optimal defensive response in chickens. Through this process, mucosal immunity plays an essential role in preventing infection and clearing the virus. It also assists in the development of longer lasting local immunity against IBV, mainly in the respiratory tract but also in the oviduct and gastrointestinal mucosal linings. The head-associated lymphoid tissues, particularly the Harderian gland, have an important role in the synthesis of IgA. Levels of this immunoglobulin in lachrymal fluid often reflect the degree of protection against IBV challenge. Beyond the head, the importance of mucosal immunity has predominantly been studied in the trachea. Though IgA has been the major focus of IBV mucosal immunity investigations, the role of mucosal-associated non-specific, innate and cellular immune responses may also be significant. Ciliary movements and non-specific substances in the mucosa, such as mucins and peptides, assist in the entrapment and removal of living and non-living antigens. Mucosal-associated innate immune responses determine cascades of downstream cellular and humoral immunity against IBV. Cellular immunity, particularly CD4+, CD8+ and other T-cells subsets, have been studied in mucosal-associated sites, especially in the trachea. The strength of cellular immunity at mucosal sites has been associated with protection against IBV. Recently, the evaluation of mucosal immunity has shifted from traditional methods to quantitative assays of mRNA transcription of immune genes. This and other molecular-based approaches will likely boost our understanding of chicken mucosal immunity against virulent and vaccine strains of IBV. It has been well accepted that mucosal immunity plays an important role in pathogenicity, vaccinal immune-take and protection conferred against virulent IBV.

Infectious bronchitis (IB) is an acute and highly contagious disease that affects broiler, breeder, and layer chickens, which results in economic losses for the poultry industry. The spread of IB virus (IBV) from an infected flock to a neighbouring flock mostly occurs by aerosol (1). The virus initially infects the upper respiratory tract, then spreads to the lower respiratory tract. However, some strains of IBV replicate in non-respiratory tissues. For example, replication in the kidneys causes nephritis, whereas replication in the oviduct causes drops in egg production and/or quality (2). The virus can also replicate in the intestines and cause enteritis (3). In cockerels, IBV has been reported to cause infertility due to testicular infection and inflammation (4).

IBV-specific antibody levels in serum, lachrymal fluid (tears), nasal and tracheal secretions have been assayed using enzyme-linked immunosorbent assay (ELISA), virus neutralization (VN) or hemagglutination inhibition (HI) tests (5-7). Humoral antibodies are important and abundantly found in blood. Several studies have shown that circulating antibody titres do not correlate with protection against IBV challenge (8, 9). In terms of cellular immunity, cytotoxic T- cells (CTL) and cytokine responses in chickens have been shown to correlate with early reduction in IBV infection and clinical signs (10-12). In addition, increased expression of CTL associated genes, like those transcribing granzymes and perforin, have been reported after IBV vaccination (13, 14) and found to be significantly associated with tracheal protection against IBV challenge (14). Thus, each component of the immune system, and the complex interactions between them, are important for an overall optimal immune induction against IBV.

Chicken lymphoid tissues are either of epithelial or mesenchymal origin and are occupied by haematopoietic cells via the blood. The primary lymphoid organs (thymus and bursa) are colonised by stem cells of haematopoietic origin that mature *in situ* to become immunologically competent T (thymus) and B (bursa) cells. This process involves the development of T and B cells that ignore host cells but respond to foreign antigens. These immunologically mature cells then re-enter circulation and seed peripheral lymphoid organs, such as the HG, and lymphoid follicles in various systemic and mucosal tissues [e.g. mucosal associated lymphoid tissues or MALT, which includes gut (GALT), bronchi (BALT), Head (HALT) etc.]. For a better understanding of the host response to microorganisms, the chicken immune responses can be divided into the following, i) passive (maternally-derived antibodies), ii) non-specific, iii) innate, iv) cellular, v) mucosal and vi) humoral.

Different aspects of IBV and immune responses have been previously reviewed (15-17). In keeping with the view that every component of the immune system is essential and intricately interact with each other, this review aims to provide an overall view on chicken mucosal immunity, and its role in the induction of mucosal-associated immune responses to virulent or vaccine strains of IBV.

## **Mucosal immune responses following IBV infection**

#### Traditionally, mucosal immune responses to IBV have been largely confined to the detection of IgA levels in the lachrymal fluid, and tracheal or respiratory tract washings. In some instances, immunohistochemistry has been used to quantify the number of IgA-bearing B-cells. In this review, these aspects will be appraised in relation to virulent and vaccine strains of IBV. Additionally, where appropriate, the mucosal-associated non-specific defences of innate and cellular immune responses will be covered.

The role of local antibodies in the defence of mucosal surfaces against IBV has been well documented (17-19). In general, local immunoglobulins (such as IgA) in the lachrymal fluid are produced by lymphocytes in the HG, which provides local protection in the upper respiratory tissues (20). As serum antibodies do not directly correlate with IBV protection (9, 21-23), mucosal immunity has been found to have an essential role in providing protection against infection, and subsequently disease, caused by IBV. IBV-specific IgA antibodies found in lachrymal fluid have been reported to have correlated with resistance to IBV reinfection (21, 22). It has also been shown that IBV-specific IgG responses are less protective than IBV-specific IgA antibodies found in lachrymal fluids (24). Another publication highlighted that IBV-specific IgA antibodies are essential to neutralise IBV on mucosal surfaces and have a local function in IBV control (21).

Currently, there are two lines of work related to mucosal immunity and exposure to IBV, i) focusing on host responses following infection with virulent IBVs, and ii) concentrating on host responses after IBV vaccination. Selected publication on these categories have been included in this review.

***Mucosal immune responses to virulent IBVs.*** Following infection, using immunoglobulin class-specific monoclonal ELISA, IBV-specific IgA and IgG have been detected in the lachrymal fluid, tracheal washes, oviduct washes, duodenal and cecal contents (7, 17, 25, 26), and in the HG through the use of enzyme-linked immunospot (ELISpot) (27). Toro et al (number of reference instead) demonstrated that IgA levels in lachrymal fluid from SPF chickens are significantly raised by four days post IBV H52 challenge (24). In another study, Ganapathy et al. (2005) have shown significantly increased IgA levels in lachrymal fluid of SPF birds at 7, 14 and 21 days post M41 challenge (7). Arkansas challenge of naïve SPF chickens induced IgA-positive lachrymal fluid as early as 5 dpc, reaching a peak by 11 dpc (28). In contrast, following inoculation of 8-week old SPF chickens with IBV vaccine or the M41 strain, there was no marked difference in IgA levels between naïve and Mass-vaccinated birds of 13 weeks of age (29). Another report found no significant increase in serum IgA levels up to 14 days post infection in week-old SPF chicks (30).

A number of researchers have reported reduction of IgA levels in infectious bursal disease virus (IBDV) or chicken anemia virus (CAV) immunosuppressed chickens. Immunosuppression caused by IBDV delayed this immune response and resulted in lower immunity levels overall when compared to IBV infection alone (27). In another study, alongside lachrymal fluid, significantly increased local IgA levels were detected in the oviduct and duodenal contents following challenge of unvaccinated birds with the IBV G strain (17).

***Mucosal immune responses to live IBV vaccine.*** For vaccination studies, IBV-specific IgA antibodies have been detected from lachrymal fluid, tracheal washes, lamina propria and tracheal epithelial cells (24, 31, 32). In particular, a major focus of IBV-specific IgA has been in relation to the Arkansas serotype. Vaccination of White Leghorn chicks with an IBV Arkansas strain resulted in detection of IgA in lachrymal fluid at 10 days post-vaccination (dpv) (32), whereas a second study identified IBV-specific IgA at 9 dpv in the HG, and later in the cecal tonsil at 14 dpv (27). However, subsequent Arkansas homologous challenge did not increase IgA levels, indicating that vaccination priming reduced the overall immune response upon challenge, and secondary exposure, or re-infection, by IBV may result in increased susceptibility of epithelia cells (33).

In comparison, Massachusetts vaccination induced IgA-positive plasma cells in the HG by 14 dpv (21, 34). Vaccination with this vaccine strain caused a significant increase in IgA levels by 12 dpv and protected the birds from Mass H52 infection at 19 dpv (24). It has also been reported that simultaneous application of Ma5 and 4/91 vaccine strains induces high levels of IgA and IgG in upper respiratory tract washings (35). Raj and Jones (36) reported that lachrymal fluid showed the highest IgA and IgG concentration on 7 dpi, but while IgA decreased to an insignificant level by 17 dpi, IgG was still measurable at 23 dpi. IgA and IgG levels in lachrymal fluid appear to correlate with protection against IBV, and may be indicative of effective IBV vaccination (14). Expression levels of the different immunoglobulins (IgM, IgG, and IgA) have been reported to be dependent on the particular chicken line (19).

**Mucosal-associated IgG antibodies**

A number of studies have reported the presence of IBV-specific IgG (also called IgY) in mucosal surfaces and fluids (7, 14, 37, 38), though at a smaller concentration than IgA. IgG is not traditionally considered as a local or mucosal immunoglobulin. However, in the event of infection and inflammation, a vast amount of IgG secretes onto mucosal surfaces, such as respiratory, gastrointestinal or reproductive tracts. This process of IgG transfer from blood to the mucosal surfaces is known as ‘transudation’ and can occur in young chicks with maternally-derived antibodies, and also in birds with high levels of IgG due to expose to IBV vaccines or field infection. The valuable contribution of mucosal-associated IgG has been reported in some mammals, for example, in *Rhesus* macaques for anti-HIV IgG (39, 40) and in mice for herpes simplex virus type 2 (41).

As previously stated, serum antibody levels do not closely correlate with tissue protection, but mucosal-associated IgG antibodies may contribute to the protection of the respiratory tract (9, 23). Thus, antibodies on mucosal surfaces could contribute to protection at the point of entry and replication. This is important for IBV, as the virus mainly enters via the respiratory route and initial replication occurs in respiratory and lymphoid tissues in the head, before proceeding to the trachea and other tissues. Infection and inflammation may alter the permeability of blood vessels, leading to leakage of serum IBV-specific IgG onto respiratory mucosa, and this may trigger neutralisation of invading IBV at mucosal surfaces. Mockett et al. (42) reported that MDA provides protection against IBV infection in newly hatched chicks from 1 to 7 days of age. In another study, chicks hatched with high MDA were shown to have significant protection against IBV Mass-type challenge at 1 day-old but not at 7 days old. IBV MDA (D388 serotype, QX genotype) neutralising antibodies provide partial protection against tracheal damage and high protection against viral replication in the kidney (43). The importance of IgG, either humoral or mucosal-associated, in controlling coronavirus infection and/or minimising pathology became much more prominent with the emergence of SARS-CoV-2 vaccines against COVID-19. Secretion of these antibodies onto the mucosa of the respiratory system was found to be the reason for the reduction in the infection of SARS-CoV-2 and disease severity (44). These reports further support the importance of humoral antibodies, and transudation of this immunoglobin into mucosal surfaces. In chicken, higher levels of IBV-specific IgG in the blood stream would assist in control of IBV infection and disease.

**Mucosal-associated non-specific, innate and cellular immunity**

The mucosal surfaces of the respiratory, gastrointestinal and reproductive tracts provide non-specific physical barriers. In poultry, the non-specific elements comprise biological secretions from Goblet cells, including mucins and antimicrobial peptides. In addition, cilia motility in the respiratory and reproductive tracts facilitate binding and removal of particles, including dust and microorganisms. Mucins, which are glycoproteins, have been the focus of research for certain poultry respiratory diseases. For example, for NDV, higher levels of MUC5AC and MUC2B have been reported in chickens to be associated with increased protection (45). The role of mucins in relation to IBV infection or vaccination is not known.

In recent years, there has been an increased interest in analysing mucosal innate immune responses in poultry, and for IBV, the trachea has been the most widely studied. Several immune pathway components have been identified, including the activation of TLR3 (31, 46-48). Increased levels of TLR, i.e. pattern recognizing receptors (PRRs), would be reflected by further changes downstream. This results in the release of a specific set of interferons, cytokines and chemokines involved in modulation of the innate (and adaptive) immune responses(49). Using qRT-PCR, sequencing, immunoproteomics and transcriptomics, chicken immune cells and other mediators such as interferons, cytokines and chemokines, can be extensively studied for enhanced development of vaccines against IBV.

A number of studies demonstrated mucosal-associated cell-mediated immune responses in birds infected with IBV. After infection with virulent IBV 793B, CD4+ and CD8+ cells were detected in sections of the trachea and lung (36). The cellular immune response is critical in the recovery from IBV infection (50). Kotani et al. (51) assessed immunophenotypes of mononuclear cells in the tracheal mucosa, and reported that CTLs at this site were proposed to be involved in the clearance of IBV. Others have reported that cytotoxic enzymes, such as granzymes and perforin, which are secreted by CTL cells such as CTLs, γδ T cells, NK cells, CD8αα and others, play an important role in the cytotoxic activity induced following exposure to IBV (52, 53).At mucosal sites, they also correlated significantly with the parameters of tracheal changes following homologous IBV challenge in a vaccine dose-dependent manner (14). To date, much concentration has been given to the trachea in understanding the mucosal-associated cellular responses to IBV. Future studies may need to be extended to include the cellular immune responses at other mucosal sites, particularly those found in the head (e.g. turbinate) and in the oviduct.

**Conclusions**

Mucosal immunity plays an essential role in preventing/ eliminating and/or controlling IBV infection at the point of entry point. Therefore, future IBV studies on pathogenicity, vaccination, and vaccination-challenge should consider inclusion of mucosal immunity indicators. Further understanding mucosal immunity mechanisms will allow the development of novel and innovative vaccination methods, including, for example, innovative adjuvants for mucosal use, nanotechnologies, and molecular biotechnologies to effectively protect chicken flocks.

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