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Infectious Bronchitis in Poultry: Constraints and Biotechnological Developments in Vaccines

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ABSTRACT

Infectious Bronchitis (IB) of chicken is a viral disease caused by a *Coronavirus* (IBV). It is worldwide distributed and characterized by its heavy economic impact on the poultry industry. The objective of this study is to elucidate the molecular aspect of the IBV, to describe the humoral and cellular immune responses, especially those played by cytotoxic T lymphocytes in the control of this infection in addition to the role played by each of the viral proteins S and N in the induction of those immune reactions. Biotechnological advances (especially gene therapy) in the IB control have been assessed by several researchers; however they are still facing some constraints. Development of new vaccines against IBV involves detailed knowledge of its antigenic structure and of the specific Cytotoxic T Lymphocytes (CTL) epitopes.

Key words: Infectious bronchitis, poultry virology, immunopathology, vaccines

INTRODUCTION

Chicken infectious bronchitis is a worldwide infectious disease affecting different poultry sectors. It was first described in 1931 in young chickens in the United States (Butcher *et al.*, 2002). It is caused by several serotypes of *Coronavirus* (IBV) which are variably distributed. Some emerging variants spread from country or primary foyer where they are isolated to another (Rafiei *et al.*, 2010) while others remain localized with no tendency for extension (Cavanagh *et al.*, 1992). The incidence of infection can reach 100% in countries practicing intensive livestock (Bayry *et al.*, 2005).

Infectious Bronchitis (IB) control is conventionally based on live and inactive vaccines but both types of vaccines have some disadvantages. Inactive vaccines stimulate only the humoral response while the stimulation and proliferation of CTL is low (Cavanagh, 2007). Live attenuated vaccines have some disadvantages, especially the overthrow of pathogenicity and genetic modifications affecting the spicules. These vaccines also guard their replication and the maintenance of vaccine virus after several cycles of replication promotes the selection of viral sub populations genetically modified “Quasispecies”, especially on their Subunit 1 (S1) (Liu *et al.*, 2009). Live vaccines may increase the mutation rate up to 1.5% (Lee and Jackwood, 2001). The appearance of mutations in the vaccine viruses after their passage on field populations is considered one of the essential factors for vaccination failure (Cavanagh *et al.*, 1992).

The development of new vaccine strategies is necessary for a better control of the disease (Yan *et al.*, 2013). More recently *Chicken infectious bronchitis virus* (IBV) control is based on recombinant vaccines produced by genetic techniques to overcome the disadvantages of conventional vaccines. Several viral and bacterial agents are genetically modified to serve as vectors expressing different genes encoding the major structural viral proteins. These recombinant vaccines provide a good protective immunity. The choice of the most suitable alternative is based on detailed knowledge of the mechanisms of infection and the nature of the protective immune response which guides the choice of protecting antigen (Nascimento and Leite, 2012).

The objective of this study is to elucidate the molecular aspect of chicken IBV, to describe the humoral and cellular immune responses, especially those played by cytotoxic T lymphocytes (Cytotoxic T lymphocytes) in the control of this infection and the role played by spike (S) glycoprotein (S) and nucleoprotein (N) in the induction of immune reactions. Biotechnological advances (especially genetic therapy) in the IB control have been assessed by several recent researchers. Development of recombinant vaccines against IBV involves detailed knowledge of its antigenic structure and of the specific CTL epitopes.

TAXONOMY AND CHARACTERISTICS OF THE IBV

The Coronaviridae family belongs to the order of Nidoviridae that affects a broad spectrum of animal species (Van Vliet *et al.*, 2002). The Coronaviridae sub-family is divided into three genera based on their genetic and serological characteristics: Alpha-Coronavirus, Beta-Coronavirus and Gamma-Coronavirus (Gonzalez *et al.*, 2003; Ulferts and Ziebuhr, 2011). The first two genera include species pathogenic to mammals (Chu *et al.*, 2011). The Coronaviridae are enveloped viruses. Their name derives from “Corona” that means the halospicules which are directed outwardly and their genome is the largest among RNA viruses with a size of about 30 Kb (Woo *et al.*, 2005; Wickramasinghe *et al.*, 2011). It consists of a single strand of positive polarity RNA (Carter and Wise, 2005). The genome encodes four essential structural proteins: The nucleocapsid “N”, the membrane protein “M”, the coat protein “C” and the spicules “S” which are characteristic of *Coronavirus* (Barcena *et al.*, 2009).

The genome of the *Coronavirus* has a general organization common with other members of the order Nidoviridae (Casais *et al.*, 2001). It is divided into several reading frameworks, ORF1 (Open Reading Frames 1) which is localized to the 5-terminal end and comprises ORF1a and ORF1b which encode respectively a polyprotein pp1a and pp1ab (Masters, 2006). The cleavage products are involved in RNA replication (De Haan *et al.*, 2002).

A framework for transition (Ribosomal frame-shifting or sequence-RFS) generates a phenomenon of “shift” (Sawicki and Sawicki, 1998) which is programmed at ORF1a. This shift is necessary for the synthesis of PP1ab after PP1a (Namy *et al.*, 2006). Several frameworks ORF2, ORF3, ORF4, ORF 5, ORF 6 and ORF 7 are separated by a short repeated sequence of the transcript (or transcription regulatory sequence TRS) (Sawicki and Sawicki, 1998). The “leader” sequence and the poly (A) are located respectively at the terminal ends 5' and 3' (Boursnell *et al.*, 1987). Two untranslated regions (UTR or untranslated-regions) lie at the terminal ends 5' and 3'. The UTR 5 is located before the reading frame ORF 1 and the UTR 3 is positioned after the last ORF and before poly (A) (Sawicki and Sawicki, 1998).

Immunopathology IB: Infection is initially followed by the recruitment of non-specific immune cells. The recognition of Antigens (Ag) is achieved through specific receptors (pattern-recognition

receptors or PRRs) of Antigen Presenting Cell (APC) (Juul-Madsen *et al.*, 2011). Among these PRRs, the Mannose-Binding Lectin (MBL) is abundant in the infectious process. It recognizes carbohydrates expressed by many pathogens (viruses, bacteria or parasites) (Juul-Madsen *et al.*, 2011). Increasing serum levels is associated with resistance to several diseases including IB (Kjaerup *et al.*, 2013). According to Juul-Madsen *et al.* (2007) a good complement activation and inhibition of the multiplication of IBV in the trachea are recorded in chickens with the highest serum rate of MBL.

IBV stimulates the production of different chemokines (CXCR4, CCR6, factors derived from stromal cell), interferon type 1 and interleukin 1 beta (IL-1 β) (Guo *et al.*, 2008) which act in synergy to activate the migration of specific immune cells to sites of viruses entering (Caron, 2010).

Cytotoxic T Lymphocytes (CTLs) play an important role in the anti-infectious poultry protection. The cytotoxic activity is predominantly provided by the TL CD8⁺. The naive LT CD8⁺ differentiates into LT memory and LT cytotoxic "CTL" which is responsible for the destruction of pathogens (Rey *et al.*, 2005). The LT CD8⁺ recognizes cells expressing the viral Ag with MHC-I, through their TCR (T cell receptor). The adhesion of LT CD8⁺ to their target cells and the creation of immunological synapse promote the release of the contents of cytoplasmic granules in the contact regions leading to lysis of infected cells. The titer of CTL is clearly correlated with the decrease in severity of disease and virus shedding. At the 10th day post-infection (p.i), CTL achieved their peak and then they gradually decline (Seo and Collisson, 1997).

The cellular response is a complex reaction which depends on MHC phenotype of CTL. Passive transfer of CTL of infected chickens with IBV to naive chickens with CTL of the same MHC allows the protection of these last. This protection is not observed with the passive transfer of CTL from uninfected chicken (Seo *et al.*, 2000). The LT CD8⁺ are primarily responsible for this protection (Seo *et al.*, 2000). The LT reduce lethality and viral portage but not completely eliminate the virus. The use of immunosuppressors reduces the multiplication and the number of lymphocyte cells. It also increases the mortality and severity of lesions as well as the multiplication of viruses (Raj and Jones, 1997).

The humoral immunity is characterized by the production of neutralizing AC (Anti-Bodies) and hemagglutination inhibition that play a significant role in the control of IBV. However, the major role is attributed to cell-mediated immunity (Collisson *et al.*, 2000). The IgMs appear quickly but with transient manner and they attest a primary infection (Martins *et al.*, 1991; Mockett and Cook, 1986). They are detectable only from the 10th day p.i and they reached their peak very quickly on the 12th day p.i. The IgGs appear late compared to IgMs, at the 15th p.i., then gradually increase up to 30 days p.i. Their presence indicates a past infection (Seo and Collisson, 1997).

The maturity of the immune system is critical to develop an active immune response. Parental sensitization influences the immune response of chickens. According to Liu *et al.* (2012), a primary sensitization of one-day-old chicks allows the appearance of IgG 9 days p.i. Primary infection beyond the 7th day of age follows a more rapid onset of IgG (6 to 9 days p.i). The choice of time of vaccination should take into consideration the duration of passive protection. According to Paul *et al.* (2008), the highest titer in maternal AC (MAC) is found in the first two weeks of age (100% of chicken from vaccinated parents are seropositive), then it is reduced between the third and fourth week.

Role of local immunity: The IB is localized primarily at the respiratory system. The local immunity is critical; it can prevent the spread of infection from the primary site of infection to other

target organs. The ACs are detectable in the wash of the trachea, oviduct, tears, ceca and duodenal contents up to the 7th p.i., (Raj and Jones, 1996; Gervelmeyer *et al.*, 1998). The highest titer is observed however in the trachea (Gervelmeyer *et al.*, 1998; Okino *et al.*, 2013). According to Raj and Jones (1996), a very good correlation is found between the titer of AC in the washing products of the oviduct and egg production in laying hens. High levels of local IgG and IgA are good indicators of optimal protection (Okino *et al.*, 2013).

The number of lymphocytes in the harder gland and lymphoid tissue associated with connective tissue (CALT: Conjunctiva-Associated Lymphoid Tissue) increases rapidly after immunization of chickens with ocular route (Gurjar *et al.*, 2013). The IFN (Interferon gamma) is also expressed earlier in lymphoid tissue associated with conjunctives and Harder gland (Gurjar *et al.*, 2013).

Immunological structures and Ag future vaccinal candidates: Different proteins (S, M and N) stimulate the production of monoclonal antibodies. However many of them do not induce viral neutralization and consequently do not play a protective role (Ignjatovic and McWaters, 1991).

The tropism of the IBV is determined by the spicules. Variations in the structure or the amino-acid composition of these glycoproteins lead to tropism modifications; thus the ability of the virus to replicate is reduced which results in a decrease of its pathogenicity (Liu *et al.*, 2009; Wickramasinghe *et al.*, 2011).

The spicules play a key role in the initiation of infectious bronchitis by binding to sialic acid of target cells (Promkuntod *et al.*, 2014). They allow the attachment, penetration and membrane fusion (Gallagher and Buchmeier, 2001) as well syncytia formation and spread of virus between surrounding host cells (Cavanagh, 1995). Membrane fusion is triggered by proteases that cause cleavage of the spicules into two subunits: “S1” which allows the receptor binding and “S2” which allows the membrane fusion (Weiss and Navas-Martin, 2005; Wickramasinghe *et al.*, 2011).

This initial step of conformational change is a good target for immunological control of *Coronavirus* infections (Holmes, 2003). Antibodies directed against spicules neutralize infection. According to Kusters *et al.* (1989), a highly conserved region involved in the infectious process, located near the N-terminal end of S2 has epitopes recognized by AC which may be important in vaccines development.

Immunological and protective role of spicules: The spicules stimulate the production of AC neutralizing and inhibiting hemagglutination. The affinity of ACs is greater to S1 than S2. The IgG anti-S has an important role in initial stages of the virus neutralization process by inhibiting binding to membrane receptors. The IgGs anti-S1 are endowed with a good neutralizing capacity as compared with anti-S2. This is related to their high affinity and efficiency to bind to the surface of the virus (Zeng *et al.*, 2006).

Furthermore, S2 are hidden by oligosaccharides, their compact structure in form of short rods and their positioning below S1, limit or reduce the accessible surface by Ig which explains the affinity difference found between the two sub units (Krokhin *et al.*, 2003). Recombinant vaccines with gene of “S1” induce protective AC which reduces tracheal and renal lesions. Their titer increases with reinfections (Song *et al.*, 1998). Thus, the incorporation of S1 genes is essential for the generation of recombinant effective vaccines (Asadpour *et al.*, 2010).

Epitopes which induce Ig production and CTL recruitment are located at the S1 and S2 (Ignjatovic and Sapats, 2005). By the use of monoclonal antibodies, glycoproteins S show eight dominant antigenic regions from S1-A to S1-F for the S1 and from S2-G to S2-H for the S2. S1-D

produces hemagglutinant AC, however regions S1-A to S1-E and S2-G are responsible for the production of the neutralizing AC (Koch *et al.*, 1990). Nevertheless S1-E region is the most immunogenic (Lin *et al.*, 2012).

Role of protein N: Nucleoproteins induce adaptive immune response to IBV. They have specific CTL epitopes on their C-terminus (Seo *et al.*, 1997). Incubation of CLT from the spleen of infected chickens in the presence of infected cells by IBV or viral vectors expressing the proteins 'N' allows the destruction of these infected cells. The expression of these epitopes with MHC molecules is essential for the destruction of infected cells by CTLs CD8+ (Seo and Collisson, 1997).

ELISA technique shows that AC are mainly directed against the nucleocapsid "N" than spicules "S" (Leung *et al.*, 2004). The protein "N" produces the highest percentage of lymphocytes compared to protein S and M (Yan *et al.*, 2013). Mice immunized by an adenovirus recombinant vaccine expressing the N protein, produces both humoral and cellular specific immune responses (Zakhartchouk *et al.*, 2005).

Conventional vaccines: Inactive vaccines are conventionally used in the control of IB. They stimulate only the humoral response while the stimulation and proliferation of CTL is low (Cavanagh, 2007). They are usually used in combination with live vaccines to enhance humoral and cellular immune response in order to obtain adequate protection (Box *et al.*, 1980; Ghadakchi *et al.*, 2003).

Live attenuated vaccines are also widely used in the control of IB; nevertheless they have some disadvantages, especially the overthrow of pathogenicity and genetic modifications. The most deadly mutations are those affecting the spicules because of their essential role in the induction of a protective immune response as they are the major antigens and vaccine candidates.

These vaccines guard their replication *in vivo* after administration. However, the maintenance of vaccine virus by several cycles of replication promotes the selection of viral sub populations genetically modified "Quasispecies". According to McKinley *et al.* (2008), these changes affect the sequence of the spicules genes. The selection comes only three days after vaccination. The virus must adapt to the microenvironment of different tissues (trachea, oviduct, etc.) which results in changes in spicules genes (Gallardo *et al.*, 2010). The selection can take place at preparations of live vaccines during their serial passages in embryonated eggs. These modifications affect especially S1 (Liu *et al.*, 2009). The appearance of mutations in the vaccine viruses after their passage on field populations is considered one of the essential factors for vaccination failure (Cavanagh *et al.*, 1992). The live vaccines are then responsible for sudden development of IB. Fortunately, the mutation rate is low. It is around 0.5% but with vaccination, it can reach 1.5% (Lee and Jackwood, 2001).

RECOMBINANT VACCINES: A NEW PREVENTIVE APPROACH

Viral vectors: Many viruses are used as viral promoters for IBV genes, to prevent infectious bronchitis. The avian poxvirus expressing the IFN(and the S1 causes rapid onset of AC, LT CD4+ and CD8+. The mortality rate, the severity of lesions as well as viral shedding are reduced (Shi *et al.*, 2011). The same observations are recorded by Zhang *et al.* (2012) with Marek's disease virus expressing the S1 gene.

DNA vaccines: DNA vaccines strongly enhance the immune response particularly those co-recombined with viral Ag and cytokines (synergistic effect). The immune response is proportional

to the number of expressed antigenic structures. Trivalent vaccines are more effective than divalent and monovalent vaccines (Tian *et al.*, 2008; Yang *et al.*, 2009; Jiao *et al.*, 2011).

Co-recombinant vaccine (with S1 and nucleoprotein N using an attenuated strain of *S. typhimurium*) strongly stimulates local immunity in the respiratory mucosa. The humoral and cellular responses are very significantly increased compared to the vaccine expressing S1 only (Jiao *et al.*, 2011). Vaccines with live attenuated bacteria allow the vaccination through the mucosal surface and specifically target APCs (OIE Terrestrial Manual, 2008).

Trivalent vaccine expressing proteins S, N and M is more effective than monovalent vaccine expressing only one of these proteins (Yang *et al.*, 2009). The same results are reported by Yan *et al.* (2013); the AC titer and the lymphocytes percentage are very high compared to vaccine with monovalent plasmids; the level of protection may reach 90%.

The use of genes that code for CTL epitopes and specific T lymphocytes (Th) is better than the use of whole virus structures. According to Tian *et al.* (2008), a DNA vaccine expressing both seven epitopes which derived from the subunits S1 and S2 and the nucleoprotein N, stimulates strong humoral and cellular immune responses and provides protection that exceeds 80%.

Polyvalent vaccines utilization is encouraged with DNA vaccines; several sequences encoding various Ag and cytokines can be introduced together into the same bacterial promoter (Ingolotti *et al.*, 2010). In fact, the vaccinia virus is characterized by the large size of its genome which allows the integration of several viral genes. The amount of viral proteins is considerable and directly related to the very high levels of virus expression (Moss, 1996).

Adjuvants: The cellular immune response is improved after intramuscular administration of DNA vaccines expressing the nucleoprotein N with IL2 which increase the IgG titer, the percentage of LT and the level of protection compared to vaccines expressing only the N protein (Tang *et al.*, 2008). The maximum clinical and necrotic protection of chickens (up to 70%) is obtained with repeated intramuscular injections of plasmids co-expressing IL2 and S1 (Zhang *et al.*, 2009).

According to Chen *et al.* (2010), the highest ratio CD4⁺/CD8⁺ is obtained with vaccines co-expressing interleukin 18 (IL18) and S1 compared with those expressing only spicules S1. The levels of protection are respectively 75 and 100%. The S1 associated with GM-CSF of chickens (granulocyte-macrophage colony stimulating factor or Ch GM-CSF) significantly enhances the protection from 73.3 to 86.7% (Tan *et al.*, 2009).

Chicks immunized in ovo with spicules S and interferon " type 1 are more resistant to infection occurring during the first weeks of age. Clinical signs and histological lesions are reduced.

The amount of DNA injected is reduced by the addition of adjuvants. With interferon ", a reduced amount of 5 micrograms of DNA is sufficient to protect chickens compared with higher doses of DNA without interferon combination (Babapoor *et al.*, 2009). *In vitro*, IFN" type 1 inhibits colonization of chickens' kidney cells by IBV. This effect is dose dependent, a dose of 100 µg mL⁻¹ can control up to 50% of viral infection (Pei *et al.*, 2001).

Fowl pox virus expressing IBV S1-Ch IFN(is more efficient than the H 120 vaccine and IBV S1 vaccine. The number of circulating LT CD8⁺ cells is greatly increased and the virus is rapidly eliminated from organs with reduced duration of persistence of Ag in the kidneys. Lesions of the internal organs are less severe and they last for a short duration (Wang *et al.*, 2009).

CONSIDERATION IN THE DEVELOPMENT OF VACCINES

Constraints related to vaccines: DNA vaccines produce a low level of AC. This disadvantage is overcome by increasing the dose of injected DNA or by reminders (Dufour, 2001).

A protocol involving a recombinant vaccine with conventional vaccines is an alternative strategy. A DNA vaccine in primary vaccination expressing the S1 followed by a booster using a live vaccine produces a better response with detectable AC after infection (Babapoor *et al.*, 2009). Also the association with inactive vaccines produces a significant increase in neutralizing IgG and CD4+/CD3+ and CD3+/CD8+ (Guo *et al.*, 2010).

A vaccination program involving a trivalent vaccine for the primary vaccine to an inactive vaccine booster, gives 100% protection. The mortality rate and viral replication are in the order of 0% and no clinical signs are observed (Yan *et al.*, 2013).

Constraints related to the virus

Selection pressure: The immune response exerts selective pressure on the virus which responds by changing its antigenic structure to escape the body's defenses. The affinity of the virus for cell receptors can change in the physical and chemical conditions of the microenvironment of the host (Toro *et al.*, 2012).

Genetic variations: The conditions of intensive farming (confinement and high density), facilitates the transmission of viruses, the circulation of different serotypes and the possibility of co-infection are favorable to produce genetic changes (Liu *et al.*, 2013). The possibility of multiple viruses to infect the same cell facilitates recombination and the emergence of new strains such as SARS-CoV (Holmes and Rambaut, 2004) and viral hepatitis in mice (MHV) (Makino *et al.*, 1986).

The recombination is most noticeable among members of the same family where the variations can reach 25% of the whole genome (Lai, 1996; Holmes, 2009). A non-homologous recombination can occur between the genomic RNA and RNA trans-infected segments (Liao and Lai, 1992).

The large size of the *Coronavirus* genome (26.4-31.7 kb) which is larger compared to that of all RNA viruses allows an important genes plasticity (Woo *et al.*, 2010).

RNA transcription process is normal however RNA_m synthesis is discontinuous and passes through many stages (Sawicki and Sawicki, 1998; Moreno *et al.*, 2008). Interactions between the Separated regions of genome cause the switch phenomenon for the transfer of newly formed RNA from site to another one which leads to high recombination frequency (Pasternak *et al.*, 2001). The Random selection of RNA templates are responsible (Pasternak *et al.*, 2006).

Replication errors and the absence of correction mechanisms in the RNA polymerase are responsible for a high rate of uncorrected mutations that accelerate virus's evolution (Jenkins *et al.*, 2002; Taylor, 2006). The lack of fidelity of RNA polymerase leads to a great genetic and antigenic heterogeneity which promotes increased viral quasispecies (Sallie, 2005).

Conclusion and prospects in the control of IB: This review summarized resent knowledge on structure, immuno-biological aspect and vaccine biotechnology in chicken IBV control. The functions of the major viral proteins of IBV and the various actors of the immune response are well illustrated, facilitating therefore to understand different points of action to prevent this infection. The results of modern vaccinology genetic researches are very motivating and promising. In fact, the control of IBV has recorded a great evolution with recombinant vaccines. The efficacy of gene therapy depends however on detailed knowledge of two main axes: The immune mechanisms during natural infection to exactly determine how chickens resist against the infection and specific protective epitopes of CTL able to be the major immunogenic vaccine candidates.

Recombinant vaccines allow a better understanding of the nature and the relationship between separated viral structural particles and their immunological functions (structure-function) and the characterization of the target proteins and the required immunity reactions that protects the body against infections (Moss, 1996). The spicules and nucleoproteins are the principal targets viral proteins of the immune response which show high protective roles and which allow the production of recombinant vaccines exclusively on these proteins. The use of recombinant polyvalent vaccines is the best solution with the development of genetic engineering techniques.

The ignorance of the immune mechanisms of the host against viral infections is usually one of the main causes of vaccine failure. The question that arises with gene therapy against IBV is how a recombinant vaccine can induce protection equivalent to that of a natural infection.

Based on this study and the evolution of the tools of gene therapy, research in vaccinology against IBV remain always news. More research in virology, molecular immunology and pathogenesis of IBV are needed to develop better vaccine protection in the coming years. Recombinant vaccines represent the future in the prevention of this infection and certainly can lead in the coming years to vaccines offering high levels of protection.

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