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Relationship Between Mucosal Antibodies and Immunity Against Avian Infectious Bronchitis Virus

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Abstract: Immunity against avian infectious bronchitis virus (IBV) was assessed in specific pathogen free (SPF) chickens vaccinated at age of three weeks with H120 attenuated vaccine strain and, after three weeks, by measuring the virus-neutralizing and viral specific antibody isotypes in the mucosa (lachrymal secretion) and systemic (serum) compartments, followed by the evaluation of the protection to challenge with the M41 virulent strain of IBV. Lachrymal virus-neutralizing (VN) and IgG or IgA anti-IBV antibodies induced by H120-vaccination and detected at challenge showed significant correlation with the protection against IBV infection. No significant correlation was observed between lachrymal IgM anti-IBV antibodies, or between serum antibodies and protection to IBV infection. Therefore, the results of this study indicate that lachrymal antibodies, especially those expressing virus-neutralizing activity and from IgG and IgA isotypes, are effectively induced by immunization with IBV attenuated vaccine strain and are associated with the tracheal protection against IBV infection, suggesting that the levels of these lachrymal antibodies can be used for monitoring the protective immunity against IBV in vaccinated chickens.

Key words: Infectious bronchitis virus, lachrymal virus-neutralizing and IgG and IgA antibodies, tracheal humoral immunity, H120 attenuated vaccine

INTRODUCTION

Avian infectious bronchitis (IB) remains a major problem in the poultry industry worldwide (Cavanagh, 2007; Cook et al., 2012). Live and inactivated vaccines are available, but they are not effective long term in controlling IBV infections, specially those caused by variant strains (20). The essential function of existing IBV vaccines is to elicit, ideally, local and systemic specific antibodies, as well as cell-mediated immunity to this virus, in order to confer efficient protection against infection with homologous IBV strain (Bijleng et al., 2004; Pei and Collison, 2005; Cavanagh, 2007; Okino et al., 2013).

The tracheal mucosa is the primary target tissue for IBV infection and there is considerable evidence indicating that local immunity in the upper respiratory tract is an important first line of resistance against IBV infection (Gomez and Raggi, 1974; Davelaar and Kouwenhoven, 1976; Otsuki et al., 1990; Cook et al., 1991; Toro and Fernandez, 1994; Mondal and Naqi, 2001; Okino et al.,

2013). Conversely, a lack of association between serum antibody levels and resistance to challenge with IBV was often reported (Raggi and Lee, 1965; Toro and Fernandez, 1994; Gelb et al., 1998; Cavanagh, 2007).

However, most of the studies about immune-protection mechanisms against IBV infection did not address the putative correlation between changes in anti-IBV antibody isotypes, including the virus-neutralizing antibodies in respiratory mucosa, that occurred before the IBV challenge in vaccinated chickens, with the protection status against this virus infection. Local IgG, IgA and IgM anti-IBV antibodies, particularly those expressing virus neutralizing activity, should have their protective roles better elucidated, specially at challenge with IBV in vaccinated chickens.

Thus, this study aimed to assess more accurately the IBV immune status in vaccinated chickens, in order to clarify the possible role of virus-neutralizing and anti-IBV antibody isotypes locally produced, specifically in

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lachrymal secretion to use these parameters as a predictor of anti-IBV protective immunity in vaccinated birds.

MATERIALS AND METHODS

Chickens: Specific-pathogen free White Leghorn-type chickens were used. These birds were divided in two groups (vaccinated and unvaccinated birds) that were housed in two positive-pressure isolators throughout the experimental period.

Vaccination and challenge: One group (nine chickens) was vaccinated, at 21 days of age, by ocular route with a live commercial vaccine, containing approximately 10^4 embryo infectious doses 50% (EID_{50})/bird of H120 attenuated IBV strain. The second group (ten chickens) was left unvaccinated and used as non-immune control group in the experimental infection with M41 strain of IBV. After three weeks, blood and tear samples were collected from these two groups of chickens before the birds were challenged by oculo-nasal routes with 10^5 EID_{50} of M41 strain of IBV per bird. Four days post-challenge, the birds were humanely sacrificed for immediate examination of tracheal explants prepared from each organ. Briefly, each of nine explants prepared from the proximal, medial and distal parts of each trachea was examined by low-power microscopy and ciliary inhibition was scored as 0 to 4: a score of 0 represented 0% of ciliostasis and scores of 1, 2, 3 and 4 represented 25, 50, 75 and 100% of ciliostasis, respectively (Andrade *et al.*, 1982). Portions of each trachea were also examined by histopathology analysis and assigned lesion scores of 0 to 3, as reported Andrade *et al.* (1982). Tracheal swabs were submitted to virus isolation in embryonic chicken eggs and two passages were performed (Gelb *et al.*, 1989). At each passage, the embryos were examined for IBV pathological effects up to 7 days post-inoculation. Allantoic fluid samples were harvested 3 days after inoculation, pooled and analyzed for the presence of virus by competitive-ELISA (Bronzoni *et al.*, 2001).

Collection of blood and lachrymal secretion samples from vaccinated and non-vaccinated chickens: Lachrymal and blood samples were collected immediately before challenge. Tear samples were harvested, by adding 25 µL of glycerol in the conjunctiva of lower eyelid of chickens. This is followed by the collection of the resultant lachrymal secretion using a micropipette with disposable tips. Volumes of 50-200 µL were usually collected from each bird. Lachrymal samples were stored at -20°C for later analysis in ELISA or virus-neutralization tests. Serum samples were obtained from clotted blood samples by a conventional procedure and stored at -20°C for the same serological tests.

Concanavalin A-Sandwich-ELISA for antibody detection (Con A-S-ELISA): The Con A-S-ELISA for the quantification of chicken anti-IBV antibody isotypes was carried out following the general protocol described by Bronzoni *et al.* (2005). Briefly, the microplates were coated with 0.25 mg/mL of Con A Type IV (Sigma Chemical Co. St. Louis, Mo., USA) diluted in PBS pH 7.4 and incubated overnight at 4°C. After washing, the microplates were blocked with a solution containing PBS supplemented with 10% skimmed milk (SM) and incubated for 45 min at 37°C. An ideal dilution of a suspension of allantoic fluid infected with M41 strain of IBV was added, following the wash procedure, onto the Con A pre-coated microplate wells and the reaction was incubated for 1 h. at 37°C. Chicken serum or tear samples in their optimum dilutions (1:50), as well as the positive or the negative reference chicken serum or tear samples, prepared in PBST-SM were added and the microplates were incubated for 1 h. at 37°C. All anti-chicken immunoglobulin peroxidase conjugates (Bethyl Laboratories, Montgomery, TX), which were specific for each immunoglobulin isotype (anti-chicken IgG, IgA or IgM), were added in their optimum dilutions to separate wells and the reaction was incubated for 1 h at 37°C.

The development of color reaction step was induced after the addition of hydrogen peroxide and ortho-phenilene-diamine (OPD) to the microplate wells. A stop solution containing HCL (1M) was added after 15 min and the plates were read in a wavelength of 490 nm. For each test serum or tear sample and each antibody isotype the mean OD (OD_{MTS}) was expressed in relation to the positive reference (serum/tear) mean OD (OD_{MPRS}) and the negative reference (serum/tear) mean OD (OD_{MNRS}) as a sample to positive ratios (S/P), according to the formula $S/P = OD_{MTS}-OD_{MNRS}/OD_{MPRS}-OD_{MNRS}$. To establish cut off values for each lachrymal and serum immunoglobulin isotype, samples from 12 uninfected and non-immunized control SPF chickens were tested. The mean S/P values plus twice the standard deviation were taken as the values above which samples were considered positive.

Virus-neutralization (VN) test: Serum and lachrymal fluid samples were individually tested in duplicate by the virus-neutralization (VN) test, using embryo kidney cells as the indicator system. A beta-procedure (constant virus against serially diluted serum or tear samples) was followed as recommended Wooley *et al.* (1976), using the M41 IBV strain as the challenge virus. Endpoints of the antibody titers were read as the reciprocal of the highest dilution that neutralized the activity of the virus and the titer was expressed as Log_{10} 1/highest dilution.

Statistical analysis: The significance of differences between the results of the two experimental groups of chickens was analyzed statistically by the two-tailed

Mann-Whitney U-test and Spearman's rank correlation was determined, using PRISM software (GraphPad Software, San Diego, CA, USA), at a 5% significance level ($p = 0.05$).

RESULTS

Pathological changes in IBV vaccination-challenge test: Experimental vaccination of SPF chickens at the age of three weeks with attenuated H120 IBV strain provided, three weeks later, complete protection to tracheal epithelial surface against challenge with M41 virulent strain of IBV. All vaccinated birds conserved, after challenge, high levels of ciliary activity as well as no virus was recovery from their tracheal samples (Table 1). On the contrary, the control group of unvaccinated birds showed a marked ciliostasis in the epithelial cells of the tracheal rings and the virus was re-isolated in 100% from their tracheal samples (Table 1).

Evident histopathologic alterations were also observed in the tracheas of unvaccinated birds, whereas low scores of tracheal lesions were recorded in vaccinated birds (Table 1). The tracheal lesions in the unvaccinated birds were characterized by extensive epithelial deciliation and desquamation. In addition, heterophils frequently infiltrated between degenerated ciliated epithelial cells and basal cells and, for some birds, many heterophils and desquamated epithelial cells were seen in lumen of the trachea. Goblet cells also demonstrated degeneration and desquamation. A severe edema, along with infiltrations of heterophils and lymphocytes and vascular congestion were also observed in the tracheal lamina propria of unvaccinated birds. In contrast, H120-vaccinated birds exhibited, four days after challenge with IBV M41 strain, absence (four out nine chickens), mild (three out nine chickens) or moderate (two out of nine chickens) focal pathological changes on the tracheal mucosa and slight lymphocyte infiltration focuses in the lamina propria of this organ (Table 1 and Fig. 1A, 1B, 1C and 1D).

Relationship between antibody responses and protection of chickens vaccinated with IBV: Low levels of anti-IBV neutralizing and anti-viral specific antibody isotypes were detected immediately before challenge with IBV M41 strain, in serum samples of vaccinated chickens (Table 1). No significant correlation was found between pre-challenge neutralizing antibody titers, detected in the systemic compartment (serum) and the protection status to IBV infection in vaccinated chickens, measured as tracheal ciliary activity and tracheal lesions (Table 1). Similar lack of correlation was observed for serum levels of IgG, IgM and IgA anti-IBV antibody isotypes and the protection against virus challenge, or when these anti-IBV immunoglobulin isotypes were related with the virus-neutralizing (VN) antibody titers. Despite this, the mean level of anti-IBV IgG isotype of vaccinated group differed statistically from that recorded for the unvaccinated group.

On the other hand, a single IBV vaccination elicited after three weeks, in the lachrymal secretion, moderate to high levels of anti-virus antibodies, which differed statistically from those recorded for non-immunized birds (Table 1). Significant negative correlation was also found between the lachrymal virus neutralizing antibody titers and the tracheal ciliostasis after virus challenge ($r = -0.86$, $p<0.05$). In addition, a significant negative correlation was observed for IBV neutralizing antibody titers and the tracheal microscopic lesions ($r = -0.77$, $p<0.05$).

The levels of lachrymal anti-IBV IgG and IgA antibodies also correlated negatively with the protection against IBV challenge evaluated by the inhibition of ciliar activity ($r = -0.81$ for IgG, $p<0.05$ and $r = -0.62$ for IgA, $p<0.05$). Moderate to high negative correlations were also found, relating the concentrations of these antibody isotypes with the development of tracheal microscopic lesions ($r = -0.79$ for IgG, $p<0.05$ and $r = -0,61$ for IgA, $p<0.05$). In addition, IgG and IgA anti-IBV isotypes detected in lachrymal secretion also demonstrated significant correlations with the VN antibody titers measured in this compartment ($r = 0.63$ for IgG and $r = 0.40$ for IgA, $p<0.05$). Despite the positive coefficient of correlation found between the lachrymal IgM anti-IBV antibodies and VN antibody titers ($r = 0.55$, $p<0.05$), no significant correlation was detected for the levels of this antibody isotype and the protection status against IBV infection.

DISCUSSION

The mechanisms of protective immunity in chickens infected with IBV are complex and, though extensively investigated, they are not fully understood (Cavanagh, 2007; Cook *et al.*, 2012). As the upper respiratory tract, specially trachea, is the primary target organ for IBV infection, the local immune responses developed in the local mucosa could be an important first line of resistance against this virus. However, the precise role of antibodies in the control of IBV infection remains controversial (Gomez and Raggi, 1974; Davelaar and Kouwenhoven, 1976, 1977; Otsuki *et al.*, 1990; Cook *et al.*, 1991; Gelb *et al.*, 1998; Pei and Collison, 2005; Cavanagh, 2007). Indeed, the accurate prediction of the resistance to IBV infection in vaccinated chickens has been hampered, since circulating antibody titers against this virus often related poorly with protection to IBV infection (Raggi and Lee, 1965; Toro and Fernandez, 1994). Thus, our results demonstrated the importance of local antibodies and expanded the findings of different authors on the immune-protection mechanisms against IBV infection (Gillete, 1981; Davelaar *et al.*, 1982; Hawkes *et al.*, 1983; Davelaar and Kouwenhoven, 1988; Toro *et al.*, 1997; Toro and Fernandez, 1994; Mondal and Naqi, 2001; Okino *et al.*, 2013). All together these data highlight the relevance of the mucosal antibodies, or of the mucosal memory antibody responses in the protection against IBV.

Table 1: Relationship of systemic and local anti-IBV antibody responses at challenge with protection of vaccinated and unvaccinated birds against infection with M41 strain of IBV

	-------------------- Ab Levels-Serum --------------------				---------- Ab Levels-Lachrymal Secretion -----------				Protection To Challenge		
	IgG	IgA	IgM	VN	IgG	IgA	IgM	VN	C.I.[a]	T.H.[b]	VI[c]
Vaccinated/Bird Number											
1	0.41	0.40	0.74	1,6	0.06	0.07	0.6	1.6	0	0	-
2	0.38	0.16	0.06	1.6	0.51	1.1	0.21	1.6	1	2	-
3	0.30	0.20	0.19	2.2	0.61	0.38	0.35	2.2	0	0	-
4	0.39	0.09	0.22	0.3	0.27	0.59	0.35	1.6	0	1	-
5	0.29	0.11	0.22	1.3	0.53	0.45	1.03	1.9	0	0	-
6	0.27	0.08	0.34	0.3	0.70	1.3	0.25	1.3	0	0	-
7	0	0	0.21	1.3	0.42	0.31	0.54	1.6	1	1	-
8	0.52	0.27	0.08	0.3	0.67	0.64	0.03	0.3	1	2	-
9	0.30	0.35	0.36	0.3	0.32	0.17	0.73	2.5	0	1	-
Mean±s.d	0.32+0.14	0.19+0.13	0.2+0.2	1.0+0.7	0.51+0.1*	0.58+0.4*	0.46+0.3*	1.6+0.6*	0.33±0.5*	0.78±0.8*	
Unvaccinated/Bird Number											
11	0.07	0.10	0.14	0.3	0.03	0.05	0.14	0.3	3	3	+
12	0.06	0.06	0.17	0.3	0.02	0.04	0.19	0.3	4	3	+
13	0.05	0.06	0.10	0.3	0.02	0.04	0.04	0.3	4	3	+
14	0.04	0.12	0.26	0.3	0.01	0.16	0.04	0.3	3	3	+
15	0.04	0.20	0.19	0.3	0.01	0.08	0.09	0.3	3	3	+
16	0.08	0.03	0.19	0.3	0.002	0	0	0.3	4	3	+
17	0.09	0.02	0.06	0.3	0.006	0.03	0.12	0.3	4	3	+
18	0.08	0	0.04	0.3	0	0.06	0.18	0.3	4	3	+
19	0.17	0	0.04	0.3	0	0	0	0.3	4	3	+
20	0.11	0	0.07	0.3	0.01	0.06	0	0.3	4	3	+
Mean±s.d	0.08+0.04	0.06+0.06	0.13+0.07	0.3+0	0.01+0.01	0.05+0.04	0.12+0.1	0.3+0	3.7+0.48	3	

Levels of anti-IBV antibodies for each isotype were determined in Con A-S-ELISA and expressed as S/P values. The titers of virus-neutralizing antibodies were determined in VN Test and expressed as Log10 (1/Dilution) and VN titer = 0.3 are negative. *Differences statistically significant ($p<0.05$), by comparing with the results from the unvaccinated group. a C.I- Ciliar inhibition scores; b T.H.- tracheal histopathology scores and c VI-Virus Isolation

In the present study, all chickens vaccinated by ocular route with attenuated IBV vaccine (H120 strain) were resistant to challenge with virulent M41 strain, based on the conservation of tracheal ciliary activity, on the virus re-isolation and on the absence of the development of relevant microscopic lesions in this organ. Furthermore, moderate to high levels of IgG and IgA anti-IBV antibodies expressing virus-neutralizing activity were detected in the lachrymal secretions of the vaccinated birds at IBV challenge day and the levels of these antibodies correlated significantly ($0.61 \leq r \geq 0.87$) with the protection against experimental infection with homologous IBV strain.

These findings indicated that local antibodies, especially those exhibiting virus-neutralizing activity and from IgG and IgA isotypes, played a critical role in the restriction of primary replication of the virus, resulting in host protection. Although the levels of specific IBV IgM antibodies in the lachrymal secretion did not correlate significantly with the protection status, the ability of these antibodies in limiting IBV infection at respiratory mucosal sites cannot be totally ruled out. In fact, the production of this anti-IBV isotype at 10 days post-vaccination, an earlier post-vaccination period than that tested here, was associated with the protection against virus challenge in layer chickens (De Wit *et al.*, 2006). On the contrary, systemic anti-IBV antibodies measured in this study, though have increased three weeks after the vaccination, they reached lower levels than those detected in lachrymal secretion and showed no significant correlation with the protection status to IBV infection.

Interestingly, there were also fewer vaccinated birds carrying lower levels (S/P = 0.30) of anti-IBV antibodies, in the lachrymal secretion (two birds for IgG, two for IgA and three for IgM) than in serum samples (five birds for IgG, seven for IgA and six for IgM) (Table 1). In addition, negative or lower levels for all antibody isotypes were not detected simultaneously in the lachrymal secretion of any vaccinated bird and one antibody isotype was, at least, increased at moderate to high levels in lachrymal secretion (Table 1). A similar result was observed for the virus-neutralizing antibodies, so that there were four out of nine vaccinated birds presenting negative VN antibody titers in serum, whereas only one exhibited this condition in lachrymal samples (Table 1).

Although our results have demonstrated the correlation between lachrymal anti-IBV antibodies and protection against IBV infection, this finding disagreed with the data recorded by Gelb *et al.* (1998). This disagreement could be associated with the fact that these authors did not determine VN antibody titers, as performed in this study and a different method of ELISA was used in that study, which was not able to discriminate the antibody isotypes, as the anti-IBV antibody levels were analyzed here. Furthermore, the protection was evaluated by Gelb *et al.* (1998) only based on the absence of virus isolation, whereas in our study, in addition to virus isolation, tracheal ciliary activity and histopathology were evaluated.

Contrarily to this divergence, the results of the current investigation agreed with the findings of Toro and Fernandez (1994) and Mondal and Naqi (2001),
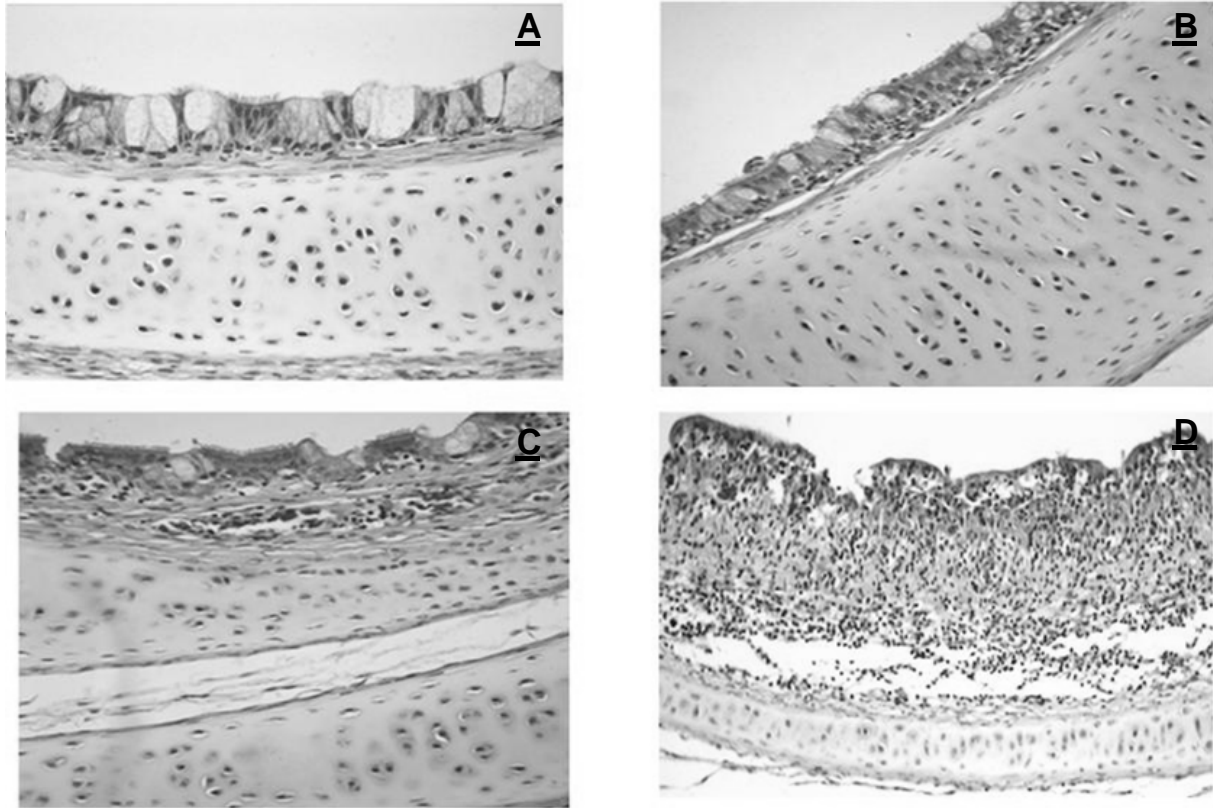
Fig. 1: Tracheal histopathology analysis of vaccinated (A, B and C) or unvaccinated (D) chickens from tissue samples collected at four days post-infection with M41 strain of IBV. (A) Normal trachea with no evidence of inflammation, HE, magnification of 40 x (Lesion score = 0). (B) Trachea with minimal congestion and cellular infiltration in lamina propria and with some areas of deciliation of mucosal epithelium, HE, magnification of 40 x (Lesion score = 1). (C) Trachea with moderate congestion, edema and cellular infiltration in lamina propria and areas of desquamation of epithelium, HE, magnification of 40 x (Lesion score = 2). (D) Trachea with severe congestion, edema and cellular infiltration in lamina propria and areas of desquamation of epithelium, magnification of 20 x (Lesion score = 3)

confirming the involvement of local specific antibodies in the control of IBV infection, particularly those exhibiting virus-neutralizing activity and belonging to IgG or IgA isotypes. In addition, our results confirmed the findings of Okino *et al.* (2013) that characterized the role of tracheal memory humoral immune responses mediated by lachrymal IgG and IgA anti-IBV antibodies, at one and five days post-infection, in the protection of tracheal mucosa against the infection with IBV M41 strain.

Thus, our results indicated that local antibodies are effectively induced by IBV attenuated vaccine in SPF chickens and these antibodies are providing a significant part of the protection for the upper respiratory tract of chickens, which is the most frequent entry site of IBV. Despite this, the cell-mediated immune responses, that were not evaluated in this study, cannot be ruled out as an additional protective mechanism of resistance against IBV infection, given that specific cytotoxic T CD8 lymphocytes have been shown to be important in the

immune-protection against this virus (Collisson *et al.*, 2000; Seo *et al.*, 2000; Okino *et al.*, 2013). Thus, the correct concept applicable for defining the most effective mechanisms of immune-protection against IBV infection is to assume that local antibodies and cellular immune mechanisms could be acting in an additive or synergistic way, in order to confer a more complete protection status against IBV infection, as reported (Okino *et al.*, 2013).

Conclusion: In conclusion, the importance of the production of local antibodies as specific primary barrier against IBV infection on the respiratory mucosal surfaces of vaccinated birds was well characterized in this study. The current results indicated also that the anti-IBV antibodies, which are induced by vaccination with attenuated vaccine strain, in lachrymal secretion and, probably, in other respiratory mucosal secretions, can be used as reliable markers to evaluate more

accurately the anti-IBV immunity, because they may be playing a relevant role, not only in the protection of the challenged birds, but in the inhibition of virus spreading within the chicken host.

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