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Original article

TURKEY HUMORAL AND CELL-MEDIATED IMMUNE RESPONSES TO A NEWCASTLE VISCEROTROPIC VACCINE AND ITS ASSOCIATION WITH MAJOR HISTOCOMPATIBILITY COMPLEX

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Summary

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Immune responses to vaccines are mainly influenced by the nature of vaccines and host variation in response to vaccination. In this study we aimed to investigate turkey humoral and cell-mediated immune responses to a Newcastle viscerotropic vaccine and its association with major histocompatibility complex (MHC). Turkeys were vaccinated with Villegas–Glisson/University of Georgia (VG/GA) attenuated vaccine against Newcastle disease. The stimulation index of lymphocyte proliferation and antigen-specific local secretory IgA responses in bile, duodenum, ileum, as well as serum IgY and IgA responses were analysed by enzyme-linked immunosorbent assay. The turkey MHC class II B locus was selected as candidate gene for detection of associations with cellular and humoral immune responses. Significant differences were observed between both cellular and humoral responses of vaccinated and unvaccinated groups. A significant positive correlation was also found between ND specific IgY and ND specific IgA titres in serum, intestine (duodenum and ileum) and trachea. Moreover, the correlation between specific IgA titres in ileum and specific bile, duodenum and trachea was positively significant. High resolution melting analysis (HRM) was used to genotype MHC class II B exon 2. Eight melting profiles (A-G) were identified, among which, profile G showed a significant association with cellular response. The profile B revealed significant association with total IgA titres in serum and ileum. These findings help our understanding of the association of turkey MHC types with immune responses. Further correlation analysis between serum and mucosal antibody titres demonstrated that the levels of IgY and IgA in serum can give an impression about the levels of secretory IgA and situation of mucosal immunity. Based on the significant effects, ND specific IgY in serum appears to be a promising indirect marker for specific IgA in serum and trachea.

Key words: cellular, high-resolution melting analysis, humoral, mucosal immunity, turkey

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral infection caused by the strains of avian paramyxovirus serotype-1 (AMPV-1) from the family *Paramyxoviridae*, subfamily Paramyxovirinae, in the genus *Avulavirus* (Mayo, 2002; Stear, 2005). Newcastle disease virus (NDV) infects approximately 236 species of domestic avian species (chicken, turkey, goose, duck, and pigeon), pet and free-living birds (Kaleta & Baldauf, 1988). Turkeys are susceptible to infection with velogenic neurotropic and velogenic viscerotropic NDV (Piacenti *et al.*, 2006), but clinical signs appear to be less severe than chickens (Box *et al.*, 1970).

Vaccination and/or quarantine and slaughter of flocks affected with NDV are the only ways for prevention and control of the disease (Alexander *et al.*, 1991). Although the advances made in the diagnosis and vaccination, the disease continues to impact negatively poultry industries (Alexander *et al.*, 2012). However, the development of vaccine-induced immunity and enhancing host antibody response by vaccination remains the most efficient strategy to control outbreaks of Newcastle disease (Luo *et al.*, 2013). Vaccine failure in birds may be due to two main reasons including low vaccine efficacy and variation in response to vaccination (Kapczynski *et al.*, 2013). Effective vaccination against Newcastle disease requires measurement of specific antibodies titres since a correlation exists between antibody response and transmission potential to susceptible birds (Maraqa, 1996). Titre of specific antibodies can then be used for determination of the critical vaccination coverage and herd immunity (Van Boven *et al.*, 2008). Beside routine measurement of serum antibodies, it is important to understand the immune response of mucosal effector sites for the specific sIgA secretion (Al-Garib *et al.*, 2003). This includes verifying the correlation between serum antibody levels and specific IgA responses in local secretions (Externest et al., 2000). The monitoring of success vaccination in a flock by demonstrating an elevation of antibody titres within a few days after vaccination is pivotal but the cell-mediated immune (CMI) responses are also important in induced protective immunity (Sharma, 1999). Several studies revealed that both antibody mediated immunity and cell mediated immunity (CMI) play important roles in protection against ND (Meulemans *et al.*, 1986).

In turkeys, the vaccine of choice intend to replicate both in the respiratory and intestinal tract that elicits adequate immune response with minimal respiratory reactions, is the Villegas–Glisson/ University of Georgia (VG/GA) strain of NDV. This strain has been isolated from the intestine of healthy turkeys (Villegas, 1998; Nunes *et al.*, 2002). Although the VG/GA vaccine is widely used in commercial turkey, assessment of the cell-mediated and mucosal immune responses of birds to vaccine still remains to be elucidated.

Variation in response to vaccination is mainly due to the variation in immune response genes (Kennedy *et al.*, 2012). The major histocompatibility complex (MHC), as a key component in immune responses, contains many genes involved in cytokines expression, complement proteins production, and the highly polymorphic loci in charge of processing and presenting endogenous and exogenous peptide antigens to T cells (Horton *et al.*, 2004). Studies with chicken breeds indicated that MHC genes were involved in controlling both humoral and cell-medi-

ated immune responses (Livant *et al.*, 2001). There is also evidence for the associations of chicken MHC with productive and reproductive traits (Nikbakht & Esmailnejad, 2015). Turkey MHC, like in chicken, is divided into two genetically unlinked regions designated as MHC-B and MHC-Y loci located on the same microchromosome (Chaves *et al.*, 2007; Delany *et al.*, 2009). The MHC-B locus (BF–BL region) is generally considered as the homolog to the mammalian MHC which contains most of the antigen processing and presenting genes (Kaufman, 2013). MHC-Y locus contains non-classical MHC genes, lectin-like loci, and other unknown genes with varied effects on disease susceptibility (Chaves *et al.*, 2010). In the case of association studies, sequence surveys typically concentrated on the peptide-binding region (PBR) encoded in exons 2 and 3 of class I and exon 2 of the class IIB genes (Babik *et al.*, 2009). To date no study has assessed the associations between MHC and immune responses of turkeys.

As mentioned, anticipated immune responses to vaccines are mainly influenced by vaccine nature and host genetic variations. So, the objectives of the current study were to investigate humoral and cell-mediated responses to VG/GA vaccine and the possible correlations between the level of IgY and IgA antibodies in serum and IgA in different mucosal sites of vaccinated turkey. Furthermore, based on the polymorphisms at turkey class IIB genes, the associations between the MHC and immune responses were analysed.

MATERIALS AND METHODS

Turkeys and housing

Ninety six 1-day-old commercial turkey poults (Premium) obtained from local market. The poults were raised in concrete floor pens covered with 8 cm of clean pine wood shavings, and each pen was equipped with one tube feeder and one automatic waterer. Throughout the study, the birds were grown following standard temperature regimens, which gradually decreased from 38 to 23 ºC. Birds were maintained on a 16L: 8D schedule and allowed to consume feed and water *ad libitum*. Air temperature was controlled according to Aviagen recommendations. Poults were divided into 2 treatment groups, with 8 replicates per treatment and 6 poults (tom) per replicate.

Vaccine

At 9 and 20 days of age 8 groups (48 birds) were given 100 µL of a VG/GA vaccine ($10^{6.5}$ EID_{50}) by ocular route and 8 groups remained unvaccinated. The experiment was approved by the Research Ethics Committee of Tehran University.

Collection of samples

Blood samples were collected at 1, 9 and 23 days of age. At day 1 and 9 birds were bled before vaccination for detection of maternal antibodies (mAb). At he day 23 birds were humanely euthanised using a carbon dioxide chamber and bile, intestinal and tracheal washes were obtained. Briefly, after necropsy bile obtained from turkeys using sterile syringes, centrifuge at 21,000×*g* for 20 min at 4 °C, and the supernatant stored at −20 °C until further analyses, while 10 centimeter portion from duodenal and ileum as well as, trachea was quickly removed, cut longitudinally and then incubated in 10 mL of PBS for 4 h on ice. The tissues then were discarded and the washes collected. All intestinal and tracheal washes were individually clarified by centrifugation at 10000×*g* for 15 min, and the supernatant obtained.

Samples were stored at −20 °C until analysis.

ELISA assay

Optimisation of the antibody titre for indirect IgY enzyme-linked immunosorbent assay (ELISA) was conducted using a checker board titration. In each microplate well, 50 µL of diluted antigen (prepared from VG/GA vaccine strain of NDV), ranging from 100 to 1.56 µg/mL (four fold) was coated overnight at 4 °C. Unbound antigens were removed by washing with PBS-T (PBS containing 0.05% [vol/vol] Tween-20) and wells were blocked by 100 µL PBS-Blotto (PBS containing 0.5 % [w/vol] nonfat dry milk) for 1 hour. After three additional washing steps, 100 µL of pooled serum was added to wells using two fold dilutions starting at 1:20 and left for 30 min at 37 °C. The plate was then washed and 100 µL of diluted (1:10000) horse radish peroxidase (HRP)-labelled goat anti-chicken IgY (AbD Serotec, Oxford, UK) was added to each well and left for 30 min at 37 °C. After washing, TMB substrate was added and the mixture was incubated in dark place for 15 min at room temperature. The reaction was then stopped by the addition of 100 µL/well of stopper solution (1% SDS). Absorbance at 450 nm was measured using a microplate reader (Stat FAX 2000, Awareness Technology, Inc., USA).

Indirect ELISA has been used to measure specific IgY antibodies in turkey serum. Microtitre plates were coated overnight at 4 °C with 50 µL antigen (7 µg/mL) in PBS per well. Plates were washed with PBST and nonspecific binding sites were blocked with 100 µL of PBS-Blotto. Plates were washed three times with PBST before adding 50 µL of serially diluted sera and processed as described above using HRP-labelled goat anti-chicken IgY as the secondary antibody.

Indirect ELISA has been used to measure IgA antibodies in turkey serum, bile, intestinal (duodenum and ileum) and trachea against Newcastle disease virus vaccine (NDV). The same above mentioned protocol was used for checker board titration and according to the results microtitre plates were coated with 50 µL (30 µg/mL) antigen in PBS per well. After washing and blocking the plates, serially diluted samples (sera, bile, intestinal and tracheal wash) were added and processed as described above except for using HRP-labeled goat anti-chicken IgA as the secondary antibody (AbD Serotec, Oxford, UK).

For measuring the total IgA, the microtitre plates were coated with 100 µL of serially diluted sera, bile, intestinal and tracheal washes. After one night, plates were washed with PBST and blocked with 100 µL of PBS-Blotto for 2 h at room temperature. Plates were washed three times with PBST before adding 100 µL of (1:10000) diluted conjugate (HRP-labeled goat anti-chicken IgA (AbD Serotec, Oxford, UK).

Lymphocyte proliferation assay

Cell proliferation was tested using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Heparinised blood samples were taken at 23 days. Peripheral blood mononuclear cells (PBMCs) were isolated and 100 µl of PBMC suspension in complete RPMI1640 was seeded in a 96-well plate (5×10^5 cells/well). For a test sample, cells were cultured with 3 µg/mL of NDV recall antigen (NDVr), as a positive control cells were cultured with 10 µg/mL of phytohaematoglutinin (PHA) (Sigma-Aldrich, St. Louis, MO, USA) and for negative

control cells were cultured without NDVr or PHA. All treatments were performed in triplicate. At 72 h of culture, 10 µL of MTT (Sigma-Aldrich, St. Louis, MO, USA) solution in PBS (5 mg/mL) was added to each well. After 4 h of incubation, coloured crystals of formazan were dissolved with a 100 µL of dimethylsulfoxide (Sigma-Aldrich, St. Louis, MO, USA). Plates were kept on orbital shaker for 5 min and optical density (O.D.) was read on a multi-well scanning spectrophotometer (ELISA reader) (Stat Fax 2000, Awareness Technology Inc., Palm City, USA) at 570 nm. The stimulation index (SI) is expressed with average OD value in the test group divided by average OD value in negative controls.

MHC typing

DNA was extracted from blood samples by using a genomic DNA extraction kit (Bioneer, Korea). Turkey MHC sequences (Genbank DQ993255 and EU522671) were used to design primers. PCR primers (F: 5'-CTGCCCGCAGCGTTCT-3'; R: 5'-CGAGACCCGCACCTTGG-3') were designed for amplification and high resolution melting analysis (HRM) of turkey MHC class II B locus, exon 2 (389 bp). Amplification was performed in a final volume of 25 µL containing 20 ng template DNA, 1.5 mM $MgCl_2$, 250 µM of each dNTP, PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1 µL EvaGreen dye (20X) and 1 U/µL of Taq DNA polymerase (CinaClon, Iran). The thermal cycling profile was: 1 cycle of 95 °C for 2 min, 30 cycles of 95 °C for 30 sec, 60.5 °C for 30 sec and 72 °C for 40 sec, with a terminal step of 5 min at 72 °C.

Melting analysis of PCR products (389 bp) in the presence of the fluorescent nucleic acid dye, Eva Green, was used to genotype MHC class II B. The PCR amplicons were subjected to HRM curve analysis. HRM curve analysis was performed in a Rotor-Gene™ 6000 thermal cycler (Corbett Life Science Pty Ltd). In order to determine the optimal melting condition for differentiation of MHC class II B exon 2 region, the PCR products were setup on 0.22 °C/s ramping between 75 °C and 95 °C. All specimens were tested and their melting profiles analysed using Rotor-Gene 1.7 software and the HRM algorithm provided. Plots of fluorescence vs temperature were normalised as described (Gundry *et al.*, 2003; Wittwer *et al.*, 2003). Rapid-cycle PCR products allowed amplification and genotyping in a closed-tube system in <15 min without probes or allele-specific amplification. The heterozygotes can be identified by the presence of a second, low-temperature melting transition. Normalisation regions of 80.47–80.97 and 89.71– 90.21 were used for analysis.

Statistical analysis

Antibody titres were expressed as the reciprocal of the highest dilution that gave a reading above the blank absorbance value. Titre data and SI were expressed as the natural log (ln) for calculation of group means and standard errors of the means (SEM). Between-group comparisons were performed by one-way analysis of variance (ANOVA) using Fisher's protected least significant difference set to the 95% confidence level. Fisher's *r*-to-*z* conversion of correlation coefficients was used to obtain the *P* values in correlation analysis. Results of statistical analyses were considered significant if they produced values of $P \leq 0.05$. The possibility of a multivariate relationship between each of the titres and the entire set of parameters, was investigated by stepwise multiple regression. Effects were considered to be

significant with probability values of <0.05. The following model was used to test the effect of serum IgY and/or IgA and estimation of least square means for each secretory IgAs:

$y_i = \mu + IgA_{trac} + IgA_{deu} + IgA_{ill} + IgA_{bile} + \varepsilon_i$

where: y_i is a vector of observations (serum ND specific IgY or IgA) for the i^{th} bird; μ a general population mean; sum of different secretory IgA titrws; and ε_i is the residual effect for the i^{th} bird with variance. All calculations and statistical analyses were carried out using the computerised SPSS version 21.

RESULTS

Immune responses

All mAb (IgA and IgY) showed a significant decrease ($P \leq 0.05$) in titres and were almost depleted by 9 days of age. The IgA mAb titre at 1-day old was 153.98±2.57 and after 9 days decreased to 51.32±1.28. The IgY mAb titre was 655.49±6.40 which decreased after 9 days of old to 218.33±2.3.

The IgY and IgA levels detected in the serum and IgA levels in bile, intestinal and tracheal washings of experimental turkeys vaccinated with ND live vaccine (VG/AG) are summarised in Table 1. Significant differences were observed between the specific ND IgY response of vaccinated and unvaccinated groups (P=0.0005). Statistically significant differences were also observed between vaccinated and unvaccinated groups for the bile ND specific IgA titres (P=0.026) and trachea ND specific IgA titres (P=0.027). No significant differences were noted in ND specific IgA in serum and intestinal washings between vaccinated and unvaccinated groups. Furthermore, the results showed no significant differences in total IgA levels between the control and the vaccinated groups.

Mitogen (PHA) and NDVr antigens were tested for their ability to activate lymphocytes of turkeys. The same cultures were evaluated for proliferation and the stimulation index (SI) levels. Significant differences were observed between SI values of vaccinated and unvaccinated groups (P=0.0005) (Table 1). The results also revealed highly significant positive correlation between SI values of PHA and NDV (r=0.955, P<0.01).

Table 1. Cellular and humoral immunity detected in experimental turkeys vaccinated with ND live vaccine (VG/AG). Data are presented as mean geometric titres ± SEM (n=95)

Immune responses	Vaccinated	Non-vaccinated	P value
PHA	2.98±0.77	2.54±0.27	0.000
NDV	2.81±0.60	2.57±0.26	0.000
Specific serum IgY	6.59±0.02	6.44±0.03	0.000
Total serum IgA	7.12±0.08	7.18±0.05	0.749
Specific serum IgA	5.06±0.02	5.04±0.03	0.691
Total bile IgA	7.44±0.05	7.38±0.11	0.603
Specific bile IgA	5.26±0.03	5.06±0.10	0.026
Total duodenum IgA	7.21±0.05	7.08±0.09	0.282
Specific duodenum IgA	4.92±0.06	5.00±0.15	0.560
Total ileum IgA	7.23±0.04	7.31±0.07	0.440
Specific ileum IgA	4.89±0.06	5.05±0.14	0.312
Total trachea IgA	7.16±0.05	7.14±0.08	0.868
Specific trachea IgA	5.48±0.05	5.23±0.08	0.027

Correlation analysis of humoral responses

As indicated in Table 2, there was a significant positive correlation between ND specific IgY and ND specific IgA titres in serum (P=0.033, r=0.672). A highly significant positive correlation (P=0.03, 0.011) was also found between ND specific titres of IgY in serum and IgA in intestine (duodenum, r=0.683; ileum, r=0.759). There was a highly significant positive correlation (P=0.005) between ND specific titres of IgY in serum and IgA titres in trachea (r=0.807).

ND specific IgA titres in bile revealed a significant correlation with specific ileal (P=0.011, r=0.616) and tracheal (P=0.028, r=0.547) IgA titres. There was also a close relationship between ND specific IgA titres in duodenum and both ileum (P=0.0005, r=0.895) and trachea (P=0.0005, r=0.942). A significant correlation was found between ND specific IgA titres in ileum and trachea (P=0.0005, r=0.956) (Table 2).

Regarding total IgA titres, significant negative correlations were established between total serum IgA and ND specific IgA in duodenum (P=0.45, r=–0.643), but positive correlation between total ileal IgA and duodenal ND specific IgA (P=0.01, r=0.620). Moreover, negative correlation was found between total IgA titres in bile and duodenum (P=0.003,

Table 2. Correlations between serum and locally secreted antibodies and cell-mediated immune responses

PHA in relation to:	r	P	*Serum ND specific IgY in relation to:*	r	P
Specific bile IgA	0.646	0.007	Specific serum IgA	0.672	0.033
Specific Trachea IgA	0.663	0.005	Total bile IgA	0.716	0.020
Total bile IgA	0.685	0.003	Specific duodenal IgA	0.683	0.030
Total duodenum IgA	–0.521	0.039	Specific ileal IgA	0.759	0.011
Total Trachea IgA	0.524	0.037	Specific tracheal IgA	0.807	0.005
ND	0.955	0.000			
ND in relation to:	r	P	*Duodenum ND specific IgA in relation to:*	r	P
Specific bile IgA	0.515	0.041	Total serum IgA	–0.643	0.045
Total bile IgA	–0.563	0.023	Total ileal IgA	0.620	0.010
Total Ileum IgA	0.748	0.001	Specific ileal IgA	0.895	0.000
Total Trachea IgA	0.709	0.002	Specific tracheal IgA	0.942	0.000
Trachea ND specific IgA in relation to:	r	P	*Ileum ND specific IgA in relation to:*	r	P
Specific bile IgA	0.547	0.028	Specific bile IgA	0.616	0.011
Total ileal IgA	0.595	0.015			
Specific ileal IgA	0.956	0.000			
Total bile IgA in relation to:	r	P			
Specific serum IgY	0.716	0.020			
Total duodenal IgA	0.689	0.003			
Total tracheal IgA	–0.817	0.000			
Total duodenum IgA in relation to:	*r*	*p*			
Total tracheal IgA	0.833	0.000			

Table 3. Effects of the levels of serum ND specific IgY and the levels of specific IgA antibodies in serum and trachea

Variable	Parameter	Estimate	SE	P	95% Confidence Interval
Serum ND specific IgY	Overall effect	3.418	1.114	0.018	0.783–6.053
	Serum ND specific IgA	0.570	0.225	0.039	0.038–1.102
	Trachea ND specific IgA	0.054	0.014	0.007	0.020–0.087

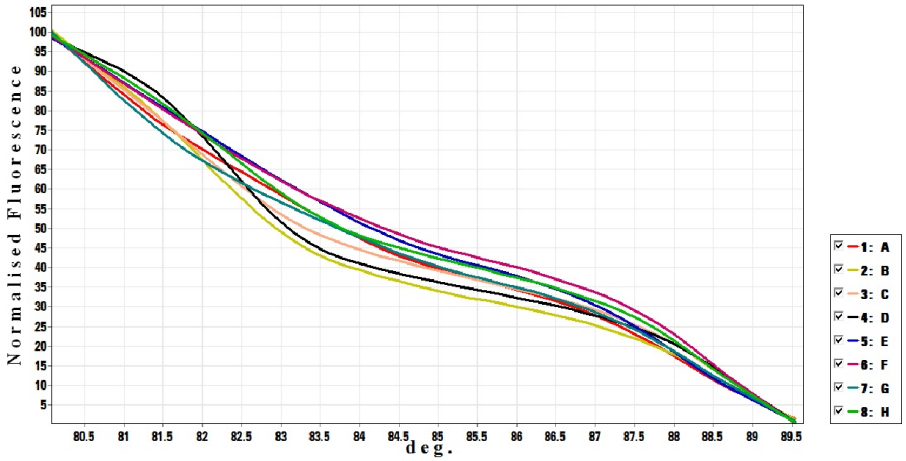

Fig. 1. Normalised HRM curve analysis of PCR products of the MHC class II B Exon 2 region showing eight profile (A, B, C, D, E, F, G and H).

r=–0.689) and bile and trachea (P=0.0005, r=–0.817). A significant correlation was found between total duodenal and tracheal IgA titres (P=0.0005, r=0.833).

Based on stepwise multiple regression analysis, with serum IgY and IgA titres as the dependent variables and the secretory IgA parameters as explanatory variables, significant effect was obtained for the ND specific level of serum IgY and the level of specific IgA antibodies in serum and trachea (Table 3).

Correlations between cell-mediated and humoral responses

There was a highly significant correlation (P=0.007) between the ND specific IgA titres in bile and intensity of the proliferative response (SI values) to PHA (r=0.646) and NDVr (r=0.515). Significant positive correlation was also found between SI values and ND-specific IgA in trachea (rPHA=0.524). Positive correlations were observed between SI and total IgA in duodenum (rPHA=0.685; rNDVr= 0.748) and trachea (rPHA=0.663; rNDVr =0.709). In addition, significant negative correlations were found between SI and total IgA titres in bile (rPHA=–0.521; rNDVr=–0.563) (Table 2).

Associations of immune responses with MHC types

Melt curve analysis (Fig. 1) showed eight heterozygotes normalised melting curves.

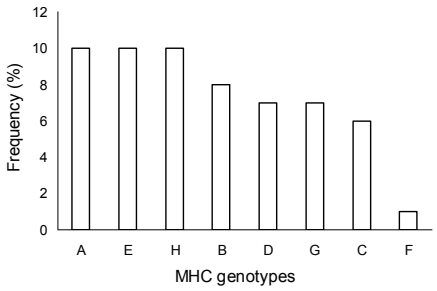

Fig. 2. MHC genotypes frequencies in commercial turkeys.

The profile A generated two major peaks at 82.2 and 89.7 ºC respectively. The profile B generated two major peaks at 83.3 and 90.2ºC respectively. Profile C generated two major peaks at 73.5 and 83 ºC and one minor peak at 90.2 ºC. The profile D generated two major peaks at 76.8 and 83.7 ºC and one minor at 89.7 ºC. The profile E generated two major peaks at 83.8 and 90.5 ºC. Profile F generated two major peaks at 76 and 83.5 ºC and one minor peak at 90.2 ºC. Profile G generated two major peaks at 78.2 and 82.5 ºC and one minor peak at 89.7 ºC. Profile H generated two major peaks at 83.7 and 90 ºC. All genotypes could be distinguished from each other by high-resolution melting analysis. Frequencies of each profile were genotyped with defined genotypes in normalised HRM graph with equal or greater than 95 genotype confidence percentage (GCP) and the distributions of these frequencies are shown on Fig. 2.

The HRM profile G showed significant association with both PHA and NDVr stimulation indices ($P \leq 0.05$). Also, there were significant associations between HRM profile B and total IgA titres in serum and ileum ($P \leq 0.05$) (Table 4).

DISCUSSION

Mucosal vaccination can evoke both systemic and mucosal immune responses, at most secretory IgA antibodies, at the same portal of entry of most infectious pathogens. Fortifying mucosal response by a vaccine is an effective way of targeting the pathogen before occurence of infection (Stear, 2005). The conjunctiva, part of the eye mucosa and mucosal barrier are possible routes for mucosal vaccines since they are exposed to the external environment and have equal proportions of dendritic cells (DCs) and mast cells in the lamina propria together with B and T cells in the epithelium (Box *et al.*, 1970; McFerran & McCracken, 1988). Previous studies have revealed successful protection by eye drop vaccination in avian and bovine models (Luo *et al.*, 2013). It has also been demonstrated that IgA and other antibodies could be induced by both live and inactivated ND vaccines not only in the serum, but also in intestine and trachea

Table 4. Significant association of MHC genotypes with immune traits in commercial turkeys

Immune trait	General mean	MHC genotype	Frequency %	Genotype effect	Standard error	T value	P value
SIPHA	2.724+0.132	G	7.4	–3.761	1.780	–2.112	0.040
SIND	2.689±0.285	G	7.4	–2.964	1.278	–2.320	0.024
Total serum IgA	6.866±0.171	B	8.4	0.561	0.258	2.175	0.035
Total ileal IgA	7.317±0.072	B	8.4	–0.347	0.110	–3.162	0.003

(Chimeno Zoth *et al.*, 2008). The results of the present study indicated that there are significant differences between the ND specific IgY antibodies as well as bile and tracheal specific IgA response of vaccinated and unvaccinated groups. Our results are in agreement with previous studies in chickens that have reported an increase in serum IgY and secretory IgA titres after ND vaccination (Al-Garib *et al.*, 2003; Perozo *et al.*, 2008). In the case of total IgA, studies in chickens similarly showed no significant differences between the control and vaccinated groups (Villegas, 1998; Pilette *et al.*, 2001; Mayo, 2002). This phenomenon has been found both in animal models and in humans, explaining the TCD4-independent production of IgA (Cardinale *et al.*, 2001). It can be suggested that in turkeys, like other species, T-cells are probably not the main source of cytokines necessary for differentiation of B cells towards IgA production. This is further confirmed by the observation of the negative correlation between cell-mediated immunity (SI of PHA and NDVr) and total IgA titres in bile ($P<0.05$).

Cell mediated immunity is suggested to play a key role in protection and has been revealed to be the first immunological response following ND vaccination (Cannon & Russell, 1986). The early protection following vaccination can be demonstrated in the presence of low levels of antibody or in the absence of detectable antibodies (Allan & Gough, 1976). Our results indicated a significantly higher cell-mediated immune response after vaccination with AG/VG vaccine, as estimated by a MTT assay using PHA and specific NDV antigen. Lymphocyte proliferation can occur as early as 2–3 days after a primary or secondary vaccination (Maraqa, 1996; Reynolds & Maraqa, 2000). There was a highly significant positive correlation between SI values of PHA and NDVr ($P<0.01$) e.g. turkey lymphocytes were indeed responsive to mitogen stimulation as well as ND vaccine. The turkey's responses to these mitogens were somewhat similar to what previously reported by Lambrecht *et al.* (2004). Collectively, it could be concluded that the AG/VG vaccine induced both cellular and humoral immune responses in turkeys.

No correlation was found between total serum IgA and total secretory IgA titres. In chickens, it has been similarly reported that the increase in the total antibodies in the intestine was not associated with increased serum IgA and IgG antibodies (Haghighi *et al.*, 2005; Ghahri *et al.*, 2013). Total IgA production mainly depends on systemic or local immune stimulation which can be widely altered by the tissue structure, pathogens and stimulators. Significant negative correlation between total bile IgA and total IgA in duodenum or trachea showed the higher level of secretory IgA in the bile. Although the lack of correlation between total blood and bile IgA in turkeys disagreed with findings in chickens (Rose *et al.*, 1981), it can be further confirmed that its level was higher than other secretions. The negative correlation between serum total IgA and specific IgA in duodenum, however, suggests a dichotomy between total IgA in serum and specific IgA in secretions.

A significant positive correlation was observed between the levels of ND specific titres of IgY in serum and IgA in serum, duodenum, ileum and trachea. This is in agreement with studies that used lentogenic virus vaccine (including VG/GA) in chickens (Perozo *et al.*, 2008). It can be a common phenotype for live mucosal vaccines that act similar to natural infections and promote specific serum IgG and

IgA responses (Brandtzaeg, 2007). Our results do not show any correlation between total or specific IgA titres of serum and secretions. Even though according to Muir *et al.* (2002), the trend in IgA antibody response at the intestinal site is mirrored in serum IgA concentration, Cihak *et al.* (1991) has reported that serum IgA levels may not correlate with those of secretory IgA in mucosal tissues (Muir *et al.*, 2002). Inconsistent IgA levels in bile vs those of the trachea have also been noticed (Cihak *et al.*, 1991). However, it can be speculated that in turkey, or at least in VG/GA vaccinated turkey, IgA antibodies produced at intestine or trachea did not show a blood dependent trend.

Observation of the correlation between specific IgA levels in bile and specific IgA in other secretions such as ileum and trachea is of great importance. Interestingly, significant positive correlations were also found between the cell-mediated response (SI values) and ND specific IgA titres in bile (P=0.007; r=0.646) and trachea (P=0.037; r=0.524). Similar to our findings, it has been reported that higher bile IgA in the chickens vaccinated with the VG/GA strain of NDV, positively correlated with the higher IgA in the intestine and trachea (Perozo *et al.*, 2008). Peyer's patches are main source of IgA producing cells for both the intestine and other mucosal tissues (Macpherson *et al.*, 2008). This could be followed by the idea of the existence of a common mucosal immune system, where sensitised lymphocytes originating in each mucosal lymphoid tissue can migrate to other mucosal tissues. Accordingly, the correlation between levels of IgA titres in intestine and trachea can be interpreted as the replication pattern of VG/GA strain in the intestine that can induce active lymphocytes (IgA producing cells) for both mucosal sites. However, in case of turkey, the assumption that antibodies produced locally in the intestinal tract are transported via the portal vein to the liver and bile and then actively transported through the epithelium (Mayo, 2002), could not be supported.

The possibility of a multivariate association between each of the measurements was investigated by stepwise multiple regression, using specific IgY and IgA levels in serum as dependent variables, and the specific secretory IgAs as explanatory variables. Significant effects were obtained only for the level of specific serum IgY and the level of serum specific IgA (P=0.039) and specific trachea IgA (P=0.007). These findings help our understanding of the correlation between serum and mucosal antibody titres. Detection the levels of serum IgY and IgA may give an impression about the levels of secretory IgA and situation of mucosal immunity. The model can be used for parameters that could not be measured in a large number of birds. Based on the significant effects, ND specific IgY in serum appears to be a promising indirect marker for specific IgA in serum and trachea. Based on the importance of quantifying the mucosal immune response to assess protection capabilities of NDV vaccines, these data would be of the great value when applied in the field.

The genetic diversity of the turkey MHC class II B exon 2 in the studied population was investigated and associations of the MHC genotypes with cell mediated and humoral responses were determined. High-resolution melting analysis of the turkey MHC class II B gene could define genotypic identity between individuals. Using this method, eight MHC genotypes (A–H) were identified in which the profile G showed significant associa-

tion with cell mediated response ($P \leq 0.05$) and profile B: with total IgA titres of serum ($P \leq 0.05$) and ileum ($P \leq 0.01$). These results can give evidence for a direct or closely linked effect of turkey MHC genes on the immune responses to ND AG/GV vaccine. Although several studies suggest that the chicken MHC are involved in cell mediated and antibody responses (Ewald *et al.*, 2007), very few experiments could be found addressing the effects of turkey MHC on immune responses (Tsai *et al.*, 1992; Nestor *et al.*, 1996). To the best of our knowledge, this study is the first investigation of the associations between turkey MHC polymorphisms and both cell mediated and humoral responses. Further investigation is necessary to determine the genotyping of MHC genes in commercial turkey and to characterise or improve avian immunity. It is critical to understand the diversity, expression and function of turkey MHC genes.

CONCLUSIONS

In summary, immune responses to vaccination are characterised by the underlying complex network of cells and humoral mediators, which in turn, are influenced by immune genes. Based on the significant effects, ND specific IgY in serum appears to be a promising indirect marker for specific IgA in serum and secretions. In addition, since the IgA titres in secretions correlated with cell mediated response, the evaluation of IgA levels in the mucosal immune response is important and can aid the NDV control strategies. Despite very little knowledge about the turkey MHC, and in contrast with chicken, it appears that turkey MHC class II B genotypes are associated with both humoral and cell-mediated immune responses. These observations in the turkey may well be relevant for other birds and related species, and open new perspectives for both basic and clinical research concerning host pathogen interactions.

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