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Investigation onto the correlation between systemic antibodies to surface glycoproteins of infectious laryngotracheitis virus (ILTV) and protective immunity

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ABSTRACT

Infectious laryngotracheitis virus (ILTV) is an alphaherpesvirus that causes upper respiratory tract disease in chickens and significant losses to the poultry industry worldwide. Both antibody and cell-mediated responses are generated against ILTV infection; however, the correlation of humoral immune response with protection against ILTV infection is debatable. To examine if whether antibody responses to individual ILTV glycoproteins are correlated with disease and protection, four ILTV glycoproteins (gD, gE, gG and gJ) were expressed as recombinant proteins and used in conjunction with commercially available recombinant gC and gI in indirect ELISAs to measure post-vaccination and/or post-challenge chicken serum antibodies. Serum optical density (OD) values detected by the whole virus, gC, gI and gJ were significantly higher in birds vaccinated with the Serva vaccine strain compared to the SA2 vaccine strain. However, the mean ODs detected by gD, gE and gG were not significantly different between the vaccine strains. Examination of post-ILTV vaccination sera found that gE was the most antigenic glycoprotein and that gC ODs were strongly correlated with those of gI and gJ, while ODs to gG had a relatively poor correlation with those of other glycoproteins. Moderate to poor correlations were found between microscopic tracheal lesion scores and ODs to individual glycoproteins. Examination of post-vaccination pre-challenge antibodies to individual glycoproteins did not find a strong correlation with protective immunity as measured by the severity of clinical signs, gross lesions, and tracheal viral load. Results from this study demonstrated that systemic antibody titers to individual ILTV glycoproteins C, D, E, G, I and J had a relatively poor correlation to protective immunity.

1. Introduction

Infectious laryngotracheitis virus (ILTV; *Gallid alphaherpesvirus* 1) is an alphaherpesvirus that infects chickens, causing an acute upper respiratory tract disease and significant losses to the poultry industry globally (Davison et al., 2009). The disease mainly occurs in densely populated poultry production areas and results in severe production losses due to increased mortality, decreased body weight, loss of egg production and increased susceptibility to other respiratory pathogens (Garcia et al., 2013). A variety of immune responses are generated following infection but not all provide protection against the disease. Early studies on ILTV have suggested that virus neutralizing antibodies can be detected 5–7 days post-infection (DPI), peak around 21 days and decrease over the next several months (Jordan, 1981.). Local antibodies can be detected in the trachea seven days PI and plateau at 10–28 days PI (Bagust et al., 1986; York et al., 1989a). Antibody titers in serum and mucosal surfaces were linked with the stage of infection (York et al., 1989b). Although antibody and cell-mediated responses are generated against herpesvirus infections, the correlation of humoral immune response in the protection against ILTV infection is debatable (Fahey et al., 1983; Fulton et al., 2000; Leong et al., 1994). There are controversial reports on the role of humoral immune response in protection against herpes simplex viruses (HSV-1 and HSV-2), while antibodies against HSV can mediate prophylactic protection in mice (Sherwood et al., 1996; Zeitlin et al., 1996).

Twelve HSV glycoprotein homologs have been identified in the ILTV

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genome, designated as gK, gN, gH, gB, gC, gJ, gM, gL, gG, gD, gI, and gE, and are considered to be responsible for stimulating humoral and cell-mediated responses (Thureen and Keeler, 2006). Glycoprotein B (UL27) is essential for viral attachment and penetration of host cells. It is involved in cell-to-cell spread and syncytium formation (Lee et al., 2011; Liang et al., 1991). Glycoprotein C (UL44) is a structural protein but non-essential for viral replication in cell culture (Kingsley et al., 1994). It plays a role in viral attachment and virulence. In most alphaherpesviruses, gC mediates viral attachment by interacting with the cellular heparin sulfate receptor (Kingsley et al., 1994). Glycoprotein D (US6) is essential for attachment for most herpesviruses and functions as a receptor binding to susceptible cells (Di Giovine et al., 2011). The gE/gI (US8/US7) heterodimer promotes cell-to-cell spreading of the virus by binding cellular receptors at cell junctions and plays a significant role in cell-to-cell spread of ILTV (Devlin et al., 2006). Glycoprotein G is non-essential but conserved in most alphaherpesviruses. It is not assembled into viral particles but is secreted by infected cells (Helferich et al., 2007). The US5 gene of ILTV encodes glycoprotein J (gJ), which was initially named as gp60 because it was identified as 60 kDa glycoprotein. The only reported function of alphaherpesvirus gJ is its ability to inhibit apoptosis and viral egress, and the mechanisms of these functions are not fully known (Fuchs et al., 2005; Mundt et al., 2011; You et al., 2018)

Enzyme-linked immunosorbent assay (ELISA) is a rapid and simple test widely used for the detection of humoral immune responses following infection/vaccination. However, commercially available ILTV ELISA use the whole virus as the detecting antigen and therefore may not be sensitive for the detection of seroconversion following virus-vectored vaccines or to provide an insight into the protective immune response induced by vaccination (Vagnozzi et al., 2012). Recently, individual ILTV surface glycoproteins have been used for ELISAs to detect antibodies to ILTV in sera from the birds vaccinated with attenuated and vectored vaccines against ILT (Chang et al., 2002; Godoy et al., 2013; Shil et al., 2012). However, the role of surface glycoproteins in their ability to reflect disease and protection has not been elucidated. This study was conducted to express individual surface glycoproteins of ILTV in their native form to evaluate their potential for the detection of a protective immune response in the birds after challenge or vaccination.

2. Materials and methods

2.1. Preparation of plasmid constructs for expression of ILTV glycoproteins in mammalian cells

Gene sequences for glycoproteins B, D, E, G, and J of the Australian ILTV vaccine strain SA2 (GenBank accession number JN59696) were optimised for expression in mammalian cells and synthesized by GenScript USA Inc. Purified glycoproteins C and I were obtained from Veterinary Diagnostic Solutions Pty Ltd (Melbourne, Australia). Prior to synthesis, transmembrane domains, and cytoplasmic tails where present were removed from the genes. Synthetic genes were cloned into the vector pCAGGS-mcs (GenScript archived vector 60,300) via *Sac*I and *Xho*I restriction (5' and 3' ends, respectively). An in-frame nucleotide sequence encoding a Flag epitope tag (DYKDDDDK) was included at the 3' end of each glycoprotein coding region. Human embryonic kidney-293 T (HEK-293 T) cell line (ATCC® CRL-3216™) were used to express the recombinant proteins using a transient expression. Initially, all the recombinant plasmids were transformed into *E. coli* DH5α cells (BIO-85026, BioLine) and transformants were plated onto Luria Burtani (LB) agar plates containing 100 μg/mL ampicillin and plates were incubated overnight at 37 °C. The resultant colonies were selected and used to inoculate LB broth containing 100 μg/mL ampicillin. The cultures were incubated overnight at 37 °C with constant shaking (220 rpm) and the plasmid DNA was extracted from them using a miniprep plasmid extraction kit (Axygen Biosciences) according to the manufacturer's instructions. DNA was quantified by measuring the absorbance at 260 nm using a NanoDrop ND-100 UV–vis Spectrophotometer (Thermo Scientific). Plasmid constructs carrying genes were digested with restriction enzymes *Sac*I and *Xho*I (New England Biolabs) at 37 °C and digested products were separated in a 0.8% agarose gel to confirm the size of the insert. The insert DNA was also subjected to nucleotide sequencing (Monash Micromon DNA Sequencing Facility) to confirm its identity.

2.2. Mammalian cell culture

HEK 293 T cells were removed from liquid nitrogen, placed in a 37 °C water bath and suspended into 9 mL of prewarmed Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of Amphotericin B (Gibco). Cells were centrifuged at 1000 × g for 5 min, the supernatant was discarded, and the cell pellet was resuspended in 30 mL of DMEM containing 10% fetal bovine serum (FBS). Cells were transferred into a 75 cm^2tissue culture flask and the culture was incubated at 37 °C. Three days later adherent cell layer was washed, and the cells were counted using trypan blue staining and a hemocytometer. Cells at 1 × 10^7/mL were frozen down in a medium containing 5% (V/V) dimethyl sulfoxide (DMSO) in FBS and stored in liquid nitrogen.

2.3. Transfection of HEK 293 T cells

HEK 293 T cells were plated onto each well of a 6-well plate at 3–4 × 10^5 cells in a 2 mL volume of DMEM supplemented with 10% FBS and the plate incubated at 37 °C until the cells reached 70% confluency. For each construct, 4 μL of FuGene HD (Promega) was mixed with 100 μL of DMEM and the mixture incubated at room temperature (RT) for 20 min. One microgram of plasmid DNA was mixed with the transfection mixture and was incubated at room temperature for 5 min. In each transfection experiment, an enhanced green fluorescent protein gene construct was used as a positive control and untransfected cells as a negative control. A 100 μL volume of transfection mixture was added to each well and the plate incubated at 37 °C in an incubator with 5% CO_2 overnight. The medium containing serum was replaced with fresh medium without serum and the plate incubated at 37 °C for another 48 h.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunostaining

SDS-PAGE was performed as described previously (Laemmli, 1970). Cell culture supernatants and cell lysates (prepared in 1% Triton X-100) were mixed with 4 × lysis buffer (62.5 mM Tris-HCl, pH 6.8,10% glycerol, 1% SDS, 1 M β-mercaptoethanol, 0.005% Bromophenol Blue), heated at 95 °C for 5 min and centrifuged at 14,000 × g for 1 min. The proteins were separated through a 10% gel by electrophoresis at 140 V for 1 h and were stained with Coomassie brilliant blue R-250 (Biorad). For immunostaining experiments, proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane which was blocked with 5% w/v skimmed milk for 1 h at RT. The membrane was incubated with a primary antibody (1/1000 dilution of anti-Flag monoclonal antibodies/ chicken anti-ILT sera) in 1 × PBS with 0.05% Tween 20 (PBST) for 1 h at RT. After probing with a secondary antibody (1/2000 dilution of HRP-conjugated goat anti-mouse/ or goat anti-chicken IgG antibodies in PBST) for 1 h at RT, the membrane was washed with wash buffer (PBST). Protein bands were detected by exposure of the membrane to tetramethylbenzidine (TMB) substrate according to the instructions provided by the manufacturer (Sigma).

2.5. Affinity purification

Purified gC and gI were obtained from the Veterinary Diagnostic Solutions Ltd (Melbourne, Australia). For other glycoproteins, anti-Flag monoclonal antibodies were chemically coupled to the cyanogen bromide (CNBr)-activated Sepharose resin using a protocol provided by the manufacturer (Sigma). One mL of resin was placed in a column and was equilibrated using equilibration buffer (1:5) (DMEM diluted in PBS) and then 10 mL of the cell culture supernatants (collected 72 h post-transfection) diluted in PBS was loaded onto it at a flow rate of approximately 100 µL/min. The resin was washed with 5–10 column volumes of wash buffer (1:5) (DMEM diluted in PBS), and proteins eluted using 0.1 M acetic acid (pH 2.8). The eluted fractions were neutralised using 2 M Tris-HCl (pH 7.4), and their protein concentration measured using NanoDrop. The size and purity of the proteins were assessed using SDS-PAGE.

2.6. Sera from experimentally vaccinated and/or challenged chickens

A total of 65 experimental chicken sera was used in this study. Thirty chicken sera were collected during a previous experiment conducted in our laboratory to compare the replication and transmissibility of an ILTV vaccine delivered via eye drop or drinking water (Coppo et al., 2012a). These included sera from chickens vaccinated with Serva (n = 10) and SA2 (n = 10) vaccine strains and unvaccinated chicken (n = 10). Also, 10 sera were obtained from a different experiment conducted to study the immunogenicity and relative pathogenicity of gG deficient ILTV in SPF chickens infected intratracheally with 10^3 median embryo infective doses (EID_{50}) (Devlin et al., 2007). These included sera from chickens vaccinated with SA2 (n = 5) and ΔgG ILTV (n = 5) vaccines. Twenty-five sera were also from a challenge experiment conducted to study the relationship between mortality, clinical signs, and tracheal pathology of ILT in chickens infected intratracheally with 10^3 EID_{50} (Kirkpatrick et al., 2006). These included sera from chickens challenged with vaccine strains SA2, A20 and ΔgG, field strains SA and QLD, and uninoculated chickens, all collected 21 days post-inoculation. Microscopic lesions were scored on a scale of 0–5 according to a method described previously (Guy et al., 1990).

2.7. ELISA

To define the optimal concentration of the glycoproteins in ELISA, each was diluted in carbonate-bicarbonate buffer (pH 9.6) by two-third (2/3) fold serial dilutions and 50 µL of each dilution was used to coat each well of a 96-well Maxisorp plate (Nalge Nunc International, IL, USA). After overnight incubation at 4 °C, the wells were washed three times with wash buffer (PBS, 0.05% Tween 20) and blocked with 1% bovine serum albumin (Sigma) in PBS at RT for 2 h and washed 3 times as before. Mouse anti-Flag monoclonal antibody (CSIRO, Australia) diluted 1/1000 in ELISA buffer (100 mM Tris-HCl pH 7.4, 500 mM NaCl, 0.1 mM Na_2EDTA, 2% w/v BSA, 3% w/v Triton X-100, 3% w/v Tween 20, 5% normal rabbit serum) was added to each well and the plate was incubated for 1 h at RT. The wells were then washed four times in wash buffer and then 50 µL of rabbit anti-mouse HRP conjugated immunoglobulin G (Merck Millipore) diluted 1/1000 in ELISA buffer was added to each well. The plate was incubated for another 1 h at RT and washed again as before. Then 125 µl of TMB substrate (Sigma) was added to each well and the plate incubated for 5 min. The reaction was stopped by adding 25 µL of 2 M H2SO4 to each well and the absorbance of each well was measured as optical density (OD) at a wavelength of 450 nm using a microplate reader (ThermoFisher). The OD values, as opposed to titers (relative units), were used for examination of antibody responses to glycoproteins to eliminate potential bias that could be introduced depending on the reference serum (from either vaccinated or challenged birds) used for interpolation of the ODs. Where comparison between antibodies against individual glycoproteins had to be made, a ratio of whole virus/individual glycoprotein ODs was calculated and used. Each glycoprotein was used at the optimum concentration in subsequent ELISAs using the same protocol (as described above) to determine antibody responses in chickens post-vaccination/challenge against each glycoprotein.

2.8. Statistical analysis

Optical density values and whole virus/individual glycoprotein OD ratios were analysed using 2 tailed *T*-tests (Minitab 18: Minitab Inc, State College, PA, USA) and results were considered statistically significant at a *P*-value < 0.05. Pearson's correlation coefficient (r) was calculated using Microsoft Excel 365 Pro plus, and a correlation coefficient of 0.90–1.00, 0.70-0.90, 0.50-0.70, 0.30-0.50 and 0.00-0.30 were considered as very strong, strong, moderate, poor, and very poor, respectively (Mukaka, 2012). The Mann-Whitney test was used to compare the median figures for histological tracheal scores (Minitab 18: Minitab Inc, State College, PA, USA). All graphs were plotted using Graph Pad Prism 7 (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Four glycoproteins were successfully expressed and purified using a mammalian expression system

Supernatants of HEK 293 T cells transiently transfected with mammalian expression vectors encoding gD, gE, gG, and gJ were subjected to SDS-PAGE and protein of approximately 42, 49, 45 and 200 kDa were detected, respectively. All the glycoproteins were of expected sizes except for gJ that appeared to be of higher molecular weight (Fig. 1 a). However, cells transfected with a plasmid vector encoding gB did not show any expression. Plasmid vectors encoding gC and gI were also included as positive controls and generated proteins of 52 and 50 kDa, respectively, as expected. All six secreted recombinant proteins were detectable in immunostaining experiments using a mouse anti-Flag monoclonal antibody and pooled chicken anti-ILT serum (Fig. 1 b, c). Similar experiments using sera from chickens vaccinated with SA2 and Serva vaccine strains revealed that gC, gE, gI, and gJ reacted with both sera; however, gD and gG only reacted with sera from SA2 vaccinated birds (data not shown). Immunoaffinity purification of the cell culture supernatant collected 72 h post-transfection of HEK 293 T cells resulted in overall yields of approximately 250, 120, 350 and 100 µg for gD, gE, gG, and gJ, respectively.

3.2. Mean ODs detected by the whole virus, gC, gI and gJ were higher for serva vaccine strain compared to the SA2 vaccine strain

A range of concentrations of each glycoprotein antigen was tested for the detection of humoral antibody response after ILTV vaccination or challenge. ELISAs were established for the glycoproteins using optimum concentrations of 220, 330, 230, 1000, 250 and 400 ng per well for gC, gD, gE, gG, gI, and gJ, respectively. The mean ODs detected using the whole virus (WH)-based commercial test and the glycoprotein-based ELISAs were compared for the vaccinated groups (vaccinated with the Serva, SA2, and ΔgG vaccines) and uninoculated bird groups (Table 1 and Fig. 2). Birds vaccinated with Serva vaccine strain had significantly higher ($P \leq 0.009$) mean ODs detected using a whole virus, gC, gI, and gJ compared to those vaccinated with SA2 vaccine strain. However, the mean ODs detected by gD, gE and gG were not significantly different between the two vaccine strains. Similarly, there were significant differences ($P \leq 0.004$) between ODs in birds vaccinated with SA2 compared to birds vaccinated with ΔgG using the whole virus and five glycoproteins except for gI. As expected, compared to all other groups, the uninoculated negative control group had significantly lower ($P \leq 0.001$) ODs detectable by a whole virus, gC, and gI. Also, as expected, the uninoculated group had significantly lower ODs

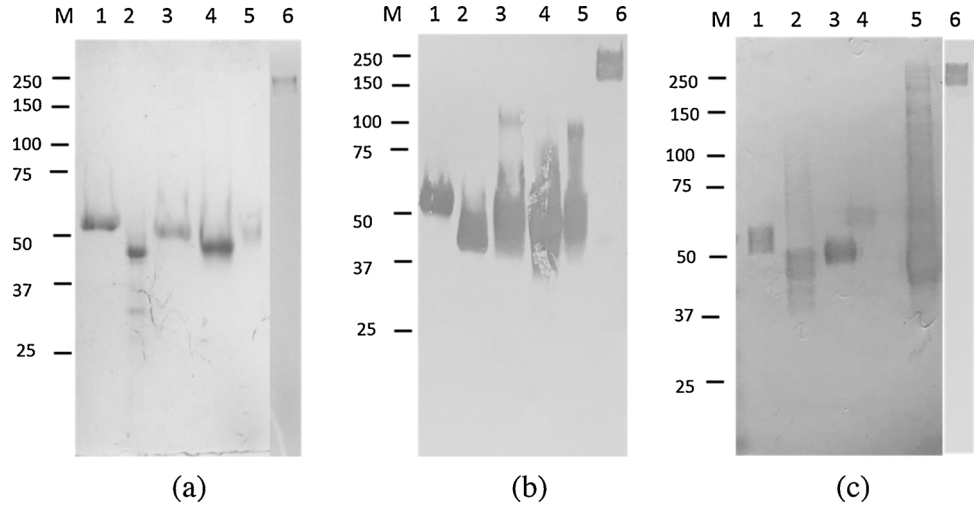

Fig. 1. SDS-PAGE (a) and Immunoblots of supernatant of the cells transfected with glycoproteins probed with mouse anti-Flag tag monoclonal IgG (b) or chicken anti-ILT (c). MW marker is precision plus protein dual colour standards from Bio-Rad (161-0374).

($P \leq 0.017$) for gD, gE and gJ compared to Serva and SA2 groups. In contrast, the uninoculated group had no significant difference with ΔgG group in gD, gE and gJ while significantly higher ($P \leq 0.001$) ODs were detected for gG than ΔgG group.

3.3. Means of the ratios between ODs to the whole virus and all glycoproteins except for gC and gI were significantly different between all vaccinated and uninoculated birds

To assess the proportion of antibodies directed to a single glycoprotein compared to the whole viral antigens, and to benchmark the level of antibody response to each glycoprotein for direct comparison, means of ratios between antibody levels to the whole virus and individual glycoproteins were calculated and used for analysis.The means of WV/gE, WV/gG and WV/gJ ratios for the Serva, SA2, and ΔgG vaccinated groups did not find a significant difference between them. However, means of the WV/gE ratios were significantly different between Serva and SA2 ($P = 0.001$), and Serva and ΔgG mutant groups ($P = 0.009$) and means of WV/gI ratio was significantly different ($P = 0.001$) between SA2 and ΔgG mutant groups. The means of WV/gD, WV/gE, WV/gG, and WV/gJ ratios were significantly different ($P \leq 0.001$) between vaccinated and uninoculated groups; however, means of WV/gC and WV/gI ratios were not significantly different between SA2, ΔgG and uninoculated control groups (Table 2).

3.4. Individual ODs to gC, gI and gJ were highly correlated

Analysis of correlation of co-efficient (r) of the ODs obtained for individual sera using ELISA based on the whole virus and the six glycoproteins showed a strong correlation (≥ 0.9) for the whole virus and gJ, gC and gI, and gC and gJ. Also, a moderate correlation (0.68-0.86) was found among all others except for gG where only poor correlations (≤ 0.38) were found with the whole virus and individual glycoproteins (Table 3).

3.5. Glycoprotein ODs were not significantly different between birds inoculated with vaccine or field strains

To examine any potential difference in glycoprotein antibody profiles between vaccinated and challenged birds, glycoprotein-based ELISAs were used to test the sera from Specific pathogen free (SPF) chickens challenged with Australian vaccine strains (SA2, A20), a vaccine candidate (ΔgG), Australian field strains [South Australia (SA), Queensland (Qld)] and a negative control group. The mean ODs against field and vaccine strains were not significantly different for any of the glycoproteins. However, significant differences ($P \leq 0.04$) were found between ODs of the negative control group and both vaccinated and field virus-challenged groups for all glycoproteins except for gG, where there was no significant difference between vaccine strains and the negative control group (Table 4 and Fig. 3). To demonstrate a potential relationship between the levels of viral replication/virulence (vaccine vs. field strains), ODs to individual glycoproteins were examined against microscopic tracheal lesion scores. Examination of the correlation between tracheal pathology and serum ODs against each glycoprotein found only poor-to-moderate correlation (gC, 0.60; gD, 0.50; gE, 0.64; gG, 0.31; gI, 0.55 and gJ, 0.64).

3.6. No strong correlation could be found between titers of antibodies to any of the glycoproteins and protective immunity

In order to investigate a possible correlation between ODs against individual glycoproteins and protective immunity, post-vaccination

Table 1
Means ± standard deviations of optical densities of sera from Serva, SA2 and ΔgG ILTV inoculated, and uninoculated control birds detected in the whole virus and six different ILTV glycoproteins ELISAs.

Groups	Number of sera	Whole Virus	gC	gD	gE	gG	gI	gJ
Serva	10	2.01 ± 0.61[a]	2.01 ± 0.31[a]	0.91 ± 0.32[a]	1.37 ± 0.27[a]	0.32 ± 0.18[a]	1.76 ± 0.42[a]	0.70 ± 0.14[a]
SA2	15	1.28 ± 0.63[b]	1.25 ± 0.75[b]	0.86 ± 0.40[a]	1.35 ± 0.39[a]	0.25 ± 0.20[a]	1.09 ± 0.62[b]	0.47 ± 0.22[b]
ΔgG	5	0.43 ± 0.20[c]	0.48 ± 0.13[c]	0.25 ± 0.15[b]	0.51 ± 0.30[b]	0.07 ± 0.03[b]	0.77 ± 0.28[b]	0.18 ± 0.07[c]
Negative	10	0.07 ± 0.03[d]	0.20 ± 0.07[d]	0.27 ± 0.08[b]	0.31 ± 0.09[b]	0.24 ± 0.06[a]	0.19 ± 0.09[c]	0.13 ± 0.02[c]
Overall vaccinated*	30	1.38 ± 0.77[e]	1.37 ± 0.75[e]	0.78 ± 0.40[c]	1.22 ± 0.45[c]	0.24 ± 0.19[a]	1.26 ± 0.62[d]	0.50 ± 0.25[d]

[a,b,c,d] In each column values with different superscript letters are significantly different ($P < 0.05$).
*Comparisons were made with negative control group only.

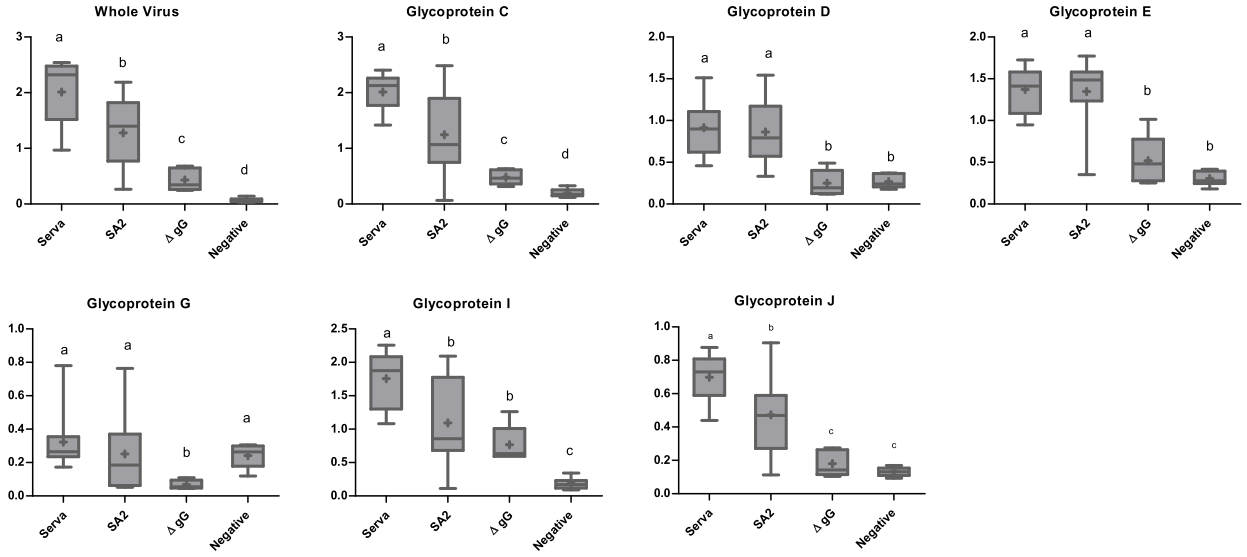

Fig. 2. Box and whisker plots of the optical density values detected using the whole virus and glycoprotein-based ELISAs. Sera were collected from chicken vaccinated with Serva (n = 10), SA2 (n = 15), ΔgG mutant (n = 5), and uninoculated birds (n = 10). Rectangles represent the interquartile range of each group, + marks indicate the mean, whiskers show the minimum and maximum values and the line inside the rectangles indicate the median. $_{a,b,c,d}$ Values with different superscript letters were statistically significantly different ($P < 0.05$).

sera (n = 10) collected during an SA2 and ΔgG vaccine efficacy study, with recorded post-challenge data for individual birds, were tested in glycoprotein ELISAs. The severity of clinical signs (no signs = 0, mild = 1, moderate = 2, severe = 3, very severe = 4), gross lesions (normal = 0, minimal changes = 1, mild = 2, moderate = 3, severe = 4, very severe = 5), and the tracheal viral load (negative = 0, positive = 1) were used to calculate a protection index which was subsequently analysed to examine the correlation between antibody response to individual glycoproteins and protective immunity (Supplementary table). A relatively poor correlation was found between protection index and ODs for individual glycoproteins (gC, 0.22; gD, 0.21; gE, 0.21; gG, -0.21; gI, -0.44 and gJ, 0.09).

Table 3
Contingency table detailing the Pearson's coefficient of correlation (r) for the optical densities detected using whole virus and glycoprotein based ELISAs. Correlation coefficient of 0.90–1.00, 0.70-0.90, 0.50-0.70, 0.30-0.50 and 0.00-0.30, were considered as very strong, strong, moderate, poor, and very poor, respectively. Underlined are the values showing the best correlation (≥ 0.80).

	Whole virus	gC	gD	gE	gG	gI	gJ
Whole virus	1.00	0.76	0.67	0.79	0.12	0.68	0.91
gC		1.00	0.73	0.82	0.33	0.92	0.92
gD			1.00	0.81	0.38	0.74	0.80
gE				1.00	0.25	0.79	0.86
gG					1.00	0.32	0.35
gI						1.00	0.90
gJ							1.00

4. Discussion

This is the first, albeit preliminary, study investigating the potential relationship between antibodies to ILTV glycoproteins and protection against viral-induced disease. Our aim was to identify any viral glycoprotein(s) that might be involved in protective immunity, and therefore can be targeted for future vaccine studies. Such glycoproteins would also have important implications for reliable detection of protective antibody responses after vaccination, especially given that current commercial serological assays use whole viral proteins and do not provide an insight into the protection status of the birds. In this study, however, no strong correlation could be found between systemic antibodies to individual glycoproteins and protective index, suggesting that the mechanism of immunity may better be linked to local antibody responses and/or cellular immunity. This is in contrast to a previous study that demonstrated that ILTV gD induces neutralizing antibodies that confer protection against a virulent virus (Basavarajappa et al., 2014). It is known that humoral immune response can play an important role in limiting the infection and reactivation of some other herpesvirus infections (Clementi et al., 2017; Ohlin and Soderberg, 2015). It is also believed that antibodies against HSV can mediate prophylaxis in mice (Chew et al., 2009). It is not known whether viral glycoproteins may have possessed epitopes that are not readily detectable in ELISA. If so, other serological assays such as virus neutralization assays may be more appropriate for predicting protective immunity. For this investigation, however, monospecific polyclonal antibodies to individual glycoproteins will be required. Combining in-vitro biological data with sequence or structure modeling could also be helpful in determining important epitopes involved in immunity (Levings et al., 2015).

Table 2
Means ± standard deviations of the ratios between optical densities for the sera from Serva, SA2 and ΔgG ILTV inoculated and uninoculated control birds detected in the whole virus (WV) and six different glycoproteins ELISAs.

Groups	WV/gC	WV/gD	WV/gE	WV/gG	WV/gI	WV/gJ
Serva	0.96 ± 0.19^{a}	2.20 ± 0.46^{a}	1.41 ± 0.25^{a}	6.50 ± 2.01^{a}	1.12 ± 0.25^{a}	2.78 ± 0.47^{a}
SA2	1.15 ± 6.82^{ab}	1.40 ± 1.05^{a}	0.86 ± 0.33^{b}	6.00 ± 7.16^{a}	1.24 ± 3.77^{ab}	2.61 ± 0.91^{a}
ΔgG	0.85 ± 0.48^{ab}	1.84 ± 0.87^{a}	0.87 ± 0.25^{b}	6.21 ± 3.76^{a}	0.54 ± 0.18^{b}	2.38 ± 0.30^{a}
Negative	0.35 ± 0.25^{b}	0.26 ± 0.19^{b}	0.22 ± 0.16^{c}	0.29 ± 0.19^{b}	0.39 ± 0.33^{b}	0.51 ± 0.27^{b}

a, b, c In each column values with a different superscript letter were statistically significantly different (P < 0.05).

Table 4
Means ± standard deviations of optical densities of sera from field, vaccine strains inoculated and uninoculated control birds detected using six different ILTV glycoproteins ELISAs and median (range) of microscopic tracheal scores of the birds in same groups.

Groups	Number of Sera	gC	gD	gE	gG	gI	gJ	Median (range) of microscopic tracheal scores
Field Strains	8	0.72 ± 0.31[a]	0.14 ± 0.05[a]	1.40 ± 0.52[a]	0.08 ± 0.01[a]	0.63 ± 0.15[a]	0.98 ± 0.34[a]	4.25 (0.00 - 5.00) [a]
Vaccine strains	11	0.82 ± 0.56[a]	0.13 ± 0.07[a]	0.59 ± 0.58[a]	0.07 ± 0.02[ab]	0.74 ± 0.62[a]	0.79 ± 0.58[a]	4.00 (0.00 - 5.00) [a]
Negative	6	0.06 ± 0.01[b]	0.07 ± 0.0[b]	0.08 ± 0.03[b]	0.06 ± 0.00 [b]	0.06 ± 0.01[b]	0.07 ± 0.01[b]	1.00 (0.00 - 1.50) [b]

a,bValues with a different lowercase superscript letters in each column were statistically significantly different ($P < 0.05$).

This is the first study to analyse the humoral immune response against a panel of ILTV glycoproteins in the hosts infected with virus strains differing in pathogenicity. Sera from chickens vaccinated with Serva vaccine failed to react or reacted poorly with gD while sera from chickens vaccinated with SA2 reacted with this glycoprotein rather strongly. This could not be attributed to the difference in the sequence of SA2 gD (which was used for expression in mammalian cells) and its counterpart in the Serva strain, since the two proteins have 98.6% sequence similarity. It is also notable that difference in gD antibodies between the two strains could potentially be related to the amount of the protein expressed in these two strains. Further examination of these strains (perhaps under both *in vitro* and *in vivo* conditions) will be required to address this presumption.

Except for gG, antibodies were detected against all glycoproteins in both vaccinated and challenged birds. This is in accordance with a previous report from our group that gG is not a potent stimulator of systemic antibody response although an ELISA based on gG could be used in conjunction with a conventional ELISA to differentiate between antibodies to a gG deficient ILTV vaccine strain and those of the field strains (Shil et al., 2012). Also, in the current study gC was found to be highly immunogenic, a finding that was contrary to a study that reported gC was not a suitable antigen for serological diagnosis (Basavarajappa et al., 2015). However, the latter study used the baculovirus expression system, reflecting that using a different expression system and/or the tag may be important for the integrity of antigenic epitopes. In our study, gC and gI appeared to be most reliable antigens for differentiating all vaccinated birds from uninoculated control birds (Table 1), suggesting that they are most antigenic glycoproteins, even though they could not provide insight into protective immunity.

Experiments conducted on the human parasite *Leishmania major* has shown that specific parasitic antigens are involved in steering the immune responses to “protective” as opposed to “disease” state (Reiner et al., 1993; Reiner and Locksley, 1995; and Sjölander et al., 1998) and that antigenic composition of vaccines should be carefully considered for successful vaccination outcome, which is protection (Mendonça, 2016). In our study differences in antibody responses to individual glycoproteins were examined to investigate whether any of the glycoproteins may be related to “protection” as opposed to “disease” state caused by the vaccine and the virulent strains respectively. Examination of antibodies induced against each glycoprotein after exposure to field and vaccine strains did not find a significant difference between the vaccine and field strains (Table 4). There was also a very poor correlation between antibody titers and microscopic tracheal lesion scores.

A mammalian expression system was used here to generate recombinant viral glycoproteins because of its advantages over bacterial

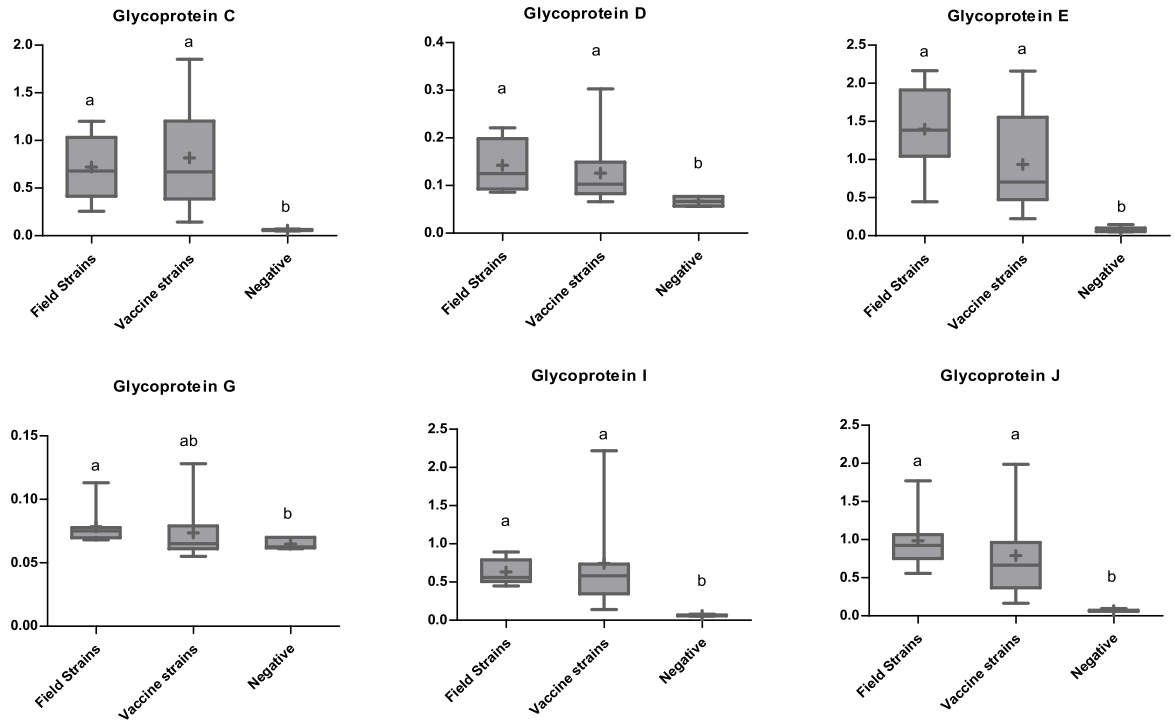

Fig. 3. Box and whisker of the optical density values detected using glycoprotein based ELISAs. Sera were collected from field (n = 8), vaccine strains (n = 11) inoculated and uninoculated birds (n = 6). Rectangles represent the interquartile range of each group, + marks indicate the mean, whiskers show the minimum and maximum values and the line inside the rectangles indicate the median. [a,b] Values with different superscript letters were statistically significantly different ($P < 0.05$).

and insect cell expression systems in terms of maintaining native structure and post-translational modification of the glycoproteins, thus providing the best reflection of antigenicity and function (White and Wimley, 1999). HEK 293 T cell lines were used because of their known capacity for production of recombinant proteins in high yield (Lin et al., 2015). While ILTV gD, gE, gG, and gJ were expressed and purified successfully using small-scale transient gene expression in HEK 293 T cells, similar experiments failed to produce a purified gB. When a similar gene construct was used in baculovirus expression system, a protein of the expected size for gB could be detected in the lysates of the expressing insect cells but not in the cell culture supernatant (unpublished data). This suggests that failure to express gB may be due to an incorrect processing, inhibiting secretion. It is puzzling that the native gB signal peptide is functional in the host (chickens) but not in the mammalian expression system used in this study.

This study investigated the potential role of antibodies to individual glycoproteins in protective immunity; however, it should be noted that protective immunity is a complex process and most likely requires interplay of several glycoproteins and multiple arms of the immune system for induction. Therefore, future studies will need to examine a combination of two or multiple ILTV glycoproteins to determine their role in protective immunity or their potential for improved serological assays.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2018.12.010.

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