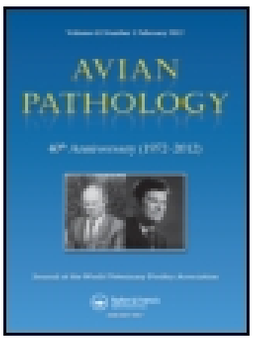

Avian Pathology

ISSN: 0307-9457 (Print) 1465-3338 (Online) Journal homepage: https://www.tandfonline.com/loi/cavp20

Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens

Ariel Vagnozzi , Guillermo Zavala , Sylva M. Riblet , Alice Mundt & Maricarmen García

To cite this article: Ariel Vagnozzi , Guillermo Zavala , Sylva M. Riblet , Alice Mundt & Maricarmen García (2012) Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens, Avian Pathology, 41:1, 21-31, DOI: 10.1080/03079457.2011.631983

To link to this article: https://doi.org/10.1080/03079457.2011.631983

Accepted author version posted online: 17 Oct 2011.
Published online: 16 Feb 2012.

Avian Pathology (February 2012) **41(1),** 21–31

Taylor & Francis
Taylor & Francis Group

Protection induced by commercially available live-attenuated and recombinant viral vector vaccines against infectious laryngotracheitis virus in broiler chickens

Ariel Vagnozzi[1,2], Guillermo Zavala[1], Sylva M. Riblet[1], Alice Mundt[1] and Maricarmen García[1]*

[1]*Department of Population Health, College of Veterinary Medicine, Poultry Diagnostic and Research Center, University of Georgia, Athens GA 30602, USA, and* [2]*Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria, Concepción del Uruguay, 3260 Entre Ríos, Argentina*

Viral vector vaccines using fowl poxvirus (FPV) and herpesvirus of turkey (HVT) as vectors and carrying infectious laryngotracheitis virus (ILTV) genes are commercially available to the poultry industry in the USA. Different sectors of the broiler industry have used these vaccines *in ovo* or subcutaneously, achieving variable results. The objective of the present study was to determine the efficacy of protection induced by viral vector vaccines as compared with live-attenuated ILTV vaccines. The HVT-LT vaccine was more effective than the FPV-LT vaccine in mitigating the disease and reducing levels of challenge virus when applied *in ovo* or subcutaneously, particularly when the challenge was performed at 57 days rather than 35 days of age. While the FPV-LT vaccine mitigated clinical signs more effectively when administered subcutaneously than *in ovo,* it did not reduce the concentration of challenge virus in the trachea by either application route. Detection of antibodies against ILTV glycoproteins expressed by the viral vectors was a useful criterion to assess the immunogenicity of the vectors. The presence of glycoprotein I antibodies detected pre-challenge and post challenge in chickens vaccinated with HVT-LT indicated that the vaccine induced a robust antibody response, which was paralleled by significant reduction of clinical signs. The chicken embryo origin vaccine provided optimal protection by significantly mitigating the disease and reducing the challenge virus in chickens vaccinated via eye drop. The viral vector vaccines, applied *in ovo* and subcutaneously, provided partial protection, reducing to some degree clinical signs, and challenge VIRUS replication in the trachea.

Introduction

Infectious laryngotracheitis virus (ILTV) is a member of the genus *Iltovirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*. ILTV is taxonomically classified as *Gallid herpesvirus 1* (Davison *et al*., 2009), and is the causative agent of the respiratory disease of infectious laryngotracheitis (ILT) in chickens. The disease occurs frequently in densely populated poultry production areas and generates severe production losses due to at least increased mortality, decreased egg production, delayed body weight gain, and predisposition to other respiratory pathogens (Guy & García, 2008). The control of the disease is based on vaccination and biosecurity. Two types of live-attenuated vaccines have been utilized to control infectious laryngotracheitis: vaccines attenuated by multiple passages in embryonated eggs or of chicken embryo origin (CEO) (Samberg *et al*., 1971); and a vaccine attenuated by multiple passages in tissue culture or of tissue culture origin (TCO) (Gelenczei *et al*., 1965). These vaccines have been proven to be effective, particularly the CEO vaccine, producing good protection against the disease (Fulton *et al*., 2000; Han *et al*., 2003; Rodríguez *et al*., 2008). However, live-attenuated ILTV vaccines, in particular that of CEO, regain virulence after consecutive passages in chickens (Guy *et al*., 1991). Both vaccines (CEO and TCO) can be transmitted from vaccinated to unvaccinated birds (Gelenczei *et al*., 1964; Hilbink *et al*., 1987; Andreasen *et al*., 1989; Rodríguez-Avila *et al*., 2007), establishing latent infections in apparently healthy chickens (Andreasen *et al*., 1989). There is strong evidence to relate recent ILTV epizootics in the USA, South America and Europe to CEO-derived strains that persisted in the field and regained virulence (Kirkpatrick *et al*., 2006; Neff *et al*., 2008; Oldoni *et al*., 2008; Chacon *et al*., 2009; Chin *et al*., 2009). This situation has encouraged the development of a new generation of ILT vaccines consisting of viral vector vaccines containing fowl poxvirus (FPV) and herpesvirus of turkey (HVT) as vectors. The HVT-LT vaccine carries the US-6 and US-7 ILTV genes encoding viral glycoproteins D and I (gI), respectively (Hein, 2008); and the FPV-LT vaccine carries the UL-27 and UL-34 ILTV genes encoding viral glycoprotein B (gB)

* To whom correspondence should be addressed:. Tel: +1 7065425656. Fax: +1 7065425630. E-mail: mcgarcia@uga.edu
Received 23 July 2011
ISSN 0307-9457 (print)/ISSN 1465-3338 (online)/12/010021-11
http://dx.doi.org/10.1080/03079457.2011.631983

and membrane-associated protein, respectively (Davison *et al.*, 2006). Compared with CEO and TCO vaccines, the advantages of these vaccines include lack of transmission from bird to bird, lack of ILTV latent infections after vaccination, and no reversion to virulence. The viral vector vaccines offer a safer vaccination alternative against ILTV and have been commercially available in the USA for approximately five years. Although they were originally licensed for subcutaneous (HVT-LT) and transcutaneous (FPV-LT) application, the growing trend of vaccinating against Marek's disease virus by the *in ovo* route and the increased incidence of ILT epornitics prompted the industry to use the ILTV vector vaccines via the *in ovo* route. The protection induced by HVT-LT and FPV-LT viral vector vaccines to broilers when applied *in ovo* with a non-commercial one-needle vaccinator indicated that these vaccines slightly reduced clinical signs when applied *in ovo* but did not reduce significantly the replication of ILTV challenge virus in the trachea (Johnson *et al.*, 2010). The objectives of the current study included: to evaluate the protection induced by ILTV viral vector vaccines when applied *in ovo* using a commercially available *in ovo* vaccination system or subcutaneously at hatch. To compare the protection efficacy of viral vector vaccines with that of the live attenuated vaccines applied via eye-drop. Protection was defined as the ability to prevent clinical signs, reduce replication of the challenge virus in the trachea, and maintain body weight gain after challenge. In addition, serum antibody responses elicited by viral vector vaccines pre-challenge and post challenge were evaluated.

Materials and Methods

ILTV vaccines and challenge isolate. The vaccines used in the present study were the viral vector HVT-LT vaccine Innovax ILT® (Intervet/Schering-Plough Animal Health, Millsboro, Delaware, USA), the viral vector FPV-LT vaccine Vectormune® FP-LT (Ceva, Lenexa, Kansas, USA), the serotype 3 HVT vaccine (Merial Inc., Gainsville, Georgia, USA), the CEO vaccine Trachivax® (Intervet/Schering Plough Animal Health), and the TCO vaccine LT-IVAX® (Intervet/Schering Plough Animal Health). The vaccines were prepared as recommended by their respective manufacturers and titrated after their delivery to confirm the dose applied. The CEO and TCO vaccines and the virulent field isolate 63140 (Vagnozzi *et al.*, 2010) were titrated in chicken kidney (CK) cells as previously described (Rodríguez-Avila *et al.*, 2007). Titres in CK cells were expressed as the 50% tissue culture infectious dose ($TCID_{50}$) based on cytopathic effect and estimated by the Reed and Muench method (Reed & Muench, 1938). The HVT-LT, HVT, and FPV-LT were titrated in secondary chicken embryo fibroblasts. The FPV-LT titration was performed in 24-well plates and titres were expressed as $TCID_{50}$ using the Reed and Muench method. The HVT-LT and HVT vaccines were titrated in 60 mm dishes, where plaques were counted as previously described (Wakenell *et al.*, 2008) and titres were expressed as plaque forming units (PFU) per millilitre.

Experimental design. The protection induced by ILTV viral vector vaccines in broilers when challenged at 35 and 57 days of age was compared with live-attenuated vaccines. In the present study, live-attenuated ILT vaccines were administered as a full dose, and the recombinant vectored vaccines were administered as a half dose to mimic broiler industry practices. The viral vector vaccines were administered *in ovo* and subcutaneously at hatch while the live-attenuated vaccines, CEO and TCO, were administered via eye drop at 14 days of age. The experimental design and treatments utilized in this study are summarized in Table 1. Three hundred broilers' eggs from a local commercial source were incubated in a small-scale hatcher (Natureform Inc., Jacksonville, Florida, USA) from 0 to 21 days. At 18 days of embryo age, the eggs were candled, infertile eggs were removed, and the fertile embryos were divided into four groups, of which two were vaccinated *in ovo* using a commercial Inovoject® vaccinator (Pfizer Animal Health, Durham, North Carolina, USA). One group of 40 embryonated eggs was vaccinated with half a dose of HVT-LT vector vaccine (Innovax ILT®; Intervet/Schering-Plough Animal Health); a second group of 40 embryonated eggs was vaccinated with half a dose of the FPV-LT vector vaccine (Vectormune® FP-LT; Ceva); a third group of 40 embryonated eggs were vaccinated with a full dose of HVT vaccine (Merial Inc.); and 120 embryonated eggs remained unvaccinated. At hatch, 90 of the non-vaccinated chicks were separated into three groups: one group of 30 chicks was vaccinated subcutaneously with half a dose of the HVT-LT vaccine; 30 chicks were vaccinated with half a dose of the FPV-LT vaccine; and the remaining 30 chicks were vaccinated subcutaneously with a full dose of the HVT serotype 3 vaccine. All vaccinated and non-vaccinated chickens were identified by wing tags and housed in separated floor pens located at the Poultry Diagnostic and Research Center (Athens, Georgia, USA). At 14 days of age, 24 non-vaccinated hatch-mates were transferred to four polycarbonate Plexiglas units with filtered-air, negative-pressure isolation units located at the Poultry Diagnostic and Research Center, and fed a standard diet and water *ad libitum*. Chickens were distributed in four units, six chickens per unit, 12 were vaccinated with the Trachivax® (CEO) vaccine (Intervet/Schering Plough Animal Health), and 12 chickens were vaccinated with the LT-IVAX® (TCO) vaccine (Intervet/Schering

Table 1. *Experimental design and treatments.*

	Vaccination			Challenge	
Treatment	In ovo	Subcutaneous	Eye drop	35 days of age	57 days of age
NVx-NCh	–	–	–	–	–
CEO	–	–	14 DO	IT/ED[a]	ND
TCO	–	–	14 DO	IT/ED[a]	ND
HVT-LT io	18 DOE	–	–	IT/ED[a]	IT/ED[b]
HVT-LT sc	–	1 DOA	–	IT/ED[a]	IT/ED[b]
HVT io	18 DOE	–	–	IT/ED[a]	ND
HVT sc	–	1 DOA	–	IT/ED[a]	ND
FPV-LT io	18 DOE	–	–	IT/ED[a]	IT/ED[b]
FPV-LT sc	–	1 DOA	–	IT/ED[a]	IT/ED[b]
NVx-Ch	–	–	–	IT/ED[a]	IT/ED[b]

DOE, days old embryo; DOA, days of age; ND, not done. [a]IT/ED, intratracheally/eye drop at a dose of 1600 $TCID_{50}$/chicken. [b]Intratracheally/eye-drop at dose 1000 $TCID_{50}$/chicken.

Plough Animal Health). Both live-attenuated vaccines were administered at a full dose via eye drop. At 34 days of age, 11 or 12 chickens from the *in-ovo*-vaccinated groups, 11 or 12 chickens from the subcutaneous-vaccinated groups, and 22 to 24 non-vaccinated chickens were transferred to 16 filtered-air and negative-pressure Plexiglas isolation units located in the same room where the CEO and TCO vaccinated chickens were housed. The *in ovo*, subcutaneously vaccinated and non-vaccinated chickens were distributed into two isolation units per group, 5 or 6 chickens per unit, identified as: non-vaccinated, non-challenged group (NVx-NCh), HVT-LT *in ovo* vaccinated challenged group (HVT-LT io), HVT-LT subcutaneously vaccinated challenged group (HVT-LT sc), HVT *in ovo* vaccinated challenged group (HVT io), HVT subcutaneously vaccinated challenged group (HVT sc), FPV-LT *in ovo* vaccinated challenged group (FPV-LT io), FPV-LT subcutaneously vaccinated challenged group (FPV-LT sc), non-vaccinated challenged group (NVX-Ch), CEO vaccinated challenged group (CEO), and TCO vaccinated challenged group (TCO). All chickens were weighed before challenge. With the exception of the NVx-NCh group, all chickens were challenged at 35 days of age with the virulent ILTV field isolate 63140 administered in a total volume of 200 µl, 100 µl intratracheally and 50 µl via eye drop per eye. Chickens in the NVx-NCh group were mock-inoculated with phosphate-buffered saline (PBS) solution instead. At 56 days of age a second group of chickens from NVx-NCh, HVT-LT io, HVT-LT sc, FPV-LT io, FPV-LT sc and NVX-Ch were weighed and transferred to 12 isolation units, two units per group, 5 or 6 chickens per unit, and were provided with feed and water *ad libitum*. With the exception of the NVx-NCh group of chickens, at 57 days of age all chickens were challenged with the virulent ILTV field isolate 63140 as described above.

Protection was evaluated by scoring clinical signs daily between 3 and 7 days post challenge (d.p.c.). The challenge virus present in the trachea was quantified by real-time polymerase chain reaction (PCR) at 3, 5 and 7 days d.p.c. Blood samples were collected before challenge and at 7 d.p.c. for antibody detection. All chickens were humanely euthanized at 7 d.p.c.

Clinical signs and body weight. Clinical signs were recorded daily from 3 to 7 d.p.c. as previously described (Vagnozzi *et al.*, 2010). Briefly, breathing patterns, conjunctivitis, and the level of depression were scored on a scale of 0 to 3: normal (0), mild (1), moderate (2) and severe (3). Mortality was given a total score of 9. The median clinical sign score per group per day was calculated, and differences among groups were analysed statistically. All chickens were weighed at 34 days of age (pre-challenge) and at 42 days of age (7 d.p.c.). The body weight gained per bird was calculated using the following formula:

$$\text{Final weight (FW)} - \text{inital weight (IW)}/\text{FW}.$$

The average weight gained for chickens challenged at 35 days of age was calculated per group. Body weight gained after challenge at 57 days of age was not recorded.

DNA extraction from trachea. Tracheal swabs were placed in 1 ml sterile PBS solution containing 2% antibiotic-antimycotic 100 × (Gibco, Grand Island, New York, USA), and 2% newborn calf serum (Gibco). All samples were stored at–80°C until processing. DNA extraction from tracheal swabs was performed using the MagaZorb® DNA mini-prep 96-well kit (Promega, Madison, Wisconsin, USA), following the manufacturer's recommendations with some modifications. Briefly, 70 µl swab suspension or supernatant from feather pulp samples was harvested after centrifugation and incubated with 7 µl Proteinase K and 50 µl lysis buffer at 56°C for 10 min in a 96-well plate; 20 µl magnetic beads was added along with 125 µl binding buffer to each well and incubated for 10 min at room temperature. The supernatant was separated and the beads were washed twice with washing buffer. Finally, the DNA was eluted from the beads with 100 µl elution buffer.

Quantitative real-time PCR for ILTV in tracheal swabs.. ILTV DNA from tracheal swabs was quantified by real-time PCR in a duplex assay normalized for the host DNA as previously described (Vagnozzi *et al.*, 2010). Briefly, the viral genome load for each sample was normalized to the amount of the host DNA (Niesters *et al.*, 2001). The relative amount of the viral DNA was calculated as the $\log_{10}$ of $2^{-\Delta\Delta Ct}$:

$$\Delta\Delta Ct_t - \Delta Ct_{bt},$$

where ΔC_t is the amount of the targeted ILTV gene normalized against the amount of the collagen gene (internal control gene) from the NVx-Ch and Vx-Ch groups of birds, and ΔCt_{bt} represents the amount of the targeted ILTV gene normalized against the amount of the collagen gene detected before challenge (Livak *et al.*, 2001).

Indirect immunofluorescent antibodies. To determine antibody titres specific to the ILTV antigens expressed by the viral vector vaccines, serum samples collected pre-challenge and 7 d.p.c. were tested with an indirect immunofluorescent antibody (IFA) assay using recombinant gB and gI proteins as antigens. Briefly, the coding sequences of gI, expressed by the HVT-LT vaccine, and gB, expressed by the FPV-LT vaccine, were amplified by PCR using high-fidelity Pfx polymerase (Invitrogen, Carlsbad, California, USA) with primers specifying unique restriction sites for cloning in the eukaryotic expression vector pcDNA3 (Invitrogen). The 3′-end primers of both glycoprotein genes contained a coding sequence for an RGS-6xHis-tag for detection with a commercially available monoclonal antibody (Qiagen, Hilden, Germany). The gB coding sequence was amplified using primers 5′-ACG GGATCC ATGCAATCCTACATCGCCGTG-3′ and 5′-CGTGCGGCCGCCTAATGGTGATGGTGATGGT-GACTTCCTCTTTCGTCTTCGCTTTCTTCTGCC-3′, and the gI coding sequence was amplified using primers 5′-ACG GGATCC ATGGCATCGCTACTTGGAACTC-3′ and 5′-CGTGCGGCCGCCTAATGGTGATGGTGATGGTGACTTCCTCT-CATTTTTATTGAGTCGGGCGAGC-3′. Recombinant plasmids were sequenced to confirm accuracy of the inserts. Expression of the recombinant proteins was initially assayed by IFA after transient transfection of LMH cells using a monoclonal antibody to RGS-6xHis (Qiagen) and convalescent sera from ILTV-infected chickens. LMH cells were seeded in 96-well plates and transfected using Mirus TransIT-LT1 transfection reagent (Roche, Madison, WI) at 60 to 80% confluency. At 48 h post transfection, cells were fixed with cold ethanol for 30 min at room temperature (RT) and air-dried. Sera were diluted in PBS at a 1:200 dilution and incubated with the fixed cells in a humid chamber for 1 h at RT. After incubation, cells were washed with PBS three times for 5 min. FITC-conjugated anti-species antibodies (Sigma-Aldrich, St. Louis, MO) were diluted in PBS containing 0.001% Evan's blue at a 1:200 dilution and incubated for 1 h at RT. Cells were washed with PBS three times for 5 min, briefly rinsed with distilled water, air-dried and mounted in 2.5% 1,4-diazabicyclo-[2.2.2]-octane in 90% glycerol. Plates were examined by conventional fluorescence microscopy using an Axiovert 40 CFL fluorescence microscope (Zeiss, Thornwood, New York, USA). Sera collected pre-challenge and at 7 d.p.c. from the NVx-NCh, CEO, TCO, HVT-LT io, HVT-LT sc, HVT io, HVT sc, FPV-LT io, FPV-LT sc, and NVx-Ch groups were tested by both gB and gI IFA. Similarly, sera were collected at 7 d.p.c. from NVx-NCh, HVT-LT io, HVT-LT sc, FPV-LT io, FPV-LT sc, and NVx-Ch groups of chickens challenged at 57 days of age were tested by gB and gI IFA. Serum samples with titres $\geq$1:200 were considered positive and the percentage of positive samples per group was reported.

Serology. Serum samples were analysed by commercial enzyme-linked immunosorbent assays (ELISAs) using the LT ELISA kit (ProFLOCK® LT ELISA Kit; Synbiotics Corp., San Diego, California, USA) following the manufacturer's instructions. ILTV neutralizing antibody titres were also determined by virus neutralization (VN) assay performed in 96-well plates with CK cells as previously described (Thayer *et al.*, 2008). Briefly, each serum sample was heated at 56°C for 30 min to destroy heat-sensitive, non-specific virus inhibitory substances. Twelve two-fold serial dilutions were performed for each serum in minimum essential medium with 2% antibiotic-antimycotic solution 100 × (Gibco) and 2% newborn calf serum (Gibco). Each dilution was mixed with 100 $TCID_{50}$ of the ILTV 63140 isolate in a 96-well plate and incubated for 60 min at 37°C. After incubation the serum–virus mix was transferred and absorbed into CK cell monolayers. The plate was incubated for 5 days at 39°C. After 5 days of incubation, the plate was examined for the presence of ILTV cytopathic effect and the VN titre

was estimated as the reciprocal of the first serum dilution were ILTV cytopathic effect was observed. The geometric mean titre for ELISA and VN was determined in serum samples for each treatment group. ELISA and VN assays were performed in serum samples collected pre-challenge (34 days of age), at 7 d.p.c. from NVx-NCh, CEO, TCO, HVT-LT io, HVT-LT sc, HVT io, HVT sc, FPV-LT io, FPV-LT sc, and NVx-Ch groups of chickens when challenged at 35 days of age, and at 7 d.p.c. from NVx-NCh, HVT-LT io, HVT-LT sc, FPV-LT io, FPV-LT sc, and NVx-Ch groups of chickens when challenged at 57 days of age.

Statistical analysis. Data were entered into an Excel spreadsheet (Microsoft® Office Excel® 2007) and analysed with SPSS Statistics 17.0 software (https://www.spss.com; SPSS-IBM Inc., Chicago, Illinois, USA). For clinical sign score analysis, the Kruskal–Wallis test was independently used to compare the median clinical sign scores for each day post challenge, and multiple pair-wise comparisons were performed for *post-hoc* comparisons. Normalized challenge viral loads [$\log_{10}(2^{-0 \triangle Ct})$] for each time point were compared independently among groups using one-way analysis of variance. When significant differences were found at the 5% level of significance, Bonferroni's method for multiple pair-wise comparisons was used to detect differences between pairs. The average weight gain was also evaluated using the analysis of variance method.

Results

Vaccines and challenge virus titres. Once the vaccines were reconstituted as recommended by the manufacturers and administered *in ovo*, subcutaneously and via eye drop, titres of the working dilutions were determined. The Innovax ILT® HVT-LT vaccine titre was 7460 PFU/ml, the Vectormune® FP-LT vaccine titre was 6.30 x $10^{3.0}$ $TCID_{50}$/ml, the HVT vaccine titre was 9000 PFU/ml and 373, 315, and 450 PFU of each vaccine were administered, respectively, *in ovo* and subcutaneously. The Trachivax® CEO vaccine titre was $1 \times 10^{3.63}$ $TCID_{50}$/ml, the LT-IVAX® TCO vaccine titre was $1 \times 10^{4.17}$ $TCID_{50}$/ml or 200 and 790 $TCID_{50}$ were administered via eye drop per bird, respectively. The vial stock of virulent field isolate 63140 at a titre of 3.98×10^4 $TCID_{50}$/ml was utilized for challenge at 35 and 57 days of age, and each chicken received approximately 8000 $TCID_{50}$ of the challenge virus.

Clinical signs score evaluation. Clinical signs scores were recorded for all groups of chickens from 3 to 7 d.p.c. Throughout both challenge experiments, 3 to 7 d.p.c., the predominant clinical signs observed were breathing difficulties and depression, followed by conjunctivitis; no mortalities were observed. For both challenge experiments the peak of clinical signs was at 5 d.p.c., and by 7 d.p.c. most chickens had cleared the conjunctivitis and only few showed signs of mild respiratory distress and depression (data not shown). The median clinical signs scores recorded at 5 d.p.c. for chickens challenged at 35 or 57 days of age are shown in Figure 1a,b, respectively. For chickens challenged at 35 days of age, clinical scores for the TCO, HVT-LT io and HVT-LT sc groups were not significantly different ($P > 0.05$) to the scores recorded for the CEO vaccinated and the NVx-NCh (negative control), or the FPV-LT io and FPV-LT sc groups. However, the FPV-LT io and FPV-LT sc groups clinical scores were significantly higher ($P < 0.05$) than those presented by the CEO vaccinated and the NVx-NCh groups. The FPV-LT io vaccinated group of chickens clinical signs scores were no different ($P > 0.05$) from the NVx-Ch (positive

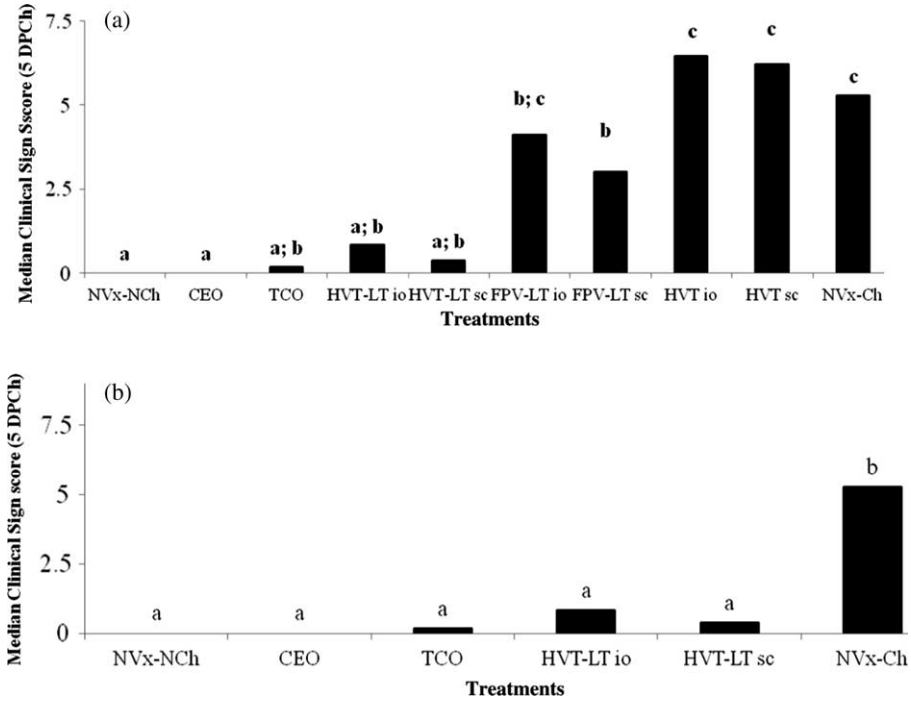

Figure 1. *Median clinical sign scores recorded at 5 d.p.c. with the virulent ILTV strain 63140. 1a: Clinical sign scores 5 d.p.c. in commercial broilers vaccinated with HVT-LT, FPV-LT in ovo (io), HVT-LT, FPV-LT subcutaneously (sc) at hatch, HVT io, HVT sc at hatch, CEO via eye drop at 2 weeks of age, TCO via eye drop at 2 weeks of age, and a non-vaccinated group (NVx-Ch) challenged at 35 days of age. 1b: Clinical sign scores 5 d.p.c. in commercial broilers vaccinated with HVT-LT, FPV-LT io, HVT-LT, FPV-LT sc and a non-vaccinated group (NVx-Ch) challenged at 57 days of age. Significant differences among treatments are presented with different lowercase letters.*

control) group scores, while the FPV-LT sc group presented scores significantly lower ($P < 0.05$) than those recorded for the FPV-LT io, HVT io, HVT sc and NVx-Ch groups (Figure 1a). As expected, no significant differences ($P > 0.05$) were observed among scores of the HVT io, HVT sc, and NVx-Ch groups. At 57 days of age, clinical scores were recorded for four vaccinated groups of chickens (HVT-LT io, HVT-LT sc, FPV-LT io and FPV-LT sc) and two non-vaccinated groups (NVx-NCh and NVx-Ch). Similarly to the chickens challenged at 35 days of age, no significant differences ($P > 0.05$) were observed between the HVT-LT io and HVT-LT sc vaccinated groups and the NVx-NCh group. The FPV-LT io and FPV-LT sc scores were significantly higher ($P < 0.05$) than scores recorded for the NVx-NCh group and were significantly lower ($P < 0.05$) than scores recorded for the NVx-Ch group (Figure 1b). By 6 d.p.c., no differences ($P > 0.05$) in clinical signs scores were observed for the NVx-NCh, HVT-LT io, HVT-LT sc, and FPV-LT sc groups of chickens challenged at either 35 or 57 days of age. However, the FPV-LT io group still presented clinical scores significantly higher ($P < 0.05$) than the NVx-NCh group either for chickens challenged at 35 or at 57 days of age. At 7 d.p.c. the clinical scores for all of the vaccinated groups were statistically similar ($P > 0.05$) to the NVx-NCh group (data not shown).

ILTV loads in trachea post challenge. Viral load levels in the trachea were quantified at 3, 5 and 7 d.p.c. by quantitative PCR and are presented in Table 2. The peak of virus replication was observed at 3 d.p.c., as indicated by 6.84 and 6.33 $\log_{10} 2^{-\Delta\Delta Ct}$ values for the NVx-Ch groups of chickens challenged at 35 and 57 days of age, respectively. At 3 d.p.c., viral loads detected in the HVT-LT io and HVT-LT sc vaccinated groups were significantly lower ($P < 0.05$) than those detected for the NVx-Ch groups of chickens challenged at either 35 and 57 days of age. At d.p.c. the HVT-LT io and HVT-LT sc groups, challenged at 57 days of age, showed viral load levels that were significantly lower ($P < 0.05$) than those of the NVx-Ch group, while when challenged at 35 days of age the HVT-LT io and HVT-LT sc vaccinated groups showed viral load levels similar ($P > 0.05$) to those detected for the NVx-Ch group. Regardless of the age when the challenge was conducted, or the day post challenge when samples were collected (3 or 5 d.p.c.), both the HVT-LT *io* and HVT-LT sc vaccinated groups of chickens presented viral load levels higher ($P < 0.05$) than levels detected for the NVx-NCh groups. At 3 d.p.c. viral load levels detected for the HVT-LT *io* and HVT-LT sc groups, when challenged at 35 days of age, were significantly higher ($P < 0.01$) than those found in the TCO and CEO vaccinated groups. However, at d.p.c. no significant differences ($P < 0.05$) were detected between the HVT-LT sc and the TCO vaccinated group, while when compared with the CEO the HVT-LT sc group presented significantly higher viral load levels. Concurrently, at d.p.c. the HVT-LT io, HVT-LT sc, FPV-LT io and FPV-LT sc groups all had viral load levels that were not significantly different to the NVx-Ch group. As expected, the viral load levels detected for the HVT io and HVT sc vaccinated chickens were significantly higher ($P > 0.05$) than those detected for the HVT-LT vaccinated chickens (*in ovo* and subcutaneously) at either 3 or 5 d.p.c., but similar to those of the NVx-Ch group ($P = 1.00$). The FPV-LT io and FPV-LT sc vaccinated groups presented similar viral loads ($P = 1.00$) to those of the NVx-Ch group at 3 and 5 d.p.c., when the challenge occurred at either 35 or 57 days of age. Viral load levels among all groups decreased significantly between 3 and 5 d.p.c., and by day 7 only the FPV-LT io, FPV-LT sc and the NVx-Ch groups of chickens had measurable viral loads.

Weight gain post challenge. The average body weight gained was recorded between 34 (1 day pre-challenge) and 41 (7 d.p.c.) days of age (Figure 2). Among the vaccinated groups, those that received the FPV-LT *in ovo* showed the lowest weight gain (0.2 kg), which was significantly lower than the weight gained by the CEO ($P = 0.044$) and the NVx-NCh ($P = 0.036$) groups, but not different to the weight gained by the TCO, HVT-LT io, HVT-LT sc or FPV-LT sc vaccinated groups of chickens.

Detection of gI and gB antibodies. In order to evaluate the antibody response to ILTV gI and gB expressed by the HVT-LT and FPV-LT vaccines, serum samples were collected at 34 days of age (pre-challenge), at 41 days of age (7 d.p.c.), and at 64 days of age (7 d.p.c.) and analysed by IFA (Table 3). A gI antibody response was

Table 2. *Trachea viral load at 3, 5 and 7 d.p.c. for chickens challenged at 35 and at 57 days of age.*

	Challenge at 35 days of age			Challenge at 57 days of age		
Group	3 d.p.c.	5 d.p.c.	7 d.p.c.	3 d.p.c.	5 d.p.c.	7 d.p.c.
NVx-NCh	0.01[a] ($\pm$0.18)^{A}	–0.27 ($\pm$0.29)^{A}	–0.18 ($\pm$0.24)^{A}	0.01 ($\pm$0.32)^{A}	0.11 ($\pm$0.13)^{A}	0.04 ($\pm$0.01)^{A}
CEO	–0.15 ($\pm$0.24)^{A}	–0.10 ($\pm$0.88)^{A}	–0.24 ($\pm$0.92)^{A}	ND	ND	ND
TCO	2.21 ($\pm$3.30)^{B}	1.02 ($\pm$2.36)^{B}	–0.38 ($\pm$0.47)^{A}	ND	ND	ND
HVT-LT io	5.09 ($\pm$1.15)^{C}	3.41 ($\pm$1.57)^{C}	0.16 ($\pm$1.30)^{A,B}	2.68 ($\pm$2.56)^{B}	2.67 ($\pm$2.01)^{B}	0.39 ($\pm$1.27)^{A}
HVT-LT sc	5.22 ($\pm$1.48)^{C}	2.41 ($\pm$1.53)^{B,C}	–0.27 ($\pm$0.41)^{A}	2.99 ($\pm$1.60)^{B}	2.49 ($\pm$1.67)^{B}	0.10 ($\pm$0.73)^{A,B}
HVT io	7.09 ($\pm$1.18)^{D}	3.63 ($\pm$0.75)^{C}	0.62 ($\pm$1.60)^{A,B}	ND	ND	ND
HVT sc	7.31 ($\pm$0.60)^{D}	3.86 ($\pm$0.80)^{C}	1.41 ($\pm$1.85)^{B}	ND	ND	ND
FPV-LT io	7.40 ($\pm$1.01)^{D}	3.56 ($\pm$0.69)^{C}	0.85 ($\pm$1.55)^{A,B}	5.90 ($\pm$0.44)^{C}	5.12 ($\pm$0.64)^{C}	1.57 ($\pm$1.52)^{A}
FPV-LT sc	6.34 ($\pm$1.21)^{D}	3.70 ($\pm$0.99)^{C}	0.84 ($\pm$1.94)^{A,B}	6.26 ($\pm$0.65)^{C}	4.76 ($\pm$0.23)^{C}	1.05 ($\pm$1.32)^{A,B}
NVx-Ch	6.84 ($\pm$1.51)^{D}	3.15 ($\pm$1.25)^{C}	0.56 ($\pm$1.41)^{A,B}	6.33 ($\pm$0.68)^{C}	4.82 ($\pm$0.41)^{C}	2.08 ($\pm$0.47)^{B}

[a]Average of $\log_{10} 2^{-\Delta\Delta Ct}$; uppercase superscript letters in each column indicate whether the data were statistically different between groups within the same time-point. Different letters indicate significant differences ($P < 0.05$). ND, not done.

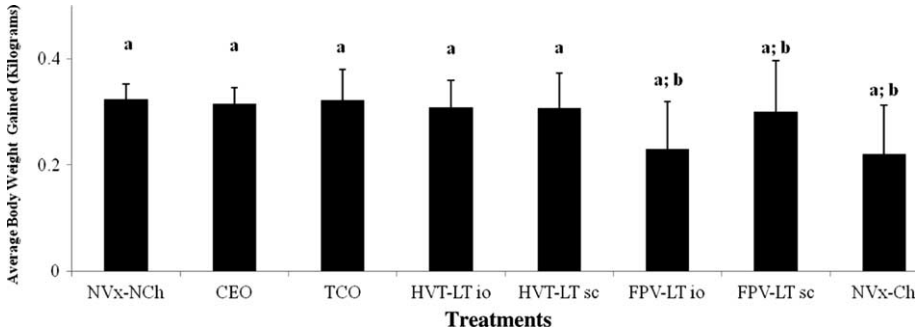

Figure 2. *Average body-weight gained after challenge at 35 days of age for each treatment group. Treatments: non-vaccinated/non-challenged (NVx-NCh), CEO vaccinated, TCO vaccinated, herpesvirus of turkey-LT in ovo vaccinated (HVT-LT io), herpesvirus of turkeys subcutaneously vaccinated (HVT-LT sc), FPV-LT in ovo vaccinated (FPV-LT io), FPV-LT subcutaneously vaccinated (HVT-LT sc), non-vaccinated/challenge.*

detected in 75% and 92% of the samples collected pre-challenge from the HVT-LT sc and HVT-LT io vaccinated groups, respectively; while a gI antibody response was detected in only 8% of the CEO and TCO vaccinated chickens. As expected, no gI antibodies were detected in serum samples collected pre-challenge from the FPV-LT or HVT vaccinated groups of chickens as neither vaccine expresses gI of ILTV. With the exception of one sample (8%) in the FPV-LT sc vaccinated group, no gB antibodies were detected in serum samples collected pre-challenge from the CEO, TCO, HVT-LT io, HVT-LT sc, and FPV-LT io vaccinated groups. As expected, no gB or gI antibodies were detected in serum samples collected pre-challenge from the NVx-Ch group. In serum samples collected 7 d.p.c. from chickens challenged at 35 days age, 89 to 100% of the samples from HVT-LT io and HVT-LT sc vaccinated groups had detectable gI antibody titres; while 60 to 90% of the serum samples from HVT-LT sc and HVT-LT io showed gI-specific antibodies when challenged at 57 days of age. Within the FPV-LT sc and FPV-LT io vaccinated groups, 44% of samples had gI antibody titres when the chickens were challenged at 35 days of age; while when challenged at 57 days of age, gI antibodies were found in 30% of the samples collected from the FPV-LT io vaccinated group and no gI antibodies were detected in samples collected from the FPV-LT sc group. Antibodies against gI were found in 92% and 50% of the serum samples collected post challenge from the CEO and TCO vaccinated groups, respectively. For the NVx-Ch group of chickens, gI antibodies were detected in 18% and 10% of the serum samples collected 7 d.p.c. in chickens challenged at 35 and 57 days of age, respectively. A gB antibody response was detected in 44% and 89% of the serum samples collected at 7 d.p.c. from FPV-LT io and FPV-LT sc vaccinated groups, when challenged at 35 days of age, respectively. When chickens were challenged at 57 days of age, gB antibodies were detected in 50% and 60% of serum samples collected from FPV-LT io and FPV-LT sc, respectively. With the exception of one serum sample from the HVT-LT io vaccinated group, no gB antibodies were detected in samples collected post challenge from the CEO, TCO, HVT-LT sc vaccinated groups, or the NVx-Ch group, when challenged at 35 days of age. Similarly, no gB antibodies were detected in serum samples collected post challenge from chickens challenged at 57 days of age from the HVT-LT io and HVT-LT sc vaccinated groups or the NVx-Ch group.

ILTV ELISA and virus neutralizing antibody titres. The ILTV antibody response elicited at 34 days of age (pre-challenge) and at 7 d.p.c. was also measured by ELISA and VN assay. With the exception of serum samples from the CEO and TCO vaccinated groups, all remaining serum samples collected pre-challenge were negative by ELISA for ILTV antibodies, including samples from chickens vaccinated with the HVT-LT and FPV-LT vector vaccines. ELISA titres in serum samples collected at 7 d.p.c. from vaccinated groups CEO, TCO, HVT-LT io, HVT-LT sc, FPV-LT io and FPV-LT sc challenged at 35 days of age showed mean titres that ranged from 1000 to 2000. Serum samples from chickens challenged at 57 days of age revealed mean titres ranging from 4000 to 5000 for the HVT-LT vaccinated groups, and from 1000 to 3000 for the FPV-LT vaccinated groups (Figure 3a). ELISA titres for serum samples collected from the NVX-Ch groups of chickens reached mean titres of 500 at 7 d.p.c. ILTV neutralizing antibodies were also determined in serum samples collected pre-challenge and post challenge (Figure 3b). Low virus neutralizing antibody mean titres of 1.0 to 4.0 were detected in serum samples collected pre-challenge from HVT-LT, FPV-LT, CEO and TCO vaccinated groups. Such values were not different from the values obtained for the NVx-NCh (negative control) group. For chickens challenged at 35 days of age, the mean virus neutralizing antibody titres detected at 7 d.p.c. for HVT-LT io, HVT-LT sc, FPV-LT io and FPV-LT sc vaccinated groups were 3.4, 9.5, 5.3, and 12.1, respectively. For chickens challenged at 57 days of age, the mean virus neutralizing antibody titres, detected at 7 d.p.c., for HVT-LT io, HVT-LT sc, FPV-LT io and FPV-LT sc were 6.5, 32.0, 13.0, and 76, respectively. Noticeable is the high standard deviation observed for mean VN titres of HVT-LT and FPV-LT subcutaneously vaccinated chickens after challenge at 57 days of age. On the other hand, virus neutralizing mean titres for CEO, TCO and NVx-Ch groups of chickens, challenged either at 35 or 57 days of age, never produced titres different to the NVx-NCh group (Figure 3b).

Discussion

The objective of the present study was to evaluate the protection induced by ILTV viral vector vaccines in broilers when challenged at 35 and 57 days of age. In the present study live-attenuated ILT vaccines were

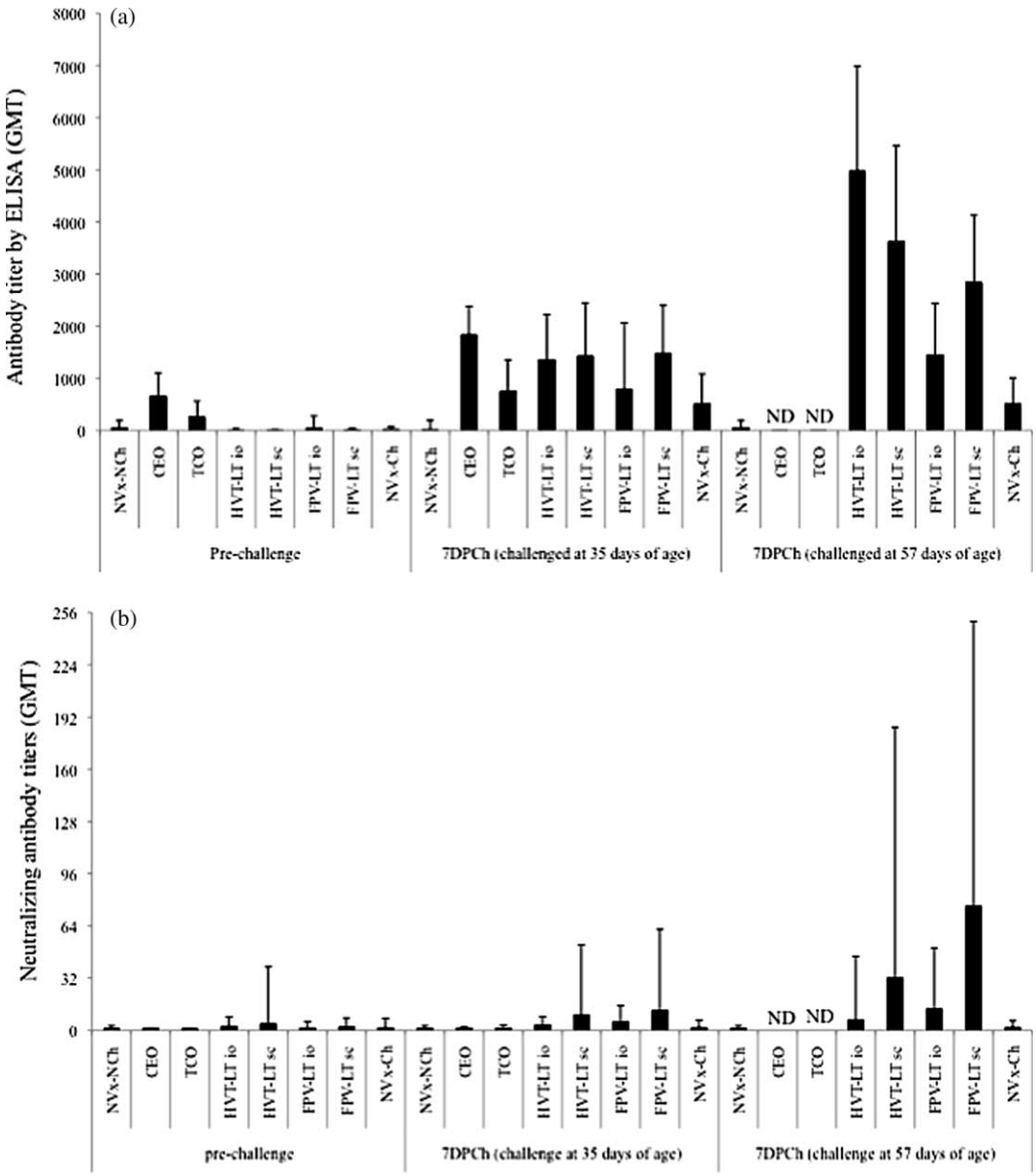

Figure 3. *ILTV antibody titres determined by ELISA and VN assay on serum samples collected pre-challenge and post challenge. 3a: ELISA ProFLOCK® LT (Synbiotics Corp., San Diego, California, USA). Geometric mean titres (GMT) were calculated for serum samples from each treatment group collected 1 day pre-challenge and 7 d.p.c. using the commercial ELISA. A treatment group with GMT higher than 500 was considered positive for ILTV antibodies by ELISA. 3b: Virus neutralizing GMT were estimated for each treatment group on serum samples collected 1 day previous to the challenge and 7 d.p.c. VN titres higher than 4.0 were considered positive.*

administered as a full dose, and the recombinant vectored vaccines were administered as a half dose to mimic broiler industry practices. The viral vector vaccines were administered *in ovo* and subcutaneously at hatch while the live-attenuated vaccines, CEO and TCO, were administered via eye drop at 14 days of age. As previously described (Oldoni *et al*., 2009), the peak of clinical signs was observed at 5 d.p.c. and the peak of challenge virus replication in the trachea was detected at 3 d.p.c. In a recent study comparing the pathogenicity of the 63140 isolate against the USDA standard challenge strain in broilers, the 63140 isolate showed characteristically earlier replication in the trachea (García *et al.*, 2010), which may explain the early peak of viral replication in this study. As previously documented (Johnson *et al.*, 2010), optimal protection was achieved by the CEO vaccine when applied via eye drop with no clinical signs recorded and no detectable challenge virus in the trachea from 3 to 7 d.p.c. Chickens vaccinated with TCO or HVT-LT presented reduced levels of clinical signs that were not statistically different from the clinical signs observed in the CEO vaccinated group. However, in contrast to the CEO vaccinated chickens, in which the lack of clinical signs was accompanied by reduction of challenge virus to levels close to the assay detection limit (Callison *et al.*, 2007), high levels of virus were detected 3 d.p.c. in HVT-LT io and HVT-LT sc vaccinated groups challenged at 35 days of age. On the other hand, HVT-LT io and HVT-LT sc vaccinated groups, when challenged at 57 days of age, had a reduction of two logarithms in viral load levels 3 d.p.c. compared with HVT-LT vaccinated groups challenged at 35 days of age, suggesting that immunity against ILT generated by

Table 3. *Detection of gI and gB antibodies by IFA in serum samples from ILTV vaccinated and challenged chickens.*

	Anti-gI (titre >1:200)[a]						Anti-gB (titre >1:200)					
	Pre-challenge		7 d.p.c. 35[b]		7 d.p.c. 57[c]		Pre-challenge		7 d.p.c. 35[b]		7 d.p.c. 57[c]	
Treatment group	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%	*N*	%
CEO	1/12	8.3	11/12	91.7	ND	ND	0/10	0.0	0/11	0.0	ND	ND
TCO	1/12	8.3	6/12	50.0	ND	ND	0/10	0.0	0/12	0.0	ND	ND
HVT-LT io	11/12	91.7	8/9	88.9	9/10	90.0	0/12	0.0	1/9	11.1	0/10	0.0
HVT-LT sc	9/12	75.0	9/9	100.0	6/10	60.0	0/12	0.0	0/9	0.0	0/10	0.0
HVT io	0/12	0.0	2/9	22.2	ND	ND	0/12	0.0	0/9	0.0	ND	ND
HVT sc	0/12	0.0	2/9	22.2	ND	ND	0/12	0.0	0/9	0.0	ND	ND
FPV-LT io	0/12	0.0	4/9	44.4	3/10	30.0	0/12	0.0	4/9	44.4	5/10	50.0
FPV-LT sc	0/12	0.0	4/9	44.4	0/10	0.0	1/12	8.3	8/9	88.9	6/10	60.0
NVx-Ch	0/12	0.0	2/11	18.2	1/10	10.0	0/12	0.0	0/12	0.0	0/10	0.00

[a]Samples with titres $\geq$ 1:200 were considered positives for either gI or gB antibodies. [b]Evaluation of serum samples from chicken groups challenged at 35 days of age and collected at 7 days post challenge. [c]Evaluation of serum samples from chicken groups challenged at 57 days of age and collected at 7 days post challenge. *N*, number of positives; %, percentage positives; ND, not done.

recombinant vaccines may take more than a month to develop. Independently of the route of administration or challenge age, the HVT-LT vaccine significantly mitigated clinical signs, although a significant reduction in virus levels was observed in chickens challenged at 57 days but not at 35 days of age.

Furthermore, the lack of protection observed in the HVT vaccinated chickens confirmed that the ILTV gI and gD glycoproteins expressed by the HVT-LT vaccine were responsible for the protection observed. Chickens vaccinated with FPV-LT presented higher clinical scores and viral load levels than the HVT-LT, CEO, or TCO vaccinated groups when challenged at 35 days of age. When chickens were challenged at 57 days of age both parameters were significantly higher than those observed for HVT-LT vaccinated chickens. Results obtained in the present study disagree with protection efficiency observed when HVT-LT and FPV-LT were administered *in ovo* and subsequently challenged at 35 days of age with the USDA strain (Johnson *et al.*, 2010). In Johnson *et al.*'s study the FPV-LT io vaccinated broiler chickens were better protected against clinical signs than the HVT-LT io vaccinated chickens. While no reduction in viral load levels was observed for any of the viral vector vaccinated group of chickens 5 d.p.c. We speculate that the differences in protection observed in both studies were due to a combination of factors, amongst which probably the most relevant is the accuracy of the *in ovo* vaccination achieved. Optimal efficiency of these vaccines strongly relies on proper *in ovo* delivery, which can be severely affected by the precision and consistency of the machine utilized for delivery (Williams & Hopkins, 2011). In the Johnson *et al.* (2010) study embryonated eggs were inoculated with a one-needle vaccinator designed for laboratory use that utilizes a system by which a single needle punches a hole in the eggshell and delivers the vaccine, all in a single shot. In the present study a commercially produced *in ovo* injection system was used, where a hole-puncher and a needle operate asynchronously and independently. We speculate that the system used in the Johnson *et al.* study may have had lower consistency and accuracy, probably resulting in a lower number of hits and thus lower protection for the vaccinates receiving either one of the viral vector vaccines. Regardless of the route of vaccination or age of challenge *in ovo* or subcutaneous vaccination with FPV-LT did not reduce viral load levels in the trachea. Tong *et al.* (2001) evaluated the protection induced by wing web vaccination with a viral vector rFPV-ILTVgB vaccine, and, similar to the commercial available FPV-LT vaccine, a significant reduction in clinical signs after challenge was observed, although challenge virus was isolated in 25 to 40% of the vaccinated chickens. These results suggest that FPV vectors may not elicit adequate local or cellular immunity to efficiently clear the challenge VIRUS from the trachea. Similarly the FPV vector vaccine encoding the haemagglutinin H5 of avian influenza (FPV-H5) showed the lowest efficacy to prevent avian influenza virus shedding from the respiratory tract as compared with avian influenza inactivated vaccines (Hsu *et al.*, 2010). Although it has not been thoroughly investigated, another factor to consider is the presence of FPV maternal antibodies limiting the protection induced by the FPV-LT vaccine. Interference of the FPV-H5 vaccination against highly pathogenic avian influenza has been documented due to pre-existing

immunity elicited by a primary FPV vaccination (Swayne *et al.*, 2000). The broiler breeder flock producing the fertilized eggs utilized in this study was vaccinated against fowlpox, as virtually all breeder flocks in the Southern USA are; however, we did not evaluate the maternal antibody status of their progeny. Body weight gain pre-challenge and post challenge was not the best parameter to measure protection among groups of chickens challenged at 35 days of age. However, it is important to point out that the FPV-LT io vaccinated group had the lowest weight gain amongst all the vaccinated groups. In other experiments conducted by our group it has been observed that body weight gained by FPV-LT *in ovo* vaccinated chickens was best when embryos were vaccinated at 19 rather than 18 days of embryo age (data not shown), and it has been documented that reactions to the FPV-LT vector vaccine can occur when administered at 17.5 to 18 days of embryo age rather than at 19 days (Williams *et al.*, 2010).

Detection of antibodies against ILTV glycoproteins expressed by viral vectors was not interpreted as a measure of protection, but rather as an indication that the chickens received the vaccine and a measure of the antigenicity of ILTV glycoprotein expressed by the vector. However, the presence of gI antibodies detected pre-challenge and post challenge in chickens vaccinated with HVT-LT indicated that the vaccine induced a robust antibody response, which coincided with a significant reduction of clinical signs. On the other hand, gB antibodies were detected only post challenge in FPV-LT vaccinated chickens, suggesting that a weaker immune response was elicited against gB by FPV-LT vaccination, which coincided with presence of clinical signs and high levels of challenge virus in the trachea. Although the exact nature of the ILTV protective immunity has not been completely elucidated, it has been documented that humoral antibody response by itself does not provide the necessary immunity to induce effective protection from disease (Fahey *et al.*, 1983, 1984; Fahey & York, 1990; Honda *et al.*, 1994). The marginal role that antibodies play in the protection against ILTV was further confirmed as no differences in ELISA titres were observed between the optimally protected CEO vaccinated and poorly protected FPV-LT vaccinated groups of chickens; in addition, measurable neutralizing serum antibodies were only detected for HVT-LT and FPV-LT vaccinated groups of chickens while no neutralizing antibody titres were detected in the CEO or TCO vaccinated group of chickens. In particular, the HVT-LT vaccine elicited a strong antibody response probably associated with the mitigation of the disease; however, to comprehensively assess the immunogenicity of the vector vaccines, the local and cell-mediated immunity would need to be evaluated.

Overall the HVT-LT vaccine was more effective than the FPV-LT vaccine in mitigating the disease and reducing levels of challenge virus when applied *in ovo* or subcutaneously, while the FPV-LT mitigated clinical signs of the disease more effectively when administered subcutaneously than *in ovo* but did not reduce the levels of challenge virus in the trachea. The CEO vaccine provided optimal protection by significantly mitigating the disease and clearing the challenge virus from the trachea when applied at a full dose via eye drop. Vaccine-induced protection against virus replication and shedding is relevant in light of the fact that field infection in broiler chickens appears to coincide with the age range at which such birds are sent to slaughter plants in many regions. Thus, the likelihood of ILTV being spread mechanically during catching and transportation may be increased if the vaccines used did not prevent viral replication in the trachea and shedding into the environment. Protection induced by viral vector vaccines was best when the challenge was performed at 57 days than at 35 days of age, suggesting that the onset of immunity for viral vector vaccines may come later than what is required to resist early exposure in the field; the average age when broiler outbreaks occur in the field is around 5 to 6 weeks of age (G. Zavala, personal communication). One possible strategy to improve the efficacy of current commercial ILTV viral vector vaccines is to co-administer or co-express immunomodulators that may enhance both humoral and cell-mediated responses and consequently may elicit an early onset of protection. Several examples on the advantages of immunomodulators have been documented. An HVT vector expressing IL2 increased the protection induced by infectious bursal disease virus and infectious bronchitis virus (IBV) live attenuated vaccines when given by *in ovo* vaccination (Tarpey *et al.*, 2007). An FPV vector co-expressing IBV S1 protein and IL-18 administered at hatch enhanced significantly the protective efficiency against IBV challenge (Cheng *et al.*, 2010), and a recombinant FPV co-expressing type I interferon and the Newcastle disease virus HN and F proteins increased the protective efficacy of the vaccine against Newcastle disease virus challenge when given *in ovo* and at hatch (Karaca *et al.*, 1998). Based on the results obtained in this study and field observations, we speculate that in their current design the ILTV viral vector vaccines can mitigate the disease to a certain degree, but due to their inability to reduce challenge virus replication in the trachea, they may not contribute to reduce sufficiently the circulation of virulent strains in areas of overwhelming field challenge were they have failed to contain outbreaks. In a recent unpublished retrospective study of 532 documented field cases associated with vaccinal laryngotracheitis occurring in a high-prevalence area, 44.5% of the cases corresponded to flocks that had received a recombinant vaccine (half dose) against ILT as the sole vaccine for protection against the disease. Only 2.8% of the cases occurred in flocks vaccinated with TCO vaccines; 2.6% of them occurred in CEO vaccinated flocks; and 0.2% of the cases were recorded in flocks vaccinated with a recombinant vaccine in the hatchery and a CEO vaccine in the field. Despite this apparent failure of recombinant vaccines to prevent outbreaks, it is important to underline that field experience has suggested consistently that losses in recombinant vaccinated and challenged flocks are only a fraction of the production losses commonly experienced in non-vaccinated challenged flocks. Currently it is not known how many or what proportion of vaccinated flocks are truly at risk in high-challenge areas, and thus further studies are needed to assess the performance of each vaccine under field conditions.

Acknowledgements

The present work was supported by the US Egg and Poultry Association Harold E. Ford Foundation (Project No. F025).

References

Andreasen, J.R., Jr, Glisson, J.R., Goodwin, M.A., Resurreccion, R.S., Villegas, P. & Brown, J. (1989). Studies of infectious laryngotracheitis vaccines: immunity in layers. *Avian Diseases*, 33, 524–530.

Callison, S.A, Riblet, S.M., Oldoni, I., Sun, S., Zavala, G., Williams, S., Resurreccion, R., Spackman, E. & García, M. (2007). Development and validation of a real-time Taqman® PCR assay for the detection and quantification of infectious laryngotracheitis virus in poultry. *Journal of Virological Methods*, 139, 31–38.

Chacon, J. L. & Ferreira, A. J. (2009). Differentiation of field isolates and vaccine strains of infectious laryngotracheitis virus by DNA sequencing. *Vaccine*, 27, 6731–6738.

Chen, H-Y, Yang, M-F., Cui, B-A., Cui, P., Sheng, M., Chen, G., Wang, S-J. & Geng, J-W. (2010). Construction and immunogenicity of a recombinant fowlpox vaccine coexpressing S1 glycoprotein of infectious bronchitis virus and chicken IL-18. *Vaccine*, 28, 8112–8119.

Chin, R.P., García, M., Corsiglia, C., Riblet, S.M., Crespo, R., Shivaprasad, H.L., Rodríguez-Avila, A., Woolcock, P. & França, M. (2009). Intervention strategies for laryngotracheitis: impact of extended downtime. *Avian Diseases*, 53, 574–577.

Davison, A.J., Eberle, R., Hayward, G.S., Mcgeoch, D.J., Minson, A.C., Pellet, P.E., Roizman, B., Studdert, M.J. & Thiry, E. (2009). The order Herpesvirales. *Archives of Virology*, 154, 171–177.

Davison, S., Gingerich, E., Casavant, N.S. & Eckroade, R.J. (2006). Evaluation of the efficacy of a live fowlpox-vectored infectious laryngotracheitis/avian encephalomyelitis vaccine against ILT viral challenge. *Avian Diseases*, 50, 50–54.

Fahey, K.J., Bagust, T.J. & York, J.J. (1983). Laryngotracheitis herpesvirus infection in the chicken: the role of humoral antibody in immunity to a graded challenge infection. *Avian Pathology*, 12, 505–514.

Fahey, K.J. & York, J.J. (1990). The role of mucosal antibody immunity to infectious laryngotracheitis virus in chickens. *Journal of General Virology*, 71, 2401–2405.

Fahey, K.J., York, J.J. & Bagust, T.J. (1984). Infectious laryngotracheitis herpesvirus infection in the chicken II. The adoptive transfer of resistance with immune spleen cells. *Avian Pathology*, 13, 265–275.

Fulton, R.M., Schrader, D.L. & Will, M. (2000). Effect of route of vaccination on the prevention of infectious laryngotracheitis in commercial egg-laying chickens. *Avian Diseases*, 44, 8–16.

García, M., Vagnozzi, A., Riblet, S.M., Zavala, G. (2010). Infection of broilers with two virulent strains of infectious laryngotracheitis virus (ILTV): Criteria for evaluation of experimental infections. *American Veterinary Medical Association*. Atlanta, GA. July 31-August 3, 2010. (Convention Notes CD-ROM no page provided).

Gelenczei, E.F. & Marty, E.W. (1964). Studies on a tissue-culture-modified infectious larygotracheitis virus. *Avian Diseases*, 8, 105–122.

Guy, J. & García, M. (2008). Infectious laryngotracheitis virus. In Y.M. Saif, J.R. Glisson, A.M. Fadly, L.R. McDougald, L.K. Nolan & D.E. Swayne (Eds). *Diseases of Poultry*, 12th ed., (pp. 137–152). Ames, AI: Blackwell Publishing.

Guy, J.S., Barnes, H.J. & Smith, L.G. (1991). Increased virulence of modified–live infectious laryngotracheitis vaccine virus following bird-to-bird passage. *Avian Diseases*, 35, 348–355.

Han, M.G. & Kim, S.J. (2003). Efficacy of live virus vaccines against infectious laryngotracheitis assessed by polymerase chain reaction-restriction fragment length polymorphism. *Avian Diseases*, 47, 261–71.

Hein, R. (2008). Recombinant HVT/LT Vaccine (INNOVAX®-ILT) field application issues, 43rd National Meeting on Poultry Health and Processing. Ocean City, MD. pp: 73–74.

Hilbink, F., Oei, H.L. & van Roozelaar, D.J. (1987). Virulence of five live vaccines against avian infectious laryngotracheitis and their immunogenicity and spread after eye-drop or spray application. *Veterinary Quarterly*, 9, 215–225.

Honda, T., Okamura, H., Taneno, A., Yamada, S. & Takahashi, E. (1994). The role of cell-mediate dimmunity in chickens inoculated with the cell-associated vaccine of attenuated infectious laryngotracheitis virus. *Journal of Veterinary Medical Science*, 56, 1051–1055.

Hsu, S-M., Chen, T.H-H. & Wang, C-H. (2010). Efficacy of avian influenza vaccine in poultry: a meta analysis. *Avian Diseases*, 54, 1197–1209.

Johnson, D.I., Vagnozzi, A., Dorea, F., Riblet, S.M., Mundt, A., Zavala, G. & García, M. (2010). Protection against infectious laryngotracheitis virus (ILTV) by in ovo vaccination by commercially available viral vector recombinant vaccines. *Avian Diseases*, 54, 1251–1259.

Karaca, K., Sharma, J.M., Winslow, B.J., Junker, D.E., Reddy, S., Cochran, M. & McMillen, J. (1998). Recombinant fowlpox viruses co-expressing chicken type I IFN and Newcastle disease virus HN and F genes: influence of IFN on protective efficacy and humoral responses of chickens following in ovo or post-hatch administration of recombinant viruses. *Vaccine*, 16, 1496–1503.

Kirkpatrick, N.C., Mahmoudian, A., O'Rourke, D. & Noormohammadi, A.H. (2006). Differentiation of infectious laryngotracheitis virus isolates by restriction fragment length polymorphic analysis of polymerase chain reaction products amplified from multiple genes. *Avian Diseases*, 50, 28–34.

Livak, K. & Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods*, 25, 402–408.

Neff, C., Sudler, C. & Hoop, R.K. (2008). Characterization of western European field isolates and vaccine strains of avian infectious laryngotracheitis virus by restriction fragment length polymorphism and sequence analysis. *Avian Diseases*, 52, 278–283.

Niesters, H. (2001). Quantification of viral load using real-time amplification techniques. *Methods*, 25, 419–429.

Oldoni, I., Rodríguez-Avila, A., Riblet, S. M. & García, M. (2008). Characterization of infectious laryngotracheitis virus (ILTV) isolates from commercial poultry by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). *Avian Diseases*, 52, 59–63.

Oldoni, I., Rodríguez-Avila, A., Riblet, S. M., Zavala, G. & García, M. (2009). Pathogenicity and growth characteristics of infectious laryngotracheitis virus (ILTV) isolates from United States. *Avian Pathology*, 38, 47–53.

Reed, L. & Muench, H. (1938). A simple method of estimating fifty percent end-points. *American Journal of Hygiene*, 27, 493–497.

Rodríguez-Avila, A, Oldoni, I., Riblet, S.M. & García, M. (2007). Replication and transmission of live-attenuated infectious laryngotracheitis virus (ILTV) vaccines. *Avian Diseases*, 51, 905–911.

Rodríguez-Avila, A‡, I. Oldoni, I., Riblet, S. M. & García, M. (2008). Evaluation of the protection elicited by direct and indirect exposure to live attenuated infectious laryngotracheitis virus (ILTV) vaccines against a recent challenge strain from the United States. *Avian Pathology*, 37, 287–292.

Swayne, D.E., Beck, J.R. & Kinney, N. (2000). Failure of a recombinant fowl poxvirus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox vaccine. *Avian Diseases*, 44, 132–137.

Tarpey, I., van Loon, A.A., de Haas, N., Davis, P.J., Orbell, S., Cavanagh, D., Britton, P., Casais, R., Sondermeijer, P. & Dundick, R. (2007). A recombinant turkey herpesvirus expressing chicken interleukin-2 increases the protection provided by in ovo vaccination with infectious bursal disease and infectious bronchitis virus. *Vaccine*, 25, 8529–8535.

Thayer, S., & Beard, C. (2008). Serologic Procedures. In *A laboratory Manual for the Isolation, Identification, and Characterization of Avian Pathogens*. 5th Ed. The American Association of Avian Pathologists, Athens, Georgia. Chapter 47, pp 222–229.

Vagnozzi, A., García, M., Riblet, S.M. & Zavala, G. (2010). Protection induced by infectious laryngotracheitis virus (ILTV) vaccines alone and combined with Newcastle disease virus (NDV) and/or infectious bronchitis virus (IBV). *Avian Diseases*, 54, 1210–1219.

Wakenell, P. & Sharma, J. (2008). Marek's Disease. In *A laboratory manual for the isolation, identification, and characterization of avian pathogens*. 5th Ed. The American Association of Avian Pathologists, Athens, Georgia. Chapter 22, pp 99–105.

Williams, C.J. & Hopkins, B.A. (2011). Field accuracy of vaccine deposition by two different commercially available in ovo injection systems. *Poultry Science*, 90, 223–226.

Williams, S.M., Smith, J.A., García, M., Brinson, D., Kiupel, M. & Hofacre, C. (2010). Histiolymphocytic and heterophilic bronchopneumonia as a severe reaction to in ovo fowl-pox vaccination. *Veterinary Pathology*, 47, 176–179.