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Author(s): Deirdre I. Johnson, Ariel Vagnozzi, Fernanda Dorea, Sylva M. Riblet, Alice Mundt, Guillermo Zavala, and Maricarmen García
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Protection Against Infectious Laryngotracheitis by *In Ovo* Vaccination with Commercially Available Viral Vector Recombinant Vaccines

Deirdre I. Johnson,[A] Ariel Vagnozzi,[A] Fernanda Dorea,[B] Sylva M. Riblet,[A] Alice Mundt,[A] Guillermo Zavala,[A] and Maricarmen García[AC]

[A]Poultry Diagnostic and Research Center, Department of Population Health, College of Veterinary Medicine, The University of Georgia, 953 College Station Road, Athens, GA 30602

[B]Department of Health Management, Atlantic Veterinary College, University of Prince Edward Island, 550 University Avenue, Charlottetown, PE Canada C1A 4P3

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SUMMARY. Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens caused by infectious laryngotracheitis virus (ILTV). The disease is mainly controlled through biosecurity and by vaccination with live-attenuated vaccines. The chicken embryo origin (CEO) vaccines, although proven to be effective in experimental settings, have limited efficacy in controlling the disease in dense broiler production sites due to unrestricted use and poor mass vaccination coverage. These factors allowed CEO vaccines to regain virulence, causing long lasting and, consequently, severe outbreaks of the disease. A new generation of viral vector fowl poxvirus (FPV) and herpesvirus of turkey (HVT) vaccines carrying ILTV genes has been developed and such vaccines are commercially available. These vaccines are characterized by their lack of transmission, lack of ILTV-associated latent infections, and no reversion to virulence. HVT-vectored ILTV recombinant vaccines were originally approved for subcutaneous HVT or transcutaneous (pox) delivery. The increased incidence of ILTV outbreaks in broiler production sites encouraged the broiler industry to deliver the FPV-LT and HVT-LT recombinant vaccines *in ovo*. The objective of this study was to evaluate the protection induced by ILTV viral vector recombinant vaccines after *in ovo* application in 18-day-old commercial broiler embryos. The protection induced by recombinant ILTV vaccines was assessed by their ability to prevent clinical signs and mortality; to reduce challenge virus replication in the trachea; to prevent an increase in body temperature; and to prevent a decrease in body weight gain after challenge. In this study, both recombinant-vectored ILTV vaccines provided partial protection, thereby mitigating the disease, but did not reduce challenge virus loads in the trachea.

RESUMEN. Protección contra la laringotraqueítis infecciosa mediante la inmunización *in ovo* con vacunas recombinantes con vectores virales disponibles comercialmente.

La laringotraqueítis infecciosa es una enfermedad respiratoria de los pollos que es muy contagiosa causada por el virus de laringotraqueítis infecciosa. La enfermedad es controlada principalmente a través de la bioseguridad y por la vacunación con vacunas vivas atenuadas. Las vacunas producidas en embriones de pollo (con las siglas en inglés CEO), aunque han demostrado ser efectivas de manera experimental, tienen una eficacia limitada para controlar la enfermedad en áreas de producción avícola con alta densidad debido a su uso sin restricciones y a su baja cobertura mediante vacunación masiva. Estos factores permitieron que las vacunas elaboradas en embriones recuperaran su virulencia, causando brotes de la enfermedad de larga duración y de consecuencia graves. Se han desarrollado una nueva generación de vacunas con base en vectores virales como el poxvirus aviar (con las siglas en inglés FPV) y el herpesvirus de pavo (HVT) que contienen genes del virus de la laringotraqueítis y que están disponibles comercialmente. Estas vacunas se caracterizan por que no se transmiten, no inducen infecciones latentes asociadas con el virus de la laringotraqueítis y no sufren reversión a la virulencia. Las vacunas recombinantes utilizando al virus herpes de los pavos (con las siglas en inglés HVT) contra el virus de la laringotraqueítis infecciosa HVT fueron inicialmente aprobadas para su aplicación subcutánea (para el virus herpes de los pavos) o transcutánea (para el virus de la viruela). El aumento en la incidencia de los brotes del virus de la laringotraqueítis infecciosa en los centros de producción de pollo engorde alentó a la industria avícola para aplicar estas vacunas con vectores de viruela aviar o de herpes virus de los pavos por inoculación *in ovo*. El objetivo de este estudio fue evaluar la protección inducida por la vacuna recombinante con vectores virales contra el virus de la laringotraqueítis, después de su aplicación *in ovo* en embriones de pollo de engorde comerciales de 18 días de edad embrionaria. La protección inducida por las vacunas recombinantes contra la laringotraqueítis se evaluó por su capacidad para prevenir los signos clínicos y la mortalidad, por la reducción en la replicación del virus de desafío en la tráquea, por evitar el aumento de la temperatura corporal, y por evitar una disminución en la ganancia de peso después del desafío. En este estudio, ambas vacunas recombinantes con vectores contra la laringotraqueítis aviar confirieron protección parcial, mitigando así la enfermedad, pero no redujeron las cargas de virus de desafío en la tráquea.

Key words: infectious laryngotracheitis virus, fowl poxvirus, recombinant viral vectored vaccines, protection, herpes virus of turkeys, *in ovo*

Abbreviations: CEO = chicken embryo origin; CEO-Ch = CEO vaccinated challenged group; Ct = cycle threshold; dpi = days postinoculation; ELISA = enzyme-linked immunosorbent assay; FANP = filtered-air vegetative pressure; FPV = fowl poxvirus; FPV-LT-Ch = FPV-LT *in ovo*-vaccinated challenged group; HVT = herpesvirus of turkey; HVT-LT-Ch = HVT-LT *in ovo*-vaccinated challenged group; IFA = indirect fluorescent antibody; ILT = infectious laryngotracheitis; ILTV = infectious laryngotracheitis virus; LMH = leghorn male hepatoma; LT = laryngotracheitis; MDV = Marek's disease virus; NDV = Newcastle disease virus; NVx-Ch = nonvaccinated challenged group; NVx-NCh = nonvaccinated nonchallenged group; PBS = phosphate-buffered saline; pfu = plaque-forming units; qPCR = quantitative real-time PCR; RT = room temperature; RRFLP = reverse restriction-fragment length polymorphism; $TCID_{50}$ = tissue culture infectious dose; TCO = tissue culture origin; USDA = United States Department of Agriculture

[C]Corresponding author. E-mail: mcgarcia@uga.edu

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens that may cause severe production losses due to morbidity, mortality, decreased egg production, weight loss, and predisposition to other avian respiratory pathogens (14). Infectious laryngotracheitis virus (ILTV) belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and is taxonomically classified as *Gallid herpesvirus 1* (9). Two types of live-attenuated vaccines have been used worldwide for the control of ILT: 1) chicken embryo origin (CEO) vaccines, and 2) tissue culture origin vaccines (TCO). The disease persists in spite of extensive use of vaccination, particularly in areas of intense broiler production. These vaccines have proven to be effective, particularly the CEO vaccine (11,12,15), producing good protection if correctly applied. However, the unrestricted use of the CEO vaccines, poor flock vaccination coverage when applied by coarse spray or in the drinking water (11,19), lax biosecurity measures, and the concomitant effects of other respiratory diseases of poultry (*Mycoplasma* spp., infectious bronchitis virus, etc.) have diminished the effectiveness of these vaccines. Under experimental conditions, it has been clearly documented that live-attenuated ILTV vaccines, in particular the CEO vaccines, regain virulence after consecutive passages in chickens (13). Both vaccines (CEO and TCO) can be transmitted from vaccinated to unvaccinated birds (1,12,19,35) and establish latency in apparently healthy chickens (1,20).

Genotyping studies of outbreak-related ILTV isolates from Europe, the United States, and South America indicated that the greater part is genetically closely related to the CEO vaccines (7,29,31). Therefore, strong evidence exists to indicate that ILTV epizootics originate from CEO-derived strains that regain virulence and persist in the field (31). A new generation of viral vector fowl poxvirus (FPV) and herpesvirus of turkey (HVT) vaccines carrying ILTV genes has been developed and the vaccines are commercially available. The fowl poxvirus vaccine carries ILTV-enveloped glycoprotein gB and UL-34 genes (10), and the HVT vector vaccine carries the ILTV-enveloped glycoprotein gI and gD genes (18). The hallmarks of these vaccines are their lack of transmission from bird to bird, a lack of ILTV latent infections, and no reversion to virulence. Therefore, these new recombinant viral vector vaccines offer a safer vaccination alternative against ILTV than do the live-attenuated vaccines (CEO and TCO). The increased incidence of ILTV outbreaks in broiler production sites across the United States (8,31), and the increased evidence of the efficacy of *in ovo* vaccination with Marek's disease virus (MDV) and FPV vaccines (2,41) encouraged the broiler industry to deliver the FPV-LT and HVT-LT recombinant vaccines *in ovo*. To offset cost, half-a-dose is routinely used in broilers. Considering that licensing of these products was originally approved for subcutaneous or transcutaneous delivery of a full dose, the effectiveness of the ILTV recombinant vaccines when administered by the *in ovo* route has not been thoroughly evaluated. The objective of this study was to evaluate the protection induced by ILTV viral vector recombinant vaccines after *in ovo* application in 18-day-old commercial broiler embryonated eggs. Protection induced by ILTV viral vector vaccines was assessed by their ability to prevent clinical signs and mortality and to reduce challenge virus replication in the trachea and the loss of body weight after challenge.

MATERIALS AND METHODS

Experimental design. One-hundred commercial broilers eggs were incubated in a small-scale hatcher (Natureform Inc., Jacksonville, FL) from 0 to 21 days. At 18 days of embryo age, the eggs were candled and infertile eggs were removed. The remaining embryos were divided in three groups for *in ovo* vaccination. One group of 18 embryonated eggs was vaccinated with the recombinant HVT-LT vaccine (Innovax ILT®, Intervet Schering-Plough Animal Health, Millsboro, DE) with a titer of 6096 plaque forming units (pfu/dose); a group of 18 embryonated eggs were vaccinated with the recombinant FPV-LT vaccine (Vectormune® FP-LT, Ceva Biomune, Lenexa, KS) with a titer of 4,000 pfu/dose; and a third group of 54 embryonated eggs remained unvaccinated. All embryos received half a dose of the FPV-LT and HVT-LT vaccines with the one-needle vaccinator from AviTech® Intellilab (Salisbury, MD). After hatching, the chickens were individually identified and moved to isolated floor pens. At three weeks of age, 18 nonvaccinated hatchmates were transferred to filtered-air negative pressure (FANP) isolators located at the Poultry Diagnostic and Research Center (Athens, GA) and were vaccinated via eye-drop with the Trachivax® (CEO) vaccine (Intervet Schering-Plough Animal Health) at a full dose of $10^{4.5}$ tissue culture infectious dose ($TCID_{50}$) per chicken. At 34 days of age, the recombinant-vaccinated and nonvaccinated chickens were transferred to FANP isolators. Once in the isolation units, chickens were weighed and divided in five groups (12 birds per group) as follows: 1) nonvaccinated nonchallenged group (NVx-NCh) as negative controls; 2) nonvaccinated challenged group (NVx-Ch) as positive controls; 3) HVT-LT *in ovo*-vaccinated challenged group (HVT-LT-Ch); 4) FPV-LT *in ovo*-vaccinated challenged group (FPV-LT-Ch); and 5) CEO-vaccinated challenged group (CEO-Ch). With the exception of the NVx-NCh group, at 37 days of age all chickens were challenged with the U. S. Department of Agriculture (USDA) ILTV strain at a dose of $10^{3.5}$ $TCID_{50}$ per chicken in a total volume of 200 µl; 100 µl intratracheally and 50 µl per eye via eye drop. Chickens in the NVx-NCh group were mock infected with phosphate-buffered saline (PBS) solution instead.

Commercial enzyme-linked immunsorbent assay (ELISA). Serum samples were collected from all treatment groups, prechallenge (5 wk of age) and 11 days postchallenge, and analyzed for the presence of ILTV antibodies by the commercial LT ELISA kit (ProFLOCK® LT ELISA Kit; Synbiotics Corp., San Diego, CA).

Indirect immunofluorescent antibodies. The coding sequences of glycoproteins gB, gC, gI, and gJ were amplified using high-fidelity PCR using Pfx polymerase (Invitrogen, Carlsbad, CA) and primers specifying unique restriction sites for subcloning in the eukaryotic expression vector pcDNA3 (Invitrogen). The 3′-end primers also contained a coding sequence for an RGS-6×His-tag for detection with a commercially available monoclonal antibody (Qiagen, Hilden, Germany). The coding sequence for gB was amplified using primers 5′-ACGGGATCC ATGCAATCCTACATCGCCGTG-3′ and 5′-CGTGCGGCCGCC-TAATGGTGATGGTGATGGTGACTTCCTCTTTCGTCTTCGC-TTTCTTCTGCC-3′, the coding sequence for gC was amplified using primers 5′-CGAGAATTCATGCAGCATCAGAGTACTGC-3′ and 5′-CGTTCTAGACTAATGGTGATGGTGATGGTGACTTCCTC-TTGTTGTCTTCCAGCACCATGC-3′, the coding sequence for gI was amplified using primers 5′-ACGGGATCCATGGCATCGC-TACTTGGAACTC-3′ and 5′-CGTGCGGCCGCCTAATGGTGA-TGGTGATGGTGACTTCCTCTCATTTTTATTGAGTCGGGCG-AGC-3′, and the coding sequence for gJ was amplified using primers 5′-AGCGGATCCATGGGGACAATGTTAGTGTTGC-3′ and 5′-CGTGCGGCCGCCTAATGGTGATGGTGATGGTGACTTCCTC-TAAAATAAATGGCGGTCCATAGCG-3′. Sequences of the recombinant plasmids were confirmed by sequencing. Expression of the recombinant proteins was assayed by indirect immunofluorescence assay after transient transfection in leghorn male hepatoma (LMH) cells (36) using a monoclonal antibody to RGS-6×His (Qiagen) and convalescent sera from ILTV-infected chickens. LMH cells in 96-well plates were transfected using Mirus TransIT-LT1 transfection reagent (Roche, Madison, WI) at 60% to 80% confluency. Forty-eight hours post-transfection cells were fixed with −20 C ethanol for 30 min at room temperature (RT) and air-dried. Antibodies diluted in PBS were incubated with the fixed cells in a humid chamber for 1 hr at RT. After incubation, cells were washed with PBS three times for 5 min. Fluorescein isothiocyanate-conjugated anti-species antibodies (Sigma-Aldrich, St. Louis, MO) were diluted in PBS (1:200) containing 0.001% Evan's

Table 1. Primers and probes utilized in duplex quantitative ILTV real-time PCR.

Gene	Probe	Primer (5′ to 3′)	Amplicon	
			Position	Size
UL 44[A] (ILTV)	Probe	FAM-CAGCTCGGTGACCCCATTCTA-BHQ1	75742–75844	103 bp
	FW	CCTTGCGTTTGAATTTTTCTG		
	RV	TTCGTGGGTTAGAGGTCTGT		
α2-collagen[B]	Probe	TxR-CCCTTAACTGAGTTCCCCAGCTACTGCAG-BHQ2	2360–2435	76 bp
	FW	GGGAACTGGAGAACCCAATTTT		
	RV	CGTGCCGCTGTCTCTACCAT		

[A]Callison *et al.*, 2007 (6).
[B]Islam *et al.*, 2004 (21).

blue and were incubated for 1 hr. Cells were washed as before and briefly rinsed with distilled water prior to air-drying and mounting in 2.5% 1,4-diazabicyclo [2.2.2] octane in 90% glycerol. Plates were inspected by conventional fluorescence microscopy using an Axiovert 40 CFL fluorescence microscope (Zeiss, Thornwood, NY). Experimental sera were serially diluted 1:2 starting with a 1:50 dilution. The highest dilution that produced a specific immunofluorescence represented the titer for the particular antigen assayed. Average titers and standard deviations for each antigen in each treatment group were calculated.

Protection parameters. Clinical signs and body temperatures were recorded daily from days 2 to 11 postchallenge. Breathing patterns, conjunctivitis, and depression were scored on a scale of 0 to three indicating normal (0), mild (1), moderate (2), and severe (3). Any mortality was given an additional score of three. The total clinical sign score (Σ of individual chicken scores) was calculated daily, per group, and graphed. The median clinical sign score per group was calculated daily and differences in clinical sign scores among groups were statistically analyzed. Body temperatures were recorded daily for all chickens postchallenge. The mean body temperature per day per group was calculated and compared to the appearance and severity of clinical sign scores. All chickens were weighed at 5 wk of age prechallenge and at 11 days postchallenge (46 days of age). The percentage body weight gained was calculated per group (mean weight postchallenge − [mean weight postchallenge/mean weight prechallenge]) × 100.

DNA extraction from trachea, esophagus swabs, and feather follicle. Trachea and esophagus swabs were placed in 1 ml sterile PBS containing 2% antibiotic–antimycotic 100× (Gibco, Grand Island, NY) and 2% newborn calf serum (Gibco). All samples were stored at −80 C until processing. DNA extraction from tracheal swab samples was performed using the MagaZorb® DNA mini-prep 96-well kit (Promega, Madison, WI) following the manufacturer's recommendations, with modifications. Briefly, 70 μl of sample (swab suspension) were incubated with 7 μl of proteinase K and 50 μl of lysis buffer at 56 C for 10 min in a 96-well plate, and 20 μl of magnetic beads were added along with 125 μl of binding buffer to each well and incubated for 10 min at RT. The supernatant was separated and the beads were washed twice with washing buffer. Finally, the DNA was eluted from the beads with 100 μl of elution buffer.

Detection of HVT and FPV genomes by PCR analysis. Vaccination with recombinant viral vectored HVT and FPV genomes was evaluated by PCR in samples collected 7 days posthatch. Feather follicles and tracheal swabs were collected from HVT–LT-vaccinated chickens and analyzed by real-time PCR (21), and trachea and esophagus swabs were collected from FPV–LT-vaccinated chickens and analyzed by conventional PCR specific for the FPV vector (42).

Quantitative real-time PCR (qPCR) for ILTV genome detection. ILT viral DNA in tracheal swabs was quantified by real-time PCR in a duplex assay normalized for the host DNA. The real-time PCR assays were carried out on the SmartCycler® II System (Cepheid, Sunnyvale, CA). Primers and probe (Table 1) were designed to amplify a 103-bp region of the UL 44 gene of ILTV (6). As an internal control (calibrator), a 76-bp region of the chicken (*Gallus gallus domesticus*) α2-collagen gene was amplified using primers and probe (Table 1) as previously described (21). The target and the internal control were measured as cycle threshold (Ct) values in a duplex reaction. The duplex reaction was set up to a final volume of 25 μl as follows: 12.5 of 2X master mix (Quanti Tec® multiplex PCR non-ROX kit; Qiagen). All primers were utilized at a final concentration of 1 μM and probes at a final concentration of 0.1 μM, 1 μl of thermolabile uracil N-glycosylase (HK-UNC Epicentre Biotechnologies, Madison, WI) and 5 μl of DNA template. The thermal cycling profile used was 50 C for 2 min, 95 C for 15 min, 40 cycles of 94 C for 15 sec, and 60 C for 45 sec.

Quantitation of ILTV genomes in tracheal swabs. The viral load of each sample was calculated from the amount of viral DNA (target) normalized to the amount of host DNA, which serves as the endogenous internal control (30). The relative amount of the viral DNA was calculated as the $\log_{10}$ of the $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_t - \Delta Ct_{bt}$); where ΔCt_t is the amount of the target gene normalized against the amount of the host gene (internal control gene) of samples from NVx-Ch and vaccinated challenge groups of birds, and ΔCt_{bt} is the amount of the target gene normalized against the amount of the host gene before challenge (25).

Genotyping of challenge virus. In order to confirm that the virus obtained 5 days postchallenge was the USDA challenge strain, reverse restriction-fragment length polymorphism (RRFLP) of the ICP4 gene noncoding region was performed using restriction enzymes *Ava*I and *Alw*I, previously demonstrated (6). This assay is capable of differentiating between the USDA challenge strain and the CEO vaccine. Two qPCR-positive tracheal swabs samples collected 5 days postchallenge from the NVx-Ch, HVT-LT-Ch, and FPV-LT-Ch groups, and one swab from the CEO-Ch group were tested by RRFLP.

Virus isolation. Virus isolation was performed in tracheal swab samples collected 5 days postchallenge in chicken kidney cells from adult, 3- to 4-wk-old specific-pathogen-free chickens, prepared as previously described (35). Samples were considered positive by virus isolation when the cytopathic effect characteristic of ILTV was observed in 1 of 3 consecutive passages; the sample was considered negative after three passages without observation of ILTV cytopathic effect. Tracheal swab samples were thawed at 37 C, mixed by vortexing, and frozen at −80 C three consecutive times and centrifuged for 3 min at 1024 × *g*. The supernatants were used for inoculation of cell monolayers.

Statistical analysis. Data were entered into an Microsoft Office Excel 2007 spreadsheet (Redmond, WA) and analyzed with Intercooled Stata® 9.2 (StataCorp LP, College Station, TX). Measures of temperatures were compared among groups independently for each day using the one-way ANOVA. When significant differences were found at the 5% level of significance, the Bonferroni's method for multiple pair-wise comparisons was used to detect difference between pairs. The same procedure was used to evaluate the PCR data. Normalized challenge virus loads [Log_{10} ($2^{-\Delta\Delta Ct}$)] were compared independently for day 5 and 8 postchallenge. The average weight gain was also evaluated using the ANOVA method. For clinical score analysis, the Kruskal–Wallis test was independently used to compare the median clinical scores for each day postchallenge, and then multiple pair-wise comparisons were performed for *post hoc* comparisons. Pearson correlation analysis was performed on clinical sign scores, viral shedding, and body temperature data.

RESULTS

Detection of HVT and FPV genomes by PCR analysis. HVT genomes were detected in 15 of 34 feather follicle samples collected

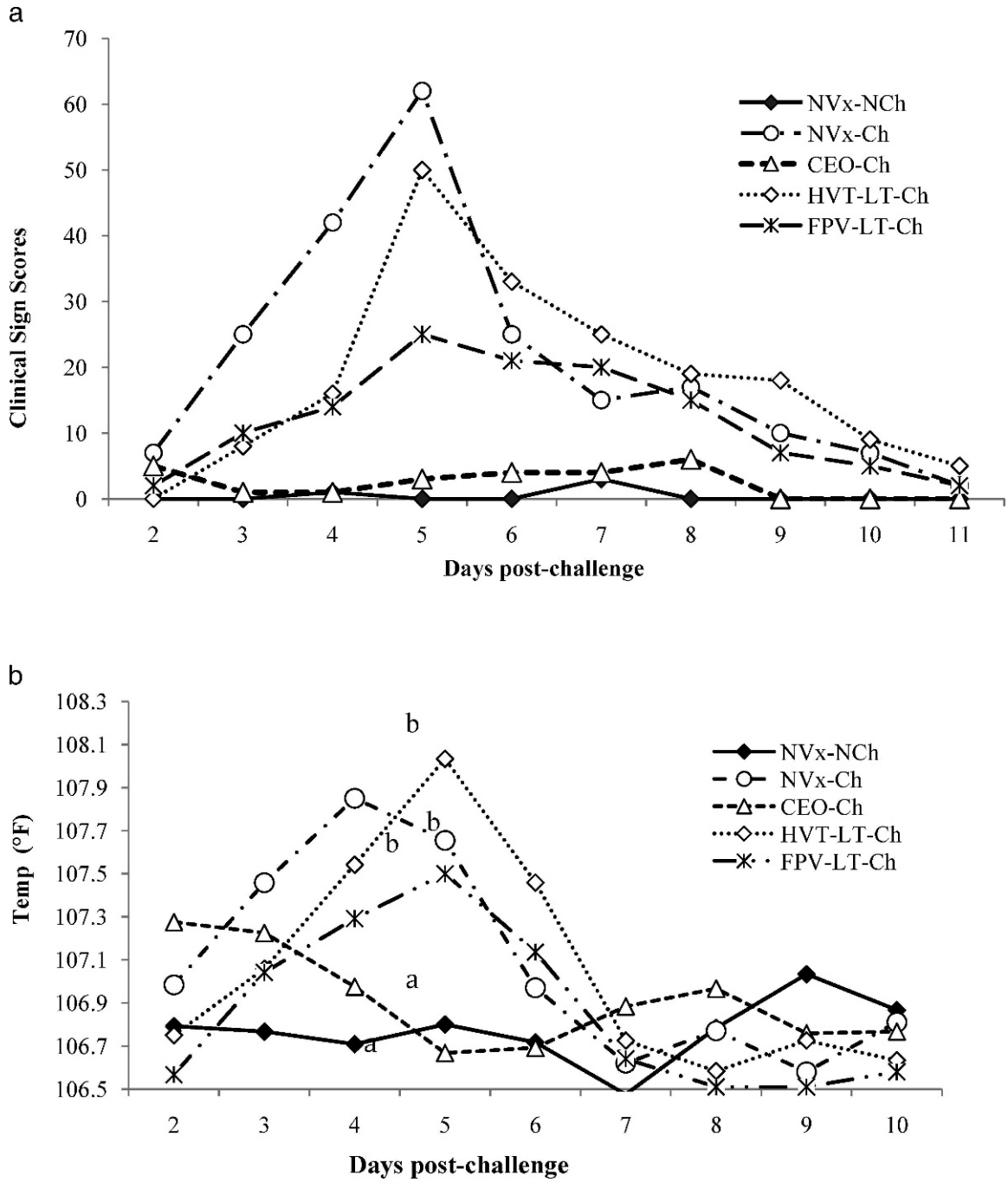

Fig. 1. Total clinical sign scores and mean body temperature recorded daily postchallenge. (1a) Clinical sign scores for NVx-NCh, NVx-Ch, CEO-Ch, HVT-LT-Ch, and FPV-LT-Ch. (1b) Mean body temperature for NVx-NCh, NVx-Ch, CEO-Ch, HVT-LT-Ch, and FPV-LT-Ch. Significant differences (ANOVA) among groups were found at $P < 0.05$ for days 2 (0.03), 3 (0.01), and 4 to 6 (<0.01); significant differences between groups are indicated by lowercase letters.

from HVT–LT-vaccinated chickens, while no HVT genomes were detected in tracheal swabs. No FPV-LT genomes were detected in trachea or esophagus swabs collected from FPV–LT-vaccinated chickens at 7 days posthatch.

Clinical signs, body temperature, and percent weight gained postchallenge. Figure 1a shows the total clinical sign score for each group of chickens recorded from days 2 to 11 postchallenge. Peak clinical signs scores for the NVx-Ch, FPV-LT-Ch, and HVT-LT-Ch groups were observed 5 days postchallenge. The group with the highest clinical sign score was the NVx-Ch, reaching a total score of 62, while the group with the lowest clinical score was the NVx-NCh, with no clinical signs observed at 5 days postchallenge. Amongst the vaccinated groups, the HVT-LT-Ch group, which received the vaccine at 18 days of embryo age, experienced the highest clinical score of 50, followed by the FPV-LT-Ch with a clinical sign score of 25, and the CEO-Ch reached a clinical sign score of three by day 5 postchallenge. Clinical signs gradually decreased for all groups of chickens from day 7 to 11 (Fig. 1a). Statistical analysis was performed on the median clinical sign score for each group from days 2 to 11 postchallenge (Table 2). Significant differences ($P < 0.05$) were observed among all groups from days 3 through 10 postchallenge. At 5 days postchallenge, the

Table 2. Median clinical signs scores at days 2 to 11 postchallenge. Daily median clinical sign scores with the same lowercase letters were not significantly different.

	Days postchallenge									
	2	3	4	5	6	7	8	9	10	11
P-values[A]	0.0609	0.0003	0.0001	0.0001	0.0001	0.0011	0.0009	0.0058	0.0390	0.6142
NVx-NCh	0	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0
Nvx-Ch	1.0	2.0[b]	3.5[b]	6.0[b]	2.0[b]	1.0[b]	2.0[b]	0.5[a,b]	0.5[a,b]	0
CEO-Ch	0	0[a]	0[a]	0[a]	0[a]	0[a]	0[a,c]	0[a]	0[a]	0
HVT-LT-Ch	0	0.5[a,c]	1.0[c]	4.0[b]	3.0[b]	2.5[b]	1.5[b]	1.0[b]	1.0[b,c]	0
FPV-LT-Ch	0	1.0[b,c]	1.0[c]	2.0[c]	2.0[b]	2.0[b]	1.0[b,c]	0[a]	0[a,c]	0

[A]Median clinical sign scores, from day 2 to 11 postchallenge, of ($P < 0.05$) were considered significant.

median clinical sign score for the CEO-Ch group showed no statistical difference from the NVx-NCh group at the 5% level of significance, while the FPV-LT-Ch group median clinical sign score was significantly different from these two groups and from the HVT-LT-Ch and NVx-Ch groups. The median clinical sign scores of the HVT-LT-Ch and NVx-Ch groups were not significantly different from each other. Figure 1b shows the mean body temperatures for each group from days 2 to 11 postchallenge. Significant differences ($P < 0.05$) were observed for the mean body temperatures among groups from days 2 through 6 postchallenge. Similar to clinical signs, the peak body temperatures were observed at day 5 postchallenge. The group with the highest mean body temperature was NVx-Ch, while the lowest mean body temperature was recorded for the NVx-NCh group. Among the vaccinated groups, the HVT-LT-Ch and FPV-LT-Ch experienced the highest mean body temperatures (42.2 C and 41.9 C, respectively), with no significant difference observed between these groups at the 5% level of significance. The CEO-vaccinated group showed a mean body temperature of 41.5 C, which was not significantly different from the mean body temperature of 41.55 C recorded for the NVx-NCh group. Table 3 shows the mean percent body weight gained postchallenge. Although there was no statistically significant difference, a trend was observed among the groups. The CEO-Ch group showed the highest mean percent body weight gained postchallenge, followed by the NVx-NCh, FPV-LT-Ch, and HVT-LT-Ch groups. The lowest percent body weight gained was observed for the Nvx-Ch group.

Trachea viral loads and genotyping of challenge virus. The challenge virus load in the trachea of chickens within all groups was evaluated by quantitative PCR. The normalized values obtained at 5 and 8 days postchallenge are shown in Figure 2. Differences among groups were more evident at day 5 (Chi-square test of homogeneity, $P < 0.001$) than at day 8 postchallenge ($P = 0.006$). The average amount of virus shed at day 5 postchallenge in the CEO-Ch group showed no significant differences from the NVx-NCh group (Fig. 2). The average viral load value calculated for the CEO group was 0.4408 ($\log_{10} 2^{-\Delta\Delta Ct}$), with 2 of 12 samples showing relatively low amounts of viral DNA (data not shown). No significant differences were detected among average viral loads ($\log_{10} 2^{-\Delta\Delta Ct}$) in NVx-Ch (5.528), HVT-LT-Ch (5.035), and FPV-LT-Ch (5.460) groups, but viral loads for these groups were significantly higher than those detected for the CEO-Ch (0.471) and NVx-NCh (0.1174) groups at 5 days postchallenge. At 8 days postchallenge, as the overall level of viral loads declined, the ANOVA test showed significant differences among all groups ($P = 0.006$) while pair-wise differences were less pronounced among groups. The amount of virus shed by chickens in the NVx-Ch ($P = 0.054$) and the HVT-LT ($P = 0.038$) groups was very close to the 5% significance level, albeit these groups remained significantly different to the NVx-NCh chickens. These differences were considered relevant given the small sample size. Positive qPCR results obtained at 5 days postchallenge were confirmed by virus isolation in 12/12, 9/12, and 8/12 tracheal swab samples from the NVx-Ch, FPV-LT-Ch, and HVT-LT-Ch groups, respectively. No virus was isolated from tracheal samples collected from the NVx-NCh and CEO-Ch groups after three passages in chicken kidney cells. RRFLP analysis on tracheal samples collected postchallenge showed that the viral DNA from samples belonging to the NVx-Ch, CEO-Ch, HVT-LT-Ch, and FPV-LT-Ch groups possessed the *Ava*I restriction site for all such groups and lacked the *Alw*I site (ΔCt for *Ava*I was ≥ 1 and ΔCt for *Alw*I was ≤ 1; data not shown). The presence of the *Ava*I restriction site in the ICP4 noncoding region is characteristic of the USDA challenge strain (6).

Table 3. Percentage body weight (% BW) gained from 35 to 46 days of age.

Treatment groups	Mean % BW[A]
NVx-NCh	62.8 (0.21)[a]
NVx-Ch	44.8 (0.90)[a]
CEO-Ch	66.2 (0.10)[a]
HVT-LT-Ch	46.9 (0.07)[a]
FPV-LT-Ch	48.2 (0.11)[a]

[A](Mean BW prechallenge [35 DOA] − mean BW postchallenge [45 DOA] / mean BW prechallenge) × 100. The % BW with same lowercase letters were not statistically significant ($P > 0.05$).

Serology. Antibody detection by commercial ELISA and indirect fluorescent antibody (IFA) testing, in sera collected pre- and postchallenge, is presented in Table 4. ELISA titers were not detected in prechallenge sera samples collected from recombinant viral vector vaccinated chickens (HVT-LT and FPV-LT), while antibodies (mean titer 512) were detected in sera collected 2 weeks post-CEO vaccination via eye-drop. Antibody titers specific to viral glycoprotein B were detected by IFA in prechallenge sera from FPV-LT and CEO-vaccinated chickens, with gB FPV-LT group titers significantly higher than CEO group titers. Glycoprotein I antibodies titers were only detected in sera from CEO vaccinated chickens; however, these were not significantly different to the negative control (NVx-NCh; Table 4). Eleven days postchallenge, mean ILTV antibody titers ranged from 2000 to 6000, as detected by commercial ELISA in all treatment groups, with the exception of the NVx-NCh group. The highest mean antibody titers detected by commercial ELISA were for the FPV-LT group, followed by the CEO-vaccinated group, and these were not significantly different to the titers obtained for the Nvx-Ch group of chickens. Antibody titers specific to gB were detected postchallenge in the CEO, HVT-LT, and FPV–LT-vaccinated groups of chickens, with the FPV–LT-vaccinated group showing significantly higher gB titers than did the HVT-LT and CEO groups. Similarly, antibody titers to gI were detected for the CEO, HVT-LT, and FPV–LT-vaccinated groups;

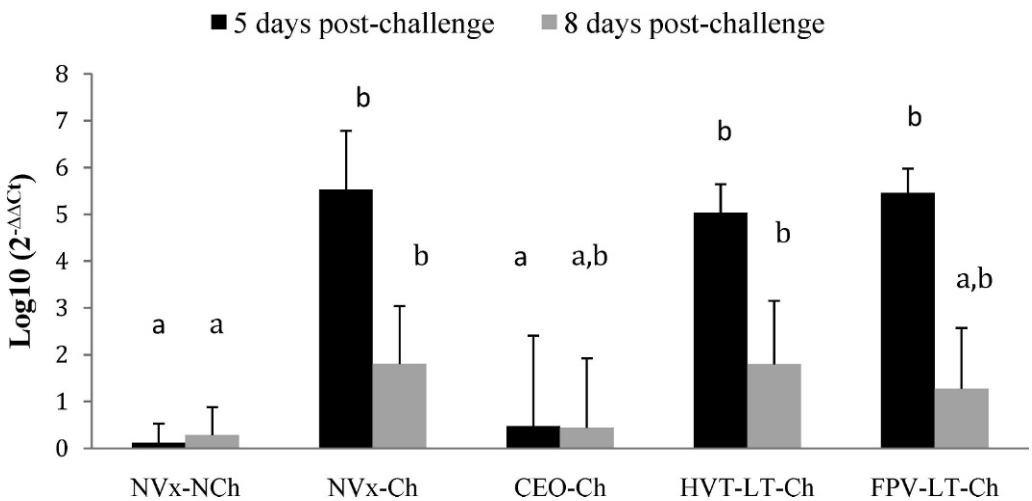

Fig. 2. Trachea challenge virus load determined by quantitative ILTV- PCR at days 5 and 8 postchallenge. The amount of the viral nucleic acid relative to the amount of chicken α2-collagen was expressed as log $_{10}$ 2−ΔΔCt. At day 5 postchallenge, the average log $_{10}$ 2−ΔΔCt values were 0.1174 (NVx-NCh), 5.528 (NVx-Ch), 0.471 (CEO-Ch), 5.035 (HVT-LT-Ch), and 5.460 (FPV-LT-Ch). At day 8 post-challenge the average values were 0.282 (NVx-NCh), 1.805 (NVx-Ch), 0.441 (CEO-Ch), 1.799 (HVT-LT-Ch), and 1.273 (FPV-LT-Ch). Significant differences among groups are indicated by different lowercase letters (groups with the same lower case letter, evaluated independently for 5 days postinoculation (dpi) and 8 dpi, did not show statistically significant differences in viral shedding). The NVx-Ch, HVT-LT-Ch, and FPV-LT-Ch groups showed statistically significant differences in viral shedding between 5 and 8 days dpi.

however, no significant differences in titers were observed among these groups. Antibody titers to gC and gJ were detected in serum samples collected postchallenge for the HVT-LT and FPV–LT-vaccinated groups of chickens; in FPV–LT-vaccinated chickens, the gC titers were numerically higher than the gJ, while in HVT-LT the opposite was observed (Table 4).

DISCUSSION

Due to their safety and the coding capacity of foreign genes, FPV and HVT have been the preferred vectors for the development of poultry recombinant vaccines (26,37). HVT- and FPV-vector vaccines against Newcastle disease (ND; 3,16,22,27,28,34), infectious bursal disease (17,24), ILTV (38,40), as well as FPV-vector vaccines against avian influenza (4,39), are commercially available. The FPV-LT was initially designed for wing-web vaccination in chickens over 8 wk of age (10). The HVT–LT-vector vaccine was designed for subcutaneous vaccination in 1-day-old chickens (18). However, due to the increased incidence of ILTV outbreaks in broilers, and the convenience of *in ovo* vaccination, these recombinant vaccines were administered extensively to the broiler embryos. The objective of this study was to evaluate the protection induced by viral vector recombinant ILTV vaccines after *in ovo* application in commercial broilers. Protection induced by recombinant ILTV vaccines was assessed by their ability to prevent clinical signs and mortality, maintain body weight gain after challenge, and reduce viral replication of the challenge virus in the trachea.

As previously observed (32), the peak of clinical signs was identified at 5 days postchallenge and confirmed by increased viral replication in the trachea and increased body temperatures among NVx-NCh chickens. As compared to the NVx-NCh group, the *in ovo*-vaccinated HVT-LT-Ch and FPV-LT-Ch group of chickens also presented a significant increase in clinical signs, body temperature, and tracheal viral loads 5 days postchallenge. Although the mean clinical sign scores for the HVT-LT-Ch at day 5 postchallenge was significantly higher than the clinical sign score for the FPV-LT-Ch group, the average viral loads for both recombinant vaccinated groups of chicken were not different ($P < 0.05$) from the average viral load estimated for the NVX-Ch group of chickens. Although there was no statistically significant difference, the mean percent body weight gained by the FPV-LT and HVT–LT-vaccinated groups of chickens were below the weight gained by the CEO-vaccinated group postchallenge. These results indicated that the ILTV recombinant viral vector vaccines applied *in ovo* mitigated the disease to certain degree; however, they did not reduce the replication of the challenge virus in the upper respiratory tract.

Significant differences for the challenge virus loads were not detected between the recombinant HVT-LT and FPV–LT-vaccinated groups and the NVx-NCh positive control, particularly at 5 days postchallenge, which marked the peak of clinical signs. The genotype of the virus found in the trachea postchallenge was easily confirmed as the USDA strain (5). Very low viral loads were detected in the negative control (NVx-NCh) group of chickens at 5 and 8 days postchallenge, indicating that the negative controls became infected due to cross-contamination during early stages of the challenge infection. In contrast to the HVT- and FPV-vectored recombinant ILT vaccines, the live-attenuated CEO vaccine reduced clinical signs and significantly reduced challenge virus loads in the trachea of chickens when applied via eye-drop. It has been clearly substantiated that the CEO and TCO vaccines replicate very effectively in the trachea when applied via eye-drop (35). Using PCR analysis specific for FPV (41) and HVT (21), it was demonstrated that neither of these two vectors replicated in the trachea of *in ovo*-vaccinated chickens. It has been shown that other viral vector vaccines, such as HVT-NDV (16,27,28,34) and FPV-NDV (23,38), provide different degrees of protection against challenge and reduction of viral shedding (16,23,27,28,34). A significant reduction in viral shedding was obtained only when HVT-NDV *in ovo*-vaccination was combined with a live-adjuvanted NDV vaccine at 1 day of age (33). We speculate that the inability of the FPV-LT and HVT–LT-vectored vaccines to replicate in the trachea, and consequently to induce a strong local immune response, might be the reason why these vaccines provide partial protection and cannot abolish the replication of the challenge virus in the trachea.

Table 4. Antibody titers against ILTV whole virus, determined by commercial ELISA, and to specific viral glycoproteins determined by IFA. Antibody titers with the same lowercase letters were not significantly different.

	Prechallenge[A]			Postchallenge[B]				
	ELISA	gB	gJ	ELISA	gB	gC	gI	gJ
NVx-NCh	0[C]/12[a]	0/5[a]	0/5[a]	0/12[a]	0/5[a]	0/5[a]	0/5[a]	0/5[a]
HVT-LT	0/12[a]	0/7[a]	0/7[a]	9/12 (2803 ± 2314)[a,b]	7/9 (622 ± 466)[a]	7/9 (722 + 577)[a,b]	9/9 (1333 ± 822)[b]	8/9 (944 ± 913)[b]
FPV-LT	0/12[a]	4/7 (29 ± 24)[b]	0/7[a]	12/12 (6022 ± 2826)[b]	7/7 (6629 ± 4540)[b]	7/7 (1371 ± 904)[b]	7/7 (1657 ± 1965)[b]	7/7 (657 ± 232)[a,b]
CEO	4/12[D] (591 ± 920)[E,b]	2/12 (8 ± 19)[a]	4/12 (17 ± 24)[a,b]	10/12 (4950 ± 4069)[b]	9/12 (325 ± 421)[a]	ND[F]	8/12 (417± 555)[b]	ND
NVx-Ch	0/12[a]	ND	ND	12/12 (3083 ± 1430)[a,b]	ND	ND	ND	ND

[A]Samples collected 35 days posthatch.
[B]Samples collected 11 days postchallenge.
[C]Number of positive samples.
[D]Samples collected two weeks post CEO vaccination.
[E]Arithmetic mean titer with the same lowercase letters are not statistically significant ($P > 0.05$).
[F]Not done.

Although no ILTV antibodies were detected by commercial ELISA in prechallenge serum samples from chickens vaccinated with FPV-LT vaccine, low gB antibodies titers were detected by IFA, indicating that chickens did receive the FPV-LT vaccine. Furthermore, serum samples from FPV–LT-vaccinated chickens collected 11 days postchallenge showed a significant increase in gB antibody titers, indicating that the FPV–LT-vaccinated chickens were effectively primed; however, this strong humoral gB response was not sufficient to protect chickens from clinical disease and challenge virus replication in the trachea. On the other hand, no gI antibodies were detected in prechallenge serum from HVT–LT-vaccinated chickens, and no differences in gI titers were observed postchallenge among HVT-LT, FPV-LT, and the CEO-vaccinated group of chickens. Therefore, in this study, the HVT-LT vaccine did not elicit a strong gI antibody response, although evidence that the chickens received the vaccine was confirmed by the presence of HVT genomes in the feather follicles. Optimal efficiency of these vaccines strongly relies on proper *in ovo* delivery; the efficiency of MDV vaccines can be severely affected by the embryonic compartment in which it is deposited during *in ovo* vaccination. MDV vaccines delivered to the embryo body or to the amniotic fluid result in excellent protective immunity, but administration of the vaccine to the air cell or allantoic sac results in poor protection (41). The site of injection may vary with embryo age. Younger embryos (<17 days) have smaller size and more fluids, which may increase the chance for the vaccine to remain in the air cell or allantoic sac, while in larger, older embryos (18 to 19 days), there may be an increased possibility for the vaccine to be delivered correctly into the embryo body or the amnios. Also, variation in embryo size can influence the delivery of the vaccine to the embryo (41). In order to limit the variability on embryo size, embryos used in this study were obtained from breeder flocks between 30 to 35 weeks of age.

Other variables that influence the proper delivery of vaccines are the gauge and length of the needle utilized for delivery (2). In this study, embryos were inoculated with a one-needle vaccinator designed for laboratory settings. This machine is probably not as consistent as the large-scale *in ovo* vaccinators utilized commercially. Overall, in this study, it was demonstrated that both recombinant-vectored ILTV vaccines provided partial protection and did not reduce challenge virus loads in the trachea. The protection induced by ILTV recombinant-vectored vaccines when applied *in ovo* was not as favorable as expected. Further studies are being conducted to evaluate the protection induced by the HVT-LT and FPV-LT, when administered with a commercial *in ovo* vaccinator, in order to evaluate the role of *in ovo* delivery in the vaccine's efficacy to protect against ILTV. Field evidence has indicated that, in regions of strong challenge, the recombinant viral vectored vaccines have failed in full protection against the disease; however, in regions with mild-to-moderate challenge, these vaccines have been efficient and provide a safer alternative than the continuous use of CEO vaccination.

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