UNDERSTANDING THE PROTECTION ELICITED BY RECOMBINANT HERPESVIRUS OF TURKEYS (rHVT) INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) VACCINE AND AN ALTERNATIVE VACCINATION STRATEGY AGAINST THE DISEASE

by

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ABSTRACT

Infectious laryngotracheitis (ILT) is an acute respiratory disease of poultry caused by gallid alpha herpesvirus I (GaHV-1) commonly recognized as Infectious laryngotracheitis virus (ILTV). Prevention and control of the disease is built on biosecurity and vaccination. Particularly, chicken embryo origin (CEO) vaccines have proven to be the most protective vaccines against the disease. However, it has been demonstrated that CEO vaccines have inherent virulence which is easily triggered if the vaccine is allowed to circulate in naive or poorly vaccinated flocks. Also, the CEO vaccines establish latency; therefore, vaccinated birds carry the virus for life and under stress the vaccine virus can be reactivated. In recent years, the use of recombinant ILT vaccines has significantly expanded as it offers a safer vaccination alternative for the control of the disease. However, experimental evidence has shown that recombinant Herpesvirus of turkey – Laryngotracheitis (rHVT-LT) vaccines induce partial protection because under challenge with virulent strains, virus replication still occurs in the trachea of vaccinated chickens. In order to better understand the role that the dose of rHVT-LT vaccine plays in protection; whether rHVT-LT vaccinated chickens can transmit virus after challenge, and how the combination of rHVT-LT and

CEO vaccines benefit the control of ILT, the objective of this work was threefold: 1) To evaluate the protection efficacy of a rHVT-LT vaccine when administered at standardized doses of 6000, 3000 and 1000 plaque forming units (PFUs) and the effect of the rHVT-LT vaccination in transmission of the challenge virus to contact naive chickens; 2) To evaluate the replication and protection efficacy of a rHVT-LT vaccine when administered at a double commercial dose (13000 PFU); and 3) To evaluate the effect of rHVT-LT vaccination in CEO replication and how this vaccination strategy enhances protection and limits challenge virus transmission to contact naive chickens. Independent of the rHVT-LT vaccine dose, vaccinated chickens showed significant reduction in clinical signs, maintained body weight gain after challenge, and lessened the challenge virus replication. However, despite reduction of challenge virus replication in the trachea, challenge virus was transmitted from rHVT-LT vaccinated chickens to contact naive chickens, whereas in CEO or rHVT + CEO vaccinated chickens transmission of the challenge virus to naive chickens was impeded. Finally, it was concluded that priming with rHVT-LT reduced CEO virus replication and the addition of a CEO vaccination provided a more robust protection than rHVT alone.

INDEX WORDS: Infectious Laryngotracheitis Virus, Vaccination, Live attenuated vaccines, rHVT-LT

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DEDICATION

This thesis is dedicated to my loving wife Ana and my wonderful son Fabrizio who have always been a constant source of support and encouragement during this journey. I love you so much!!!

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CHAPTER 1

INTRODUCTION

Infectious laryngotracheitis (ILT) is a viral respiratory disease of chickens that result in severe production losses due to mortality, morbidity and/or decreased egg production. Severe forms of the disease are characterized by gasping, expectoration of bloody mucus, and high mortality due to asphyxia (10). The disease is caused by Gallid alpha herpesvirus I (GaHV-1) a member of the genus *Iltovirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, commonly recognized as Infectious laryngotracheitis virus (ILTV) (4).

Control of the disease is built on biosecurity and vaccination. Modified live attenuated vaccines, produced in chicken embryos (chicken embryo origin, CEO) or tissue culture (tissue culture origin, TCO), have been used for years to control ILT. Nonetheless, live vaccine viruses are capable of spreading (3,11,21), have residual virulence, particularly the CEO vaccines, which increases after bird to bird passages (14). The ability of modified live attenuated vaccines to establish latent infections and to sporadically reactivate leading to renewed virus shedding, is also a matter of concern in the control of the disease (17). As a response to the frequent ILT epizootics related to CEO vaccines, recombinant vaccines using either fowlpox virus (FPV) and/or herpesvirus of turkey (HVT) as vectors expressing ILTV genes are commonly used. The recombinant (r) rFPV-LT vaccine expresses the ILTV glycoprotein (g) B (gB) and the UL-32 genes, while there are two rHVT-LT vaccines, either expressing the ILTV gI and gD genes, or the ILTV gB gene. The ILTV glycoproteins expressed in the vector vaccines constitute important

immunogenic antigens that can elicit both humoral and cell-mediated immunity (25). The gD is a highly conserved herpesvirus structural glycoprotein and functions as a viral ligand for cell entry receptors to mediate fusion (9). The gI is important in cell-to-cell spread of the virus (6) and the gB is involved in fusion and entry into the host cell (2). The main advantage of the recombinant vaccines is that they offer a safer vaccination alternative because their lack of transmission and spread from bird to bird, the absence of ILTV latent infections, and the inability to revert to virulence (7).

Between the two available vectors utilized for recombinant vaccines against ILT, the herpesvirus of turkey (HVT) which is classified as a *Meleagrid herpesvirus 1* (MeHV-1) (22), also known as the serotype 3 within the Marek's disease virus (MDV) group, is the most frequently used vector owing to its capacity to limit interference by maternally derived antibodies (24), its safety (7), and its ability to establish persistent viremia (8,20,26). Furthermore, because HVT replicates in a highly cell-associated manner in lymphocytes, it is suggested that it would induce a long-lasting cell-mediated immune response (15). On the other hand, Fowlpox virus (FPV), belongs to the genus *Avipoxvirus*, and has also been employed for the delivery of viral antigens in several experimental and commercial vaccines for chickens (1). However, the major disadvantage of the use of FPV vectored vaccines is that the presence of maternal antibodies could interfere with the vaccination of young birds (16) and previous pox viral exposure affects its protection efficacy (5). Protection studies against ILT comparing rHVT-LT and rFPV-LT, determined that chickens vaccinated with rHVT-LT were better protected, presenting lower clinical signs scores and challenge virus genome load levels in trachea than rFPV-LT vaccinated chickens after a virulent ILTV challenge (19,23).

Although rHVT-LT vaccines present a safer vaccination option than CEO vaccines and mitigate the signs of the disease, studies have shown that vaccination with rHVT-LT did not limit challenge virus replication in the trachea as effectively as the CEO vaccines (18,23). It is suspected that the induction of a limited local respiratory immune response maybe the reason why rHVT-LT vaccines are not as effective as CEO vaccines in curtailing viral replication in the trachea after challenge (13,18). It was shown that after *in ovo* or subcutaneous vaccination with a rHVT-LT vaccine, the expression of ILTV and HVT genes in the spleen and feather follicles was robust, whereas gene expression in the lungs was limited. The authors concluded that reduced expression of ILTV and HVT genes in the lung might result in a weak mucosal immune response in the respiratory tract against ILTV (13).

Due to the failure of rHVT-LT vaccinated poultry to efficiently block challenge virus replication, it is believed that in endemic areas rHVT-LT vaccinated flocks although apparently healthy, may recurrently shedding virus after a field challenge (23), but the former has not been proven under experimental conditions. The protection efficacy of ILTV recombinant vaccines can also be significantly affected by the use of fractional doses and improper handling during the administration of these vaccines. Previous studies have demonstrated the negative effect of diluting recombinant HVT vaccines in the protection elicited against Marek's disease (MD) (12); nonetheless, the protection efficacy against ILT when rHVT-LT vaccine is administered at fractionated doses has not been evaluated. Although it has not been proven it is believed that protection efficacy of rHVT-LT vaccines might be enhanced by increasing the vaccine dose because it would boost vector vaccine replication and consequently increase the expression of ILTV glycoproteins. Another feasible approach to expand protection against ILT is to implement a combined vaccination strategy of rHVT-LT and CEO vaccines. The rationale of this vaccination

plan is based on the assumption that chickens previously vaccinated with rHVT-LT will reduce CEO vaccine replication and the booster of a CEO vaccination will improve reduction of the challenge virus replication, limiting the circulation of both CEO and challenge viruses in the environment, and consequently the outbreaks of the disease.

Therefore, this work attempts to answer whether the rHVT-LT dose is correlated with protection; whether rHVT-LT vaccinated chickens can transmit virus after challenge, and how the combination of rHVT-LT and CEO vaccines help in the control of ILT. The specific objectives of this work are: 1) To evaluate the protection efficacy of a rHVT-LT vaccine when administered at standardized doses of 6000, 3000 and 1000 plaque forming units (PFUs) and the effect of the rHVT-LT vaccination in transmission of the challenge virus to contact naive chickens; 2) To evaluate the replication and protection efficacy of a rHVT-LT vaccine when administered at a double commercial dose (13000 PFU) and 3) To evaluate the effect of rHVT-LT vaccination in CEO replication and how this vaccination strategy might enhance protection and limit challenge virus transmission to contact naive chickens.

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CHAPTER 2

LITERATURE REVIEW

ETIOLOGY

Classification

Infectious laryngotracheitis virus (ILTV), the causative agent of the infectious laryngotracheitis disease (ILT), is taxonomically classified as Gallid alpha Herpesvirus-1 (GaHV-1), belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Iltovirus* (25).

Viral Structure

ILTV is an enveloped virus which possess a linear double stranded DNA genome contained within an icosahedral particle with a hexagonal nucleocapsid (80 – 100 nm) similar to other herpesviruses (24). The ILTV genome is approximately 150 kb in size consisting of 77 predicted open reading frames (ORFs) with unique long (UL) and unique short (US) regions and inverted repeats flanking the US region. The nucleocapsid of ILTV contains 162 elongated hollow capsomers, which contain 150 hexons and 12 pentons. The envelope surrounding the nucleocapsid is a lipid bilayer, which is associated with the outer surface of the tegument (26). Unique to herpesviruses, tegument is a structure that occupies the space between the nucleocapsid and the envelope and contains many virus encoded proteins called tegument proteins (53). The surface of the envelope contains viral glycoproteins, namely glycoprotein (g) B, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM (41) which are responsible for virus entry, transport of the nucleocapsid, and cell to cell spread of the virus. In addition to their roles in host range and pathogenicity, the ILTV's

glycoproteins are immunogenic and responsible for stimulating humoral and cell-mediated immune responses (130,132,133). Based on antibody responses in chickens and the reactivity of monoclonal antibodies raised against the whole virus, the envelope glycoproteins gJ and gC have been characterized as the most abundant surface glycoproteins of ILTV (40,123).

Viral Replication

Although the ILTV replication cycle has not been completely elucidated, it is assumed that the ILTV replication cycle is comparable to the replication cycle of its herpesvirus prototype, herpes simplex virus-1 (HSV-1) (41). ILTV glycoproteins (g) such as gB, gC, gD, gH and gL are expected to be involved in the process of attachment to host cell receptor (115). Until know only the ILTV gC has been thought to mediate the entry of ILTV into the host cell (91). Unlike HSV-1, ILTV entry is believed to be heparan sulfate independent as ILTV gC lacks the heparan binding domain (77,91). Following attachment, the virus initiates fusion of the envelope with the host cell plasma membrane. The nucleocapsid is released into the cytoplasm and transported to the nuclear membrane. Viral DNA is liberated from the nucleocapsid and migrate to the nucleus, through nuclear pores, where transcription and replication of viral DNA occurs (44). The viral replication process is highly regulated and there are three major categories of genes expressed at different periods of viral transcription and translation. The expression of immediate early (IE) genes is self-regulated and occurs at the beginning of ILTV infection. IE gene products are mostly transcription factors and are expressed independently on *de novo* protein. Early (E) genes are generally involved in viral DNA metabolism and replication, and are dependent of *de novo* protein synthesis. Late (L) genes are partially or completely dependent on the replication of viral DNA and generally encode structural and other proteins involved in virion assembly (85).

Viral nuclear egress begins with translocation of the capsid through the nuclear membrane after pro-capsid packaging of monomeric DNA, followed by addition of an envelope from the inner membrane of the host cell nucleus and movement to the lumen of the endoplasmic reticulum into vacuoles within the cytoplasm (52). Mature capsid-less particles are produced in the *trans*-Golgi region of the cytoplasm, where assembly of tegument and secondary envelopment occur, and infectious virions are subsequently released by exocytosis (41). An *in vitro* infection study has determined that the ILTV replication initiates with the formation of infectious progeny viral particles after 8 to 12 hours following infection and the maximum viral replication is observed within 24 to 30 hours post-infection (94).

PATHOBIOLOGY

Host

Infectious laryngotracheitis is universally described as an infection of chickens, although there are reports of natural infections in pheasants, peafowl and partridges (11,23). Natural and experimental infections were also reported in turkeys. Turkeys might have an age-dependent resistance, since lesions were only seen in younger birds (93,127). Sub-clinical infection and seroconversion have been observed in ducks infected through intratracheal and intranasal routes (129). Starlings, sparrows, crows, doves, pigeons and guinea fowl appear to be refractory to the infection, as well as non-galliform birds and mammals (44)

Transmission

The natural routes of entry for ILTV are via the upper respiratory and ocular routes. Ingestion can also be a mode of infection, although exposure of the nasal epithelium following ingestion is required with this route (100). The main mode of transmission of ILTV is horizontal

by direct or indirect contact and there is no evidence of vertical transmission (44). Sources of ILTV are clinically affected chickens, latent infected carrier chickens, contaminated dust, litter, and fomites (34).

Pathogenesis

ILTV infects mainly the upper respiratory tract, lungs, conjunctiva, and air sacs (3,4). As ILTV infection frequently led to the formation of mucoid plugs/casts in the trachea predisposing to chicken asphyxia and mortality (5), it was hypothesized that ILTV stimulates the hypersecretion of mucus in the trachea. Mucins, specifically MUC5AC and MUC5B, were initially believed to cause the tracheal thickening of the mucosa, due to inflammation, which results in a reduced tracheal lumen diameter and obstruction. However, Reddy *et al*. (98) demonstrated that production of MUC5AC and MUC5B were barely observed in the trachea, larynx, trachea and bronchi, and in the tracheal plugs/casts of ILTV infected chickens. Whereas mucoid plugs/casts produced during acute ILTV infection were mainly composed of DNA-fibrous structures probably generated by heterophils and other inflammatory cells.

Furthermore, ILTV was shown to infect leukocytes (15) and macrophages *in vitro* (12) which may hint at the possible mechanism by which a systemic infection by ILTV is established. Following virus entry to susceptible epithelial cells of the respiratory tract and the conjunctiva, ILTV starts the lytic phase of replication approximately three to five days post infection leading to clinical manifestations. The lytic infection is followed by the establishment of latency of ILTV in the trigeminal ganglion (125). Experimentally, homing of the virus to the trigeminal ganglion has been demonstrated 4 to 7 days after intratracheal infection (3). Stress can trigger viral reactivation, leading to migration of ILTV to the trachea, where production and excretion of viral

particles is renewed (64). Therefore, long lived birds could be considered ILTV reservoirs and contributors to the spread of the virus (55,62,101).

Clinical Signs and Lesions

Clinical signs are typically observed 6 - 12 days after natural infection, whereas under experimental conditions they can be identified as early as 2 - 4 days post infection (71). There are two clinical forms of the disease (severe and mild). Clinical signs associated with the severe form include conjunctivitis, nasal discharge, depression, dyspnea, gasping, expectoration of bloody mucous, high morbidity and variable mortality (5 to 70%), whereas clinical signs in mild forms involve nasal discharge, conjunctivitis, sneezing, coughing and mild tracheitis with low morbidity and mortality (0.1 – 2.0%) (5). Decreased in feed consumption, lower egg production and reduced eggshell thickness have also been observed (97).

Gross lesions of ILTV infection are characterized by mucoid inflammation, degeneration, and necrosis. In severe forms of the disease, diphtheritic changes may be seen as mucoid casts that fill the entire length of the trachea. Severe hemorrhages into the trachea may result in blood casts, and mucous can be mixed with blood and necrotic tissue. In mild forms of the disease, gross lesions may be seen in the conjunctiva and throughout the respiratory tract, although lesions are more commonly observed in the larynx and the upper trachea, as a mild inflammation or excess of mucous to a severe hemorrhagic tracheitis (105). In very mild cases, gross lesions may consist only of edema and congestion of the conjunctiva, the infraorbital sinus, and mucoid tracheitis. The length of infection varies with the severity of lesions, with flocks typically recovering within 10 - 14 days after the initial manifestation of clinical signs (44).

As for gross lesions, microscopic changes also vary with regard to the severity and stage of disease. Early microscopic changes in the tracheal mucosa involve goblet cell loss and

infiltration of mucosa with inflammatory cells. Respiratory and conjunctival epithelial cells become enlarged and edematous as the infection progresses. As early as 3 dpi, intranuclear inclusion bodies are found in epithelial cells and are present only at the beginning of the infection. As the infection progresses, epithelial cells in the respiratory tract enlarge, the trachea loses cilia and becomes edematous. Consequently, the affected epithelia desquamate, exposing the lamina propria and subsequently its blood vessels protrude to the tracheal lumen, causing hemorrhage (56,95).

IMMUNITY

There is enough evidence today to conclude that the immune systems of avian species diverge from that of mammalian species and untested extrapolation from mammalian systems to avian will not provide the required knowledge for understanding host - pathogen relationships. In comparison with mammals, birds lack lymph nodes, but they have an avian-specific primary lymphoid organ, the bursa of Fabricius, which is the site of development of their B-cell receptor repertoire (73). Additionally, in comparison to mammals chickens have different repertoires of Toll-like receptors (TLRs) (10,22,113), defensins (84), cytokines (74), chemokines (66,74), and antibodies (72). Chickens also lack functional eosinophils and the avian functional equivalent of the mammalian neutrophil is the heterophil. Despite all these differences, the basic principle of innate immune responses driving appropriate adaptive immune responses to clear initial infection and provide immunological memory remains constant for all vertebrate species (73).

Innate Immune Response

As in mammals, the avian innate immune response has its own receptors (pattern recognition receptors [PRRs]), its own effector cells (e.g. neutrophils/heterophils, natural killers

[NK] cells and dendritic cells [DC]), and produces cytokines and chemokines that drive inflammatory responses and presents pathogen antigens to the adaptive immune response, in the context of the major histocompatibility complex (MHC) via antigen-presenting cells (APC) (73).

Understanding the mechanisms of innate responses against ILTV infection have been limited to few studies. A microarray analysis suggested that ILTV infection of chicken embryonic lung cells induced upregulation of genes related to cellular growth and proliferation, apoptosis, cell signaling and inflammation. In particular upregulation of cytokine genes such as interleukin (IL)-6, IL-8, IL-15, chemokines CXC K60, CCL17 and CCL20 were detected following ILTV infection suggesting that inflammation plays a pivotal role during the early phase of infection (82). Vagnozzi *et al*. (120) evaluated the transcription levels of interferon (IFN)-γ, IFN-β, IL-1β, IL-6, IL-8 in CEO vaccinated chickens at 6 and 12 hours post infection. After challenge, a significant increase in IFN-γ was evidenced, whereas expression of the pro-inflammatory cytokines (IL-1β, IL-6, IL-8) and type I IFN β were either slightly diminished or were unaffected. Therefore, the authors propose that the rapid expression of IFN-γ may play an important role in limiting early virus replication.

Activation of chicken toll-like receptors (TLRs) with synthetic ligands has also been investigated in their potential to limit ILTV replication. Thapa *et al*. (114) found that *in ovo* delivered of CpG oligonucleotide (CpG-ODN), which is a well-defined TLR9 ligand, significantly reduces ILTV infection which was associated with the increase of macrophages and the transcription upregulation of the IL-1β gene in the lung. Additionally, Abdul-Cader *et al*. (1) identified that *in ovo* delivered of CpG-ODN was capable of eliciting significant cellular responses characterized by innate (KUL01+ cells) and adaptive (IgM+ B cells and CD4+ and CD8α+ cells) immune responses at day 1 post-hatch which may be associated with a reduction of clinical signs,

mortality and cloacal viral genome load in chickens infected with ILTV via intratracheally at 1 day of age. Similarly, lipoteichoic acid (LTA) treatment via *in ovo* at 18 days of embryonation can lead to antiviral response against pre-hatch ILTV infection *in vivo*, which is associated with expansion of macrophage populations and expression of IL-1β and myeloid differentiation primary response gene (MyD88) in the lung via toll-like receptor 2 (TLR2) activation (58). On the other hand, stimulation with the TLR4 ligand (lipopolysaccharide [LPS]) *in vitro* in an avian macrophage cell line (MQ-NCSU) did not induce a type 1 interferon response, but it did induce the expression of CD14 and nitric oxide (NO) suggesting that LPS can be a potential innate immune stimulant that can be used against ILTV infection; however further evaluations *in vivo* are warranted (57).

Adaptive Immune Response

Although under some circumstances innate immune responses can suffice to control infection, adaptive immune responses are normally required to eliminate pathogens, and these generally lead to immunological memory, either as a result of primary infection with a pathogen or in response to vaccination (73).

Humoral and cell mediated immunity (CMI) responses have been described following ILTV infection. Although antibodies are produced against the virus, the humoral immune responses do not guarantee protection against ILTV infection (38,99). This observation was evidenced by the ability of bursectomised chickens, unable to produce specific antibodies, to resolve primary infections as efficiently as chickens retaining functional bursa of Fabricius. Additionally, it has been demonstrated that mucosal antibodies are not essential in preventing viral replication (38). Virus neutralizing antibodies can be detected within 5 to 7 days post infection, however there is no correlation between levels of neutralizing antibodies and resistance to

challenge (108). Passive transfer of maternal antibodies to the progeny has also been demonstrated (60), but no significant differences were observed between chickens hatched from hyperimmune and non-vaccinated parents regarding protection against challenge (37). More recently, a study was conducted to examine whether antibody responses to individual ILTV glycoproteins are correlated with disease and protection. Four ILTV glycoproteins (gD, gE, gG and gJ) expressed as recombinant proteins and two commercially available recombinant gC and gI were used in order to evaluate post-vaccination and/or post-challenge chicken serum antibodies (ELISA). Overall, results from this study demonstrated that systemic antibody titers to individual ILTV glycoproteins C, D, E, G, I and J had a relatively poor correlation with protective immunity (103).

On the other hand, cell mediated immunity (CMI) plays a major role in resistance to infection (99,131). Fahey *et al*. (39) showed that protection against ILTV infection could be transferred by spleen cells and peripheral blood leukocytes from previously infected congenic immune donors. Later, Honda *et al*. (63) corroborated this information, showing that thymectomized vaccinated chickens were poorly protected after challenge, inferring that the key component of protection was mediated by the CMI.

Intratracheal inoculation with a virulent ILTV strain showed an early upregulation of the IFN-γ gene at 1-day post-infection in the Harderian gland; whereas, the trachea exhibited an increase transcription of IL-2 and IFN-γ genes at 5 to 9 days post-infection. The early increase of the IFN-γ gene transcription in the Harderian gland suggests a strong innate immune response probably triggered by activated NK cells. While the late upregulation of IL-2 and IFN-γ gene transcription suggests the expansion of the adaptive immune response in the trachea (118). More recently, *in vitro* infection studies using tracheal organ tissue (TOC) specimen cultures and blood derived monocytes (BDM) and *in vivo* infection studies in specific-pathogen-free chickens were

used to examine the establishment of infection and inflammation by ILTV and determine how gG influences that response to infection (20). Glycoprotein G (gG) is a broad-range viral chemokine binding protein conserved among most alphaherpesviruses, including ILTV. A number of studies comparing the immunological parameters between infection with gG expressing and gG-deficient ILTV strains have demonstrated that expression of gG is associated with increased virulence, modification of the amount and the composition of the inflammatory response, and modulation of the immune responses toward antibody production and away from cell-mediated immune responses (29,33). Coppo *et al*. (20) showed that gG expression influenced the transcription of some cytokines and chemokines of which the transcription of the chicken IL-8, chCXCLi1 and chCXCLi2, was consistently affected by the expression of gG. In a tissue environment where inflammatory signals were already present, the expression of gG was associated with a reduction in the transcription of chCXCLi1 and chCXCLi2, especially at very early time points of infection. The expression of gG in the BDM ILTV infection model also resulted in a reduction in the transcription of chCXCLi1 and chCXLi2 at both 6 and 24 hours post infection.

EPIDEMIOLOGY

ILTV has been identified in most countries and remains a serious threat wherever susceptible poultry populations are grown (8). A trend toward growing denser poultry populations in shorter cycles, rearing different types of poultry (layers and broilers) in the same area, combined with poor biosecurity are some of the factors that have contributed to the increase of ILTV outbreaks worldwide (17,44,92). Backyard flocks may also serve as a source of ILTV to commercial poultry (44). As vertical transmission has not been demonstrated (44), horizontal transmission is the sole mechanism of virus spread which is facilitated by exposure of susceptible

flocks to contaminated equipment, personnel, clothing and shoes, improperly disposed contaminated litter, manure and infected carcass (17,34). An epidemiological study conducted during an ILTV outbreak in California emphasized the importance of biosecurity measures, which included extended downtime between flocks, cleaning and disinfection, and improvements in vaccination programs (17). One potential source for spread of the virus is during the transport of infected and/or vaccinated birds to the processing plant (92,124). As expected wind might play a significant role in the dissemination of ILTV. Farms located within the wind stream of an infected flock are 10 times more likely to be affected by the disease (69). Seasonality plays a role in the incidence of the disease as higher numbers of cases are observed during winter (December-February), as opposed to the lower prevalence during summer and fall (92). It is believed that sensitivity of the ILTV by both light and heat may play a role in the seasonality of the disease (34).

Although many factors are involved in the epidemiology of the disease, molecular epidemiology studies confirm that viruses derived from CEO vaccines and wild type viral strains are equally responsible for outbreaks of the disease (86). The methods used for differentiation of ILTV strains include restriction endonuclease analysis of viral DNA (54), DNA hybridization assays (80), and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (88). Within the United States, 9 groups with unique PCR-RFLP patterns have been identified using genes, such as ORFB-TK, ICP4, UL47/gG, and gM/UL9 (89). The resulting groupings consisted of the USDA reference strain in group I, the TCO vaccine strain in group II, field isolates closely related to the TCO vaccine in group III, CEO vaccine strains and CEO identical commercial poultry isolates in group IV, commercial poultry isolates closely related to the CEO vaccine in group V, commercial poultry isolates in group VI, and unique backyard flock isolates in groups VII, VIII, and IX. Overall, in the US the majority of isolated field strains of

ILTV have been described to be genetically close to vaccine strains (89). On the other hand, in Australia five classes of strains were originally compiled based on PCR-RFLP differentiation using a combination of gG, TK, ICP4, and ICP18.5 target genes. Class 1 consisted of the SA-2 and A20 CEO vaccines as well as related strains, classes 2 and 3 of vaccine-unlike field strains, class 4 of the Australian CSW virulent field strain, and class 5 of vaccine-like and –unlike field strains (79). In 2011, four new classes were identified, including class 6 strains isolated from the region of Victoria, the Nobilis (Serva) ILT vaccine in class 7, and the SA-2, A20, Serva recombinants in classes 8 and 9 (9,81). Similarly, molecular studies based on PCR-RFLP in Europe (87), Asia (14,76) and South America (13) suggested the potential displacement of wild-type strains with vaccine variants.

More recently, Spatz *et al*. (111) investigated the application of Oxford Nanopore MinION technology for rapid sequencing of the amplicons generated in a single-allele assay. Using phylogenomic analysis of 27 full genomes of ILTV, a single allele (ORF A/ORF B) was identified containing single nucleotide polymorphisms (SNPs) that could differentiate ILTVs into genotypes congruent with the phylogenetic partitioning. The allelic variations allowed for the cataloging of the 27 strains into 5 genotypes: vaccinal TCO, vaccinal CEO, virulent CEO-like, virulent US and virulent US backyard flocks from 1980 to 1990, correlating with the PCR-RFLP genotypes I/ II/ III (TCO), IV (CEO), V (virulent CEO-like), VI (virulent US) and VII/VIII/IX (virulent US backyard flock isolates). Overall, there was a 90% correlation between the genotyping results of the single-allele assay (Oxford Nanopore MinION) and the multi-allele assay (PCR-RFLP).

VACCINATION

Currently, there is no effective treatments in reducing the severity of lesions or minimizing the clinical signs of disease. Vaccination is the current approach to induce resistance to the disease in large susceptible populations of birds. Vaccination limits the spread of the challenge virus and ameliorates and decreases the duration of disease. Vaccines against ILTV are either live attenuated vaccines or recombinant viral-vectored vaccines (44).

Live attenuated vaccines

Chicken Embryo Origin (CEO) vaccine

In 1958, Benton *et al*. (7) first studied the use of a field strain of lower virulence as a vaccine for administration via the respiratory tract. The less virulent strain applied intranasally was capable of protecting birds against challenge. However, when the virus was administered by atomization, it produced adverse reactions characterized by acute respiratory distress and mortality. Later, this strain came to be known as the chicken embryo origin (CEO) Cover vaccine strain. In 1969, another CEO attenuated virus, the Hudson strain, was validated for ocular, intranasal, or intra-tracheal administration. In 1966, an Australian field isolate was attenuated by serial passages in chicken embryos and resulted in the CEO SA2 vaccine strain. The SA2 vaccine strain was further attenuated in chicken embryo cell culture and gave rise to the A20 vaccine strain (78).

CEO vaccines are preferred in vaccination programs due to their rapid onset of immunity, and excellent protection efficacy against clinical signs and challenge virus replication (70,121). The CEO vaccines are able to confer complete protection after one-week post immunization, (5), and are the vaccines of choice utilized to stop outbreaks of the disease in broilers (34). However,

the CEO vaccines can regain virulence, inducing severe respiratory disease and mortality (55). When flocks are poorly vaccinated, herd immunity is not achieved and the vaccine virus is allowed to spread from vaccinated to unvaccinated birds facilitating reversion to virulence (101). On the other hand, it is of value to point out that transmission of CEO vaccines from vaccinated to contact exposed chickens does not confer protection against challenge (102).

Herpesviruses are known to establish latency and then reactivate as a result of environmental stress (5,55). CEO vaccines, although attenuated, establish latency. Certain stressors, like moving chickens to new housing or the start and peak of lay can induce reactivation of latent ILTV (64). The reactivation leads to a new cycle of virus lytic replication in the trachea and a potential shedding and transmission of the virus (65,67).

Controlled field experiments strongly suggest that virulence of CEO vaccines is exacerbated by poor mass vaccination practices (42). Non-uniform drinking water immunization is likely to be caused by suboptimal doses of the vaccine received by each bird, or may be due to some birds failing to drink any vaccine-containing water within the time when viable vaccine is available in the water lines (34). Therefore, under reduced vaccination coverage, the opportunities for the vaccine to spread to unvaccinated chickens are quite significant (135). Spray vaccination has been repeatedly reported to cause severe reactions in chickens (18,96). Eye-drop vaccination has been shown to provide more uniform immunity than vaccination via drinking water and spray (45). However, eye drop vaccination is not always feasible to be implemented under field condition due to the extra costs associated with the vaccine administration (135).

Tissue Culture Origin (TCO) vaccine

In 1964 the first tissue culture origin (TCO) vaccine was developed after fifty consecutive passages of the virulent ASL L-6 strain in primary cultures of chicken cells (46). Consecutive

passages of the ASL L-6 strain in primary chicken cell cultures changed the phenotype of the lesions this virus produced on the chorioallantoic membrane (CAM) of embryos and concurrently reduced its virulence in susceptible chickens. In the experimental challenge, it was demonstrated that the TCO vaccine provided birds immunity after ocular or intranasal application. On the other hand, spray and water administration of the modified virus resulted in poor protection (46). Later, the same group of researchers extended this study to evaluate the ASL L-6 strain stability. Results of 10 consecutive back-passages in susceptible birds showed no increase in virulence nor changes in CAM lesions induced by the virus (47).

In 1991, Guy *et al*. (55) demonstrated that after 20 consecutive bird to bird passage of the TCO vaccine only a mild respiratory disease was reported. These findings were in contrast with the severe respiratory disease and mortality produced by chicken passages of the CEO vaccines. Using real-time PCR assays it was evidenced that TCO vaccines can be horizontally transmitted to non-vaccinated birds and the onset of replication and transmission of the TCO vaccine was slower and limited as compared to that of the CEO vaccines (101). Consequently, outbreaks related to TCO immunizations are fairly rare (89,110) in contrast to CEO vaccine-related strains which have been frequently isolated from severe outbreaks of the disease (88,89).

In a protection study carried out by Vagnozzi *et al*. (121), chickens vaccinated with TCO showed reduced levels of clinical signs statistically not different from clinical signs observed in the CEO and rHVT-LT vaccinated groups of chickens. However, reduction of the challenge virus replication for the TCO vaccinated group was not as significant as the decline in challenge virus replication observed for the CEO vaccinated group of chickens. In a more recent study by García *et al*. (43), as compared to the CEO vaccinated group of chickens the TCO vaccinated group presented a more limited reduction of both clinical signs and challenge virus replication.

Inactivated Vaccines

Experimental inactivated ILTV vaccines have been produced from whole virus propagated in chicken embryos (6,37). These vaccines have demonstrated to induce varying degrees of serological responses and protection after challenge. Barhoom *et al*. (6) showed that experimental vaccination of chickens with inactivated ILT vaccine elicited satisfactory serological response and protection to challenge both under laboratory and field conditions. Conversely, Fahey *et al*. (37) revealed that one intramuscular injection with an experimental inactivated ILT vaccine induced low serological responses, with no significant protection to ILT. Additionally, Fahey *et al*. (37) demonstrated that a second injection of inactivated vaccine only marginally increased the titre of humoral antibody, but seemed to reduce the degree of respiratory distress. However, the levels of protection afforded by the inactivated vaccine were not significant compared with a live commercial ILT vaccine.

Recently, in a long-term commercial layer study by Palomino-Tapia *et al*. (90) the protection efficacy of a non-commercial oil-based inactivated vaccine administered either by itself or in combination with recombinant and TCO vaccines was evaluated after challenge at 35 and 74 weeks of age (WOA). Results at 35 WOA showed that the inactivated vaccine by itself did not limit clinical signs of the disease and challenge virus replication. Additionally, when administered in combination with recombinant vaccines (rFPV-LT and rHVT-LT) or the TCO vaccine, the inactivated vaccine did not enhance the base protection conferred by the recombinant vaccines or the TCO vaccine when administered by themselves. Similarly, at 74 weeks of age, inactivated vaccine did not confer protection against challenge when administered by itself. However, improvement in reduction of challenge virus replication but not in clinical signs was observed when the inactivated vaccine was administered in rHVT-LT and TCO vaccinated chickens.

Recombinant Vaccines

Recombinant vaccines for poultry were first reported in 1982. Herpesvirus of turkeys (HVT), Newcastle disease virus (NDV), adenovirus (Ad), and fowlpox virus (FPV) are some of the vectors used for the development of poultry recombinant vaccines (68). The advantage of using viral vectors with large DNA genomes (e.g. FPV, HVT, adenovirus) is that it allows the insertion of larger genome fragments and consequently multiple genes. On the other hand, RNA genomes (e.g. NDV) have a more limited capacity for foreign genetic material (2).

For ILTV there are two types of viral vector vaccines commercially available: the fowl-pox virus (FPV) and serotype 3 Marek's disease virus (MDV), also known as herpesvirus of turkeys (HVT). Additionally, new candidate vectors are experimentally being studied: La Sota strain (NDV) and serotype 1 Marek's disease virus (MDV). The hallmark of this type of vaccines is their lack of transmission and reversion to virulence, and the opportunity for mass application at the hatchery between 18 to 19 days of embryonation (via *in ovo*) or via subcutaneous injection at 1-day of age (42).

Recombinant HVT-LT (rHVT-LT)

HVT is the most frequently used vector vaccine in poultry, and is characterized by its ability to establish persistent infection in the host (35). It has been described that HVT establishes a persistent viremia in chickens for at least 8 or even 30 weeks following vaccination (107), offering the advantage of delivering foreign antigens in vaccinated birds during an extended period of time (117). Therefore, rHVT vaccines are expected to induce a long-lasting immunity. Furthermore, because HVT replicates in a highly cell-associated manner in lymphocytes, it is suggested that this viral attribute would induce a high degree of cell-mediated immune response and prevent interference with maternal derived antibodies (61).

Currently, there are three commercially available recombinant (r) HVT-vectored vaccines against ILTV. One rHVT-LT vaccine carries ILTV glycoproteins D and I (gD and gI) genes (Innovax-ILT, Merck Animal Health, Madison, NJ) (70), the second rHVT-LT vaccine carries the ILTV gB gene (Vectormune HVT-LT, Ceva Animal Health, Lenexa, KS) (36), and most recently a double rHVT-ND-LT (Innovax-ND-ILT, Merck Animal Health, Madison, NJ) vaccine carrying the NDV fusion (F) gene and the ILTV gD and gI genes is available (48). The ILTV glycoproteins expressed by the vectors are important immunogenic antigens that can elicit both humoral and cell-mediated immunity (130). The gD is a highly conserved herpesvirus structural glycoprotein and functions as a viral ligand for cell entry receptors to mediate fusion (41). The gI is important in cell-to-cell spread of the virus (28) and the gB is involved in fusion and entry into the host cell (19). Studies have demonstrated that both rHVT-LT and rHVT-ND-LT vaccines are genetically stable and safe for use in chickens (36,48,121). Additionally, rHVT-LT does not appear to spread laterally from vaccinated chickens to non-vaccinated chickens (36). Available studies indicate that both rHVT-LT and rHVT-ND-LT vaccines competently diminish clinical signs of the disease (48,70,121); however, rHVT-LT does not efficiently limit the replication of the challenge virus in the upper respiratory tract compared to the CEO vaccines (70,121). Although it has not been proven it is suspected that induction of limited local respiratory immune responses maybe the reason why rHVT-LT vaccines are not as effective as CEO vaccines in limiting viral replication in the trachea after challenge (50,70). It has been shown that after *in ovo* and subcutaneous vaccination with rHVT-LT vaccine, the expression of ILTV and HVT genes in spleens and feather follicles was robust, whereas gene expression in the lungs was limited. The authors concluded that reduced expression of ILTV and HVT genes in the lung might result in a weak mucosal immune response in the respiratory tract against ILTV (50). Furthermore, there is evidence to indicate that

the development of protective immunity after *in ovo* vaccination with rHVT-LT is age dependent and requires at least 4 - 6 weeks to fully develop (36,121). Experimentally, it has been shown that chickens vaccinated via *in ovo* with rHVT-LT were better protected when challenged at 57 days of age (DOA) rather than when challenged at 35 DOA (121).

Recombinant FPV-LT (rFPV-LT)

The first experimental recombinant fowlpox virus (rFPV) expressing glycoprotein B (gB) of ILTV (rFPV-gB) was developed in China. Evaluation of this recombinant fowlpox vaccine in SPF and commercial chickens reduced clinical signs of the disease but did not prevent replication of the challenge virus (116). Similar results were found with a recombinant fowlpox virus (rFPV) co-expressing the NDV fusion (F) and hemagglutinin-neuraminidase (HN) genes and ILTV gB genes. After challenge with ILTV, both the rFPV-gB and rFPV-gB-F-HN protected against mortality and 70% of the chickens were protected from respiratory signs (112). Additionally, the immunogenicity of two experimental ILTV recombinant fowlpox viruses (rFPV-gB and rFPV-gB/IL18) containing ILTV gB and chicken interleukin-18 (IL-18) were examined in a challenge model. After challenge the $CD4^+/CD8^+$ ratio and the level of proliferative response of the T cells were higher in chickens immunized with rFPV- gB/IL18 than chickens vaccinated with the rFPV-gB vaccine. In the former study it was concluded that the protective efficacy of the rFPV-gB vaccine could be enhanced by simultaneous expression of chicken IL-18 (16).

Currently, a recombinant FPV-vectored vaccine expressing genes from ILTV is commercially available (Vectormune FP-LT, Ceva Animal Health, Lenexa, KS). This vaccine expresses the ILTV glycoprotein B (gB) and UL-32 genes and is registered for its use via wing-web at 8 weeks of age or older. In the field, problems associated with inadequate protection have been observed due to the improper administration of the vaccine or previous pox viral exposure

(27). rFPV-LT vaccine has also been widely used via *in ovo*; however, bronchopneumonia and neurological signs in chickens between 3 to 10 days of age have been reported. After a retrospective analysis, the authors concluded that the affected chickens were *in ovo* vaccinated between 17.5 to 18.0 days of embryonation, whereas chickens vaccinated between 18.5 or 19.0 days of embryonation experienced very limited clinical signs (126).

Experimental studies using the commercial rFPV-LT vaccine delivered *in ovo* have showed partial protection against clinical signs, and compared to CEO vaccinated chickens a very limited capacity to reduce the replication of the challenge virus in the trachea (70,121). Furthermore, protection comparisons between rFPV-LT and rHVT-LT vaccinated chickens showed that rFPV-LT vaccinated groups had significantly higher clinical signs and challenge virus genome load levels in trachea than rHVT-LT vaccinated chickens (121).

Other recombinant viral vector vaccines for ILT (Experimental studies)

Recombinant NDV-LT (rNDV-LT)

The use of respiratory viruses as vectors for the development of ILTV recombinant vaccines should facilitate mass application and should enhance protection through the production of a mucosal respiratory tissue local immune response (2). Within respiratory viruses, LaSota strain of Newcastle disease virus (NDV) is a naturally occurring low-virulence strain, which is already utilized world-wide as an effective live vaccine. This vaccine strain induces strong local and systemic immune responses against NDV and has been proven to be safe and stable (51). Therefore, the use of a recombinant NDV-LT would be an interesting approach to gain protection and flexibility in the vaccination programs. Additionally, the use of NDV and ILT as a recombinant vaccine overcome the disadvantage of using together live vaccines against these two diseases due to the vaccine interference problems (119).

Using a reverse genetics approach, three experimental recombinant Newcastle disease viruses (rNDVs) designated rNDV-gB, rNDV-gC, and rNDV-gD were generated, each expressing ILTV glycoprotein (g)B, gC, and gD genes, respectively. Each immunized chicken received the vaccines at 2 weeks of age via the oculonasal route with a dose of $10^{5.2}$ $TCID_{50}$/200ul and protection was measured in term of clinical signs and challenge virus replication. Immunization with rNDV-gD induced higher protection and levels of neutralizing antibodies than the rest of the experimental recombinant vaccines. The superior protective efficacy of rNDV-gD vaccine compared to rNDV-gB or rNDV-gC vaccine was possibly attributed to the higher levels of envelope incorporation and infected cell surface expression of gD than gB or gC. Additionally, the protection efficacy conferred by the experimental rNDV-gD was comparable to the protection elicited by the commercial rHVT-LT (gB) and CEO vaccine (75).

Similarly, distinct Newcastle disease virus (NDV) recombinant, also based in the LaSota (LS) vector, expressing gB or gD of ILTV was developed. Birds vaccinated via the oculonasal route at 1 day of age, with either the rLS/ILTV-gB or rLS/ ILTV-gD (10^6 $TCID_{50}$/100ul) virus and challenged (Strain 63140) at either 21- or 28-days post vaccination displayed few or very mild clinical signs. However, the rLS/ILTV-gB construct was more effective than the rLS/ILTV-gD in decreasing the amount of challenge virus shed in the conjunctiva and the trachea of SPF chickens. Due to the viral shedding reduction patterns, only the rLS/ILTV-gB vaccine candidate was examined in subsequent protection studies using maternal antibody-negative 3-week-old commercial broiler chickens, where the rLS/ILTV-gB vaccine provided protection against clinical disease similar to the live attenuated commercial vaccines. However, reduction of viral shedding was inferior than that achieved by the live attenuated vaccines (136). Later, protection elicited by the rLS/ILTV-gB and rLS/ILTV-gD vaccine candidates when administered once or twice at 1 or

10 days of age (DOA) in the presence of NDV and ILTV maternally derived antibodies (MDA) was assessed. In the presence of MDA both candidate vaccines conferred protection against clinical signs and significantly reduced ILTV challenge virus replication; however, the protection elicited by the rLS/ILTV-gD was superior to that of rLS/ILTV-gB. Furthermore, booster vaccination at 10 DOA with rLS/ILTV-gD and rLS/ILTV-gB did not improve protection efficacy of the prime vaccination at 1 day of age. Overall this study indicated that the presence of maternal antibodies did not interfere with the ability of rLS/ILTV gB and gD vaccines to elicit protective immunity against infectious laryngotracheitis (134).

Recombinant Serotype 1 MDV-LT

MEQ-deleted BAC clone of MDV (BACΔMEQ) was shown to be a superior vaccine compared to the attenuated serotype 1 CVI988 vaccine in the control of Marek's disease (109). This strain was used as the basis for the new experimental MDV-LTV vector vaccine. In this work, two recombinant vaccines carrying either the LT virus (LTV) gene gB (gB; BACΔMEQ-gB) or LTV gene gJ (gJ; BACΔMEQ-gJ) were developed. Results demonstrated that both bivalent vaccines (BACΔMEQ-gB and BACΔMEQ-gJ) replicated well in chickens and were safe for commercial meat-type chickens bearing maternal antibodies against MDV. BACΔMEQ-gB protected as well as a commercial rHVT-LT vaccine against an ILT challenge. However, BACΔMEQ-gJ did not show adequate protection against the ILT challenge, nor increased protection when administered in combination with the BACΔMEQ-gB strain (49).

Additionally, the development of recombinant vaccines based on Serotype 1 MDV are able to confer a competitive advantage in term of applicability and protection against MDVs. First, it is possible to co-administer a recombinant (r) Serotype 1 (CVI988) and rHVT-LT together without

a deleterious interaction (68). Moreover, serotype 1 MDV virus is much more efficient in term of protection than HVT against highly virulent MDVs (128).

Recombinant ILT-NDV (rILT-NDV)

A naturally avirulent ILTV strain (LHLJ/120305) served as the vector for the construction of an experimental recombinant ILTV virus where the US9 ILTV gene was deleted and substituted by the fusion protein (F) gene of NDV genotype VII (ILTV-ΔUS9-F). A single vaccination via eye drop, with the ILTV-ΔUS9-F at a dose of 10^4 PFU provided complete protection against an ILTV challenge (WG Strain), where no chickens died or showed any clinical signs during the experiment. In addition, no viral shedding was detected in oropharyngeal swabs from 4 to 20 days post challenge (106).

ILTV Gene Deleted Vaccines

In an effort to produce more stable viruses that can maintain attenuation, instead of vaccines attenuated by passages in cells or embryos, new ILTV recombinant viruses attenuated by deletion or alterations of genes involve with virulence (gG, gJ, TK, UL0, UL47 or ORF C) have been investigated (29,30,43,59,83,123). In particular, the glycoprotein G gene deleted strain (ΔgG) (30) and the open reading frame (ORF) C gene deleted strain (ΔORFC) (43) emerged as vaccine candidates due to their degree of attenuation and ability to grow in cell culture. The ILTV glycoprotein G was identified as a virulence factor which functions as a viral chemokine binding protein that modulates the host's adaptive immune response. Once gG is secreted from the ILTV infected cells it reduces the migration of heterophils to the site of infection, consequently it hampers the T-cell responses, and skew the adaptive immune response to favor the humoral arm which results in increased antibody production. Therefore, the deletion of gG appears to result in

a shift in the immune response from a humoral (non-protective) to a cell-mediated (protective) response (33).

ΔgG virus strains of ILTV have been studied *in vivo*, and vaccination via eye-drop and drinking water have been validated (21,30,31). When delivered by eye drop and drinking water, this deletion mutant has displayed levels of safety and efficacy comparable with those of other commercially live attenuated ILT vaccines (21,31). Although horizontal transmission of the ΔgG strain from vaccinated to naive birds was evident, the spread of virus did not show increased virulence (32). Adequate safety and efficacy were also reported when the ΔgG strain was administered at 3 doses (10^2, 10^3, 10^4 pfu) via *in ovo* at 18 days of incubation; however, a limited number of embryos were evaluated (83). Consequently, further assessment of *in ovo* vaccination with the ΔgG strain is warranted.

An ILTV recombinant virus with deletion of the open reading frame C (ΔORFC) was also constructed and evaluated as a potential ILTV live attenuated vaccine (43). Previous studies demonstrated that deletion of individual genes within a cluster of five ORFs unique to iltoviruses (ORFs A through E) located at the 5' end of the unique long (UL) region of the genome indicated that deletion of individual ORFs (A, B, C, D and E) did not affect viral replication in cell culture (122). Among the five ORFs that are unessential for virus replication *in vitro*, the gene encoding ORF C is of particular interest since complete genome sequence analysis of the TCO vaccine strain revealed that it contained a nonsense mutation near the 3' end of ORF C (44). Therefore, the former information suggests that the ORF C protein might be associated with viral virulence. In order to be developed, the ΔORF C was constructed from the virulent USDA reference strain favored to be a well-characterized virulent ILTV strain. *In vivo* evaluation showed that the ΔORF C recombinant virus presented limited replication in trachea after eye-drop vaccination and limited horizontal

transmission. Compared to the CEO and TCO vaccines the level of protection induced by the ΔORF C strain when administered via eye-drop was similar to that elicited by TCO vaccination, but lower than that induced by CEO vaccination (43). Later, the safety and efficacy of the ΔORFC was evaluated when delivered *in ovo* to maternal antibody negative (MAb-) and maternal antibody positive (MAb+) embryos. The ΔORF C strain remains unsafe for *in ovo* administration as it caused elevated mortalities (10%) during the first week of age in SPF chickens (MAb-). Additionally, the protective efficacy of the ΔORF C strain in presence of maternal antibodies was weakened as compared to the protection elicited in the absence of maternal antibodies. The authors concluded that the reduced protection observed in MAb+ chickens most likely resulted from the interference of maternally derived antibodies with the ΔORF C strain (104).

Commercial Vaccination Strategies in the US

Vaccination practices for ILTV differ according to the type of production, presence of outbreaks of the disease, and the market necessities.

Vaccination in Broilers

Most vaccination regimes for light-weight broilers apply rHVT-LT vaccines to avoid post-vaccinations reactions caused by CEO that result in performance penalties. Heavy-weight broilers are more regularly vaccinated with CEO vaccine or a combination of rHVT-LT and CEO vaccines. Vaccination with CEO in broilers constitute the priority choice in the face of outbreaks and it is regularly administered by drinking water between 7 and 12 days of age.

Vaccination in Breeders

Broiler breeders are usually vaccinated via eyedrop with CEO or TCO vaccine either once or in endemic areas, they may be vaccinated twice between 4 to 5 weeks of age and again between 10 to 12 weeks of age via drinking water or eye drop.

Vaccination in Layers

Most commercial layer flocks in the United States are initially immunized with a rHVT-LT vaccine at 1 DOA, via subcutaneously followed by eye-drop vaccination with CEO or TCO, or CEO administered in the drinking water between 8 and 12 weeks of age.

BIOSECURITY

ILTV could initiate an outbreak in a number of ways. Firstly, ILTV could persist in chicken barn environment for months under favorable temperature conditions. Secondly, since ILTV infection in birds is a lifelong infection, it is suggested that the backyard flocks provide a constant source of ILTV (5).

Strict adherence to hygiene and biosecurity are capable to minimize the spread of the virus. The importance of site quarantine in preventing the movement of potentially contaminated personnel, feed, equipment, and birds is central to successful prevention and control of LT. Measures to control dogs, cats and rodents also should be in place (44). In addition, inactivation of the virus outside of the host is easily attained using heat or disinfectants (5). It is recommended that all potentially contaminated carcasses, feathers, feed, water and litter should be kept within the poultry house, and the house heated to 38 °C for 100 hours. Buildings and equipment should be washed and then sprayed with disinfectants such as phenolics, sodium hypochlorite, iodophors, or a quaternary ammonium compound (44).

Additionally, procedures such as the installation of air scrubbers, changes in house ventilation rates, and ionization systems shown to reduce dust concentrations, could reduce or eliminate infectious particles from getting in or out of farms (92).

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CHAPTER 3

PROTECTION EFFICACY OF A RECOMBINANT HERPESVIRUS OF TURKEYS (HVT) VACCINE AGAINST INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) ADMINISTERED *IN OVO* TO BROILERS AT THREE STANDARDIZED DOSES

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ABSTRACT

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens that produces significant economic losses to the poultry industry. The disease is caused by Gallid alpha herpesvirus-1 (GaHV-1), commonly known as the infectious laryngotracheitis virus (ILTV). Vaccination remains necessary for the control of the disease. Due to the inherent virulence of live attenuated vaccines, in particular that of the chicken embryo origin (CEO) vaccines, the use of ILT viral vector recombinant vaccines has significantly expanded worldwide as a safer vaccination strategy. However, the protective efficacy of recombinant ILT vaccines can be compromised by the use of fractional doses and improper handling and administration of the vaccine. The objective of this study was twofold: 1) to evaluate the protective efficacy induced by a commercial recombinant HVT-LT (rHVT-LT) vaccine when administered *in ovo* to broilers at three standardized doses (6000 plaque-forming units [PFU], 3000 PFU, and 1000 PFU), and 2) to assess the potential of rHVT-LT–vaccinated chickens to spread virus to contact chickens after challenge. Independently of the vaccine dose, vaccinated chickens showed significant reduction in clinical signs, maintained body weight gain after challenge, and diminished the challenge virus replication in the trachea. However, despite reduction of challenge virus replication, challenge virus was transmitted from rHVT-LT–vaccinated (6000/Ch, 3000/Ch) to contact-naive chickens. This study is the first to demonstrate that rHVT-LT vaccination did not prevent spread of challenge virus to contact birds.

Key words: rHVT-LT, vaccination, in ovo, plaque forming units, infectious laryngotracheitis

Abbreviations: CEF = chicken embryo fibroblast; CEO = chicken embryo origin; Ch = challenged; CK = chicken kidney cells; dpch = days post challenge; g = glycoprotein; ILTV = infectious laryngotracheitis virus; MD = Marek's disease; NVx = nonvaccinated; NVx/Ch = nonvaccinated challenged; NVx/NCh = nonvaccinated nonchallenged; PFU = plaque forming units; qPCR = quantitative PCR; rFPV = recombinant fowlpox virus; rFPV-LT = recombinant fowlpox virus-laryngotracheitis; rHVT = recombinant herpesvirus of turkeys; rHVT-LT = recombinant herpesvirus of turkey-laryngotracheitis; RT-PCR = real-time PCR; $TCID_{50}$ = tissue culture infective dose; TCO = tissue culture origin.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a viral respiratory disease of chickens that results in severe production losses for the poultry industry. Severe forms of the disease are characterized by gasping, expectoration of bloody mucus, and high to moderate mortality due to asphyxia (11). The disease is caused by Gallid alpha herpesvirus 1 (GaHV-1), a member of the genus *Iltovirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, commonly recognized as infectious laryngotracheitis virus (ILTV) (2). Control of the disease is built on biosecurity and vaccination. Modified live attenuated vaccines, produced in chicken embryos (chicken embryo origin, CEO) or tissue culture (tissue culture origin, TCO), have been used for years to control ILT. Nonetheless, live vaccine viruses are capable of spreading (1,5,21) and it has been demonstrated that their residual virulence, particularly of the CEO vaccines, increases after bird-to-bird passages (10). The ability of modified live attenuated vaccines to establish latent infections and to sporadically reactivate, leading to renewed virus shedding, is also a matter of concern in the control of the disease (13). Recent epizootics of the disease in the United States and Australia have been related

to virulent CEO-derived strains that became the source of long-lasting outbreaks (18). As a response to the frequent ILT epizootics related to CEO vaccines, a new generation of recombinant vaccines using fowlpox virus (FPV) and herpesvirus of turkey (HVT) as vectors were developed. The recombinant fowlpox virus-laryngotracheitis (rFPV-LT) vaccine expresses the ILTV glycoprotein B (gB) and the UL-32 genes; however, there are two rHVT-LT vaccines, one expresses the ILTV glycoprotein (g)I and gD genes (7) and the second one expresses the ILTV gB gene (3). The advantages of these vaccines are their lack of transmission from bird to bird, the absence of ILTV latent infections, and the inability to revert to virulence, thus offering a safer vaccination alternative against ILT (3).

The rHVT-LT is characterized by the ability of the HVT vector to establish persistent viremia in chickens (25) and, because HVT replicates in lymphocytes in a highly cell-associated manner, it is suggested that these vaccines would induce a long-lasting cell mediated immune response (12). Comparison of recombinant ILTV vaccines efficacy demonstrated that chickens vaccinated with rHVT-LT presented lower clinical signs and challenge virus load in the trachea compared to chickens vaccinated with the rFPV-LT vaccine (23).

Although ILTV recombinant vaccines present a safer vaccination strategy and mitigate the signs of the disease, studies have shown that vaccination with recombinant ILTV vaccines did not limit challenge virus replication in the trachea (15,23). It has been suggested that in endemic areas, recombinant vaccinated flocks, although apparently healthy, may be recurrently shedding virus (23), but the former has not been proved under experimental conditions. The protection efficacy of ILTV recombinant vaccines can also be significantly affected by the use of fractional doses and improper handling and administration of these vaccines. Previous studies have demonstrated the negative effect of diluting recombinant HVT vaccines in the protection elicited against Marek's

disease (MD) (6); nonetheless, the protection efficacy against ILT when administering fractionated doses has not been evaluated. The objective of this study was two-fold: 1) to evaluate the protection efficacy induced by commercial rHVT-LT vaccine (carrying the ILTV glycoprotein I and D genes) when administered in ovo to broilers at three standardized doses (6000, 3000, and 1000 plaque-forming units [PFU]), and 2) to assess the potential of rHVT-LT–vaccinated chickens to spread virus to contact chickens after challenge. The protection induced by the rHVT-LT vaccine was assessed by the ability of vaccinated chickens to prevent clinical signs of the disease, to lessen challenge virus replication in the trachea, and to avoid body weight loss after challenge. Challenge virus transmission from vaccinated (Vx) chickens was evaluated by the introduction of nonvaccinated (NVx) contact chickens.

MATERIALS AND METHODS

Challenge virus. The virus used for challenge was the virulent ILTV strain 1874C5, which belongs to genotype group VI (19). Challenge virus was titrated in chicken kidney (CK) cells prepared from 3- to 4- wk-old chickens. The median tissue culture infective dose ($TCID_{50}$) titer was calculated by the Reed and Muench (20) method.

Vaccine and vaccine titration. Innovax®-ILT (rHVT-LT; Merck Animal Health, Madison, NJ), which expresses the ILTV gD and gI, was used in this experiment (7). The rHVT-LT vaccine was titrated in a confluent monolayer of secondary chicken embryo fibroblast (CEF) cells seeded in 60-mm plates. Three, 10-fold dilutions of the reconstituted vaccine were made in Ham's F10 medium (Corning Inc, Corning, NY) and 100 µl of each dilution was added to four plate replicates of CEF monolayers. Inoculated monolayers were incubated at 37 C and 5% CO_2 and cultured media was changed and substituted with F10 media (1x) and 2% fetal bovine serum

(FBS) (Atlanta Biologicals Inc, Flowery Branch, GA) after 24 hr postincubation. At 5 days postincubation, plaques were counted under light microscopy. Virus titers were calculated as PFU per dose (100 μl) and diluted to achieve 6000, 3000, and 1000 PFU per dose.

Experimental design. Two hundred and fifty broiler eggs were obtained from a commercial hatchery and incubated in a small-scale hatcher (Natureform, Jacksonville, FL). At 18.5 days of embryonation, eggs were randomly divided in five groups of 30 eggs each and one group of 90 eggs. Within the five groups of 30 eggs, two groups were manually injected *in ovo* with vaccine diluent and identified as NVx, and three groups of 30 eggs were manually vaccinated with the rHVT-LT vaccine at the standardized doses of 6000, 3000, and 1000 PFU per embryo. The group of 90 eggs were hatched separately and served as naive contact chickens at challenge. An additional hundred eggs were injected *in ovo* with 0.1% Coomassie blue dye to evaluate the accuracy of the site of injection and to determine the stage of embryo development at the time of vaccination (24). After hatch, chickens were tagged and distributed in five groups (6000 PFU, 3000 PFU, 1000 PFU, NVx, and Contact). At 21 days of age, 3–10 chickens per group were euthanatized by CO_2 inhalation. Spleen samples were collected to evaluate the presence of HVT DNA. At 25 days of age, 16 chickens per group (6000 PFU, 3000 PFU, 1000 PFU, NVx) were bled and serum samples were collected to evaluate ILTV gI antibody levels. At 28 days of age, groups of Vx (6000, 3000, and 1000 PFU) and NVx chickens were weighed and distributed in five colony houses in groups of 16 chickens per house. Groups were identified as nonvaccinated nonchallenged (NVx/NCh), which served as a negative control; nonvaccinated challenged (NVx/Ch), which served as a positive control; and 6000 PFU/challenged (6000/Ch); 3000 PFU/challenged (3000/Ch); and 1000 PFU/challenged (1000/Ch).

Each colony house had an area to hold birds of approximately 97 ft2 (Poultry Diagnostic Research Center, Athens, GA). Not all the colony houses had the same air ventilation system. Houses that held the 6000/ Ch and 3000/Ch groups of chickens had a front air inlet fan and an exhaust air vent fan. The houses that held the 1000/Ch, NVx/Ch, and NVx/NCh group of chickens had two natural ventilation air inlets and one HEPA filtered air vent with an extractor. The Vx and the NVx/Ch groups of chickens were challenged at 28 days of age. Each chicken received the challenge virus at a dose of $10^{3.8}$ $TCID_{50}$ in a total volume of 200 µl; 50 µl was delivered in each eye and 100 µl was delivered intratracheally. The NVx/NCh group of chickens was mock-inoculated with tissue culture media in a similar fashion. At 3–9 days postchallenge (dpch), clinical signs were scored as previously described by Vagnozzi *et al.* (23) and at 3, 5, 7, and 9 dpch, tracheal swabs were collected to quantify challenge virus genome load by real-time PCR (RT-PCR). At 4 dpch, four chickens from each group were sacrificed and cranial segments of the trachea were collected, placed in 10% buffered neutral formalin, and processed for histopathologic examination. To assess the potential of rHVT-LT–vaccinated chickens to spread challenge virus to contact-naive chickens, groups of four NVx age-paired broilers were introduced into Vx and NVx challenge groups at 0, 4, 8, and 12 dpch. Contact-naive chickens were evaluated for clinical signs of the disease and tracheal swabs were collected every 4 days, from 4 to 20 dpch, to assess transmission of the challenge virus by determining viral genome load in the trachea. During the length of the experiment, chickens were fed a standard diet and provided water ad libitum. Temperature, relative humidity, and air speed in the area within the colony house holding the chickens were measured with a Kestrel 3000 meter (Nielsen-Kellerman, Boothwyn, PA). The room temperature and relative humidity were collected at chicken level and air speed was measured at the air inlets and vents. These parameters were measured twice per day per colony

house, at 7–10 a.m. and 3–5 p.m., from days 14–22 postchallenge. The average ratio of air speed inlet:air speed vent was estimated for each colony house.

This study was performed under the Animal Use Proposal A2016 10-010-R1 approved by the Animal Care and Use Committee (IACUC) in accordance with regulations of the Office of the Vice President for Research at the University of Georgia.

Clinical signs and mortality. Clinical signs were evaluated as previously described by Vagnozzi *et al*., (23). Briefly, signs of dyspnea, conjunctivitis, and lethargy were scored on a scale of 0–3, indicating normal (0), mild (1), moderate (2), and severe (3). A score of 6 was recorded for each mortality. Total clinical sign per chicken and the average score per time point per group of chickens were calculated. Clinical signs were evaluated at 3–9 dpch in Vx/Ch and NVx/Ch chickens and at 4, 8, 12, 16, and 20 dpch in contact-naive chickens.

Body weight gain. The increase of weight in grams for each chicken before challenge at 28 days of age (initial weight) and 7 dpch (final weight) was calculated using the following formula: Body weight gain = final weight (FW) – initial weight (IW) and the average weight gain during challenge for each group of chickens (6000/Ch, 3000/Ch, 1000/Ch, NVx/Ch, NVx/NCh) was estimated.

DNA extraction. Spleen samples were placed in 2-ml lysing bead matrix tubes containing 1.4-mm ceramic spheres, and tracheal swabs were placed in 2-ml microcentrifuge tubes containing 1 ml of phosphate-buffered saline (PBS) with 2% antibiotic-antimycotic (Invitrogen, Waltham, MA) and 2% newborn calf serum (Gibco, Waltham, MA). Spleen samples were homogenized in the FastPrep-24™ 5G instrument (MP Biomedicals, Santa Ana, CA) and tracheal swabs were vortexed and stored at –80 C until processing. DNA extraction was performed using the MagaZorb® DNA extraction mini-prep kit (Promega, Madison, WI) following the manufacturer's

recommendations with some modifications. Briefly, 7 µl of proteinase K (PK) solution was loaded into a 96-well plate, 70 µl of sample and 50 µl of lysis buffer were added per well, and the plate was incubated at 56 C for 10 min. After incubation, 10 µl of magnetic beads and 125 µl of binding buffer were added per well, followed by shaking for 10 min at room temperature. The supernatant and magnetic beads were separated with a magnetic stand, supernatant was discarded, beads were washed twice with 250 µl of washing buffer, and DNA was eluted from the beads with 100 µl of elution buffer after 10 min incubation at room temperature with shaking.

Duplex Real Time-PCR. HVT viral genome load was quantified by Real Time (RT)-PCR in a duplex reaction where, viral DNA was amplified with primers that targeted the region of the SORF1 gene, and the host DNA was amplified by primers that target the chicken α2 collagen gene as previously described by Islam *et al*. (14). ILTV viral genome load was also quantified by RT-PCR in a duplex assay (22). Briefly, the RT-PCR assay for ILTV also consisted of a duplex reaction where viral DNA was amplified with primers that targeted the region of the UL44 ILTV gene (glycoprotein C), and chicken DNA was amplified by primers that target the chicken α2 collagen gene. The duplex reaction for ILTV was set up to a final volume of 25 µl as follows: 12.5 µl of 2x master mix (TaqMan® Universal Master Mix II with UNG, Applied Biosystems), 1.25 µl of collagen primers to a final concentration of 0.5 µM, 1.25 µl of ILTV primers to a final concentration of 0.5 µM, 1.25 µl probes to a final concentration of 0.1 µM, and 5 µl of DNA template. In both PCR methods, the thermal cycling profile used was 50 C for 2 min, 95 C for 10 min, 40 cycles of 95 C for 15 sec, and 60 C for 60 sec. The relative amount of HVT or ILTV genomes detected per sample was expressed as the $\log_{10} 2^{-\Delta\Delta Ct}$ (17). The $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of the challenge virus replication was calculated using the following formula: $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of challenge virus replication = ($\log_{10} 2^{-\Delta\Delta Ct}$ NVx/Ch) – ($\log_{10} 2^{-\Delta\Delta Ct}$ vaccinated groups/Ch).

Trachea microscopic lesions. Microscopic lesions in cranial segments of tracheas were scored on a scale of 0–5, as previously described by Guy *et al*. (9). Briefly, normal trachea epithelium received a score of 0; normal epithelium with mild to moderate lymphocytic infiltration and absence of syncytia and intranuclear inclusion bodies received a score of 1; normal epithelium with mild to moderate lymphocytic infiltration and few foci of syncytia with intranuclear inclusion bodies received a score of 2; normal or affected epithelium with moderate to marked hyperemia, moderate to marked lymphocytic infiltration, and numerous syncytia with intranuclear inclusion bodies received a score of 3; absence of normal epithelium and sporadic presence of syncytia with inclusion bodies received a score of 4; total lack of epithelium with rare appearance of syncytia with intranuclear inclusion bodies received a score of 5.

Serology. Serum samples were collected from 16 chickens per group at 25 days of age previous to challenge. Serum samples were analyzed for the detection of antibodies against the ILTV gI using an indirect ELISA kit (IDvet, Grabels, France). The test was performed following the manufacturer's recommendations.

Statistical analysis. Data were analyzed with the statistical program GraphPad Prism 7 (GraphPad Software, La Jolla, CA). The D'Agostino-Pearson test was used for the assessment of normality. Data on clinical signs, body weight gain, HVT and ILTV viral genome load, temperature, relative humidity, and air speed were analyzed by a one-way ANOVA test. In all cases a Tukey test was selected for a post hoc analysis. Tracheal microscopic lesions and antibody titers were compared using the Kruskal-Wallis test and Dunn's test was performed as a multiple comparison procedure.

RESULTS

Monitoring rHVT-LT vaccination by RT-PCR and serology. One hundred percent of the spleens collected from rHVT-LT–vaccinated chickens (6000, 3000, 1000 PFU) at 21 days of age were positive for HVT genomes. As expected, spleens collected from the NVx group of chickens were negative for HVT genomes. No significant differences ($P > 0.05$) in mean HVT viral genome load were found in the spleen of Vx groups of chickens (6000 PFU = 2.23, 3000 PFU = 2.16, 1000 PFU = 2.32; Fig. 3.1a). Fifty percent (6000 PFU), 56% (3000 PFU), and 50% (1000 PFU) of the serum samples collected at 25 days of age from the rHVT-LT–vaccinated groups of chickens were positive (titer > 611) for gI antibodies. No significant differences ($P > 0.05$) in median antibody titers were observed among the Vx groups of chickens (6000 PFU = 657, 3000 PFU = 832.5, 1000 PFU = 590.5) while serum samples collected from the NVx group of chickens were negative for gI antibodies (Fig. 3.1b).

Clinical signs. When compared to the NVx/Ch group of chickens, Vx challenged groups (6000/Ch, 3000/Ch, and 1000/ Ch) showed significant reduction ($P < 0.05$) in clinical signs, but no differences in clinical signs ($P > 0.05$) were found among Vx challenged groups of chickens from days 3 to 9 postchallenge. The mean peak of clinical signs was observed at day 5 post challenge (6000/Ch = 1.31, 3000/Ch = 1.28, 1000/Ch = 1.90, NVx/Ch = 5.19). The most prevalent clinical signs among Vx groups of chickens were dyspnea and lethargy while the NVx/Ch group presented conjunctivitis in addition to dyspnea and lethargy. At the end of the study, a cumulative mortality of 6.3% and 18.8% was observed in the 1000/Ch group and NVx/Ch group, respectively. No mortalities were detected in the 6000/Ch and 3000/Ch groups and no clinical signs were evidenced in the NVx/NCh group of chickens (Fig. 3.2a).

Body weight gained. Mean body weight gained at 7 dpch for vaccinated (6000/Ch, 3000/Ch, 1000/Ch) and nonvaccinated (NVx/NCh, NVx/Ch) groups of chickens are shown in Figure 2b. The NVx/NCh group of chickens gained an average weight of 380.9 g, which is similar ($P > 0.05$) to the body weight gained by Vx groups of chickens (6000/Ch = 303.5 g, 3000/Ch = 312.7 g, 1000/ Ch = 323.8 g) and which is significantly higher ($P < 0.05$) than 157.9 g, the average weight gained by the NVx/Ch group of chickens (Fig. 3.2b).

Challenge virus genome load. At 3 dpch, at the peak of the challenge virus replication in the trachea (Fig. 3.2c), the Vx/Ch groups of chickens showed a significant ($P < 0.05$) reduction in viral genome load compared to the NVx/Ch group of chickens. The viral genome load reduction in the Vx/Ch groups was 3.0 $\log_{10}$ (6000/Ch), 3.7 $\log_{10}$ (3000/Ch), and 3.5 $\log_{10}$ (1000/Ch). No significant differences ($P > 0.05$) in viral genome load were observed among Vx/Ch groups of chickens at 3, 5, and 7 dpch. The NVx/NCh group did not show any levels of viral replication in trachea (Fig. 3.2c).

Microscopic lesions in trachea. No differences ($P > 0.05$) in median microscopic lesion scores were detected among the Vx groups of chickens (6000/Ch, score = 2.0; 3000/Ch, score = 2.0; 1000/Ch, score = 2.5). A trachea microscopic lesion score of two was defined as still having the presence of normal epithelium with mild to moderate lymphocytic infiltration and few foci of syncytia with intranuclear inclusion bodies. Significant differences ($P < 0.05$) in median trachea microscopic lesions were found between the NVx/Ch (score = 4.50) and the NVx/NCh (score = 0.50) groups of chickens; however, no differences ($P > 0.05$) in microscopic lesion scores were detected between the Vx groups of chickens and the NVx/Ch or the NVx/NCh group of chickens (Fig. 3.2d).

Clinical sign scores and challenge virus genome load in contact naive groups of chickens. Replication of the challenge virus in contact-naive chickens was evidenced by detection of a high viral genome load in the trachea of contact chickens. The peak of viral replication for groups of contact-naive chickens introduced at 0 dpch to the 6000 PFU/Ch, 3000/Ch, and the NVx/Ch groups was consistently observed after 8 days postexposure. The peak of viral replication for contact chickens introduced at 4, 8, and 12 dpch to the 6000/Ch and NVx/Ch groups of chickens was consistently observed at 4 days postexposure (Fig. 3.3a,d). For contact chickens introduced to the 3000/Ch group at 4 dpch, the peak of viral replication was delayed to 8 days postexposure but, similar to chickens introduced at 8 and 12 dpch to the 6000/Ch and NVx/Ch groups, contact chickens introduced to the 3000/Ch group showed the peak of viral replication at 4 days postexposure (Fig. 3.3b). Viral replication in contact chickens exposed to the 1000/Ch group was either absent or very limited (Fig. 3.3c). Contact chickens introduced to the 1000/Ch group at 0 and 4 dpch showed a marginal viral genome load in the trachea by days 8 and 12 postexposure, respectively, while no viral genomes were detected in the tracheas of contact chickens introduced at 8 and 12 dpch (Fig. 3.3c).

Contact chickens showed clinical signs of the disease (Fig. 3e–h) after exposure to Vx (6000/Ch, 3000/Ch) and NVx/Ch groups. Overall, clinical signs were more severe in contact-naive chickens than in Vx/Ch chickens. The peak of clinical signs for groups of contact chickens introduced to the 6000/Ch or NVx/Ch groups at 0 dpch appeared 8 days postexposure (Fig. 3.3e,h) while for contact chickens introduced to the 3000/Ch group at 0 dpch, the peak of clinical signs appeared at 12 days postexposure (Fig. 3.3f). Contact chickens introduced to the 6000/Ch, 3000/Ch, and NVx/Ch groups at 4 dpch showed the peak of clinical signs at 8 days postexposure (Fig. 3.3e, 3.3f, 3.3h). For contact chickens introduced at 8 dpch to the 6000/Ch and NVX/Ch

groups, the peak of clinical signs appeared 4 days postexposure (Fig. 3.3e,h) while contact chickens introduced to the 3000/Ch group of chickens the peak of clinical signs was delayed to 8 days postexposure (Fig. 3.3f). Contact chickens introduced at 12 dpch to the 6000/Ch, 3000/Ch, and NVX/Ch groups of chickens showed the peak of clinical signs 8 days postexposure (Fig. 3.3e,f,h). Clinical signs scores observed for contact chickens exposed to the 1000 PFU/Ch group were lower than clinical signs scores observed for contact chickens exposed to the 6000/Ch, 3000/Ch, and NVX/Ch groups at 0, 4, 8, and 12 dpch (Fig. 3.3g).

Temperature, humidity, and air speed. No differences ($P > 0.05$) in temperature and relative humidity were found among colony houses between 14 to 22 dpch; the average temperature detected at the chicken level among all colony houses was an average of 74.5 F and the relative humidity average was 55%. However, significant differences ($P < 0.05$) in air speed ratio were detected among colony houses. In the colony house that held the 6000/Ch group of chickens, the average inlet:vent air speed ratio detected was 1.8:1.0, indicating a higher air speed in the intake than the exhaust. In the colony house that held the 3000/Ch group of chickens, an average inlet:vent air speed ratio of 1.0:1.0 was detected, indicating air speed of the intake and the exhaust were the same. In the colony houses that held the 1000/Ch and NVx/Ch groups of chickens, the average inlet:vent air speed ratio detected was 0.4:1.0, indicating a lower air speed in the intake than in the exhaust.

DISCUSSION

The objective of this study was twofold: 1) to evaluate the protection efficacy induced by a commercial rHVT-LT vaccine when administered *in ovo* to broilers at three standardized doses

(6000, 3000, and 1000 PFU), and 2) to assess the potential of rHVT-LT–vaccinated chickens to spread virus to contact-naive chickens after challenge. In recent years, the use of recombinant ILT vaccines has increased worldwide, as these vaccines provide a safer and practical vaccination alternative in the control of ILT. Previous studies have demonstrated the negative effect that diluting HVT vaccine doses has on MD protection (8), and it has been suggested that diluting recombinant rHVT-LT vaccines could not only compromise protection against MD (6) but also against ILT. In our study the protection efficacy of the rHVT-LT vaccine against an ILTV challenge was evaluated when administered at three standardized PFU doses. The 6000-PFU dose was chosen as a full dose because that is closer to the average titer of rHVT-LT serials released by the manufacturer; the 3000 PFU could be considered as a half dose and 1000 PFU less than a one-quarter dose.

The protection induced by the rHVT-LT vaccine was assessed by the ability of Vx chickens to prevent clinical signs of the disease, to lessen challenge virus replication in the trachea, to avoid body weight loss after challenge, and to halt challenge virus transmission. In previous studies it was shown that vaccination with a full or fractionated (nonstandardized) dose of rHVT-LT decreased clinical signs and maintained body weight gain after challenge (3,23). Similarly, in our study, independent of the vaccine dose (6000, 3000, and 1000 PFU) administered, Vx chickens showed a reduction in clinical signs and maintained body weight gain after challenge. Likewise, a reduction in viral genome load in the trachea ranging from 3.0 to 3.7 $\log_{10}$ was observed in the Vx groups (6000/Ch, 3000/Ch, and 1000/Ch). Therefore, the rHVT-LT vaccine dose neither improved nor diminished protection. Evaluation of rHVT replication in the spleen at 21 days postvaccination showed no differences in HVT genome load among groups of Vx chickens, which suggests that the vaccine replication was equally efficient regardless of the dose administered. Similarly, in a

previous study by Gimeno *et al*. (7), at 1 wk after *in ovo* vaccination of specific-pathogen-free (SPF) chickens, similar HVT genome load were detected in the spleen of rHVT-LT and HVT–Vx chickens, even though the dose of rHVT-LT used was lower. Comparable gI antibody titers were elicited after *in ovo* vaccination with rHVT-LT (6000, 3000, and 1000 PFU) by 25 days of age, which indicated that parallel humoral immune responses were developed independent of vaccine dose administered. It is of value to point out that although the three groups of Vx chickens developed comparable gI antibodies titers, only 50%–56% of the chickens from each vaccinated group had seroconverted by 25 days of age. Although systemic antibody production is not associated with protection against the disease (4), the tardy anti-gI seroconversion may hint of a delay in the onset of immunity. It has been shown that broiler chickens vaccinated *in ovo* with rHVT-LT were better protected when challenged at 57 days of age rather than when challenged at 35 days of age (23).

With respect to protection, it is noticeable that chickens vaccinated with 1000 PFU showed a significant reduction in clinical signs and maintained body weight gain after challenge; however, the use of a 1000 PFU dose (< one-quarter dose) must be carefully examined. In our study, aspects of the *in ovo* vaccination were cautiously controlled; nevertheless, under field conditions there are many factors that can negatively affect the vaccine titers during the preparation and administration of the vaccine; i.e., mixing with antibiotics, lack of proper cold chain for the vaccine, time period between vaccine reconstitution and administration, and/or the inadequate administration of the vaccine (8). Therefore, under field conditions it is not recommended to administer such a low dose.

The ability of ILT vaccination to halt virus replication has been evaluated by virus isolation, assessment of genome viral load, and/or the presence of lesions in the trachea or conjunctiva. Regardless of the method used, it has been documented that vaccination with CEO

vaccines induces a fast decline of challenge virus replication in the trachea; this decline can reach from 5.0 $\log_{10}$ to 6.8 $\log_{10}$ reduction as compared to the NVx/Ch group of chickens (15,16,23). In our study, independent of the vaccine dose, chickens vaccinated with rHVT-LT showed challenge virus genome load reduction ranging from 3.0 to 3.7 $\log_{10}$ during the peak of virus replication. Therefore, we can infer that, independent of the vaccine dose used, reduction of challenge virus replication in rHVT-LT–vaccinated chickens was not as effective as what has been previously shown for CEO-vaccinated chickens. The presence of microscopic lesions, syncytial cells, and intranuclear inclusion bodies in the trachea of vaccinated challenged groups (6000/Ch, 3000/Ch, and 1000/Ch), with no obvious clinical signs of the disease, further confirmed the cytolytic replication of ILTV in rHVT-LT– vaccinated chickens.

It has been speculated that in recombinant vaccinated flocks the virus can circulate in apparently healthy group of birds (23). However, the bird-to-bird transmission of challenge virus from rHVT-LT–vaccinated chickens has not been previously documented. In this study we observed a significant transmission of challenge virus from rHVT-LT–vaccinated chickens to contact-naive chickens, which confirmed that vaccination with rHVT-LT did not reduce the challenge virus replication to sufficient levels to halt virus spread. The challenge virus was effectively transmitted from Vx (6000/Ch and 3000/Ch) and NVx/Ch groups to contact-naive chickens. In comparison to the vaccinated groups of chickens, the contact-naive chickens developed more-severe clinical signs. Even though challenge virus replication was demonstrated for the 1000/Ch vaccinated group of chickens (Fig. 3.3c), minimal virus transmission was observed to contact-naive chickens. Temperature, relative humidity, and air speed were contemplated as potential factors that influenced the lack of virus transmission within the 1000/Ch group of chickens. The temperature and relative humidity of colony houses remained constant throughout

the experiment, including the 1000/ Ch group of chickens. However, differences in ventilation among colony houses were obvious. Ventilation in the colony house that held the 1000/Ch groups of chickens was characterized by a lower air speed intake and a much higher air speed exhaust through a HEPA filter, which may have hindered virus transmission. The use of HEPA filters in the poultry house is not a feasible option because to achieve proper ventilation it requires adequate natural air supply or ventilation systems. However, further studies are warranted to better understand the association between air exchange and viral transmission.

Overall, the present study demonstrates that the rHVT-LT dose did not influence the level of protection against the ILTV challenge. Independently of the dose, protection elicited by the rHVT-LT vaccine was considered incomplete, as the challenge virus replication from vaccinated groups of chickens was not sufficiently halted, stimulating virus spread and provoking disease in contact-naive chickens. Therefore, the rHVT-LT vaccine in its current design is not the optimal tool to control outbreaks of the disease because it is not able to effectively reduce virulent virus circulation in areas of intensified field challenge. This study is the first to demonstrate that rHVT-LT–vaccinated chickens, although apparently healthy after challenge, can still spread virus.

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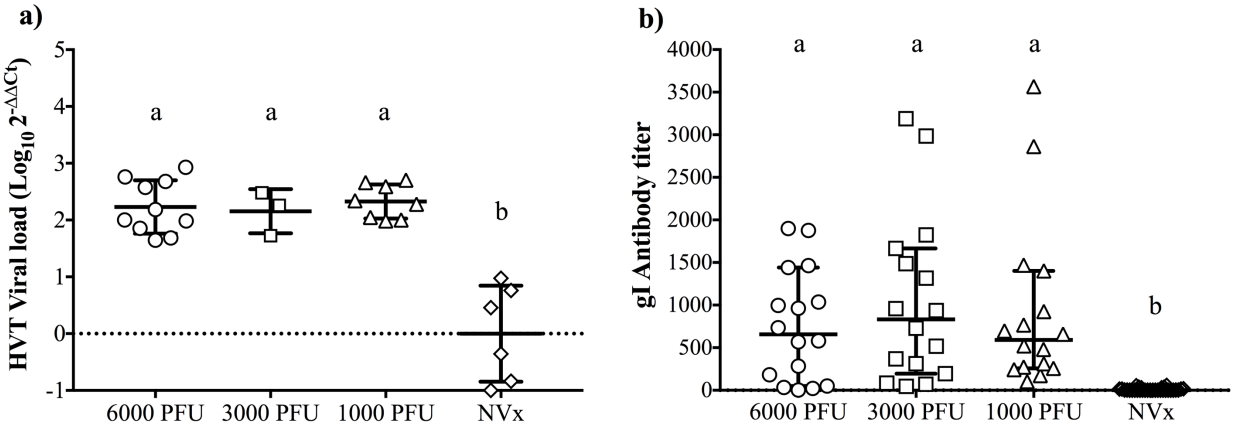

Fig. 3.1. Monitoring rHVT-LT vaccination by RT-PCR and serology. (a) HVT viral genome load ($\log_{10}\ 2^{-\Delta\Delta Ct}$) in spleen at 21 days of age. Individual viral loads are presented by geometric symbols, and horizontal and vertical lines represent the mean and the SD, respectively. (b) gI antibody titers in serum samples at 25 days of age. Individual gI titers are presented by geometric symbols, and horizontal and vertical lines indicate the median and the 95% confidence intervals (CI) for the median, respectively. Different lowercase letters indicate significant differences among groups ($P < 0.05$).

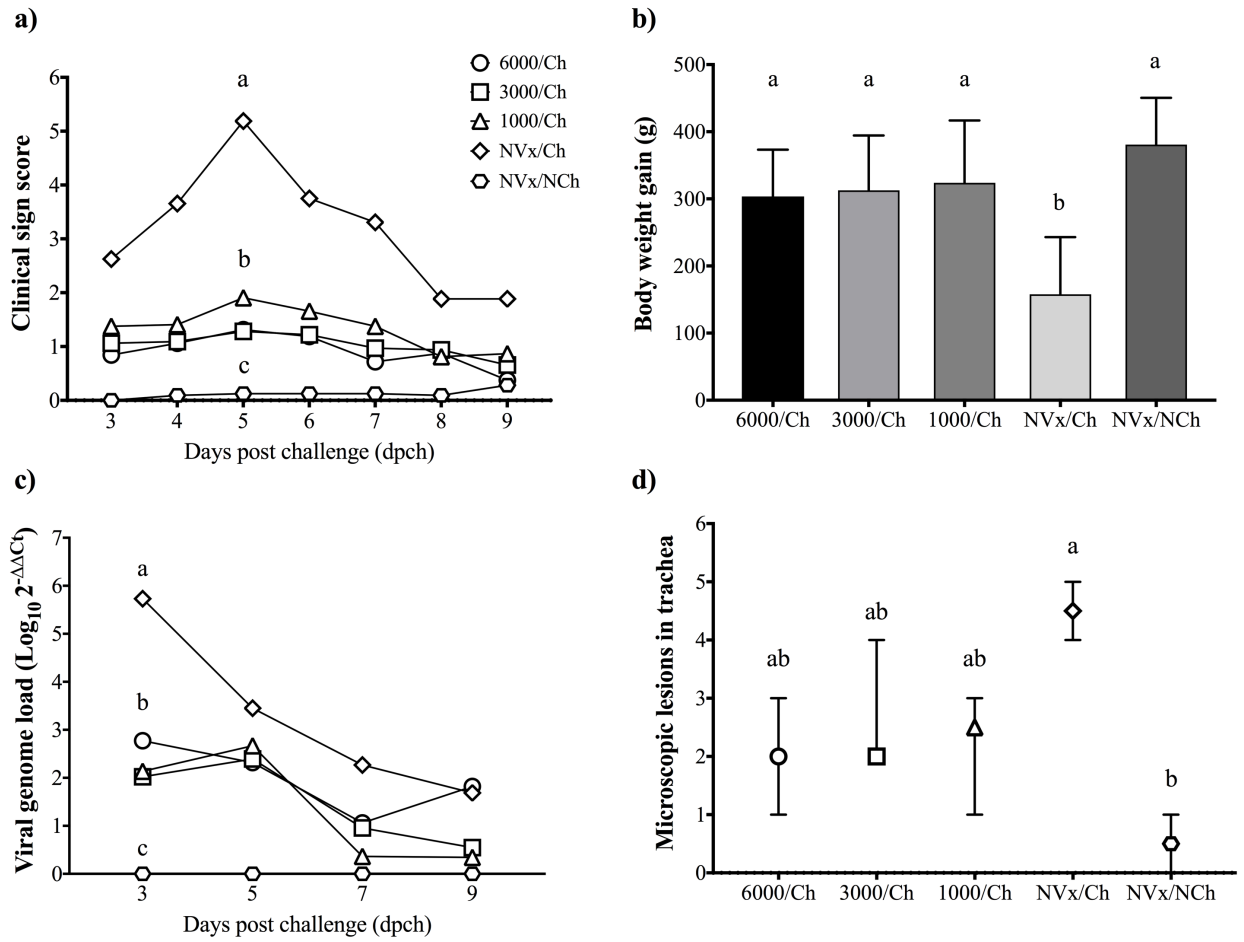

Fig. 3.2. Clinical signs, body weight, challenge virus load, and microscopic lesions in trachea from rHVT-LT–vaccinated and NVx/Ch chickens. (a) Mean clinical signs scores recorded 3–9 dpch are represented by geometric symbols at each time point (n = 16). (b) Column bars represent the mean body weight gained after 7 dpch (35 days of age) and horizontal lines indicate the standard deviation (SD) (n = 16). (c) Mean viral genome load ($\log_{10} 2^{-\Delta\Delta Ct}$) at 3, 5, 7, and 9 dpch are represented by geometric symbols for each group of chickens (n = 16). (d) Median microscopic lesion scores in trachea are represented by geometric symbols with vertical lines indicating the 95% the confidence interval (CI) for the median (n = 4). Different lowercase letters indicate significant differences among groups ($P < 0.05$).

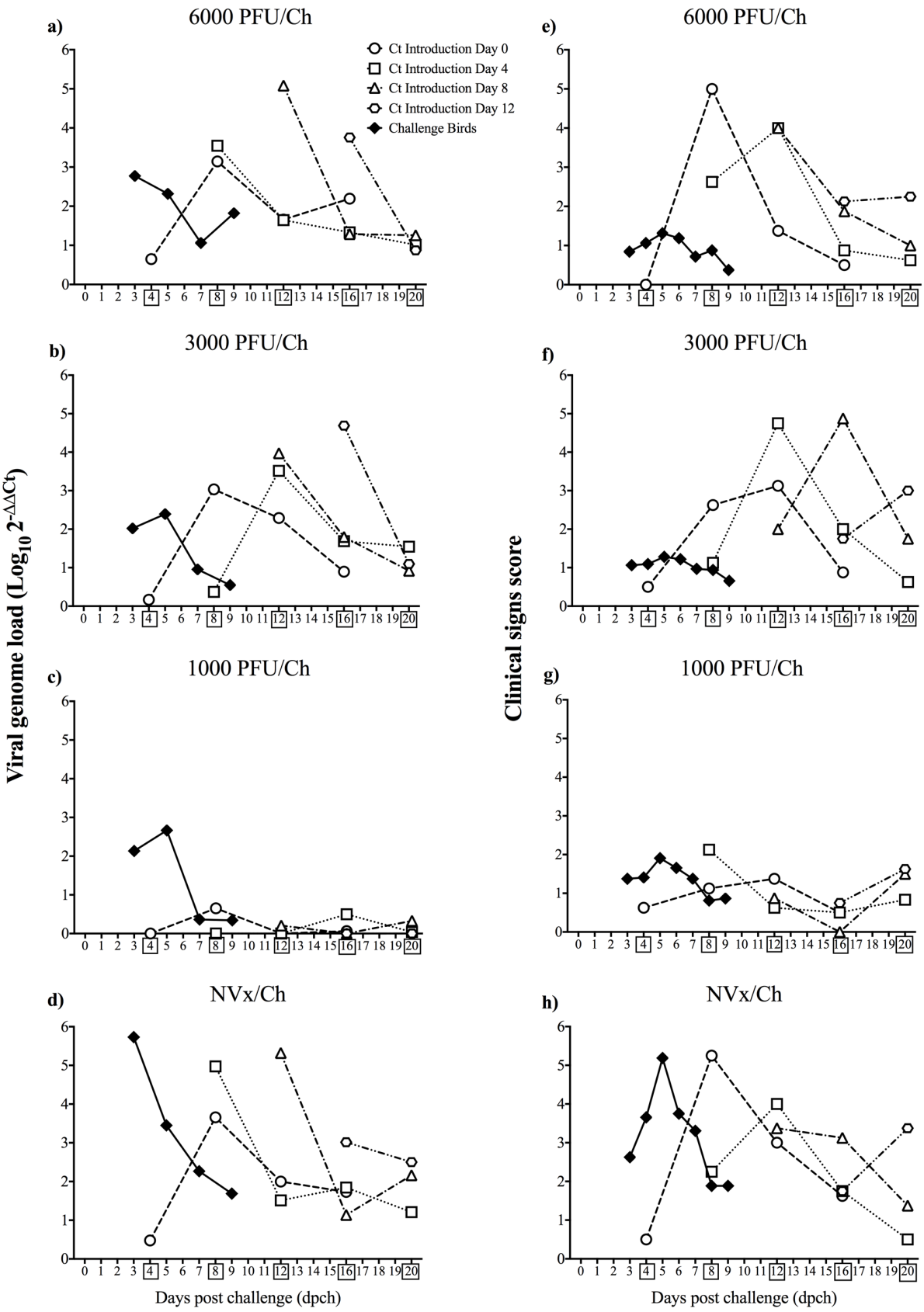
a)
6000 PFU/Ch
Ct Introduction Day 0
Ct Introduction Day 4
Ct Introduction Day 8
Ct Introduction Day 12
Challenge Birds
b)
3000 PFU/Ch
c)
1000 PFU/Ch
d)
NVx/Ch
e)
6000 PFU/Ch
f)
3000 PFU/Ch
g)
1000 PFU/Ch
h)
NVx/Ch
Viral genome load (Log10 2-ΔΔCt)
Clinical signs score
Days post challenge (dpch)
Days post challenge (dpch)

Fig. 3.3. Viral genome load and clinical signs scores in contact-naive chickens after sequential introduction to rHVT-LT–vaccinated and NVx/Ch chickens. Four contact-naive chickens were introduced at 0, 4, 8, and 12 dpch and evaluated for viral genome load and clinical signs at 4, 8, 12, 16, and 20 days postexposure. Mean challenge virus genome load and clinical signs in contact naive chickens are indicated with open geometric symbols connected by dashed lines while mean viral genome load and clinical sign scores for Vx and NVx/Ch groups are represented with solid square symbols connected by solid lines. Numbers inside the square in the X-axis indicate the time points for clinical signs evaluation and viral load sampling in the contact-naive chickens.

CHAPTER 4

REPLICATION AND PROTECTION EFFICACY OF A RECOMBINANT HERPESVIRUS OF TURKEYS (HVT-LT) AGAINST INFECTIOUS LARYNGOTRACHEITIS (ILT) ADMINISTERED *IN OVO* AT A DOUBLE COMMERCIAL DOSE

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ABSTRACT

Infectious laryngotracheitis (ILT) is a highly contagious respiratory disease of chickens that produces significant economic losses to the poultry industry. The main approach to disease control is vaccination with live attenuated and/or viral vector recombinant vaccines. Although the protective efficacy of recombinant ILT vaccines is lesser than that provided by chicken embryo origin (CEO) vaccines, their implementation has significantly expanded as it offers a safer vaccination alternative. Currently, there are two recombinant HVT-LT (rHVT-LT) vaccines, either expressing the ILTV glycoproteins I & D (gI & gD), or the ILTV glycoprotein B (gB) gene. Although it has not been proven, it is believed that increasing the rHVT-LT dose would enhance the vector replication and the insert expression which consequently will positively impact protection against ILT. Therefore, the aim of the study was to evaluate the replication and protection efficacy of a recombinant HVT-LT (rHVT-LT) vaccine when administered *in ovo* at 13000 plaque forming units (PFU), equivalent to a double commercial dose as compared to a full dose of 6500 PFU. At 7 and 14-days post *in ovo* vaccination no differences in HVT replication were observed in feather pulp, spleen, trachea and conjunctiva in vaccinated groups of chickens that received 6500 PFU or 13000 PFU. Independent of the vaccine dose, vaccinated challenged chickens showed significant reduction in clinical signs, maintenance of body weight gain, and reduced challenge virus replication in the trachea. Our results demonstrated that the use of a rHVT-LT vaccine in a double dose did not enhance the protection conferred by a single standard dose against an ILTV challenge, suggesting that the highest protection conferred by the rHVT-LT vaccine is fully achieved at its standard dose.

Key Words: rHVT-LT, vaccination, *in ovo*, PFU, recombinant, replication, protection

Abbreviations: CEO = chicken embryo origin, ILT = Infectious laryngotracheitis, ILTV = Infectious laryngotracheitis virus, PFU = plaque forming units, rHVT = recombinant Herpesvirus of turkeys, rHVT-LT = recombinant Herpesvirus of turkey – laryngotracheitis, $TCID_{50}$ = tissue culture infective dose, TCO = tissue culture origin

INTRODUCTION

Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens characterized by gasping, swollen heads and expectoration of bloody mucus. Infectious laryngotracheitis is distributed worldwide and causes significant economic losses due to mortality and decreased egg production (9). The disease is caused by *Gallid alpha herpesvirus 1* (GaHV-1) a member of the genus *Iltovirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, commonly recognized as Infectious laryngotracheitis virus (ILTV) (6). Control of the disease is built on biosecurity and vaccination. Two types of live attenuated vaccines, a chicken embryo-origin (CEO) and a tissue culture origin (TCO), have been used with relative success for years to control the disease. However, live attenuated vaccines particularly CEO are capable of spreading (5,10,25) and due to their inherent residual virulence after bird- to-bird passages they can regain virulence (13). The ability of live attenuated vaccines to establish latent infections and to sporadically reactivate, leads to shedding of virus, which is a matter of concern for disease control (15). Recent epizootics of the disease in the United States (US) and Australia have been related to CEO-derived strains that regained virulence and became the source of long-lasting outbreaks (20). As a response to the frequent ILT epizootics related to CEO vaccines, recombinant vaccines are being used as a safer vaccination alternative. The advantages of using recombinant vaccines to protect against ILT are their lack of transmission from bird to bird, the absence of ILTV latent infections after vaccination

and the inability to revert to virulence (7). Within the available viral vectors, the Herpesvirus of turkey (HVT) which is classified as the serotype 3 within the Marek's disease virus (MDV) group, has been the most commonly used vector owing to its capacity to limit interference by maternally derived antibodies (31), its safety (7), and its ability to establish persistent infection and prolonged duration of immunity (8,23,34). The virus was originally isolated from healthy turkeys (32) and has been used extensively as a safe and efficacious vaccine against Marek's disease (MD) (11,21). Currently, there are two recombinant HVT-LT (rHVT-LT) vaccines, either expressing the ILTV glycoproteins I & D (gI & gD) (12), or the ILTV glycoprotein B (gB) gene (7). The ILTV glycoproteins constitute important immunogenic antigens that can elicit both humoral and cell-mediated immune responses (33).

Although ILTV recombinant vaccines offer a safer vaccination alternative and reduce the impact of the disease, experimental evidence has demonstrated that rHVT-LT vaccines induce partial protection because challenge virus replication still occurs in the trachea of vaccinated chickens (17,28). Thus, it is imperative to consider alternative approaches to maximize the protection conferred by rHVT-LT vaccine. Although it has not been proven, it is believed that increasing the rHVT-LT dose would enhance the vector replication and the insert expression which consequently will positively impact protection against ILT. Therefore, the aim of the study was to evaluate the protection efficacy of a rHVT-LT vaccine when administered at a double commercial dose of 13000 plaque forming units (PFUs). This study will provide insight on the threshold of protection conferred by rHVT-LT (gI & gD) related to vaccine dose.

MATERIALS AND METHODS

Challenge virus. The ILTV virulent strain 1874C5, isolated from a broiler outbreak and belonging to genotype group VI (22) was propagated and titrated in chicken kidney (CK) cells prepared from 3 to 4 weeks old SPF chickens. The median tissue culture infective dose ($TCID_{50}$) based on cytopathic effect was calculated by the Reed and Muench (24) method.

Vaccine and vaccine titration. Innovax®-ILT (rHVT-LT) (Merck Animal Health, Madison, NJ) is a cell-associated live recombinant Herpesvirus of turkey (HVT, serotype 3), expressing the glycoproteins I and D (gI & gD) of infectious laryngotracheitis virus (12). rHVT-LT vaccine was titrated in confluent monolayer of secondary chicken embryo fibroblasts (CEF) cells seeded in 60 mm plates. Three 10-fold dilutions of the reconstituted vaccine were made in Ham's F10 medium (Corning Inc, Corning, NY) and 100 µl ml of each dilution was added to four plate replicates of CEF monolayers. Inoculated monolayers were incubated at 37 C and 5% CO_2 and cultured media was changed and substituted with F10 media (1x) and 2% fetal bovine serum (FBS) (Atlanta Biologicals Inc, Flowery Branch, GA) after 24 hours post-incubation. Virus growth was evaluated daily and at 5 days post incubation plaques were counted under light microscopy. Virus titers were expressed as plaque forming units (PFU) and diluted to achieve 6500 and 13000 PFU per dose (100 µl).

Experimental design. Two hundred SPF eggs were acquired from a commercial source (Charles River Laboratories Inc., Wilmington, MA) and incubated at 99.5°F and 55% relative humidity (RH) in a small-scale hatcher (Natureform Inc., Jacksonville, FL) at the Poultry Diagnostic Research Center (PDRC). At 19.0 days of embryonation eggs were randomly divided in four groups of 30 eggs each, where two groups were manually *in ovo* vaccinated with the rHVT-LT vaccine at the standardized doses of 6500 and 13000 PFU per embryo and the remaining two

groups were manually injected with vaccine diluent and identified as non-vaccinated (NVx). Additionally, 80 embryos were injected with 0.1% coomassie blue dye to evaluate the accuracy of the site of injection and to determine the stage of embryo development at the time of vaccination (29). Proper vaccine delivery included embryos in which the vaccine was injected into the amniotic sac or into the embryo body. After hatch, the percent of hatchability was estimated and chickens were tagged and distributed in four groups (6500 PFU, 13000 PFU, NVx1, NVx2). At 7 and 14 days post *in ovo* vaccination, five chickens per group at each time point were euthanatized by CO_2 inhalation and spleen, trachea, feather pulp and conjunctiva were collected to evaluate the presence of HVT genomes. At 25 days of age, the two vaccinated and one non-vaccinated group of chickens were weighed and then challenged (6500/Ch, 13000/Ch, NVx/Ch) with the ILTV virulent strain 1874C5 at a dose of $10^{3.8}$ $TCID_{50}$ in a total volume of 200 µl; 50 µl was delivered in each eye and 100 µl was delivered intra-tracheally. On the other hand, the remaining non-vaccinated group was weighted and mock-inoculated (NVx/NCh) with tissue culture media in a similar fashion. At 3 to 7 days post-challenge (dpch) clinical signs were scored, and at 3 and 5 dpch tracheal swabs were collected to quantify challenge virus genome load by real-time PCR. Body weight gain was measured over a 7-day period after challenge. During the length of the experiment, chickens were fed a standard diet and provided water at libitum. All the experiments conducted in this study were performed under the Animal Use Proposal A2016 10-010-R1 approved by the Animal Care and Use Committee (IACUC) in accordance with regulations of the Office of the Vice President for Research at the University of Georgia.

Clinical signs and mortality. Clinical signs of conjunctivitis, dyspnea and lethargy were scored from 3 to 7 days post challenge as previously described by Vagnozzi *et al*., (28). Absence of clinical signs was given a score of 0; mild, a score of 1; moderate, a score of 2; and severe, a

score of 3. Any mortality seen was given a total score of 6. Total clinical sign score was calculated for each chicken and a mean clinical sign score was assigned for each group of chickens at each time point.

Body weight gain. Body weight was measured at 25 days of age (initial weight) and 7 days post challenge (final weight) for each group of chickens (6500/Ch, 13000/Ch, NVx/Ch, NVx/NCh). The body weight gained was calculated using the following formula: Body weight gain = final weight (FW) – initial weight (IW).

DNA extraction. Tissue samples of spleen, feather pulp, conjunctiva and trachea for the detection of HVT genome load were placed in 2 ml lysing bead matrix tubes containing 1.4 mm ceramic spheres and homogenized in the FastPrep-24™ 5G instrument (MP Biomedicals, Santa Ana, CA). Swab samples of trachea for the evaluation of ILTV viral genome load were placed in 2 ml plastic microcentrifuge tubes and vortexed. Tubes contained 1 ml of phosphate-buffered saline (PBS) with 2% antibiotic-antimycotic (Invitrogen, Waltham, MA) and 2% newborn calf serum (Gibco, Waltham, MA). All samples were stored at –80 °C until processing. DNA extraction was performed using the MagaZorb® DNA extraction mini-prep kit (Promega, Madison, WI) following the manufacturer's recommendations with some modifications. Briefly, 7 µl of proteinase K (PK) solution was loaded into a 96-well plate, 70 µl of sample and 50 µl of lysis buffer were added per well, and the plate was incubated at 56 °C for 10 min. After incubation, 10 µl of magnetic beads and 125 µl of binding buffer were added per well followed by shaking for 10 min at room temperature. The supernatant and magnetic beads were separated with a magnetic stand, supernatant was discarded, beads were washed twice with 250 µl of washing buffer, and DNA was eluted from the beads with 100 µl of elution buffer after 10 min incubation at room temperature with shaking.

Duplex real-time PCR. HVT viral genome load was quantified by real-time PCR in a duplex reaction where viral DNA was amplified with primers that targeted the virus genome region of the SORF1 gene, and host DNA was amplified by primers that target the chicken α2 collagen gene as previously described by Islam *et al*., (16). ILTV viral genome load was also quantified by real-time PCR as previously described by Vagnozzi *et al*., (27). Briefly, the real-time PCR assay for ILTV consisted of a duplex reaction where viral DNA was amplified with primers that targeted the region of the UL44 ILTV gene (glycoprotein C), and host DNA was amplified by primers that target the chicken α2 collagen gene. The duplex reaction for ILTV was set up to a final volume of 25 µl as follows: 12.5 µl of 2x master mix (TaqMan® universal master mix II with UNG, Applied Biosystems), 1.25 µl of collagen primers to a final concentration of 0.5 µM, 1.25 µl of ILTV primers to a final concentration of 0.5 µM, 1.25 µl probes to a final concentration of 0.1 µM and 5 µl of DNA template. In both PCR methods, the thermal cycling profile used was 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 seconds, and 60 °C for 60 sec. The relative amount of viral DNA per sample was calculated as the $\log_{10} 2^{-\Delta\Delta Ct}$ (18), where ΔCt is the amount of the ILTV target gene normalized against the housekeeping gene – chicken collagen. The $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of the challenge virus replication was calculated using the following formula: $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of challenge virus replication = ($\log_{10} 2^{-\Delta\Delta Ct}$ NVx/Ch) – ($\log_{10} 2^{-\Delta\Delta Ct}$ vaccinated groups/Ch).

Statistical analysis. D'Agostino-Pearson test was used for the assumption of normality. Comparisons among groups for clinical signs, body weight gain and ILTV genome load were performed by one-way ANOVA test and the Tukey test was selected for a post hoc analysis. Kruskal-Wallis test was used for comparisons of differences in medians for HVT genome load between the two doses and the non-vaccinated group and Dunn's test was utilized as a multiple

comparison procedure. Wilcoxon signed-rank test was applied to compared HVT genome load between 7 and 14 days post vaccination. All data was analyzed with the statistical software GraphPad Prism 8 (GraphPad Software, La Jolla, CA). The level of significance was considered at $P < 0.05$.

RESULTS

Safety of rHVT-LT vaccine. *In ovo* vaccination with a double dose (13000 PFU) did not affect hatchability and body weight gain at 7 days of age. Additionally, no clinical signs or adverse reaction were observed after vaccination with the rHVT-LT double dose (Table 4.1).

Replication of rHVT-LT vaccine. No significant differences ($P > 0.05$) in HVT viral genome load were detected between vaccinated chickens with 6500 and 13000 PFU in feather pulp, spleen and trachea tissues at 7 and 14 days post *in ovo* vaccination (Fig. 4.1a, 4.1b, 4.1c). On the other hand, conjunctiva samples were only taken at 14 days post vaccination, showing no significant differences ($P > 0.05$) in HVT replication to both doses (Fig. 4.1d). Additionally, higher HVT viral genome load ($P < 0.05$) were observed in feather pulp samples (Fig. 1a) as compared to spleen (Fig. 4.1b), trachea (Fig. 4.1c) and conjunctiva (Fig. 4.1d) collected either at 7 or 14 days post vaccination. With regard to the increasing of viral genome load from 7 to 14 days post vaccination, feather pulp was the only tissue to show a significance increase ($P < 0.05$) in replication as age increased. No HVT viral genomes were detected in the NVx group of chickens.

Clinical signs. From 3 to 7 days post challenge, vaccinated challenged groups (6500/Ch and 13000/Ch) showed significant reduction ($P < 0.05$) in clinical signs compared to the NVx/Ch group, whereas no significant difference ($P > 0.05$) in clinical signs were observed between the vaccinated challenged chickens at both doses. The peak of clinical signs was evidenced at 5 days

post challenge (6500/Ch, score = 0.8; 13000/Ch, score = 0.5; NVx/Ch, score = 5.1) (Fig. 4.2a). Predominant clinical signs observed among vaccinated challenged groups of chickens were mild dyspnea while for the NVx/Ch group of chickens moderate to severe dyspnea, lethargy and conjunctivitis was observed. At the end of the study, a cumulative mortality of 47.1% was observed for the NVx/Ch group, whereas no mortalities were recorded for the vaccinated challenged groups of chickens (6500/Ch and 13000/Ch). No clinical signs were seen in the NVx/NCh group.

Body weight gain post challenge. By 7 days post challenge, vaccinated challenged groups (6500/Ch and 13000/Ch) maintained their body weight gain as compared to the NVx/NCh group. Both the 6500/Ch and 13000/Ch groups recorded body weight gain of 97.5 g and 99.8 g, respectively that were significantly higher ($P < 0.05$) than the body weight gained by the NVx/Ch group of chickens (5.7 g) (Fig. 4.2b).

Challenge virus genome load. At 3 to 5 days post challenge, vaccinated challenged groups (6500/Ch and 13000/Ch) showed significantly lower ($P < 0.05$) challenge virus replication in the trachea as compared to the NVx/Ch group, while no significant differences ($P > 0.05$) were detected in challenge virus load between the 6500/Ch and 13000/Ch groups of chickens at either 3 or 5 days post challenge (Fig. 4.2c, 4.2d). At 3 days post challenge, at the peak of challenge virus replication, the viral genome load reduction in the vaccinated challenged groups was 4.6 $\log_{10}$ (6500/Ch) and 4.8 $\log_{10}$ (13000/Ch). As expected challenge virus genomes were not detected in the NVx/NCh group of chickens.

DISCUSSION

A better understanding of the kinetics of the rHVT-LT vaccine replication and protection when administered *in ovo* at higher than the standard dose was required as we aim towards a more

effective way to control ILT. *In vivo* replication has been proposed to be a key factor in the ability of the HVT vaccines to elicit a strong protection against Marek's disease (MD) (30). Although it has not been proven, it is speculated that increasing the rHVT-LT vaccine dose (PFU) can not only enhance the protection against Marek's disease (MD) but also against ILT due to the increase expression of the inserted ILTV glycoproteins. The ILTV glycoproteins inserted in the vector vaccines are important immunogenic antigens that can elicit both humoral and cell-mediated immunity (33).

In our study, no differences ($P > 0.05$) in HVT replication were observed in feather pulp, spleen, trachea and conjunctiva between 6500 PFU and 13000 PFU vaccine dose, which means that HVT replication was equally efficient at both doses and the level of HVT replication was not related to the initial dose administered. Independent of the vaccine dose, HVT viral genome load was higher in feather pulp compared to the other evaluated tissues, which agrees with this being the site of fully productive viral replication (3). Based on the results of our study, feather pulp appears to be the most appropriate sample to monitor rHVT-LT vaccine replication by real-time PCR as it is very reliable and minimally invasive (2). Additionally, feather pulp was the only tissue where a significant increase in viral load was observed from 7 to 14 days post vaccination. In the present study we did not evaluate HVT replication in feather pulp at 21 days post vaccination, however in previous experiments in our laboratory we were not able to find differences in replication between 14 and 21 days post vaccination (manuscript in preparation). On the other hand, the amount of HVT recovered from the spleen did not increase with the age of the bird, following a plateau pattern from 7 to 14 days post vaccination. Likewise, chicken studies performed by Tan *et al*., (26) showed a similar level of HVT replication in spleen from 7 to 21 days post vaccination. HVT replication was also assessed in trachea and conjunctiva where lower

levels of HVT viral genome load were found, which may be explained due to the fact that trachea and conjunctiva are not the target tissue for HVT replication. In addition, we hypothesized that presence of HVT genome load in trachea and conjunctiva post vaccination could be also related to the migration of the vaccine virus through peripheral blood lymphocytes (PBL) after a primary replication in the spleen. HVT is nonpathogenic in chickens, but it does induce a viremia which is correlated with induction of protective immune responses (4).

The protection induced by the rHVT-LT vaccine was assessed by the ability of vaccinated chickens to prevent clinical signs of the disease, to lessen challenge virus replication in the trachea and to avoid body weight loss after challenge. In previous studies it was shown that vaccination with rHVT-LT decreased clinical signs of the disease and the replication of the challenge virus (7,19,28). In our study, independent of the vaccine dose (6500 PFU vs 13000 PFU) we observed a reduction in clinical signs and a reduction of challenge virus replication of 4.6 $\log_{10}$ (6500 PFU) and 4.8 $\log_{10}$ (13000 PFU), indicating that the use of the vaccine at a double dose did not significantly improve the protection of the rHVT-LT vaccine. This result might suggest that the highest performance achieved by rHVT-LT vaccines is fully obtained at its standard dose, constituting the threshold of protection for the vaccine. Based on the aforementioned, the current design of the rHVT-LT vaccine can reduce the incidence of clinical disease but due to the inability to diminish efficiently the challenge virus replication in the trachea, they may not block the circulation of field challenge virus in endemic areas (27). We suspect that the reduced replication of the rHVT-LT vaccine in the trachea as compared to the feather pulp, might result in a limited local immune response in the upper respiratory tract, which consequently will affect the reduction of the challenge virus replication.

One possible strategy to improve the efficacy of rHVT-LT vaccines is to co-administer

immunomodulators that enhance immune responses. Activation of chicken toll-like receptors (TLRs) with synthetic ligands has been investigated in their potential to limit ILTV replication. *In ovo* delivered of TLR21 ligand CpG DNA is capable of eliciting significant cellular responses characterized by innate (KUL01+ cells) and adaptive (IgM+ B cells and CD4+ and CD8α+ cells) immune cells at day 1 post-hatch which may be associated with a reduction of ILTV infection in terms of clinical signs, mortality and viral genome load (1). Similarly, lipoteichoic acid (LTA) treatment of embryonic day 18 (ED18) eggs can lead to antiviral response against pre-hatch ILTV infection *in vivo* and is associated with expansion of macrophage populations and expression of IL-1β and myeloid differentiation primary response gene 88 (MyD88) in the lung via TLR2 activation (14). Another feasible approach to expand the protection of rHVT-LT is to use them jointly with a CEO vaccine as part of a vaccination strategy.

Our results demonstrated that in recombinant HVT-LT vaccines the use of a double dose did not expand the replication and protection conferred by a single standard dose against an ILTV challenge suggesting that the maximum level of protection obtained by the recombinant HVT-LT is fully achieved at its standard dose.

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Table 4.1 Safety of a rHVT-LT vaccine used at a double dose

Group	% Hatchability	Clinical Signs or adverse reaction	Body Weight Gain (g) (95% CI)*
6500 PFU	90% (27/30)	No	39.6[a] (37.6 – 41.5)
13000 PFU	90% (27/30)	No	40.0[a] (37.3 – 42.7)
Non-Vaccinated (NVx)	90% (54/60)	No	41.3[a] (39.5 – 43.1)

Values with different superscript letters indicate significant differences ($P < 0.05$)
* 95% confidence interval (95% CI) of the mean

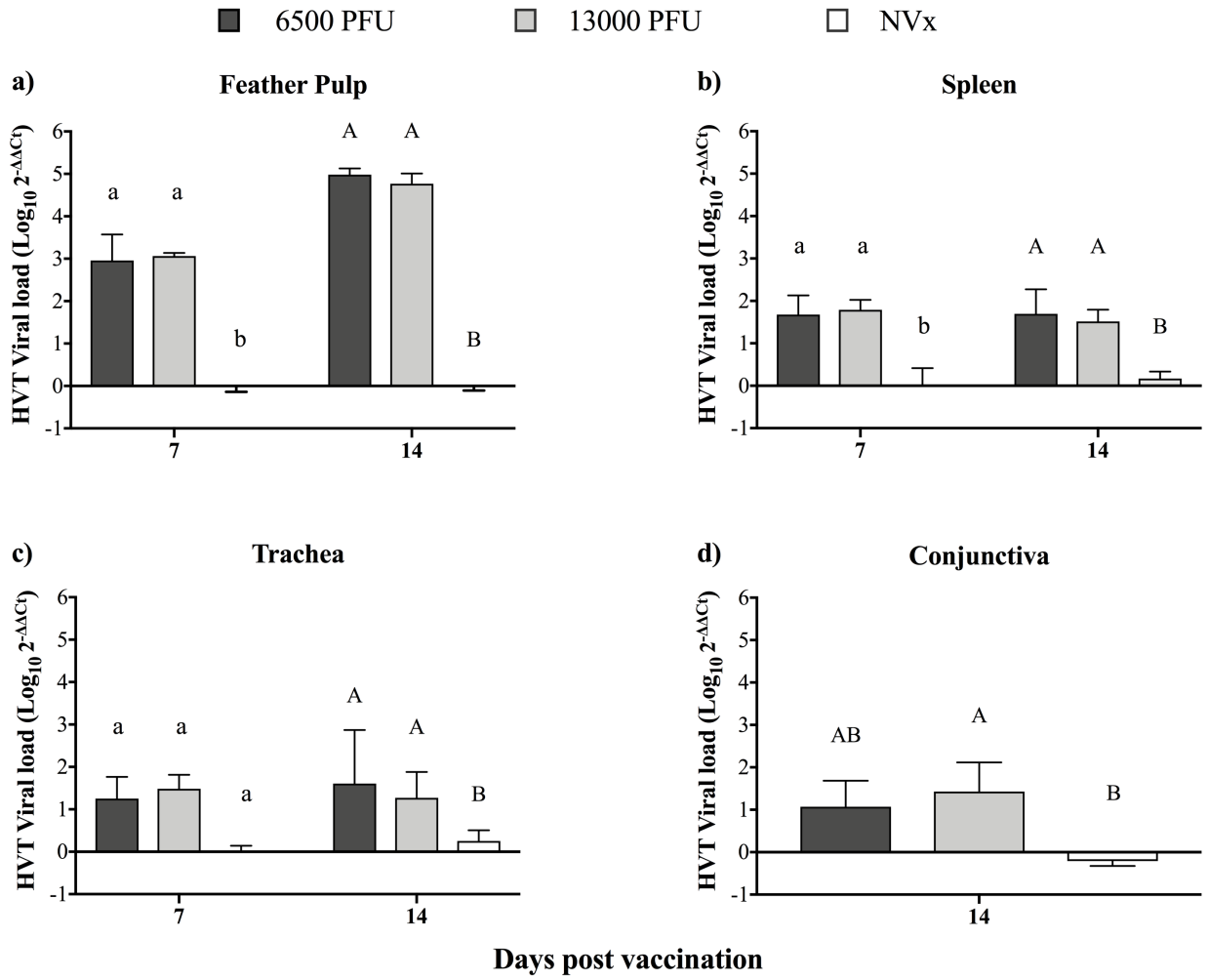

Fig. 4.1. Replication of rHVT-LT vaccine in feather pulp, spleen, trachea and conjunctiva (n = 5). Median genome load is represented by columns bars with the 95% confidence intervals (CI) of the median. a) HVT viral load in feather pulp at 7 and 14 days post vaccination. b) HVT viral load in spleen at 7 and 14 days post vaccination. c) HVT viral load in trachea at 7 and 14 days post vaccination. d) HVT viral load in conjunctiva at 14 days post vaccination. Different letters indicate significance differences among groups ($P < 0.05$).

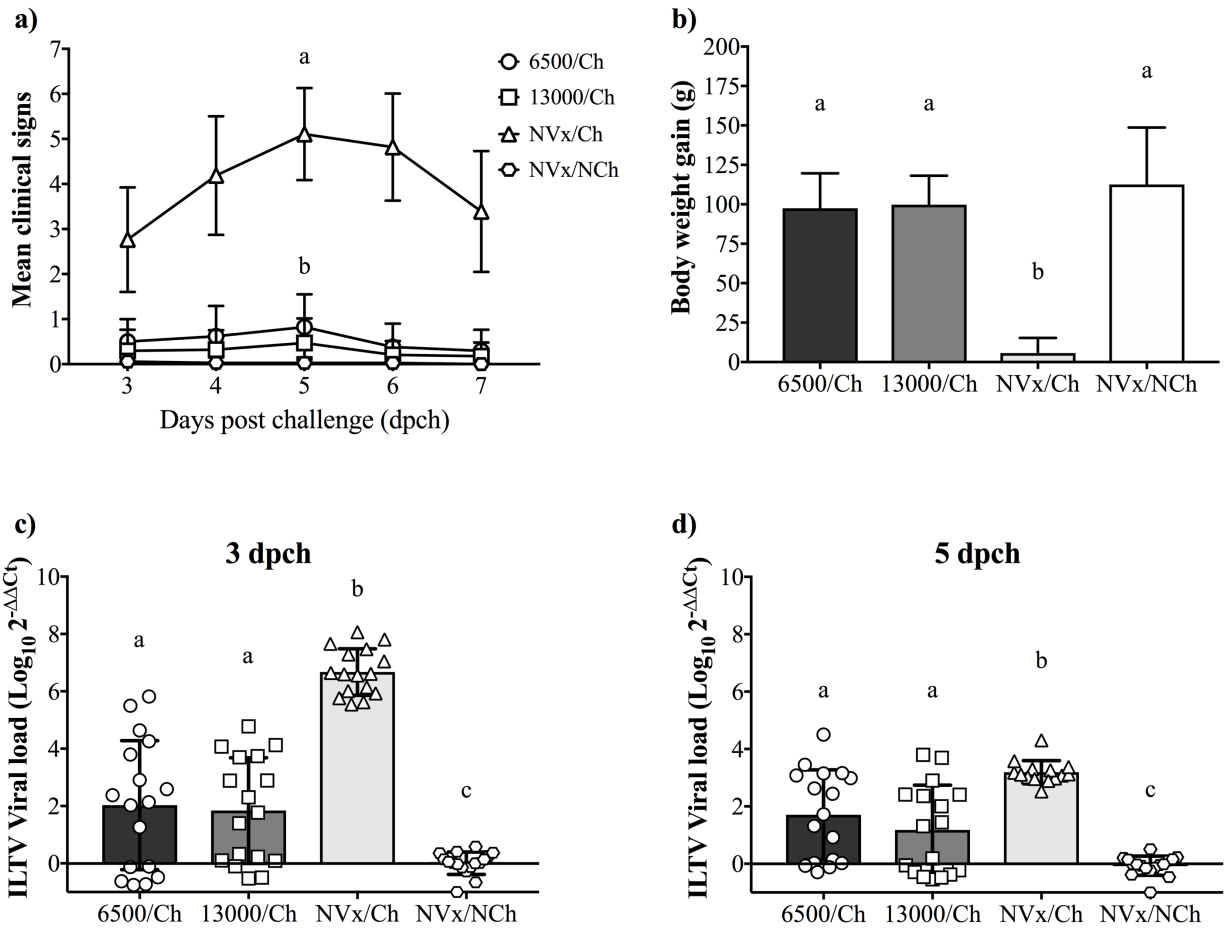

Fig. 4.2. Clinical signs, body weight gain and trachea viral load after challenge (n = 17). a) Mean clinical signs score at 3 to 7 days post ILTV challenge represented by geometric shapes showing the mean value at each time point; b) Mean body weight gain (g) at 7 days post ILTV challenge represented by bars with standard deviation (SD) error bars plotted from the mean; c) Individual and mean trachea viral load ($\log_{10} 2^{-\Delta\Delta Ct}$) at 3 days post ILTV challenge; d) Individual and mean trachea viral load ($\log_{10} 2^{-\Delta\Delta Ct}$) at 5 days post ILTV challenge. Mean viral load is represented by bars with standard deviation (SD) error bars plotted from the mean and individual values are represented by geometric symbols. Different letters indicate significance differences among groups ($P < 0.05$).

CHAPTER 5

EVALUATION OF VACCINATION AGAINST INFECTIOUS LARYNGOTRACHEITIS (ILT) WITH RECOMBINANT HERPESVIRUS OF TURKEY (rHVT-LT) AND CHICKEN EMBRYO ORIGIN (CEO) VACCINES APPLIED ALONE OR IN COMBINATION

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ABSTRACT

The chicken embryo origin (CEO) infectious laryngotracheitis (ILT) live attenuated vaccines, although capable of protecting against disease and reducing challenge virus replication can regain virulence. Recombinant ILT vaccines do not regain virulence but are partially successful at blocking challenge virus replication. The objective of this study was to evaluate the effect of rHVT-LT vaccination in CEO replication and how this vaccination strategy enhances protection and limit challenge virus transmission to contact naive chickens. The rHVT-LT vaccine was administered at 1 day of age subcutaneously and the CEO vaccine was administered at 6 weeks of age via eye-drop or drinking water. CEO vaccine replication post vaccination, challenge virus replication and transmission post-challenge were evaluated. After vaccination, only the group that received the CEO via eye-drop developed transient conjunctivitis. A significant decrease in CEO replication was detected for the rHVT-LT + CEO groups as compared to groups that received CEO alone. After challenge, reduction in clinical signs and challenge virus replication was observed in all vaccinated groups. However, among the vaccinated groups, the rHVT-LT group presented higher clinical signs and challenge virus replication. Transmission of the challenge virus to contact naive chickens was only observed in the rHVT-LT vaccinated group of chickens. Overall this study found that priming with rHVT-LT reduced CEO virus replication and the addition of a CEO vaccination provided a more robust protection than rHVT alone. Therefore, rHVT-LT + CEO vaccination strategy constitutes an alternative approach to gain better control of the disease.

Key words: Infectious laryngotracheitis, rHVT-LT, CEO, vaccination, replication, transmission

INTRODUCTION

Infectious laryngotracheitis (ILT) is an economically important respiratory disease of poultry that affects the poultry industry worldwide. The disease is caused by *Gallid alpha herpesvirus 1* (GaHV-1) commonly recognized as Infectious laryngotracheitis virus (ILTV) (Davison *et al*., 2009). The disease occurs frequently in densely populated poultry production areas. Severe forms of the disease are characterized by gasping, expectoration of bloody mucus, and high mortality due to asphyxia (García *et al*., 2013). Control of the disease is built on biosecurity and vaccination. Modified live attenuated vaccines, produced in chicken embryos (chicken embryo origin, CEO) or tissue culture (tissue culture origin, TCO) have been used for 60 years to control the disease. Despite the excellent capability of CEO vaccines to protect against clinical signs and mortality, the CEO vaccines regain virulence after bird-to-bird passage (Guy *et al*., 1990; Guy *et al*., 1991) which is facilitated by the swift transmission of these vaccine strains (Rodriguez-Avila *et al*., 2007; Coppo *et al*., 2012). In the field the use of CEO vaccines has been associated with adverse effects such as significant vaccination reactions which result in losses in body weight gain and feed conversion (Zavala, 2011). CEO-derived viruses of increased virulence are associated with multiples epizootics of the disease in the United States, South America and Europe (Neff *et al*., 2008; Oldoni *et al*., 2008; Chacón *et al*., 2009). As a response to the frequent ILT epizootics associated with CEO related viruses, a new generation of viral vector recombinant vaccines using the fowlpox virus (FPV) and herpesvirus of turkey (HVT) that express ILTV immunogenic proteins were developed, and are currently in use worldwide. There are three recombinant ILTV vaccines commercially available, a rFPV-LT vaccine which expresses the glycoprotein B (gB) and the UL-32 genes of ILTV, and two rHVT-LT vaccines, that express the gI and gD genes (Johnson *et al*., 2010; Gimeno *et al*., 2011), or the gB gene of ILTV (Esaki *et al*.,

2013). The HVT and FPV recombinant vector vaccines are characterized by their lack of bird to bird transmission (Esaki *et al*., 2013) and most importantly these vaccines are very stable and do not revert to virulence (Bublot *et al*., 2006). Experimental evidence has shown that recombinant vaccines reduce clinical signs of the disease and maintain bird performance, but are not as effective as the live attenuated vaccines in diminishing shedding of the challenge virus (Johnson *et al*., 2010; Vagnozzi *et al*., 2012).

Since the introduction of ILT recombinant vaccines, some multi age layer and heavy weight broiler complexes have adopted the use of a combined vaccination strategy including recombinant vaccines administered in the hatchery followed by live attenuated vaccines applied in the field to improve safety and expand protection against the disease. Although this strategy has been demonstrated to be successful under field conditions, the foundation of its benefits has not been studied. The objective of this study was to evaluate the effect of rHVT-LT vaccination on CEO replication and the protection efficacy of a rHVT-LT vaccine when administered alone or in combination with CEO vaccination administered via eyedrop or drinking water. The protection efficacy was assessed by the ability of vaccinated chickens to prevent clinical signs of the disease, to avoid body weight loss, and to lessen challenge virus replication and the subsequent transmission to contact naive chickens.

MATERIALS AND METHODS

Vaccines and vaccine titration. The Innovax®-ILT (rHVT-LT) (Merck Animal Health, Madison, NJ) vaccine was titrated in confluent monolayers of secondary chicken embryo fibroblasts (CEF) seeded in 60-mm plates. Three consecutive 10-fold dilutions of the reconstituted vaccine were made in Ham's F10 medium (1x) (Corning Inc, Corning, NY). Five plates of CEF

cells were inoculated with 200 µl (5 replicates) of each dilution. Inoculated CEF plates were incubated at 37 C and 5% CO_2. After 24 hours post incubation, culture media (5 ml) was changed and substituted with F10 media (1x) and 2% fetal bovine serum (FBS) (Atlanta Biologicals Inc, Flowery Branch, GA). Five days post incubation viral plaques were microscopically counted. Virus titers were calculated as plaque forming units (PFU) per dose (200 µl) and diluted to achieve 3000 PFU/dose. The Laryngo-Vac® (Zoetis, Parsippany, NJ) chicken embryo origin (CEO) vaccine was titrated in 96-well plates of chicken kidney (CK) cells prepared from 3 to 4 weeks old SPF chickens as previously described by Rodríguez-Avila *et al.* (2007). A 1000 dose Laryngo-Vac vial was diluted in 30 ml of commercial diluent, six 10-fold dilutions (10^{-1} to 10^{-6}) were performed and five replicates of each dilution were inoculated (100 µl) in CK cells. Titers in CK cells were expressed as the 50% tissue culture infectious dose ($TCID_{50}$) based on cytopathic effect (CPE) at five days post inoculation produced by ILTV replication. Titer was estimated using the Reed and Muench method (Reed & Muench, 1938).

Challenge virus. The strain 1874C5 which belongs to genotype group VI (Oldoni *et al*., 2009), isolated from a broiler outbreak, was used for challenge of vaccinated and non-vaccinated groups of chickens. Challenge virus was titrated and propagated in chicken kidney (CK) cells prepared from 3 to 4 week old SPF chickens. The median tissue culture infective dose ($TCID_{50}$) titer was calculated by Reed & Muench (1938) method as described above.

Experimental design. Three hundred specific pathogen free (SPF) eggs were obtained from a commercial source (Charles River Laboratories Inc., Wilmington, MA) and incubated at 99.5°F and 55% relative humidity (RH) in a small-scale hatcher (Natureform Inc., Jacksonville, FL) at the Poultry Diagnostic and Research Center (PDRC, University of Georgia, Athens, GA). At 1 day of age, a total of 210 SPF chickens were distributed into seven colony houses (PDRC),

30 birds per house. Three groups of 30 chickens were manually vaccinated subcutaneously (SC) with a standardized dose of rHVT-LT (3000 PFU/dose) vaccine, two groups of 30 chickens were mock inoculated with a commercial vaccine diluent subcutaneously, and two groups of 30 chickens remained not inoculated. At 14 and 21 days of age (doa), nine rHVT-LT vaccinated and nine non-vaccinated chickens were euthanized by CO_2 inhalation. Spleen and feather pulp were collected to evaluate the presence of HVT nucleic acid by real-time PCR. At 38 doa, four groups of 24 chickens each were weighed and vaccinated with the CEO vaccine via drinking water ($10^{4.6}$ $TCID_{50}$) or via eye drop ($10^{4.4}$ $TCID_{50}$). One of the rHVT-LT vaccinated groups received the CEO vaccine via drinking water (dw) (rHVT-LT + CEOdw), a second rHVT-LT vaccinated group received the CEO vaccine via eye drop (ed) (rHVT-LT + CEOed), and two groups of non-vaccinated chickens received the CEO vaccine via drinking water (CEOdw) or via eye drop (CEOed). To assess coverage of the drinking water vaccination, a commercial dye was added to the vaccine diluent. Chickens were allowed to drink the vaccine for a period of 90 minutes. The presence of dye in the tongue and crop were examined to determine exposure to the vaccine after drinking water vaccination. At 4 and 7 days post CEO vaccination, tracheal and conjunctiva swabs were collected to monitor the replication of the CEO vaccine. At 5 days post CEO vaccination clinical signs were recorded to evaluate vaccine reactions and body weight gain was measured 7 days post CEO vaccination. At 55 doa, vaccinated groups (rHVT-LT, CEOdw, rHVT-LT + CEOdw, CEOed, rHVT-LT + CEOed) and one non-vaccinated (NVx) group of chickens were challenged with virulent strain 1874C5 at a dose of $10^{3.8}$ $TCID_{50}$. The challenge virus was administered in a total volume of 200 µl split in 50 µl per eye and 100 µl was delivered intra-tracheally. The non-vaccinated non-challenged (NVx/NCh) group of chickens was mock-inoculated with tissue culture media in a similar fashion. At 3 to 7 days post-challenge (dpch)

clinical signs were scored, and tracheal swabs were collected at 3 and 5 dpch to quantify challenge virus genome load by real-time PCR. At 4 dpch four chickens from each group were sacrificed, and cranial segments of the trachea were collected and placed in 10% buffered neutral formalin for histopathological examination. Body weight gain at 7 dpch was estimated for vaccinated, NVx/Ch, NVx/NCh groups of chickens. The day of challenge contact naive chickens were introduced to all vaccinated challenged groups and the NVx/Ch group (4 birds/group). At 4, 8 and 12 days post introduction of contact naive chickens, clinical signs were evaluated and tracheal swabs were collected to determine viral genome load and assess virus shedding from vaccinated to contact chickens. During the length of the experiment, chickens were fed with a standard diet and provided water *ad libitum*. All the experiments conducted in this study were performed under the Animal Use Proposal A2016 10-010-R1 approved by the Animal Care and Use Committee (IACUC) in accordance with regulations of the Office of the Vice President for Research at the University of Georgia.

Clinical signs and mortality. Clinical signs were scored as previously described by Vagnozzi *et al*. (2012). Briefly, signs of dyspnea, conjunctivitis and lethargy were scored on a scale of 0 to 3, indicating normal (0), mild (1), moderate (2), and severe (3). Mortality received a score of 6. Total clinical sign score was calculated for each chicken and a mean clinical sign score was assigned for each group of chickens at each time point.

Body weight gain. Body weight gain was measured over a 7-day period, before and after CEO vaccination (38 – 45 doa) and before and after ILTV challenge (55 – 62 doa). The increase of weight in grams for each chicken between the two-time points was calculated using the following formula: Body weight gain = final weight (FW) – initial weight (IW) and the mean body weight gain per group was reported.

DNA extraction. All samples were resuspended in 1 ml of phosphate-buffered saline (PBS) with 2% antibiotic-antimycotic (Invitrogen, Waltham, MA) and 2% newborn calf serum (Gibco, Waltham, MA). Briefly, spleen and feather pulp were collected and placed in lysing bead matrix tubes containing 1.4 mm ceramic spheres and homogenized in the FastPrep-24™ 5G instrument (MP Biomedicals, Santa Ana, CA). Tracheal and conjunctiva swabs were collected and placed in 2 ml microcentrifuge tubes and vortexed. Samples were stored at −80 °C until processing. DNA extraction was performed using the MagaZorb® DNA extraction mini-prep kit (Promega, Madison, WI) following the manufacturer's recommendations with some modifications. Briefly, 7 µl of proteinase K (PK) solution was loaded into a 96-well plate, 70 µl of sample and 50 µl of lysis buffer were added per well, and the plate was incubated at 56 °C for 10 min. After incubation, 10 µl of magnetic beads and 125 µl of binding buffer was added per well followed by shaking for 10 min at room temperature. The supernatant and magnetic beads were separated with a magnetic stand, supernatant was discarded, beads were washed twice with 250 µl of washing buffer, and DNA was eluted from the beads with 100 µl of elution buffer after 10 min incubation at room temperature with shaking.

Duplex real-time PCR. Viral genome loads of HVT in spleen and feather follicles were quantified by real-time PCR in a duplex reaction where viral DNA was amplified with primers that target the HVT SORF1 gene and chicken DNA was amplified with primers that target the chicken α2 collagen gene as previously described by Islam *et al*. (2004). ILTV viral genome load in trachea and conjunctiva swabs was also quantified by real-time PCR in a duplex reaction where viral DNA was amplified with primers that target the UL44 ILTV gene (glycoprotein C) and chicken DNA was amplified by primers that target the chicken α2 collagen gene as previously described by Vagnozzi *et al*. (2012). The relative amount of viral DNA per sample was calculated

as the $\log_{10} 2^{-\Delta\Delta Ct}$ as previously described by Livak & Schmittgen, (2001). The $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of CEO and challenge virus replication were calculated using the following formulas: $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of CEO = ($\log_{10} 2^{-\Delta\Delta Ct}$ CEO) – ($\log_{10} 2^{-\Delta\Delta Ct}$ rHVT-LT + CEO) and $\log_{10} 2^{-\Delta\Delta Ct}$ reduction of challenge virus replication = ($\log_{10} 2^{-\Delta\Delta Ct}$ NVx/Ch) – ($\log_{10} 2^{-\Delta\Delta Ct}$ vaccinated groups/Ch).

Microscopic lesions. Tracheal microscopic lesions were scored from 0 to 5 as previously described by Guy *et al*. (1990). Briefly, microscopic scoring was performed as followed: Normal epithelium received a score of 0. Normal epithelium with mild to moderate lymphocytic infiltration but no detection of syncytia with intranuclear inclusion bodies received a score of 1. Normal epithelium with mild to moderate lymphocytic infiltration and few foci of syncytia with intranuclear inclusion bodies received a score of 2. Affected epithelium with moderate to marked hyperemia and lymphocytic infiltration with numerous syncytia with intranuclear inclusion bodies received a score of 3. Areas with absence of epithelium and occasional presence of syncytia with intranuclear inclusion bodies received a score of 4. No residual epithelium remaining and syncytia with intranuclear inclusion bodies rarely found received a score of 5.

Statistical analysis. Data was analyzed with the statistical program GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). D'Agostino-Pearson test was used for the assessment of normality. Mean differences among groups for clinical signs, body weight gain and ILTV viral genome load were performed by a One-Way ANOVA test and in all cases a Tukey's test was selected for a *post hoc* analysis. The Kruskal-Wallis test was used for comparisons of differences in medians for microscopic lesions in trachea and Dunn's test was performed as a multiple comparison procedure. Mean rHVT viral genome load was analyzed by the Student's t-test. The level of significance was considered at $P < 0.05$.

RESULTS

Detection of HVT genomes to assess recombinant vaccine replication. Seventy-eight percent of the feather pulp samples collected from rHVT-LT vaccinated chickens at 14 and 21 doa were positive for HVT genomes (Figure 5.1(a)). Whereas, among spleen samples collected at 14 and 21 doa, 100% and 89% were positive for HVT genomes. Feather pulps and spleens obtained from non-vaccinated (NVx) chickens were negative for HVT genomes. No significant differences ($P > 0.05$) in HVT genome load were detected in feather pulp and spleen at 14 or 21 doa (Figure 5.1(b)).

Clinical signs post CEO vaccination. At five days post CEO vaccination, the CEOed vaccinated group of chickens presented an average clinical sign score that was significantly higher ($P < 0.05$) than average clinical sign scores observed for the rHVT-LT, CEOdw, rHVT-LT + CEOdw, and rHVT-LT + CEOed vaccinated groups of chickens. The predominant clinical sign observed in the CEOed vaccinated group of chickens was conjunctivitis which was present in 76% of the chickens between 4 to 6 days post-vaccination. Although not as frequent as conjunctivitis mild signs of dyspnea and lethargy were also observed in the CEOed vaccinated group. The rHVT-LT, CEOdw, rHVT-LT + CEOdw, and rHVT-LT + CEOed showed no signs of conjunctivitis and had significantly fewer ($P < 0.05$) clinical signs compared to the CEOed group (Figure 2(a)).

Body weight gain post CEO vaccination. Groups vaccinated with rHVT-LT alone, rHVT-LT + CEO (rHVT-LT + CEOdw, rHVT-LT + CEOed) or CEO alone (CEOdw, CEOed), showed no significant differences ($P > 0.05$) in body weight gain after 7 days post CEO vaccination (Figure 2(b)).

Viral genome load in trachea and conjunctiva post CEO vaccination. CEO vaccine genome load in trachea and conjunctiva were quantified at 4 and 7 days post CEO vaccination

(Figure 5.2(c) & 5.2(d)). At 4 days post CEO vaccination, trachea viral genome load from groups vaccinated with rHVT-LT + CEO (rHVT-LT + CEOdw, rHVT-LT + CEOed) were significantly lower ($P < 0.05$) than viral genome load detected in trachea of CEO (CEOdw, CEOed) vaccinated groups. At 4 days post CEO vaccination the reduction of CEO viral genome load for rHVT-LT + CEOdw was 2.7 $\log_{10}$ and for rHVT-LT + CEOed was 3.6 $\log_{10}$. At 7 days post CEO vaccination the CEO genome load for rHVT-LT + CEOed group was lower ($P < 0.05$) than the CEO genome load for the CEOed vaccinated group. While the CEO genome load for CEOdw and rHVT-LT + CEOdw groups showed no differences ($P > 0.05$) (Figure 5.2(c)). In the conjunctiva, the CEO genome load at 4 and 7 days post vaccination was significantly lower ($P < 0.05$) for the rHVT-LT + CEOed group than for the CEOed vaccinated group (Figure 5.2(d)). The reduction of CEO viral genome load for the rHVT-LT + CEOed group was 3.2 $\log_{10}$ at 4 days post vaccination. No differences in CEO viral genome load ($P > 0.05$) were observed between the CEOdw and rHVT-LT + CEOdw groups at 4 and 7 days post vaccination.

Clinical signs post challenge. From 3 to 7 dpch all the vaccinated challenged groups (rHVT-LT/Ch, CEOdw/Ch, CEOed/Ch, rHVT-LT + CEOdw/Ch, rHVT-LT + CEOed/Ch) showed significant reduction ($P < 0.05$) in clinical signs as compared to the NVx/Ch group (Figure 5.3(a)). The peak of clinical signs was achieved at 5 dpch, at this time point the rHVT-LT/Ch group presented higher average clinical signs score ($P < 0.05$) than the rest of the vaccinated challenged groups, whereas no significant differences ($P > 0.05$) in clinical signs were observed among CEOdw/Ch, CEOed/Ch, rHVT-LT + CEOdw/Ch, and rHVT-LT + CEOed/Ch vaccinated groups of chickens (Figure 5.3(b)). Prevalent clinical signs observed among vaccinated challenged groups of chickens were mild dyspnea and lethargy, while for the NVx/Ch group of chickens severe dyspnea and lethargy, conjunctivitis and mortality were equally prevalent. A cumulative mortality

of 29% was observed for the NVx/Ch group of chickens (Figure 5.3(c)). No clinical signs were evidenced in the NVx/NCh group.

Body weight gain post challenge. Compared to the NVx/NCh group of chickens, by 7 dpch (62 doa) body weight gain was maintained ($P > 0.05$) in all vaccinated groups of chickens. A significant decrease ($P < 0.05$) in body weight was observed for the NVx/Ch group by 7 dpch (Figure 5.3(d)).

Challenge virus genome load in the trachea post challenge. Compared to the NVx/Ch group of chickens, at 3 (Figure 5.3(e)) and 5 (Figure 5.3(f)) dpch all vaccinated groups showed a significant ($P < 0.05$) reduction in challenge virus genome load in the trachea. Among vaccinated groups of chickens, the rHVT-LT/Ch group exhibited significantly higher viral genome load ($P < 0.05$) than groups of chickens vaccinated solely with the CEO vaccine or groups that received rHVT-LT + CEO. During the peak of virus replication, at day 3 post challenge, the reduction of challenge virus genome load was 3.8 $\log_{10}$ (rHVT-LT), 5.6 $\log_{10}$ (CEOdw), 5.4 $\log_{10}$ (rHVT-LT + CEOdw), 5.7 $\log_{10}$ (CEOed) and 5.7 $\log_{10}$ (rHVT-LT + CEOed).

Microscopic lesions in trachea post challenge. No differences were detected in microscopic trachea lesions scores ($P > 0.05$) among vaccinated challenged groups of chickens. Median lesion scores among vaccinated challenged groups ranged from 0.5 to 1.5 (Table 5.1). Two out of 4 trachea sections collected from the rHVT-LT vaccinated group of chickens showed lesion scores of 2. This score corresponds to the presence of few syncytia foci and intranuclear inclusions which appear in normal epithelium with mild to moderate lymphocytic infiltration. The NVx/Ch group ($P < 0.05$) presented the highest microscopic lesions with a median score of 4.5 (Table 5.1).

Challenge virus genome load and clinical signs for contact naive chickens. Challenge virus and clinical signs were demonstrated for contact naive chickens introduced to NVx/Ch and

rHVT-LT/Ch groups but not for contact chickens introduced to CEOdw/Ch, CEOed/Ch, rHVT-LT + CEOdw/Ch, and rHVT-LT + CEOed/Ch groups. The peak of viral genome load (Figure 5.4 (a)) and clinical signs (Figure 5.4 (b)) for contact chickens introduced to the NVx/Ch group were observed at 8 days post introduction. Whereas for contact chickens introduced to the rHVT-LT/Ch group the peak of viral genome load (Figure 5.4 (a)) and clinical signs (Figure 5.4 (b)) were delayed to 12 days post introduction. The predominant clinical signs observed in contact naive chickens were moderate conjunctivitis, dyspnea and lethargy. However, no mortalities were recorded for contact naive chickens introduced to the NVx/Ch and rHVT-LT/Ch groups of chickens.

DISCUSSION

In the United States since the introduction of ILT recombinant vaccines, some multi age layer and heavy weight broiler complexes have adopted the use of a combined vaccination strategy including recombinant vaccines administered in the hatchery followed by live attenuated vaccines applied in the field to improve protection against the disease. However, many poultry producers use only CEO vaccines for the control of the disease because it is more economical than recombinant vaccines. The uninterrupted use of CEO vaccines perpetuates the circulation of CEO viruses which can become the source of outbreaks. Therefore, introducing safer vaccination alternatives in multi-age complexes presents a unique challenge. This study explored the use of rHVT-LT and CEO vaccine in a combined program to determine whether this combination could demonstrate improved control of ILT. After CEO vaccination mild to moderate vaccine reactions were observed in groups of chickens that received the CEO vaccine by itself. These vaccine reactions were not observed in groups of chickens that received the rHVT-LT + CEO vaccine

combination. In particular the group of chickens that received the CEO vaccine via eye drop developed a transient conjunctivitis which appeared by day 4 and disappeared by 7 days post vaccination. It is of value to point out that conjunctivitis reaction may be accentuated and prolonged under field conditions due to compound effects of high ammonia and the dusty environment leading to poor performance. In contrast to the CEOed vaccinated group of chickens, the CEOdw vaccinated group showed very limited vaccine reaction. Different than in the field where poor vaccination coverage by drinking water administration may occur, in this small-scale experiment drinking water vaccination was closely monitored reaching at least 98% coverage. Due to the proper vaccination coverage achieved in this experiment there was no opportunity for vaccine back passages. With regards to the CEO vaccine replication, both rHVT-LT + CEOdw and rHVT-LT + CEOed vaccinated groups of chickens showed lower CEO viral genome load in trachea than chickens vaccinated only with the CEO vaccine. This result confirmed that prior vaccination with rHVT-LT reduced the CEO vaccine replication and suggests that the combination of rHVT-LT + CEO vaccines in the long term may reduce the circulation of CEO viruses. Therefore, this combined vaccination strategy offers a safer alternative than the uninterrupted use of uniquely CEO vaccine in poultry facilities.

Vaccine protection after challenge was assessed by the ability of vaccinated chickens to prevent clinical signs of the disease, to lessen challenge virus replication in the trachea, to avoid body weight loss after challenge, and to halt challenge virus transmission to contact naive birds. Several studies have provided clear evidence that both CEO and rHVT-LT vaccines significantly decrease clinical signs of the disease after challenge (Fulton *et al*., 2000; Vagnozzi *et al*., 2012; Esaki *et al*., 2013). However, measurements of challenge virus genome load reduction in the trachea for rHVT-LT and CEO vaccinated groups of chickens are considerably variable among

experiments. Studies have estimated that challenge virus genome load reduction in the trachea range from 1.8 $\log_{10}$ to 3.7 $\log_{10}$ for rHVT-LT vaccinated groups of chickens (Vagnozzi *et al*., 2012; Maekawa *et al*., 2019), while for CEO vaccinated groups challenge virus genome load reduction ranged from 5.0 $\log_{10}$ to 6.8 $\log_{10}$ (Johnson *et al*., 2010; Vagnozzi *et al*., 2012). In this study, the group of chickens that received only the rHVT-LT vaccine achieved a challenge virus genome load reduction of 3.8 $\log_{10}$, while groups of chickens that received only the CEO vaccine or combined rHVT-LT + CEO vaccinations ranged from 5.4 $\log_{10}$ to 5.7 $\log_{10}$. The capacity of CEO vaccination to halt replication of the challenge virus was further evidenced by the absence of challenge virus transmission to contact naive chickens introduced to CEO or rHVT-LT + CEO vaccinated groups, while challenge virus transmission was only recreated for the rHVT-LT vaccinated group. Although it has not been proven, it is suspected that induction of a limited local upper respiratory immune response maybe one of the reasons why the rHVT-LT vaccine is not as effective as the CEO vaccine in reducing challenge virus replication in the trachea (Gimeno *et al*., 2011; García, 2017). During hatchery vaccination, rHVT-LT vaccine can spread to the trachea i*n ovo* when the embryo inhales or swallows the vaccine from the amniotic fluid (Wakenell *et al*., 2000), or through intramuscular *(in ovo)* or subcutaneous deposition where the vaccine is absorbed by the tissues and ultimately reach the bloodstream. However, we hypothesize that in rHVT-LT vaccinated chickens the amount of antigen that is directed to the trachea maybe suboptimal. Therefore, the antigen capture and presentation process in the trachea and the upper respiratory tract may be affected, subsequently interfering with the activation of effector and memory T cells. Experimental evidence in CEO vaccinated chickens showed a significant increase in IFN-γ as early as 6 hours post challenge (Vagnozzi *et al*., 2016). It is highly probable that an effective memory T cell response act as a main contributor to the rapid production of IFN-γ which may play

an important role in limiting early virus replication. Defining the host immune responses elicited by rHVT-LT in the trachea will be pivotal in the quest to improve the protection against ILTV induced by HVT vector vaccines.

Notably, vaccination with rHVT-LT was not as effective to limit the replication of the challenge virus while it was quite effective in blocking the replication of the CEO vaccine. This disparity can be attributed to the level of attenuation of the CEO vaccine as compared to the challenge virus (1874C5 Strain, Genotype VI), and also the route of the virus inoculation which influences the outcome of infection (Beltrán *et al*., 2017). In this study the CEO vaccine was delivered via eye-drop, whereas the challenge virus was administered via ocular and intratracheal. It has been shown that ILTV virulent strains have the ability to alter and delay the local innate responses when administered via the intratracheal route (Vagnozzi *et al*., 2018).

When rHVT-LT vaccines were first introduced, the idea was to gradually eliminate the demand for CEO vaccination. However, the poultry industry has learned how to maximize performance and protection using both CEO and rHVT-LT vaccines. Overall this study found that priming with rHVT-LT reduced CEO virus replication and the addition of a CEO vaccination provided a more robust protection than rHVT alone. Therefore, rHVT-LT + CEO vaccination strategy constitutes a valuable approach to gain better control of ILT.

Disclosure statement

No potential conflict of interest was reported by the authors.

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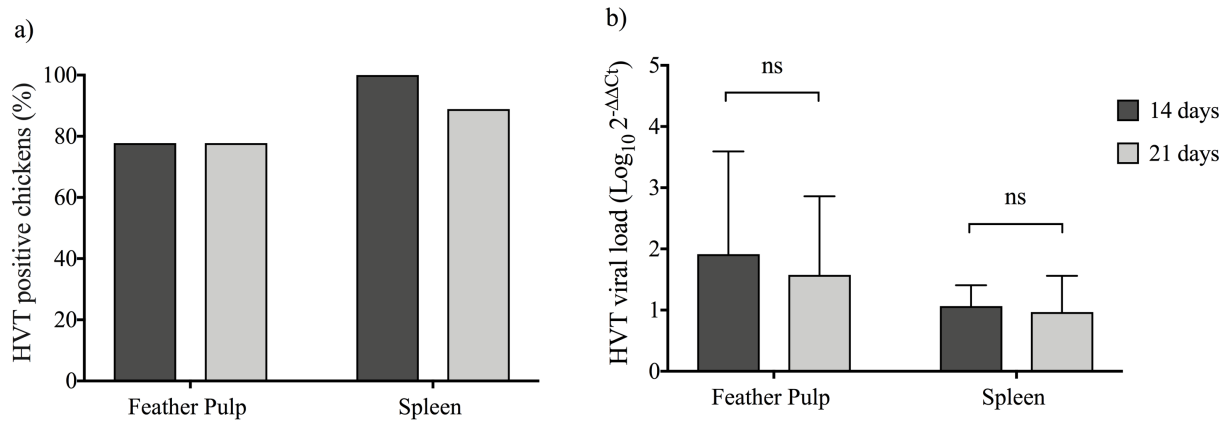

Figure 5.1. Detection of HVT genomes in feather pulps and spleen (n = 9). a) Percentages of HVT genome positive chickens in spleen and feather pulp at 14 and 21 doa represented by bars. b) Mean HVT viral genome load in spleen and feather pulp at 14 and 21 doa represented by bars with vertical lines showing the standard deviation (SD). No significant difference (ns) ($P > 0.05$) was observed in HVT viral genome at 14 and 21 doa in both tissues.

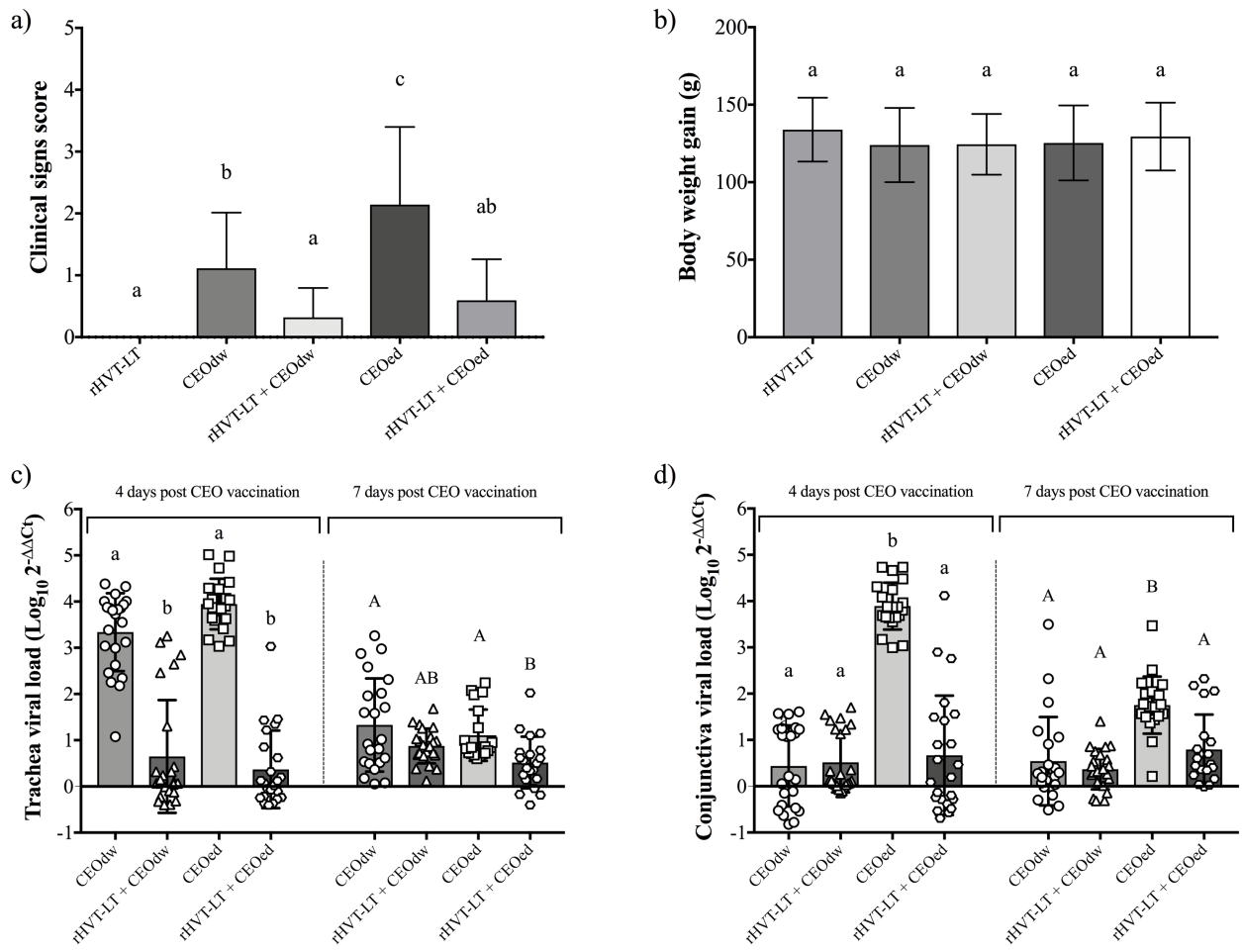

Figure 5.2. Clinical signs, body weight gain, trachea and conjunctiva viral genome load post CEO vaccination (n = 22). a) Mean clinical signs scores at 5 days post CEO vaccination represented by bars with standard deviation (SD) error lines plotted from the mean. b) Mean body weight gain (g) at 7 days post CEO vaccination represented by bars with standard deviation (SD) error lines plotted from the mean. c) Mean trachea viral load ($\log_{10} 2^{-\Delta\Delta Ct}$) at 4 and 7 days post CEO vaccination represented by bars with standard deviation (SD) error lines plotted from the mean and individual values represented by geometric symbols. d) Mean conjunctiva viral load ($\log_{10} 2^{-\Delta\Delta Ct}$) at 4 and 7 days post CEO vaccination represented by bars with standard deviation (SD) error lines plotted from the mean and individual values represented by geometric symbols. Different letters indicate significance differences among groups ($P < 0.05$).

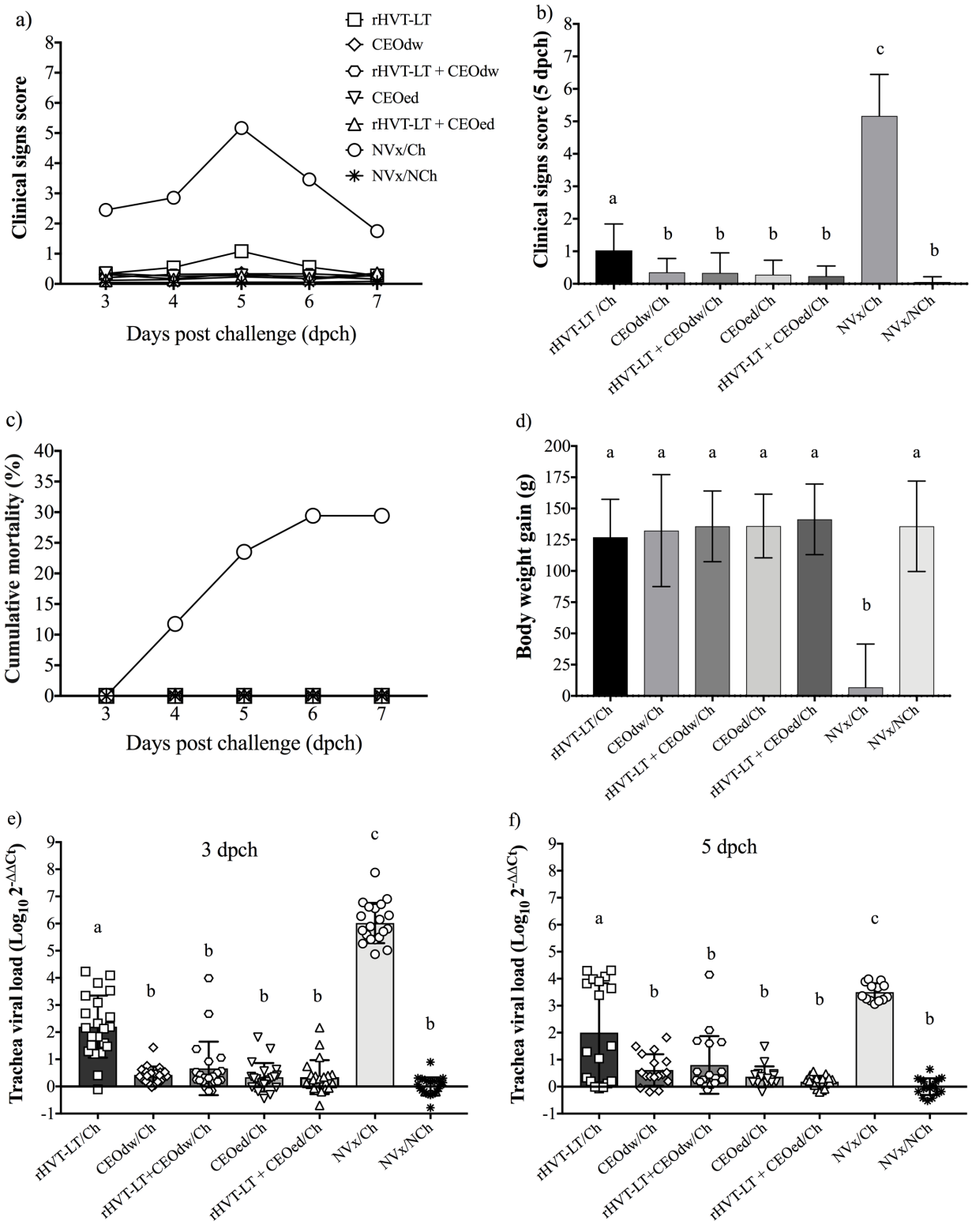

Figure 5.3. Clinical signs, cumulative mortality, body weight gain and trachea genome viral load post challenge (n = 22). a) Mean clinical signs scores represented by geometric symbols at 3 to 7

dpch. b) Mean clinical signs score at 5 dpch represented by bars with standard deviation (SD) error lines plotted from the mean. c) Daily cumulative mortality (%) at 3 to 7 dpch represented by geometric symbols. d) Mean body weight gain (g) at 7 dpch represented by bars with standard deviation (SD) error lines plotted from the mean. e) Mean trachea viral load ($\log_{10} 2^{-\Delta\Delta Ct}$) at 3 days post challenge, and f) 5 dpch. The mean is represented by bars with standard deviation (SD) error lines plotted from the mean while individual values are represented by geometric symbols. Different letters indicate significance differences among groups ($P < 0.05$).

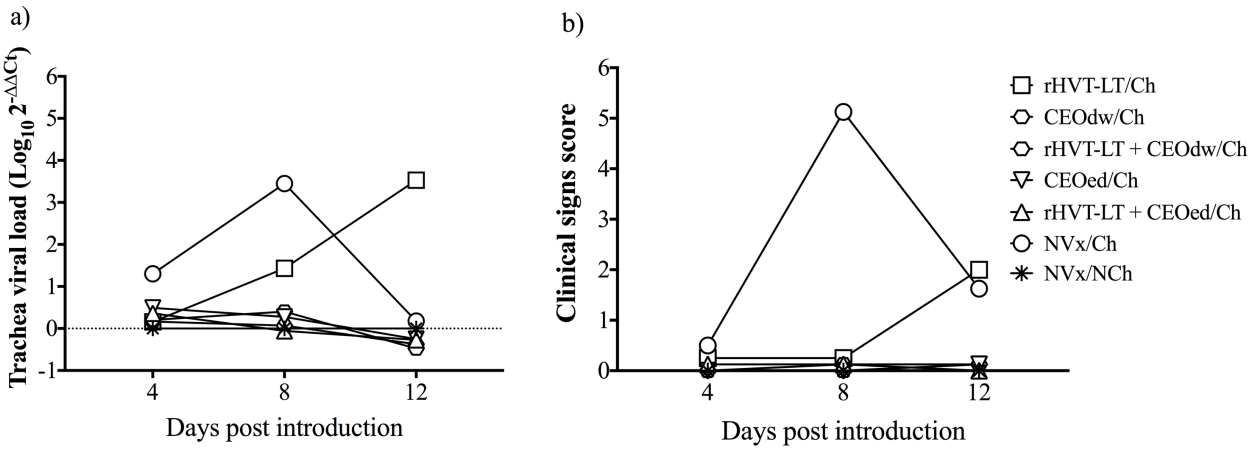

Figure 5.4. Contact naive chickens mean viral genome load and clinical signs scores. Four contact naïve chickens were introduced to vaccinated challenged and NVx/Ch groups at 0 days post challenge and evaluated at 4, 8 and 12 days post introduction. a) Mean viral genome load, and b) mean clinical signs scores post introduction are represented by geometric symbols.

Table 5.1. Median microscopic lesion score in trachea at 4 days post challenge.

Group	Score (Range)
rHVT-LT	1.5[a] (1 - 2)
CEOdw	0.5[a] (0 - 1)
rHVT-LT + CEOdw	0.5[a] (0 - 1)
CEOed	0.5[a] (0 - 1)
rHVT-LT + CEOed	0.5[a] (0 - 1)
NVx/Ch	4.5[b] (4 - 5)
NVx/NCh	0.0[a] (0 - 1)

[a]Different letters indicate significant differences ($P < 0.05$)

CHAPTER 6

CONCLUSIONS

In recent years, the use of recombinant ILT vaccines has significantly expanded as it offers a safer vaccination alternative for the control of the disease. However, experimental evidence has shown that rHVT-LT vaccines induce partial protection because challenge virus replication still occurs in the trachea of vaccinated chickens. Therefore, this study was focused on better understanding the role that the rHVT-LT dose plays in protection, whether rHVT-LT vaccinated chickens can transmit virus after challenge, and how the combination of rHVT-LT and CEO vaccines improve the control of ILT.

The objective of the first study was to evaluate the protection efficacy of a rHVT-LT vaccine when administered at standardized doses of 6000, 3000 and 1000 plaque forming units (PFUs) and the effect of the rHVT-LT vaccination in transmission of the challenge virus to contact naive chickens. Independent of the rHVT-LT vaccine dose (1000, 3000, 6000 PFU), vaccinated chickens showed significant reduction in clinical signs, maintained body weight gain after challenge, and reduced the challenge virus replication in the trachea. However, since a cumulative mortality of 6.3% was observed in the 1000 PFU group, a minimum rHVT-LT dose of 3000 PFU is recommended to induce protection. On the other hand, despite reduction of challenge virus replication in the trachea, challenge virus transmission from rHVT-LT vaccinated chickens (3000 & 6000 PFU) to contact naive chickens, was successfully recreated and maintained. Even though challenge virus replication was demonstrated for the 1000 PFU vaccinated group, minimal virus

transmission was observed to contact naive chickens. We hypothesized that the lack of viral transmission in the 1000 PFU group might be associated with a lower air speed intake and a much higher air speed exhaust in that house. Overall, these finding supports the belief that rHVT-LT vaccination, does not halt challenge virus transmission and most importantly, clinical asymptomatic rHVT-LT vaccinated chickens can shed and transmit virus after challenge.

The aim of the second study was to evaluate the replication and protection efficacy of a rHVT-LT vaccine when administered with a double commercial dose (13000 PFU). No differences in protection efficacy were detected among chickens vaccinated with rHVT-LT at 6500 and 13000 PFU. Both groups of vaccinated chickens showed significant reduction in clinical signs, maintained body weight gain after challenge, and reduced the challenge virus replication. In addition, at 7 and 14-days post *in ovo* vaccination no differences in HVT replication were observed in feather pulp, spleen, trachea and conjunctiva with the two evaluated doses. Contrary to what was previously believed, augmented doses of rHVT-LT between 6000 and 13000 PFU, neither increased vaccine replication nor protection efficacy.

In the third study, we evaluated the effect of rHVT-LT vaccination in CEO replication and how this vaccination strategy enhances protection and limits challenge virus transmission to contact naive chickens. An alternative vaccination strategy was tested where the rHVT-LT vaccine was administered at 1 day of age followed by CEO vaccination at 6 weeks of age. After CEO vaccination, only the group that received the CEO via eye-drop developed transient conjunctivitis. A significant decrease in CEO replication was detected for the rHVT-LT + CEO groups as compared to groups that received CEO alone. After challenge reduction in clinical signs and challenge virus replication was observed in all vaccinated groups. However, among the vaccinated groups, the rHVT-LT group presented higher clinical signs and challenge virus replication.

Transmission of the challenge virus to contact naive chickens was only observed in vaccinated chickens with rHVT-LT alone. It was concluded that priming with rHVT-LT reduced CEO virus replication and the addition of a CEO vaccination provided a more robust protection than rHVT alone.

Overall, these studies comprehensively revealed some drawbacks and advantages of vaccination with rHVT-LT vaccine. Based on the results obtained, the current design of the rHVT-LT vaccines offers adequate protection against clinical signs. However, they fail to limit the circulation of virus under strong challenge conditions, making the epidemiological control of the disease more difficult. Since increasing the dose did not expand protection, further studies are required to improve immunogenicity of the present recombinant ILT vaccines. One possible strategy to improve the efficacy of rHVT-LT vaccines is to co-administer adjuvants that enhance immune responses. Activation of chicken toll-like receptors (TLRs) with synthetic ligands as pathogen-associated molecular patterns (PAMPs) has been investigated in their potential to limit ILTV replication, where CpG oligonucleotide (TLR21 ligand), lipoteichoic acid (TLR2 ligand) and lipopolysaccharide (TLR4 ligand) have been demonstrated to boost the immune response against an ILTV infection. Another approach is to develop T-cell epitope vaccines, based on the idea that immunodominant T-cell epitopes are better inductors of specific immune responses. Finally, evidence was provided that combining rHVT-LT and CEO vaccines is a sound vaccination strategy that pools the benefits of either vaccine. Therefore, further studies are warranted to better characterize the additive protection conferred by the combination of the current available vaccines against ILT, as a way to gain better control of the disease.