

REPLICATION, TRANSMISSION, AND PROTECTION OF LIVE-ATTENUATED INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) VACCINES

by

ANDRES RODRIGUEZ AVILA

(Under the Direction of Maricarmen García)

ABSTRACT

Infectious laryngotracheitis virus (ILTV) is associated with serious economic losses due to clinical signs, mortality, decreased egg production, and predisposition to other avian pathogens. The virus is a member of family *herpesviridae*, subfamily *Alphaherpesvirinae*, and it is taxonomically classified as *Gallid herpesvirus 1*. Although it was the first poultry pathogen controlled by vaccination, ILTV is still a major problem in areas where dense bird populations exist. Currently, there are two main types of ILTV live vaccines commercially available, those attenuated by sequential passages in chicken embryos (CEO) or by sequential passages in tissue culture (TCO). The replication, transmission, and protection of the CEO and TCO vaccines were evaluated using vaccinated, contact-exposed, and sentinel specific pathogen free chickens. No differences were observed in the ability of the CEO and TCO vaccines to replicate in the upper respiratory tract, to transmit to contact-exposed birds, and to induce protection against the challenge virus. However, chickens contact-exposed to vaccinates were not protected against challenge.

INDEX WORDS: Infectious laringotracheitis virus; ILTV; ILT; chicken embryo origin vaccine-CEO; tissue culture origin vaccine-TCO; real time polymerase chain reaction; genome copy number; virus isolation replication; transmission; protection.

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ANDRES RODRIGUEZ AVILA

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ANDRES RODRIGUEZ AVILA

Major Professor: Dr. Maricarmen García

Committee: Dr. John Glisson
Dr. Charles L. Hofacre

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

Dedico este trabajo a las personas más importantes de mi vida, mis padres. Por medio de la educación y el cariño que me brindaron es que he llegado tan lejos en mi vida. También quiero dedicar este trabajo a mi hermano Alejandro y su esposa Gilma, quienes han creído en mí siempre y sin dudarlo me han apoyado para lograr mis sueños.

“I dedicate this thesis to the most important persons in my life, my parents. Because of the education and love that they provided to me, I have come so far in my life. As well, I would like to dedicate this journey to my brother Alejandro and his wife Gilma, who have always believed in me, and unconditionally have supported me in the road to achieve my dreams.”

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CHAPTER 1

INTRODUCTION

Respiratory diseases of poultry result in great production losses for the industry due to the severity of clinical signs. Infectious laryngotracheitis virus (ILTV) is included among the respiratory pathogens that can infect chickens and cause important economic losses. The disease can be present in two epizootic forms. The severe form is characterized by high morbidity, and moderate to high mortality; whereas the mild form, commonly present nowadays in the developed poultry industries, is responsible for the presence of clinical signs including, tracheitis, sinusitis, conjunctivitis, general depression, watery eyes, and low mortality. Although recognized as the mild presentation of the disease it produces weight loss and decreased egg production and has had a significant economic impact for the industry in the past decade.

Over the years the prevention and control of ILTV has been attempted by vaccination despite knowing that this can result in latently infected carrier birds, allowing the vaccine strains to persist in the field and recover their pathogenicity. The route of vaccine application is essential to ensure protection and to avoid persistence of vaccine derived strains in the field. The eye-drop vaccination route has been confirmed as the better and safer method of vaccine application; even though, the industry prefers mass application methods, due to lower costs, regardless of the risk that these practices can generate the emergence of outbreaks.

Modified-live vaccines have been used for fifty years to control ILTV outbreaks. In 2005 the US Animal Health Association (USAHA) published in the proceedings of their annual

meeting, that more important than vaccination are the implementation and enforcement of good biosecurity practices. However, lax biosecurity and improper vaccination strategies have barred the control and eradication of the disease.

The currently utilized modified- live vaccines, chicken embryo origin (CEO) and tissue culture origin (TCO), have been shown to induce protection against a variety of field strains when applied in susceptible chickens, preventing clinical signs and mortality. Most commercial layers and broiler breeders in the US, particularly those that are raised in locations at high risk of exposure, are vaccinated against ILTV either with CEO or TCO. Broilers are vaccinated only in the face of outbreaks, using CEO vaccines applied via the drinking water or by coarse spray. Experimental studies and field observations have allowed a wide evaluation of both commercially available live-attenuated vaccines, CEO and TCO. The first aim of this study was to compare the replication and transmission of the CEO and TCO vaccines at different time points post-inoculation in both chickens vaccinated by eye drop and contact exposed.

During recent ILTV epizootics it was demonstrated by polymerase chain reaction, restriction fragment length polymorphism (PCR-RFLP), and sequence analysis that some field isolates were genetically different. These new field isolates were classified into six molecularly different groups most of which were distinguishable from the vaccines (CEO and TCO). The PCR-RFLP provided the framework to analyze and compare the pathogenicity and growth characteristics of US poultry isolates from the different genotypes and how they differ from the vaccines. Groups V and VI viruses, which are genetically different to the vaccines, were more pathogenic than other viral groups including the CEO vaccine. Group V and VI viruses have been related with recent outbreaks; therefore, the protection induced by the vaccines against these types of viruses needed be evaluated. In order to assess the protection provided by CEO

and TCO against these newly identified isolates, the second aim of these study was to evaluate protection induced by CEO and TCO vaccines against group VI isolates.

Overall in this study the replication, transmission and protection of ITLV vaccines (CEO and TCO) were evaluated. Both vaccines replicated in the upper respiratory tract and transmitted to contact-exposed non-vaccinated chickens. Both vaccines provided protection, as evaluated by clinical signs, challenge virus transmission, and body weight gained. As demonstrated by the results of this work, vaccines can spread from vaccinated to susceptible chickens; however, in the challenge model, the chickens that were contact-exposed to vaccinated chickens were not protected against the challenge virus. Therefore, it is extremely important, in order to achieve good protection, that a flock receives a uniform vaccination to avoid the presence of susceptible chickens in the house.

CHAPTER 2

LITERATURE REVIEW

Infectious laryngotracheitis virus (ILTV) is a highly contagious respiratory pathogen primarily of chickens that may result in severe production losses due to morbidity, mortality, weight loss, decreased egg production and predisposition to other avian pathogens. Two forms of the disease have been recognized, severe epizootics characterized by signs of respiratory depression, gasping, expectoration of bloody mucous and high mortality; and mild forms manifested by mild tracheitis, sinusitis, conjunctivitis, general bird depression and low mortality. Although the overall cost of ILTV outbreaks has not been accurately determined, the economic significance of the disease is mostly related to increase mortality, weight loss, decrease in egg production, and vaccination cost (55).

History of the Disease

The disease was first reported by May and Tittsler when they described an outbreak on a Rhode Island farm (92). However, other reports suggest that it may have existed previously (12, 64). The disease was first identified as avian diphtheria or infectious bronchitis, and the term laryngotracheitis was first utilized in 1930 (13, 52). The name infectious laryngotracheitis virus was assigned by the special committee on Poultry Diseases of the American Veterinary Medical Association in 1931 (15). ILTV was the first poultry viral disease for which an effective vaccine was utilized.

Etiology

Infectious laryngotracheitis virus belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Iltovirus* (33). The virus is genetically different from other alphaherpesviruses based on viral DNA sequences (33, 93). The virus is taxonomically classified as *Gallid herpesvirus I* (33, 117). ILTV has an icosahedral viral particle with a hexagonal nucleocapsid (80 – 100 nm) composed of 162 elongated hollow capsomers similar to other herpesviruses (32, 138). The diameter of the complete viral particle is between 195–250 nm and it has an irregular envelope surrounding the nucleocapsid with viral glycoprotein spikes on its surface.

Infectious laryngotracheitis virus has a linear double stranded DNA genome present in two isomeric forms (87, 89). The buoyant density of the viral genome is estimated to be around 1.704 g/ml, consistent with other herpesviruses (102). Recently, the complete nucleotide sequence of the ILTV genome was assembled from 14 different published sequences (129). The assembled ILTV genome was shown to consist of a 148-kb molecule having a unique long (UL) region of 113 kb, and a unique short (US) region of 13 kb; the UL and US regions were shown to be flanked by two 11-kb inverted repeats. The ILTV genome contains a total of 77 predicted open reading frames; 62 of these are located in the UL region, 9 in the US region and 3 in the inverted repeat.

Early studies by York et al. (149, 150), identified five major envelope glycoproteins, with molecular weights of 205, 160, 115, 90, and 60 KD, to be the major immunogens of ILTV, responsible for stimulating humoral and cell-mediated immune responses (146). Subsequently, characterization of ILTV glycoproteins utilizing monospecific antisera or monoclonal antibodies has been undertaken in several laboratories. Several glycoproteins that are homologous to those

of human herpes simplex virus (HSV) have been characterized; these are designated glycoprotein B (gB) (103), gC (83, 136), gN (43), gM (43, 44), gG (86), and gJ (136), and in the genome a total of 12 open reading frames homologous to HSV-1 glycoprotein genes have been identified (129).

Virus Replication

Infectious laryngotracheitis virus replication seems to be similar to that of other alphaherpesvirus such as HSV (54, 104, 116). Envelope glycoproteins mediate entry of the virus into host cells by attaching to cell receptors and fusing the viral envelope to the cell membrane. Glycoprotein C (gC) mediates the initial attachment of HSV-1 by binding to cellular heparan sulfate proteoglycans, the primary host cell surface receptor for this virus (82). Kingsley et al (83) characterized glycoprotein C of ILTV to be smaller than that of other alphaherpesviruses. The shorter gC of ILTV lacks the heparin binding domain found in other alphaherpesviruses. Therefore, it appears that ILTV does not use heparan sulfate as its primary host cell receptor (82). The host cell receptor for ILTV is yet to be discovered. After fusion of the viral particle to the cell membrane the viral nucleocapsid is released into the cytoplasm and transported to the nuclear membrane, viral DNA is released from the nucleocapsid and migrates into the nucleus where transcription and replication of viral DNA occurs. During transcription of ILTV DNA three classes of genes are expressed: alpha (immediate early genes), beta (early genes), and gamma (late genes) (68). Alpha genes have regulatory functions and control the expression of beta and gamma genes. Transcriptions of beta genes follow, and generally encode proteins needed for replication of viral DNA. The gamma genes are transcribed up to 32 hours post-infection, and code for structural proteins that are expressed during and after viral DNA

replication (68, 104). It is believed that herpesvirus DNA replication occurs by a rolling circle mechanism with the formation of concatemers (16). Procapsids are formed in the nucleus with subsequent packaging of newly cleaved monomeric viral DNA. DNA-filled nucleocapsids bud from the nuclear membrane and acquire an envelope by migration through the inner lamellae of the nuclear membrane (54). The enveloped virions then migrate through the lumen of the endoplasmic reticulum into vacuoles and are released by exocytosis or cell lysis (54).

Followed by replication, at 7 to 10 days post-infection, ILTV can establish latency (7). During latent infection a limited number of viral genes are expressed and these transcripts are called latency-associated-transcripts (LATs). LATs usually originate from the right end of the unique long region or the inverted repeat flanking sequences. LATs are non-polyadenylated nuclear RNAs that are transcribed in opposite orientation to the immediate early genes; the LATs of ILTV have not been identified (151). The trigeminal ganglia are known to be an important site for latency of ILTV (7).

Strain Classification

It is clearly recognized that outbreak related ILTV strains vary in virulence. Some strains are highly virulent producing high morbidity and mortality in naive chickens, while strains of low virulence, which produce from mild to unapparent infection, have been reported (29, 75, 105, 106, 125, 130). In different laboratory systems ILTV strains have also shown differences in virulence and replication. Some strains produce increased mortality in chicken embryos (74), and others produce different plaque size and morphology in cell culture (118) and on the chorioallantoic membrane (CAM) of embryonated chicken eggs (106). Mortality patterns in embryonated chicken eggs were proposed as a biological system for the differentiation of ILTV

strains as they correlated closely with virulence (74). However, the correlation between *in vitro* and *in vivo* growth characteristics of the different ILTV strains and its relation to virulence is not known. More importantly, the biological differentiation of ILTV strains, particularly between outbreak circulating strains and modified-live vaccine viruses, remains an important practical problem, and an accurate standard protocol to determine the pathotype of ILTV strains has not been established.

Based on virus-neutralization, immunofluorescence tests, and cross-protection studies ILTV strains appear to be antigenically homogenous (29, 126). However, minor antigenic variation among strains has been observed through findings that some strains are neutralized poorly by heterologous antisera (106, 119, 126). Since the proteins and epitopes responsible for these minor antigenic differences have not been identified, as an alternative, changes in nucleic acid sequences among viruses have been widely utilized to differentiate among closely related viral strains. The methods utilized for differentiation of ILTV strains include restriction endonuclease analyses of viral DNA (59, 61, 87, 89), DNA hybridization assays (88), polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) analyses (PCR-RFLP) (24, 28, 31, 48, 51, 84, 100), PCR-RFLP and gene sequencing (60), and gene sequencing by itself (98). Restriction endonuclease cleavage of viral DNA and electrophoretic separation of DNA fragments has been shown to distinguish among different ILTV strains (87, 89), and has been used extensively in epidemiological studies to differentiate modified-live vaccine viruses from field outbreak isolates (5, 24, 28, 31, 48, 51, 59, 61, 62, 78, 79, 100). Polymerase chain reaction-RFLP analysis of the infected cell protein 4 (ICP4) gene has been shown to discriminate between vaccine and non-vaccine isolates from Taiwan (24) and Northern Ireland (51). In both reports, outbreak-related viruses obtained prior to the introduction

of modified-live ILTV vaccines were identified as non-vaccine viruses, while vaccine viruses were identified as the cause of outbreaks after the implementation of ILTV vaccination. Using a single nucleotide polymorphic site, previously identified in the ICP4 gene (51), a PCR-RFLP assay allowed the detection and differentiation of vaccine and non-vaccine viruses from field cases in the United Kingdom (31). In another study, PCR-RFLP combined with nucleotide sequence analysis of the glycoprotein G (gG) and the thymidine kinase (TK) genes allowed the differentiation of non-vaccine from vaccine isolates in Korea (60), and the analysis of both these genes allowed the identification of a viral isolate that may have originated from a recombination event between a vaccine and a non-vaccine isolate. Kirkpatrick et al. (84) utilized PCR-RFLP to differentiate among isolates of ILTV strains in Australia. They showed that reliable differentiation of ILTV strains required examination of multiple genes, and that most of the recent ILTV outbreaks in Australia were not caused by vaccine strains (84). In a recent study ILTV isolates from the United States were classified in six different PCR-RFLP groups and it was found that vaccine related, as well as, non-vaccine related isolates were associated with outbreaks of the disease (100). Nucleotide sequence analyses of the UL47 and gG genes allowed the identification of vaccine and non-vaccine viruses involved in ILTV outbreaks in Ontario (98).

Epidemiology of the disease

Infectious laryngotracheitis virus has worldwide distribution and in regions of highly dense poultry populations the reemergence and longer epidemics of the disease is a recently observed trend (19). In areas of intensive production such as in the United States, Europe, China, Southeast Asia, and Australia the disease is mostly controlled by the use of modified-live virus

vaccines. Seasonal incidence varies between states and outbreaks; however, since the development of intensive broiler production in the US, ILTV tends to occur throughout the year. Persistence of cases into the summer months and after cessation of broiler vaccination schemes also seems to be an emerging problem. Usually, during ILTV epidemics one poultry production area has been involved; however, the distribution of cases throughout different geographical areas of a state also seems to be increasing (34). Transmission between flocks has primarily been associated with proximity and a breakdown in biosecurity. Research has also shown that after backpassage in the field the chicken embryo origin (CEO) vaccine virus and, more rarely, the tissue culture origin (TCO) vaccine virus can revert to pathogenicity causing outbreaks of the disease (34). Direct or indirect contact with backyard fowl or game fowl has not been proven a common risk factor. Live haul trucks carrying flocks with active disease to the processing plant are considered a source of epidemics and the spreading of litter as soil fertilizer has also been connected with the spread of the disease (34).

Although, under experimental conditions, turkeys have been found to be susceptible to ILTV infection and the virus can be propagated in turkey embryos (143), within commercial poultry species, chickens have been recognized as the primary host of ILTV. Among non-commercial poultry, pheasants, partridges, and peafowl have been shown to be susceptible (30). Starlings, sparrows, crows, doves, ducks, pigeons, and guinea fowl appear to be resistant to the disease (11, 20, 123); although sub-clinical infection and seroconversion in ducks has been reported (144).

Infectious laryngotracheitis can be transmitted directly by inhalation, ingestion, or through the conjunctiva. Transmission studies determined that four days were required for the virus to replicate and transmit to other birds (37, 115). Mechanical transmission is one of the major

vectors to spread the infection and can occur via contaminated equipment, litter, and poultry workers (15, 39, 49, 81). Vertical transmission of ILTV has not been demonstrated (55).

Pathogenicity and Pathology

Clinical signs of the disease generally appear after six to twelve days of natural exposure (80, 124). Clinical signs of severe disease are characterized by conjunctivitis with closed eyes, nasal discharge, depression, sneezing, gasping, marked dyspnea, expectoration of bloody mucous, high morbidity and variable mortality (5 to 70%) (12, 29, 80, 106, 111, 139). Causes of mortality may be the result of a decrease on feed consumption and mucous plugs in the tracheal lumen producing asphyxiation. Gross lesions are characterized by mucoid inflammation, degeneration, and necrosis of the trachea. Diphtheritic changes are common and may be seen as mucoid casts that extend the entire length of the trachea. Severe hemorrhages into the trachea lumen may result in blood casts, and mucous can be mixed with blood and necrotic tissue (55).

In recent years mild forms of the disease have been frequently observed in high density populated poultry producing areas (29, 90, 106, 124, 125, 139). Clinical signs characteristic of the milder forms include decreased egg production, weight loss, watery eyes, mild tracheitis, swelling of infraorbital sinuses, persistent conjunctivitis, low morbidity and very low mortality (0.1 to 2%) (90, 125).

The curve of infection varies with the severity of lesions; usually, most chickens recover in 10 to 14 days (55). In mild forms of the disease gross lesions may be found in the conjunctiva and throughout the respiratory tract, but they are more commonly observed in the larynx and trachea, as a mild inflammation or excess of mucous to a severe hemorrhage tracheitis. In very

mild presentations, gross lesions may consist only of edema and congestion of the conjunctiva, the infraorbital sinus, and mucoid tracheitis (35, 90).

Microscopic lesions in the trachea change depending on the stage of the disease. As early as three days post infection intranuclear inclusion bodies are found in epithelial cells and are present only at the beginning of the infection (9, 57, 107, 135). As the viral infection progresses, epithelial cells in the respiratory tract enlarge, the trachea loses cilia and becomes edematous. The accumulation of lymphocytes, histocytes, and other multinucleated cells form syncytia; plasma cells migrate into the mucus and sub-mucosa after 2 or 3 days post-infection (PI). Late microscopic changes in the trachea are characterized by cell destruction and desquamation of the mucosal surface resulting in the loss of the epithelia covering and leaving a thin layer of basal cells.

In ILTV infection, virus replication is not restricted to the trachea. Other mucous membranes, where both vaccine and field viruses commonly replicate, are the conjunctiva and the respiratory sinuses. In certain cases viral replication has been detected in the air sacs and lungs as well; however, ILTV is characteristically highly cytolytic for the trachea, the virus is usually isolated from tracheal secretions during 6 to 8 days PI (9, 67, 108, 114), and remains at very low levels up to 10 days PI (142). No clear evidence exists for a viremic phase of infection.

Unapparent infection of the respiratory tract is a trait of ILTV persistence. Earlier observations by Komarov and Beaudette (85) and Gibbs (50) demonstrated a "field" carrier rate of approximately 2% for periods up to 16 months after a disease outbreak. Later, latent tracheal infections were demonstrated for similar periods of time in 50% or more of infected chickens (7, 133). As with other alphaherpesvirus, extra tracheal spread of ILTV to the trigeminal ganglia has been reported (9). The virus has been detected in the trigeminal ganglia of chickens 4 to 7

days after tracheal exposure. Intermittent and apparently spontaneous shedding of ILTV between 7 and 20 wk after infection (70, 71), and viral reactivation 15 months after vaccination had been reported (77). Hughes *et al.* (72) reported the re-excretion of ILTV from latently infected chickens following the stress of re-housing and the onset of production. Using polymerase chain reaction (PCR), the trigeminal ganglion was identified as a site of ILTV latency (142), and it was determined that latent infections of the trachea and the trigeminal ganglion can be simultaneously established by vaccine and challenge strains early after infection (62).

Immunity

Humoral and cell mediated immunity (CMI) responses are present after ILTV infection. Although antibodies are produced against the virus, the humoral immune response does not play a major part in the mechanism of protection (40, 112). Local secretory antibodies participate in recovery from infection but there is not correlation between the synthesis of these antibodies and absence of clinical signs (40, 41). In addition, it has been demonstrated that mucosal antibodies are not essential in preventing viral replication in vaccinated chickens (105). Virus neutralizing antibodies become detectable within 5 to 7 days PI and peak around 21 days PI, however there is no an association between levels of neutralizing antibodies and resistance to challenge (126). Maternal antibodies are transmitted to the offspring via the egg (18); nevertheless, this type of antibody does not confer protection to infection or interfere with vaccination (42, 128). On the other hand, CMI responses to ILTV infection, though they have not been well studied, are considered the main immune response responsible for protection (112, 150), Fahey *et al.* (41) demonstrated that protection against ILTV infection could be transferred by spleen cells and

peripheral blood leukocytes from congenic immune donors, which further verified that CMI responses are the main line of protection. The duration of the CMI response is not known.

Diagnosis

When clinical signs are severe a tentative diagnosis of ILTV can be made; however, in most cases other respiratory pathogens produce similar clinical signs. Particularly in mild forms of the disease a laboratory diagnosis for ILTV is essential. Diagnostic methods for ILTV include rapid detection methods as histopathological examination of intra-nuclear inclusion bodies, detection of ILTV antigens in tracheal tissues or respiratory mucus, detection of viral DNA or serology and virus isolation as a confirmatory test (132).

The most frequently utilized rapid test for ILTV diagnosis is histopathology examination of fixed tissues. Histopathology examination remains the standard method for the rapid diagnosis of ILTV. It has been shown that visualization of inclusion bodies may be less sensitive than virus isolation because intranuclear inclusion bodies appear exclusively during the early stages of infection (55). The advantage to histopathology is that it usually provides a definitive diagnosis within 24 hours. The disadvantages are that a trained pathologist is needed to provide an accurate diagnosis; the inclusion bodies are present only at an early stage of infection, and other avian viruses produce inclusion bodies (34, 55).

Virus isolation is the gold standard method for ILTV diagnosis. The best samples for virus isolation attempt include: tracheal swabs, tracheal scrapings, larynx, and eye conjunctiva swabs. These samples must be collected early in the course of infection. The virus can be isolated by inoculation of suspensions of respiratory and conjunctival exudates, or homogenates of appropriate tissues via the choroallantoic membrane (CAM) route of 9 to 12 day old

embryonated chicken eggs or chicken embryo kidney (CEK), chicken embryo liver (CELi), and chicken kidney (CK) cells (55). The most frequent method utilized for virus isolation is the inoculation of embryonated eggs via the CAM. Chorioallantoic membrane plaques or pocks can be observed as early as two days post-inoculation, and generally plaques have opaque edges and a central depress area of necrosis (66, 76). In cell culture (CEK, CELi, CK) the characteristic cytopathic effect produced by ILTV can be observed as early as 24 hours post-infection. ILTV cytopathic effect is typified by swelling of cells with displacement of chromatin, rounding nuclei, and formation of multinucleated giant cells or syncytia. One disadvantage of ILTV isolation in chicken cell cultures is that this virus is easily overgrown by other viruses such as adenovirus or/and reovirus (141). Virus isolation may take three to four passages before plaque formation or cytopathic effect appears in the CAM or cell culture. In a comparison study of CAM inoculation and a variety of cell cultures systems, Hughes and Jones (69) found that CELi cells were the most sensitive system for ITLV isolation, closely followed by CK cells. Both systems, CELi and CK cells, were superior to CAM inoculation.

Although virus isolation is a sensitive technique, definitive identification of ILTV is required after isolation, and it can be accomplished using specific antigen detection in infected cells by fluorescently labeled specific antibodies (FA) (136, 140), immunoperoxidase (IP) (57, 125, 130), polymerase chain reaction (PCR), or electron microscopy (EM). Electron microscopy has been utilized to detect ILTV upon visualization and morphological identification of the virus in tracheal scrapings (69, 134). This method is successful only when large numbers of viral particles are present in the sample. Viral antigens can be detected in tracheal tissues from day 2 to 14 PI either using FA or IP procedures (9, 57, 67, 140). Both FA and IP detection methods require a source of ILTV specific antibody; these antibodies have been prepared for use

with either animal immunization procedures (9, 67, 130, 140) or use of monoclonal antibody technology (2, 136, 150). Another procedure for detection of ILTV antigens in tracheal exudates is enzyme-link immunoabsorbant assay (ELISA) (97, 145). Antigen-capture ELISA was determined to be faster than virus isolation and more accurate than FA (145).

Molecular techniques have been added to the group of diagnostic assays for detection of ILTV nucleic acid including, restriction fragment length polymorphism (RFLP), in-situ hybridization, polymerase chain reaction (PCR), PCR-RFLP, and real-time PCR (1, 4, 22, 24, 28, 31, 73, 101, 141). These methods have been shown to be more sensitive than virus isolation and allow viral DNA detection in samples when other microorganisms are present (141). RFLP analysis of viral genomes has been used to differentiate field from vaccine strains and to provide evidence for strain variability among ITLV isolates (5, 59, 78, 87, 89, 100).

Since the development of PCR (96), this technique has played an important role as a research tool and in field identification. The principal applications of PCR are to detect small amounts of viral nucleic acid in clinical samples and to trace viral infection. Conventional PCR based assays have been used to successfully detect ILTV DNA from the trachea of experimentally (1), naturally infected chickens (73, 141), and from extra-tracheal sites such as the conjunctiva (4), and the trigeminal ganglia (62, 142). In addition, conventional PCR has proven to be useful to detect ILTV infected birds during both severe (141) and mild forms (125) of the disease.

Recently, Callison et al. (22) described a real time PCR assay capable of detecting and quantifying viral DNA expressed as genome copy number (GCN) $\log_{10}$ from tracheal and conjunctival swabs of naturally and experimentally infected birds. In this study (22) it was

demonstrated that viral genome copy number (GCN) values higher than 5 log₁₀ strongly correlated with positive virus isolation results.

Serological methods used to diagnosis ILTV by antibody production include, agar-gel immunodiffusion (AGID), virus neutralization (VN), indirect fluorescent antibody (IFA) testing, and ELISA. Virus neutralization (VN) test was first described to detect ILTV-specific antibodies in chicken serum using embryonated chicken eggs (21). Afterwards the use of cell cultures and microwell plates was described facilitating the measurement of ILTV neutralizing antibodies (26, 113, 118).

Enzyme-linked immunosorbent assay systems have been developed for detection and quantitation of ILTV-specific antibodies using purified whole virus as an antigen (94, 97, 148). Direct comparison of the serological assays demonstrated that all were valid systems for detecting and quantifying ILTV-specific antibodies (3). Although ELISA has some advantage over other serological techniques, such as higher sensitivity, rapid results and high throughput capabilities, some non-specific reactions have been reported (10).

Recently, an ELISA for detection of ILTV-specific antibodies was developed that utilized a recombinant *Escherichia coli*-expressed ILTV glycoproteins, gE and gp60 (23). It was shown that this recombinant-based ELISA differentiated between ILTV-vaccinated and unvaccinated/unexposed chickens, but sensitivity and specificity were not reported. The recent development of deletion mutants lacking immunogenic glycoproteins as possible vaccine candidates (38, 44, 45, 46), and the availability of ILTV- glycoproteins specific antibodies (136) might be suitable to facilitate the serological diagnosis, opening the possibility to develop ELISA technology capable of differentiating vaccinated from infected animals.

Even if the classical clinical signs of ILTV are present other respiratory diseases can produce the same scenario; therefore, a definitive diagnosis has to be achieved. Diseases which may produce clinical signs similar to ILTV include acute respiratory presentation of influenza virus, infectious bronchitis, diphtheritic form of avian poxvirus infection, and Newcastle disease virus, as well as other respiratory bacterial and fungi pathogens including, mycoplasmas, fowl cholera, and *Aspergillus spp.*

Control and Prevention

Since there is no effective treatment for ILTV, the disease is controlled and prevented by good biosecurity practices and vaccination. Although ILTV was the first poultry disease for which a successful vaccine was developed (14), it still remains a major problem in areas where dense bird populations exist. To control ILTV outbreaks, the most effective approach is a coordinated effort to achieve a rapid diagnosis, institution of the correct vaccination program, and prevention of virus spread to other production areas. Vaccination limits viral spread and abbreviates the duration of the disease when applied in the face of an outbreak (8). The first vaccines utilized for ILTV prevention were virulent viral strains administered onto the cloacal membrane, either by drop or brush (8). During the past forty years, the attenuation of virulent strains by sequential passages in cell culture or chicken embryos has been the source of live attenuated viruses utilized to generate protection when applied via infraorbital sinus (147), intranasal instillation (17), feather follicles (95), eye-drop (127), orally through drinking water (121), and by coarse spray (63). The route of vaccination is extremely important since some of the available live attenuated vaccines provide different grades of protection, particularly when applied by coarse spray or via the drinking water (47, 63). Therefore, careful attention must be

directed to dose and routes of vaccine application to ensure adequate immunization. Eye drop vaccination has been demonstrated to provide the most uniform protection and less severe reactions as compared to other vaccination methods (47, 63). Raggi and Lee (110) found that ILTV vaccines must contain a titer greater than 10^2 plaque-forming units/ml to induce satisfactory immunity when administered by routes other than the oral route, and virus concentration of 10^5 embryo infective dose was necessary to induce adequate protection through oral vaccination (65).

Although the administration of modified-live vaccines in the drinking water or by spray are the favored methods for rapid and mass application, several problems have been associated with these routes. When the procedure is not performed properly it may result in a large proportion of the flock failing to develop protective immunity or developing a rolling reaction (114). Failures with spray vaccination can be due to small droplet size spray or the use of excessive dose resulting in deep penetration of the respiratory tract and consequently producing severe reactions (27, 109). On the other hand, the use of low dosage in spray application can result in adverse reactions due to uneven flock vaccination that results in the back passage of the vaccine virus (34). Vaccination via the drinking water requires the vaccine virus to contact nasal epithelial cells by aspiration of virus through the external nares or choanae, which does not necessarily occur in chickens vaccinated through drinking water route (114).

Currently, there are two main types of modified-live vaccines commercially available, those attenuated by sequential passages in chicken embryos (chicken embryo origin-CEO), or by sequential passages in tissue culture (tissue culture origin-TCO). Experimental studies and field observations have allowed a wide evaluation of both commercially available modified-live vaccines, CEO and TCO. Laryngotracheitis vaccine viruses have been shown to spread readily

from vaccinated to non vaccinated chickens (6, 25, 63, 115, 121). Although modified-live vaccines provide adequate protection when administered properly, a variety of adverse effects including insufficient attenuation, production of latently infected carriers (7), and increased virulence as a result of bird-to-bird passage (56) have been previously reported and documented in the field (34). Spread of vaccine viruses may be avoided by using individual vaccination methods that ensure simultaneous infection with vaccine virus of all susceptible chickens and by reinforcing biosecurity measures on the farm.

Experimental evidence has indicated the involvement of modified-live vaccine viruses in outbreaks (34, 56, 58, 59, 100). Although virulence of all vaccine viruses was lower compared to the field isolates (58), vaccine viruses were shown to be indistinguishable from these isolates based on DNA-restriction endonuclease analyses (59), however, the virulence of vaccine viruses increased after bird-to-bird sequential passages, in the case of CEO causing severe respiratory disease and mortality, and in the case of TCO causing a milder respiratory response at the same backpassage level (56). Guy et al. (56) suggested that increased virulence of modified-live vaccine viruses might occur as a result of poor mass vaccination methods and lax biosecurity conditions that permit the sequential passage of vaccine viruses in the field.

Infectious laryngotracheitis virus vaccination programs vary depending on the type of bird production and the prevalence of the disease. Most commercial layers and broiler breeders in the US, particularly those that are raised in locations at high risk of exposure, are vaccinated against ILTV either with TCO vaccine by the eye-drop route, or with CEO vaccine applied in the drinking water, via eye drop or coarse spray (34). In the US, broilers are vaccinated only in the face of outbreaks, using CEO vaccines applied via the drinking water or by coarse spray (34, 55). This vaccination strategy has shown mixed (34). In the face of an outbreak in commercial

pullets, layers, and broiler-breeders vaccination may be also used successfully in reducing the spread of the disease within flocks. To obtain the best result the vaccine should be administered immediately after the diagnosis of an outbreak is confirmed (34).

The largest challenge for modified live vaccines to control ILTV is in the multi-age-layer flocks. When the vaccine is not properly administered, the presence of susceptible birds in multi-age layer flocks will result in the constant circulation of virus, and these viruses are considered the source of vaccine related outbreaks (34). In a previous report it was demonstrated that vaccine application via eyedrop route provided more uniform protection following a single dose compared with spray and drinking water routes (47). In contrast to this report (47), a second dose of modified-live vaccines may be unsuccessful in maintaining protection levels because the replication of vaccine virus can be neutralized by existing immunity (40, 147).

Even though vaccination with the live attenuated vaccines is widely utilized in breeders and layers, outbreaks in broiler flocks have been recognized in recent years as an emerging problem. Therefore, vaccination is necessary when these flocks are in the vicinity of ILTV outbreaks, in the middle of the outbreak, or when the disease has previously occurred on that farm (34, 55).

Vaccines based on recombinant DNA technology have been developed for ILTV, and they hold promise for the development of control and eradication programs. In one approach, immunogenic envelope proteins were expressed in the avian virus vector herpes virus of turkeys or attenuated fowl pox virus. The obtained recombinant viruses were shown to protect experimentally immunized chickens against a challenge infection with virulent ILTV (36, 120, 131). However, these recombinant viruses require individual application and are not suitable for mass application.

A second alternative is the use of stably attenuated ILTV mutants by direct deletion of virulence determinant genes. These mutants may then be capable of inducing protective immunity without the ability to produce disease (45, 53, 91, 99, 122, 137). An advantage of deletion mutant vaccines is that they can be used for mass application with low amounts of virus, therefore lowering cost. An additional advantage of deletion mutant vaccines is that they can be easily differentiated from field viruses genetically and by serology, and due to lower replication rates *in vivo* they may not transmit to unvaccinated chickens.

A recombinant fowlpox virus-vectored vaccine for immunization of chickens against LTV is commercially available in the United States (36). This vaccine is used for immunization of multi-age layer flocks. It is administered by wing-web inoculation of chickens that are at least eight weeks of age and at least four weeks prior to onset of egg production.

The future eradication of this disease can be feasible by enforcing biosecurity measures, using safer vaccines, and implementing diagnostic surveillance that can easily identify and differentiate the presence of vaccines or challenge viruses. In addition, in densely populated poultry areas the control of the disease greatly depends on a rapid and accurate diagnostic system followed by communication between the poultry industry and the government.

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CHAPTER 3
REPLICATION AND TRANSMISSION OF LIVE-ATTENUATED INFECTIOUS
LARYNGOTRACHEITIS VIRUS (ILTV) VACCINES ¹

¹ Andrés Rodríguez-Avila, Ivomar Oldoni, Sylva Riblet, and Maricarmen García. Accepted by *Avian Diseases*. Reprinted here with permission of Publisher, 27/07/2007.

Keywords: Infectious laryngotracheitis virus; chicken embryo origin vaccine; genome copy number; Infectious laryngotracheitis virus; tissue culture origin vaccine.

Abbreviations: CEO = chicken embryo origin; GCN: genome copy number; ILTV = infectious laryngotracheitis virus; ReTi-PCR = real time PCR; SPF = specific pathogen free; TCID₅₀ = 50% tissue culture infectious doses; TCO = tissue culture origin; VI = virus isolation.

SUMMARY

The aim of this study was to evaluate the replication of live attenuated infectious laryngotracheitis virus (ILTV) vaccines in selected tissues and their ability to transmit to contact-exposed birds. Four-week old specific pathogen free (SPF) chickens were eye drop-inoculated with the tissue culture origin (TCO) and chicken embryo origin (CEO) vaccines. Contact-exposed chickens were housed in direct contact to eye drop-inoculated chickens from the first day post-inoculation. Virus isolation and real time PCR were used to detect the presence of live virus and viral DNA, respectively, in the trachea, trigeminal ganglia, eye conjunctiva, cecal tonsils, and cloaca, from eye drop inoculated and contact exposed birds, at days 2, 4 to 10, 14, 18, 21, 24, and 28 post-inoculation. No differences were observed in the ability of the TCO and CEO vaccines to replicate in the examined tissues. Both vaccines presented a localized replication in the eye conjunctiva and the trachea. Both vaccines were capable of transmitting to contact-exposed birds, attaining peaks of viral DNA as elevated as those observed in inoculated birds. The CEO vaccine replicated faster and reached higher viral genome copy number (GCN) than the TCO vaccine in the conjunctiva and trachea of eye drop inoculated and contact exposed birds. The DNA of both vaccine viruses migrated to the trigeminal ganglia during early stages of infection. Although the CEO and TCO vaccines were not recovered from the cecal tonsils and the cloaca, low levels of viral DNA were detected in these sites during the peak of viral replication in the upper respiratory tract.

INTRODUCTION

Infectious Laryngotracheitis (ILT) is a viral upper respiratory disease of chickens responsible for serious production losses in the poultry industry due to moderate mortality and decreased egg production (14). Infectious laryngotracheitis virus (ILTV) or Gallid Herpesvirus 1 (GHV-1) (27) is a highly contagious avian pathogen that belongs to family *herpesviridae*, subfamily *alphaherpesvirinae*. Since first described by May and Tittsler in 1925 (24), the severe form of the disease has been characterized by clinical signs including watery eyes, hemorrhagic conjunctivitis, nasal discharge, respiratory rales, gasping, marked dyspnea, and expectoration of blood-stained mucous. Morbidity and mortality can vary depending on the viral strain, and the severe epizootic form of the disease cause morbidity up to 100% and mortality of 70% (14). Although it was the first poultry pathogen controlled by vaccination, ILT is still a major problem in areas where dense bird populations exist (3). Virulent viral strains were initially employed for vaccination and administered onto the cloacal membrane, either by drop or brush (6). During the past 40 years, the attenuation of virulent strains by sequential passages in tissue culture and embryonated eggs has been the source of live-attenuated vaccines (13, 29) and different application methods have been evaluated and utilized in the field. These methods include mass applications in the drinking water (30), spray vaccination (29), or individual-application by eye-drop (18). The route of vaccination is extremely important since some of the available live-attenuated vaccines provide different grades of protection, particularly when applied by coarse spray or the drinking water (12, 18). Eye-drop vaccination has been demonstrated to provide a more uniform protection (12), and less severe reactions as compared to spray vaccination (18).

Currently, there are two main types of ILTV live vaccines commercially available, those attenuated by sequential passages in chicken embryos (chicken embryo origin-CEO) or by sequential passages in tissue culture (tissue culture origin-TCO). Experimental studies and field observations have allowed a wide evaluation of both commercially available live-attenuated vaccines, CEO and TCO. These attenuated vaccines induce protection, preventing clinical signs and mortality (12, 13, 17), both can persist in apparently healthy birds (2, 20), and can spread from bird to bird (2, 13, 18). Gain of virulence after bird – to - bird passage has been reported, in the case of CEO causing severe respiratory disease and mortality, and in the case of TCO causing a milder respiratory response at the same back passage level (15).

Most commercial layers and broiler breeders in the US, particularly those that are raised in locations at high risk of exposure, are vaccinated against ILTV either with TCO vaccine by the eye-drop route, or with CEO vaccine applied in the drinking water, eye drop or coarse spray. In the US, broilers are vaccinated only in the face of outbreaks, using CEO vaccines applied via the drinking water or by coarse spray (11). Although vaccination with the live-attenuated vaccines is widely utilized in breeders and layers, most of the outbreaks occur in broilers. Molecular epidemiology studies suggest that the majority of broiler outbreak strains in the US are closely related to the CEO vaccines, while outbreaks with TCO type isolates are rare (16, 22, 23, 25). Despite the available field and experimental evidence indicating that live-attenuated vaccines behave differently, the replication and transmission of currently employed CEO vaccines and TCO vaccine have not been comprehensively compared.

To properly evaluate the replication of vaccine strains clearly established viral detection methods are required. Virus isolation is considered the reference standard method to verify an active viral infection. The sensitivity of virus isolation methods, using either chicken embryo

cells (liver, kidney, and lung), embryonated eggs, or adult chicken kidney cells was compared for their ability to propagate ILTV from the trachea of suspected ILT field outbreaks (19). Chicken embryo liver and adult chicken kidney cells demonstrated to have the best sensitivity of all the systems tested, and to be equally satisfactory for the isolation of ILTV from clinical samples (19). In addition to virus isolation, PCR has been a reliable diagnostic and research tool utilized for the detection of viral DNA from trachea, conjunctiva, and trigeminal ganglia (1, 17, 21, 33, 35). Recently, a real time PCR (ReTi-PCR) assay was developed for detection and quantification of viral nucleic acid in tracheas from experimentally and naturally infected birds. Viral genome copy number (GCN) values higher than $5 \log_{10}$ strongly correlated with virus isolation results (7). In this study samples from eye conjunctiva and trachea were collected as these been identified as the main sites of viral replication (4), and from the trigeminal ganglia as the main site of viral latency (33). In addition, cecal tonsils and cloacal swabs were collected to evaluate the possibility of viral shedding through the cloaca. The overall objective of this study was to compare the replication and transmission of the CEO and TCO vaccines at different time points post-inoculation in both eye drop inoculated and contact exposed chickens using virus isolation and quantitative ReTi-PCR.

MATERIALS AND METHODS

Chickens. Two separate trials were performed to compare the replication of the CEO and TCO vaccines. Ninety-six white leghorn specific pathogen free (SPF) chickens were obtained from Merial (Gainesville, GA) for each trial. The chickens were housed in stainless steel cages with filtered-air and positive-pressure at the Poultry Diagnostic and Research Center (PDRC,

Athens, GA), and fed a standard diet and water *ad libitum*. At four weeks of age, birds were divided into four groups of 24 chickens per cage, 12 of which were inoculated by eye drop, and 12 were contact-exposed to the inoculated chickens. Wing bands were used to identify contact-exposed chickens. Chickens were inoculated by eye-drop with the TCO or CEO live attenuated vaccine in separate experiments using the recommended dose per bird (0.033 ml). In the same room a total of 48 chickens were divided into four cages, 12 chickens per cage, and were utilized as negative controls during each experiment.

Vaccine viruses and vaccine titration. The live attenuated ILTV vaccines used in this experiment were Schering Plough (Omaha, NE) ILT-Vax® (TCO) (serial number 89364, expiration day May 26, 2009) and the Schering Plough (Millsboro, DE) Trachivax® (CEO) (serial number LT37/06, expiration day October 11, 2007). Vaccine titration was performed before and after inoculation in 96 well plates using chicken kidney (CK) cells prepared from 3-4 week old chickens (8×10^5 per ml) in five replicates from 10^{-1} to 10^{-9} dilutions. The 50% tissue culture infective dose (TCID₅₀) was estimated by the Reed and Muench method (26).

Sample collection and processing. Samples were collected from two eye drop-inoculated and two contact-exposed chickens at 2, 4 to 10, 14, 18, 21, 24, and 28 days post-inoculation. For the negative control group, samples were collected from one chicken every day from day 2 to 28 post-inoculation. Chickens were euthanized by CO₂ gas inhalation (Institutional Animal Care and Use Committee). Before euthanization, conjunctiva and cloacal swabs were collected and placed in 1 ml of sterile phosphate buffered saline solution (PBSS) containing a 2% antibiotic-antimycotic 100X (Gibco, Grand Island, NY) and 2% newborn calf

serum (Gibco, Grand Island, NY). After euthanasia, the trachea was dissected from the larynx to the bronchial bifurcation. The larynx and trachea epithelium was scraped. Scrapings were re-suspended in 1 ml of PBSS. The head was removed and the trigeminal ganglia extracted. After extraction, the trigeminal ganglia were minced and resuspended in 1 ml of PBSS. The intestines were exposed and the cecal tonsils were dissected, cut longitudinally, washed with PBS, minced, and re-suspended in 1 ml of PBSS. All samples were stored at -80°C until processing for virus isolation and DNA extraction.

Cell Culture. CK cells were prepared from 3-4 week old SPF chickens (Merial, Gainesville, GA). Chickens were euthanized by cervical dislocation (Institutional Animal Care and Use Committee). Kidneys were removed, washed with PBS, and minced to remove red blood cells. Kidney cells were disassociated in a 0.25% trypsin solution (Cellgro, Herndon, VA) at 37°C , stirred for 12 minutes, and trypsin was changed three to four times as needed. The cell suspension was centrifuged at 256 xg at 4°C for 12 minutes and cell pellets were resuspended in incomplete media after centrifugation. Cell density was adjusted to 8×10^5 cells per ml in complete media containing 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and 2% antibiotic-antimycotic 100X (Gibco, Grand Island, NY). Cells were seeded at 1ml per well into 24 well plates. After 24 hours the complete medium was replaced with fresh medium and plates were used 48 hours after seeding.

Virus Isolation. Chicken kidney Cells were inoculated in duplicate with 150 μl of sample per well in the 24 well plates. Samples from trachea, trigeminal ganglia, and cecal tonsils were inoculated directly into cells and incubated at 37°C , 5% CO_2 for five days. All samples were

passed three consecutive times in CK cells. Samples were considered positive by VI, when the cytopathic effect (CPE) characteristic of ILTV was observed, and were considered negative after three passages without observation of ILTV CPE. Before inoculation, samples were frozen and thawed three times. Samples were thawed at 37 °C, vortexed, and frozen for 24 hours at –80 °C. After the last thawing samples were vortexed and centrifuged for two minutes at 7500 rpm. The supernatant obtained was used to inoculate CK cells. Cloacal and eye conjunctiva swabs were filtered before inoculation.

DNA Extraction. DNA extraction was performed using the Qiaamp Mini kit (Qiagen, Valencia, CA) with modifications from the manufacturer's recommendations. Briefly, 100 µl of swab or tissue suspension was incubated with 10 µl of proteinase K and 400 µl of lysis buffer at 56° C for 10 minutes. After incubation, 100 µl of 100% ethanol was added to the lysate. Samples were washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

Real Time PCR Taqman Assay (ReTi-PCR). ReTi-PCR was executed as previously described (7). Primers and probe utilized in the assay are located in the viral glycoprotein C (gC) gene, and were synthesized by IDT (Coralville, IA) and BioSearch Technologies (Novato, CA). The final amplification reaction volume was 25 µl including; 12.5 µl of 2X master mix (Quantitect Probe PCR kit, Qiagen, Valencia, CA), primers to a final concentration of 0.5 µM, probe to a final concentration of 0.1 µM, 1 µl of HK-UNG (Epicentre, Madison, WI), 2 µl of water, and 5 µl of DNA template. The reaction was amplified with the Smart Cycler (Cepheid, Sunnyvale, CA) using a program of 50° C, 2 minutes; 95° C, 15 minutes; and 40 cycles of 94° C,

15 seconds; 60° C, 60 seconds with optics ON. For each ReTi ILTV assay reaction, the threshold cycle number (C_T value) was determined to be the PCR cycle number at which the fluorescence of the reaction exceeded 30 units of fluorescence. The GCN per amplification reaction was estimated using the standard curve equation ($y = -0.289x + 12.487$) and expressed as $\log_{10}$. The GCN $\log_{10}$ value reported per sample was either the average of two samples, when viral DNA was detected in both, or the value obtained for one sample.

RESULTS

Vaccine titrations. The CEO and TCO vaccines were titrated before and after chicken inoculation. The CEO vaccine titer in CK cells was $10^{5.32}$ and $10^{5.27}$ TCID₅₀/ml, before and after inoculation. The TCO vaccine titer in CK cells was $10^{5.75}$ and $10^{5.67}$ TCID₅₀/ml, before and after inoculation. A final dose per bird of $10^{4.27}$ and $10^{4.17}$ was applied of the CEO and TCO vaccines, respectively.

Virus Isolation. From all samples collected during the CEO and TCO experiments, vaccine viruses were isolated only from eye conjunctiva and trachea in inoculated and contact-exposed chickens. Vaccines were not rescued from trigeminal ganglia, cecal tonsils, or cloaca either from inoculated or contact-exposed chickens. Results on VI for the CEO and TCO experiments are presented in Tables 3.1 and 3.2. The CEO virus was isolated at days 2, 4, 5, and 6 from eye conjunctiva, and at days 2, 4, and 5 from trachea of inoculated chickens. In contact-exposed chickens the CEO virus was isolated at days 7, 8 and 9 from eye conjunctiva, and at days 8 and 9 from the trachea (Table 3.1). The TCO virus was isolated at days 4 and 6 from eye

conjunctiva, and at day 6 from trachea of inoculated chickens. In contact-exposed chickens the TCO virus was isolated at day 9 from both eye conjunctiva and trachea (Table 3.2). Parallel samples collected from negative controls were negative by VI after three passages in CK cells for both experiments.

Real Time PCR Taqman Assay (ReTi-PCR). Vaccine viral DNA was quantified and expressed as GCN $\log_{10}$ per sample (Fig. 3.1). CEO and TCO viral DNA was found in all samples collected from inoculated and contact-exposed chickens at similar times during infection (Fig. 3.1). Results of viral quantification by ReTi-PCR are summarized below for each tissue:

Eye conjunctiva. Viral DNA was detected in CEO inoculated chickens consecutively from days 2 to 14 (Fig. 3.1a), and in contact-exposed chickens from days 5 to 14 post-inoculation (Fig. 3.1b). The peak of GCN for inoculated and contact-exposed chickens, was attained at days 4 ($10^{6.2}$) and 8 ($10^{5.7}$) post-inoculation. Viral DNA was detected in TCO inoculated chickens from day 4 to 14, and in contact-exposed chickens from days 5 to 14 post-inoculation. The peak of GCN was reached at days 6 ($10^{5.1}$) and 9 ($10^{5.7}$) for TCO inoculated and contact-exposed chickens (Fig. 3.1a and 3.1b).

Trachea. Viral DNA was detected in CEO inoculated chickens consecutively from days 2 to 7, and at day 10 (Fig. 3.1c). In contact-exposed chickens viral DNA was detected at days 4, 5, 8, 9, and from days 14 to 21 post-inoculation (Fig. 3.1d). The peak of GCN for inoculated chickens was attained at day 4 (10^6). In contact-exposed chickens the GCN reached a peak at day 9 ($10^{4.3}$). Viral DNA was detected in TCO inoculated chickens at days 2, 4, 6, 7, and 14 (Fig. 3.1c), and in

contact-exposed chickens at days 9, 18 and 21 (Fig. 3.1d). Viral GCN peaks were also detected at day 6 (10^5), and at day 9 ($10^{5.1}$) from inoculated and contact-exposed chickens, respectively.

Trigeminal ganglia. A GCN range from $10^{1.62}$ to $10^{2.2}$ was detected in the trigeminal ganglia of CEO inoculated chickens at days 2, 4, and 6 (Fig. 3.1e), and in contact-exposed chickens at days 4, 5, 8 and 9 (Fig. 3.1f). In the same way, a GCN range of $10^{1.7}$ to $10^{2.46}$ was detected in the trigeminal ganglia of TCO inoculated chickens at days 4, 6, and 8 (Fig. 3.1c), and at days 4 and 9 in contact-exposed chickens (Fig. 3.1f).

Cecal tonsils. In CEO inoculated chickens a GCN range of 10^2 to $10^{2.7}$ was detected from days 2 to 6, and at day 21 (Fig. 3.1g), and in contact-exposed chickens at days 8 and 18 post-inoculation (Fig. 3.1h). In TCO inoculated chickens viral DNA was detected at days 6 ($10^{2.2}$) and 8 ($10^{1.94}$) (Fig. 3.1g), and in contact-exposed chickens at day 18 ($10^{2.7}$) post-inoculation (Fig. 3.1h).

Cloaca. In CEO inoculated chickens viral DNA was detected from only one sample at day 5 ($10^{2.6}$) (Fig. 3.1g), and in two samples from contact-exposed chickens at days 4 ($10^{2.1}$) and 9 ($10^{1.7}$) post-inoculation (Fig. 3.1h). In TCO inoculated chickens viral DNA was detected at days 8 ($10^{1.44}$) and 9 ($10^{1.71}$) (Fig. 3.1g), and in contact-exposed chickens at days 4 ($10^{1.8}$) and 9 ($10^{2.1}$) (Fig. 3.1h).

Parallel samples collected at similar times post-inoculation from negative control chickens during both experiments were all negative by Re-Ti PCR.

DISCUSSION

The replication and transmission of the TCO and CEO vaccines was evaluated in specific pathogen free (SPF) chickens after the administration of the vaccines via eye-drop exposure. The estimated TCID₅₀ titer in CK cells was similar for both vaccines. The replication of the vaccines for the trachea, eye conjunctiva, trigeminal ganglia, cecal tonsils and cloaca was initially assessed by virus isolation. Vaccine viruses were only isolated from trachea and the eye conjunctiva, from either inoculated or contact-exposed chickens. Indicating that of the tissues examined, these were the main sites of replication of the vaccine viruses. The CEO virus was recovered from inoculated chickens from eye conjunctiva and trachea as early as two days post-inoculation, while the TCO vaccine virus was first recovered at day 4 from the eye conjunctiva, and at day 6 from the trachea. The CEO virus was recovered from 4 of 8 chickens during days 2 to 6, whereas the TCO virus was isolated from only 1 of 8 chickens during the same time frame post-inoculation. The earlier recovery and frequency of isolation of the CEO vaccine virus from inoculated and contact-exposed chickens demonstrated that the CEO vaccine virus replicates and spreads faster than the TCO vaccine.

Real Time PCR (Re-Ti PCR) was a very useful tool in the evaluation of the vaccine replication and transmission. Callison *et al.*, (7) previously reported that a viral GCN equal or higher to $10^{5.0}$ was required per sample for successful virus isolation in chicken embryo chicken kidney cells from the trachea of experimentally and naturally infected chickens. In this study virus isolation, using CK cells, was possible in samples with a GCN equal or higher than $10^{4.3}$. These results suggest that in samples with GCN lower than $10^{4.3}$, either actively replicating virus

was absent, or the sensitivity of the virus isolation system utilized was not sufficient to detect lower levels of virus.

Earlier experiments have demonstrated that the viral replication in the eye conjunctiva is clearly associated with the route of vaccine inoculation (4, 29). In this study both vaccine viruses replicated rather efficiently in the eye conjunctiva as demonstrated by virus isolation and confirmed by GCN values from inoculated as well as contact-exposed chickens. In contact-exposed chickens both vaccine viruses replicated in the eye conjunctiva attaining peaks of viral DNA as elevated as those observed in inoculated chickens, indicating that the eye conjunctiva serves as a site of viral replication for both vaccines. The GCN values obtained during the course of the experiment for the eye conjunctiva, in CEO and TCO inoculated chickens, represented a normal curve of vaccine virus replication where the virus initially replicates to high numbers and is gradually eliminated by the immune system (Fig. 3.1a). On the other hand, the GCN values obtained for the CEO contact-exposed chickens from the eye conjunctiva mimics a natural infection curve (Fig. 3.1b). The virus infects the chickens through a natural route of entry, followed by a consistent increase in viral DNA until attaining the peak of viral replication, with a subsequent decrease in viral DNA when the virus is cleared from the eye conjunctiva. As opposed to CEO a gradual increase in TCO viral DNA was not observed in contact-exposed chickens during early stages of infection (Fig. 3.1b), further indication that the TCO vaccine replicates less aggressively than CEO.

Active replication in the trachea, after ocular inoculation, was demonstrated for both vaccine viruses during the first week post-inoculation. Both vaccines reached a peak of viral DNA in the trachea between 4 and 6 days post-inoculation. Viral DNA from both vaccines disappeared from the trachea only to appear in low levels at days 10 and 14. The disappearance

of the virus from the trachea after the first week post-replication has been previously reported for pathogenic (4) and vaccine strains (35), and coincides with the intermittent appearance of neutralizing antibodies in the trachea (9, 35). The pattern of detection of viral DNA in the trachea of contact-exposed chickens was considerably different than the pattern observed in inoculated chickens. Compared to inoculated chickens, where a peak of viral DNA was attained; in contact-exposed chickens, an intermittent appearance of viral DNA was observed. This is probably the outcome of the individual variation among chickens exposed to air borne transmission, quantity and frequency of the exposure, and eventually the role of local immunity (10). Moreover, different than in inoculated chickens where low levels of viral DNA reappeared in the trachea at 10 and 14 days post-inoculation, in the trachea of contact-exposed chickens CEO and TCO viral DNA persisted up to 21 days.

The extra-tracheal spread of ILTV to the trigeminal ganglia, and the ability of field and vaccine strains to establish a latent infection in this site has been clearly documented (5). It is believed that similar to other alpha-herpesviruses, ILTV migrates from the eye to the trigeminal ganglia via the neural pathways (31, 32). The detection of viral DNA in the trigeminal ganglia of inoculated chickens demonstrated that both vaccines could reach the ganglia. In contact-exposed chickens, viral DNA from both vaccines was detected in the trigeminal ganglia as early as 4 days post-exposure, before the detection of viral DNA in the eye conjunctiva. The early detection of viral DNA in the trigeminal ganglia of contact-exposed chickens may reflect the ability of the virus to reach the ganglia through the nasal cavity enervations after inhalation of air borne virus.

This result further confirms the involvement of the trigeminal ganglia during the early pathogenesis of ILTV infection (5). Bagust *et al.*, (5) reported the re-isolation of the pathogenic strain CSW-1 from the trigeminal ganglia 6 days after conjunctival exposure. In this study the

absence of virus isolation from the trigeminal ganglia may reflect the lack of sensitivity of the chicken kidney cells to detect the vaccine viruses in the ganglia during early stages of infection. On the other hand, the ReTi-PCR assay was capable of detecting viral DNA in the ganglia during the early stages of infection, but not during late stages of infection. Using nested-PCR Han and Kim (17) detected vaccine viral DNA in the trigeminal ganglia 21 days post-inoculation, indicating that the sensitivity of nested PCR was required for the detection of latently infected chickens.

Similarly to the trigeminal ganglia, neither vaccine virus was isolated from the cecal tonsils nor cloaca; however, low levels of viral DNA were detected in the cecal tonsils as early as two days post-inoculation, and 5 days post CEO inoculation in the cloaca. Viral DNA in the cecal tonsils and cloaca was detected at the same time points when elevated GCN values were detected in the eye conjunctiva and trachea. It has been reported that different strains of ITLV from the United States, including the parental strain of the CEO and TCO vaccines utilized in this study, have the ability to infect macrophages (8). An explanation for the presence of viral DNA in the cecal tonsils, and consequently in the cloaca is that macrophages and/or other cells of the immune system carry the virus during the peak of viral replication, or viral DNA may also reach the cloaca by direct gut transmission. The lack of virus isolation, and the low levels of viral DNA detected in the cecal tonsils and cloaca, suggests that neither vaccine virus actively replicates in these sites during early stages of infection. Further studies are necessary to determine if active shedding of the virus through the cloaca is a characteristic of more pathogenic field isolates and consequently of importance to understand the epidemiology of the disease.

Overall this study it was demonstrated that ILTV vaccines had a similar replication pattern, both presented a localized replication in the eye conjunctiva and the trachea, and both were capable of transmitting to contact-exposed chickens. The earlier recovery, frequency of isolation, and higher viral GCNs detected in inoculated and contact-exposed chickens proved that the CEO vaccine replicates and spreads faster than the TCO vaccine.

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Table 3.1. Virus isolation for chicken embryo origin (CEO) inoculated and contact-exposed chickens.

Days Post-Inoculation	Eye conjunctiva		Trachea	
	Inoculated	Contact-exposed	Inoculated	Contact-exposed
2	1/2 ^A	0/2	1/2	0/2
4	2/2	0/2	1/2	0/2
5	2/2	0/2	2/2	0/2
6	2/2	0/2	0/2	0/2
7	0/2	2/2	0/2	0/2
8	0/2	2/2	0/2	1/2
9	0/2	2/2	0/2	1/2
10	0/2	0/2	0/2	0/2
14	0/2	0/2	0/2	0/2
18	0/2	0/2	0/2	0/2
21	0/2	0/2	0/2	0/2
24	0/2	0/2	0/2	0/2
28	0/2	0/2	0/2	0/2

^A Number of samples positive for virus isolation per day.

Table 3.2. Virus isolation for tissue culture origin (TCO) inoculated and contact-exposed chickens.

Days Post-Inoculation	Eye conjunctiva		Trachea	
	Inoculated	Contact-exposed	Inoculated	Contact-exposed
2	0/2 ^A	0/2	0/2	0/2
4	1/2	0/2	0/2	0/2
5	0/2	0/2	0/2	0/2
6	2/2	0/2	1/2	0/2
7	0/2	0/2	0/2	0/2
8	0/2	0/2	0/2	0/2
9	0/2	1/2	0/2	1/2
10	0/2	0/2	0/2	0/2
14	0/2	0/2	0/2	0/2
18	0/2	0/2	0/2	0/2
21	0/2	0/2	0/2	0/2
24	0/2	0/2	0/2	0/2
28	0/2	0/2	0/2	0/2

^A Number of samples positive for virus isolation per day.

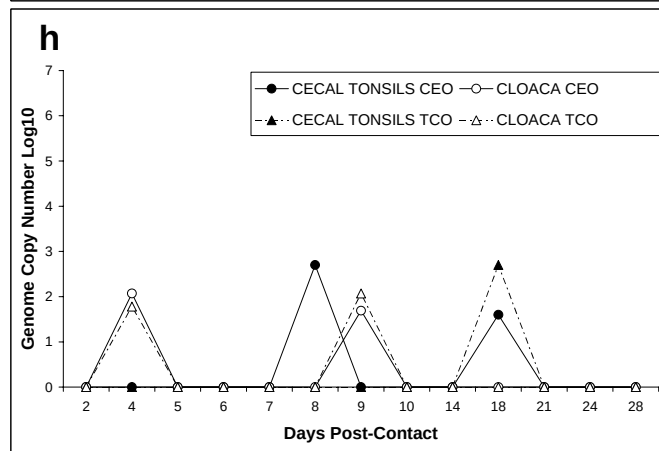
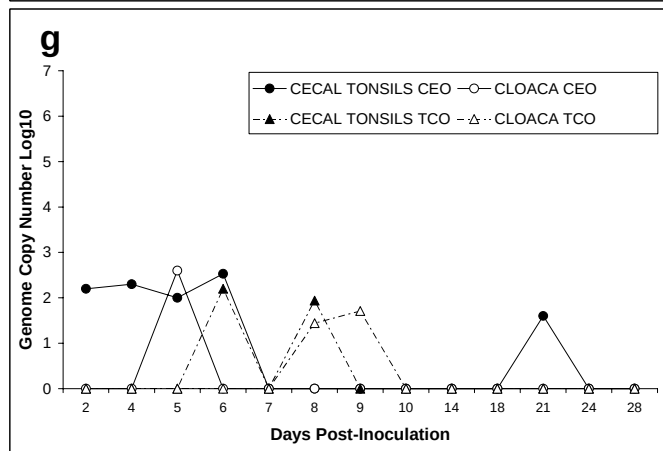
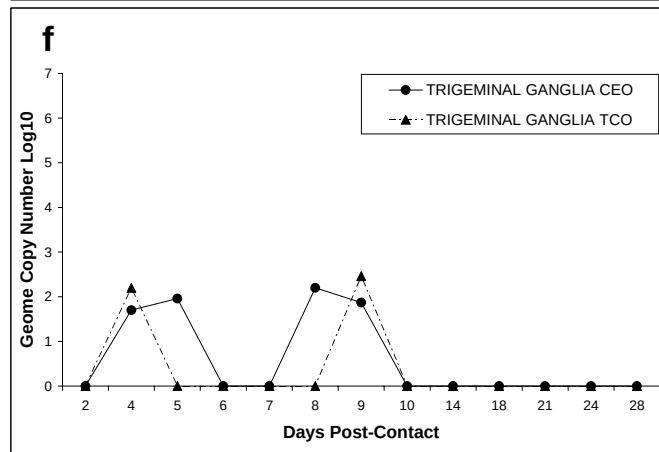
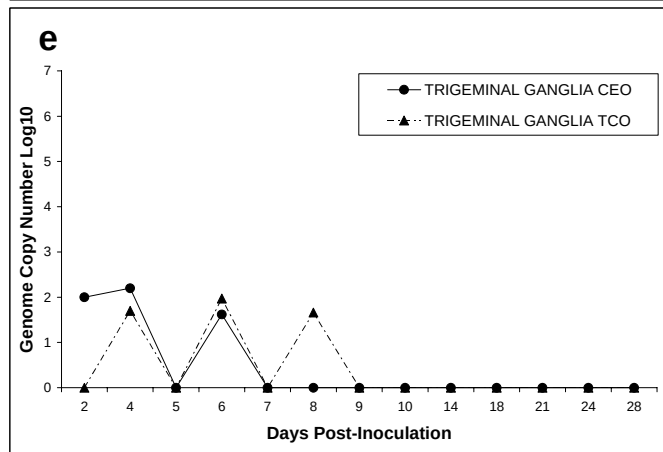
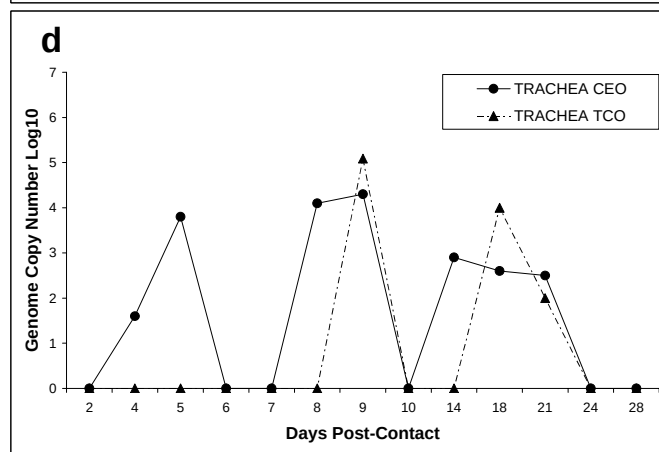
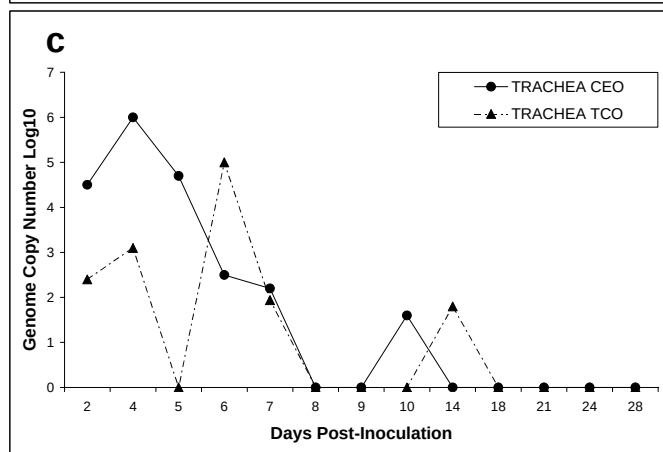
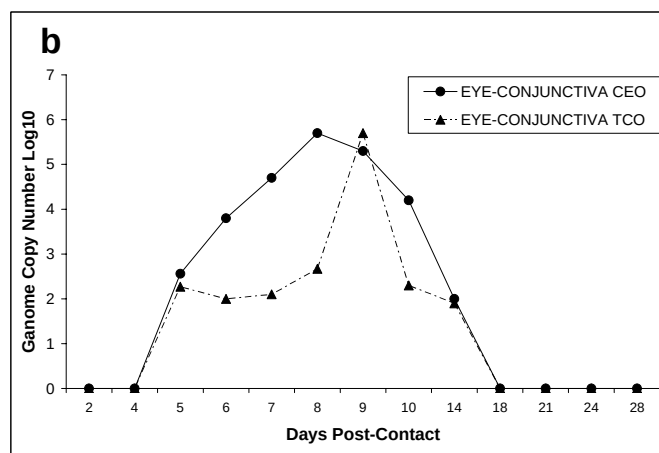
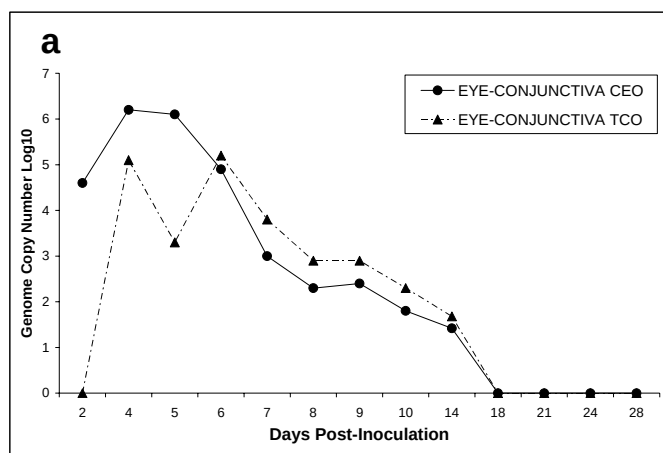


Figure 3.1. Viral genome copy number $\log_{10}$ detected per sample by Real Time PCR Taqman Assay (ReTi-PCR) from CEO and TCO vaccines inoculated and contact-exposed birds. Graphs a, c, e and g correspond to samples from inoculated chickens and graphs b, d, f, and h correspond to samples from contact-exposed chickens. Graphs are separated by tissues tested: a, and b eye conjunctiva; c and d trachea; e and f trigeminal ganglia; g and h cecal tonsils and cloaca.

CHAPTER 4
CHALLENGE STUDY FOR EVALUATION OF LIVE ATTENUATED VACCINES
AGAINST INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) ¹

¹ Andrés Rodríguez-Avila, Ivomar Oldoni, Sylva Riblet, and Maricarmen García. Submitted to Avian Pathology, 10/23/2007.

ABSTRACT

Infectious laryngotracheitis virus (ILTV) is a highly contagious agent that causes an acute respiratory infection in chickens. The disease affects growth, egg production and leads to significant economic losses during periodic outbreaks. Live attenuated vaccines (chicken embryo origin [CEO] and tissue culture origin [TCO]) have been widely used to control the disease in the United States of America (USA). It is believed that most of the outbreaks in the USA are caused by vaccine related isolates that persist in the field. In a recent study, some field viruses were characterized as genotypically different (Group VI) from the vaccines. The objective of this study was to evaluate the protection elicited by the CEO and TCO vaccines against a field isolate from group VI in vaccinated and contact-exposed chickens. Protection was assessed after four weeks of vaccination by scoring clinical signs and mortality, quantifying weight gained, and evaluating viral shedding to sentinel chickens by real time PCR and virus isolation from day 2 to 12 post-challenge. Significant evidence was obtained from the evaluated parameters to demonstrate that CEO and TCO eye drop vaccinated chickens were protected, while chickens contact-exposed to vaccinates were not protected against challenge. The data obtained from sentinel chickens suggested that the CEO and TCO vaccinated chickens did not shed the challenge virus up to 12 days post-challenge. The presented challenge model is a reliable tool to evaluate protection induced by live attenuated ILTV vaccines, and it can be applied to evaluate the safety and efficacy of the newly developed ILTV vaccines.

INTRODUCTION

Infectious laryngotracheitis is a highly contagious disease of chickens that may cause severe production losses due to morbidity, mortality, decreased egg production, weight loss, and/or predisposition to other respiratory avian pathogens (Guy & Bagust, 2003). Infectious laryngotracheitis virus (ILTV) belongs to family *herpesviridae*, subfamily *alphaherpesvirinae*, and it is taxonomically classified as *Gallid herpesvirus 1* (Davison, 2006). The severe form of the disease is characterized by watery eyes, hemorrhagic tracheitis, conjunctivitis, nasal discharge, respiratory rales, gasping, marked dyspnea, and expectoration of blood-stained mucous. Morbidity and mortality can vary depending on the viral strain. The severe epizootic form of the disease causes morbidity up to 100% and mortality of 70% (Guy & Bagust, 2003).

The two main types of ILTV live attenuated vaccines commercially available in the United States are those attenuated by sequential passages in chicken embryos (chicken embryo origin [CEO]), and those attenuated by sequential passages in tissue culture (tissue culture origin [TCO]). These attenuated vaccines induce protection, preventing clinical signs and mortality (Gelenczei & Marty, 1964; Fulton *et al.*, 2000; Han & Kim, 2003). Both can persist in apparently healthy birds (Andreasen *et al.*, 1989; Hughes *et al.*, 1989) and can spread from vaccinated to unvaccinated birds in close contact (Gelenczei & Marty, 1964; Hilbink *et al.*, 1987; Andreasen *et al.*, 1989; Rodriguez-Avila *et al.*, 2007). The route of vaccination is extremely important since some of the available live attenuated vaccines provide different grades of protection, particularly when applied by coarse spray or in the drinking water (Hilbink *et al.*, 1987; Fulton *et al.*, 2000). Eye-drop vaccination has been demonstrated to provide a more

uniform protection (Fulton *et al.*, 2000), and less severe reactions as compared to spray vaccination (Hilbink *et al.*, 1987).

Outbreaks of mild to moderate forms of the disease are common in commercial layer flocks worldwide, while sporadic outbreaks of ILT in broiler flocks have also been recognized as an emerging problem in several countries including the USA (Davison, 2005). Molecular epidemiology studies suggest that the majority of broiler outbreak strains in the USA are closely related to the CEO vaccines, while outbreaks with TCO type isolates are rare (Guy *et al.*, 1989; Keller *et al.*, 1992; Keeler *et al.*, 1993). Oldoni & Garcia (2007) reported the use of polymerase chain reaction and restriction fragment polymorphisms (PCR-RFLP) to examine the genotype of 25 isolates from commercial poultry, back yard flocks, and the commonly utilized commercially available live attenuated vaccines (CEO and TCO). In this study, commercial poultry isolates were genotyped into four groups (III, IV, V, VI). Groups III, IV and V were closely related to attenuated live vaccine strains, while group VI isolates were characterized as different from the vaccine strains (Oldoni & Garcia, 2007). The group VI viral genotype was first identified in 2004 in a single USA state, and during 2006 and 2007 was again identified in outbreaks in two states (Oldoni *et al.*, 2007). Furthermore, when compared to the CEO vaccine, group VI isolates were more pathogenic, and showed decreased ability to replicate in chicken kidney cells (Unpublished data, Oldoni *et al.*). The protection efficacy of live attenuated vaccines has been evaluated against a variety of ILTV strains by clinical signs, mortality, viral recovery and spread (Gelenczei & Marty, 1964; Hilbink *et al.*, 1987; Fulton *et al.*, 2000; Han & Kim 2003). The protection induced by CEO and TCO vaccines against most current USA isolates has not been evaluated. The objective of this study was to evaluate the protection induced by these vaccines

against a current group VI ILTV isolate. Protection was assessed scoring clinical signs and mortality, quantifying weight gain, virus isolation, serology, and viral shedding.

MATERIALS AND METHODS

Experimental design. A total of one hundred and sixty leghorn specific-pathogen-free (SPF) chickens were obtained from Merial (Gainesville, GA, USA). The chickens were distributed in 8 polycarbonate plexiglass isolation units with filtered-air and positive-pressure at the Poultry Diagnostic and Research Center (PDRC, Athens, GA, USA), and fed a standard diet and water *ad libitum*. At four weeks of age, two groups of 20 chickens were vaccinated, one with TCO (TCOVx) and the other with CEO (CEOVx) (refer to Virus strains and titration). The day after vaccination, 10 chickens from each vaccinated group were moved into two units containing 10 non-vaccinated chickens each. Two more groups of 10 non-vaccinated chickens were placed together with the remaining 10 vaccinated chickens in each unit. Finishing with four units containing a total of 20 chickens, 10 vaccinated and 10 non-vaccinated. The non-vaccinated chickens were utilized as contact-exposed chickens (CT-TCOVx and CT-CEOVx) to vaccinates. Wing bands were used to identify contact-exposed chickens. Chickens were vaccinated via eye-drop using the recommend dose per bird (0.033 ml). The remaining 80 chickens were randomly distributed into four groups of 20 chickens per isolation unit.

Four weeks after vaccination, the 40 contact-exposed chickens, 20 CT-TCOVx and 20 CT-CEOVx, were removed from the isolation units holding vaccinated chickens (TCOVx and CEOVx) and placed in two different isolation units. At the same time, 20 TCOVx, 20 CEOVx, 20 CT-TCOVx, 20 CT-CEOVx, and 10 non-vaccinated (NVx) chickens were challenged (Ch).

Chickens were challenged by inoculation of a final dose per chicken of $10^{3.02}$ 50% tissue culture infective dose (TCID₅₀) in a total volume of 200 µl, 50 µl in each eye and 100 µl in the trachea.

One day post-challenge, 50 non-vaccinated chickens were divided in five groups of 10 chickens each and placed in the four units holding vaccinated chickens and in the unit holding non-vaccinated-challenged chickens (NVx-Ch). These newly placed non-vaccinated chickens were used as sentinels for the vaccinated-challenged groups (SE-TCOVx-Ch and SE-CEOVx-Ch), and the non-vaccinated-challenged group (SE-NVx-Ch). Twenty chickens were used as a non-vaccinated-non-challenged group (NVx-NCh).

Samples Collection. Tracheal swabs were collected at day 9 after vaccination from five chickens of each CT-TCOVx and CT-CEOVx groups for real time PCR. Trachea and eye-conjunctiva swabs were collected from two chickens of the SE-TCOVx-Ch and SE-CEOVx-Ch groups and from two chickens of the SE-NVx-Ch and NVx-NCh groups every day from day 2 to 12 post-challenge. Swabs were placed in 1 ml of sterile phosphate buffered saline solution (PBSS) containing 2% antibiotic-antimycotic 100X (Gibco, Grand Island, NY, USA) and 2% newborn calf serum (Gibco, Grand Island, NY, USA). All samples were stored at -80 °C until processing for virus isolation and DNA extraction.

Virus strains and titration. The live attenuated ILTV vaccines used in this study were the Schering Plough (Omaha, NE, USA) ILT-Vax® (TCO) (serial number LX06/07, expiration date January 24, 2009) and the Schering Plough (Millsboro, DE, USA) Trachivax® (CEO) (serial number LT51/07, expiration date August 23, 2008). The challenge virus utilized in this study was identified as 2/A/04/BR, isolated from broilers, and classified by multiple PCR-RFLP

as a member of group VI genotype (Oldoni & Garcia, 2007). It was plaque purified in the chicken liver tumor cell line LMH (Kawaguchi *et al.*, 1987), and passed three times on chicken kidney (CK) cells. Vaccine and challenge virus titrations were performed in 96 well plates of CK cells prepared from 3 - 4 week old chickens, using a final concentration of 8×10^5 cells per ml in five replicates from 10^{-1} to 10^{-10} dilutions. The TCID₅₀ titer was estimated by the Reed and Muench method (Reed & Muench, 1938).

Clinical signs and body weight. Clinical signs were scored every day from day 2 to 12 post-challenge in TCOVx-Ch, CEOVx-Ch, CT-TCOVx-Ch, CT-CEOVx-Ch, NVx-Ch, and NVx-NCh groups from five chickens per group. To score clinical signs from the same bird every day, chickens were identified using vegetable colors sprayed on the chicken wings feathers. Breathing patterns, conjunctivitis, and the level of depression were evaluated and scored daily for all groups of chickens. Breathing patterns were scored on a scale of 0 (normal breathing), 1 (open mouth breathing), and 2 (gasping with an extended neck). Conjunctivitis was scored on a scale of 0 (normal), 1 (swollen and partial closure of the eyes), and 2 (complete closure of the eyes). The level of depression was scored on a scale of 0 (normal behavior), 1 (mildly depressed), and 2 (severely depressed). Mortality was given a score of three. All chickens were weighed the day before vaccination (four-weeks of age), the day pre-challenge (eight-weeks of age), and at day 12 post-challenge. The average weight gain and clinical signs scores were calculated for each group.

Virus Isolation. Virus isolation was performed in adult chicken kidney (CK) cells as previously described (Rodríguez-Avila *et al.*, 2007). Briefly, cells were seeded at 100 µl per

well into 96 well plates. After 24 hours, cells were inoculated in triplicate with 70 µl of sample per well. All samples were passed three consecutive times in CK cells. Samples were considered positive by virus isolation when the cytopathic effect (CPE) characteristic of ILTV was observed, and they were considered negative after three passages without observation of ILTV CPE. Before inoculation, samples were frozen and thawed three times. Samples were thawed at 37 °C, vortexed, and frozen at –80 C. After thawing, samples were vortexed and centrifuged for three minutes at 1024 x g, and the supernatant was used to inoculate CK cells.

DNA Extraction. The DNA extraction was executed using the MagaZorb DNA Mini-prep 96-well kit (CORTEX BIOCHEM_{TM}, San Leandro, CA, USA) according to the manufacturer instructions.

Real Time PCR Taqman Assay (ReTi-PCR). ReTi-PCR was performed as previously described (Callison *et al.*, 2007). The primers and probe utilized in the assay were located in the viral glycoprotein C gene, and were synthesized by IDT (Coralville, Iowa, USA) and BioSearch Technologies (Novato, California, USA). The genome copy number (GCN) log₁₀ per amplification reaction was estimated using the standard curve equation ($y = -0.289x + 12.487$) generated from the gC plasmid and expressed as log₁₀. The GCN log₁₀ value reported was the average of two samples.

Serology. Ten blood samples were collected before vaccination (four-weeks of age), pre-challenge (eight-weeks of age), and 12 days post-challenge per group. Sera were analyzed with a commercial LT ELISA kit (ProFLOCK[®] LT ELISA Kit, Synbiotics Corp., San Diego, CA).

Statistical Analysis. The Kruskal-Wallis test with post-hoc pair-wise comparisons using the Mann-Whitney test was used to analyze and compare the data obtained from clinical signs scores. One-way ANOVA with post-hoc multiple comparisons using Fisher's least significant difference (LSD) was used to compare and analyze percentage of body weight gained. The Fisher's exact test was used to compare and analyze the incidence of mortality.

RESULTS

Virus titration. The CEO and TCO vaccines were titrated before and after vaccination. The CEO vaccine titer in CK cells was $10^{4.70}$ and $10^{4.59}$ TCID₅₀/ml before and after vaccination. The TCO vaccine titer in CK cells was $10^{4.92}$ and $10^{4.79}$ TCID₅₀/ml before and after vaccination. A final dose of $10^{3.21}$ and $10^{3.39}$ per chicken was applied of CEO and TCO vaccines, respectively. The titer of the group VI challenge virus in CK cells was $10^{3.77}$ and $10^{3.69}$ TCID₅₀/ml pre- and post-challenge. A final viral dose of $10^{3.02}$ TCID₅₀/200 µl was applied per chicken.

Real Time PCR Taqman Assay (ReTi-PCR) and Virus Isolation. Viral DNA was detected in all five tracheal swabs collected at day 9 after vaccination from each chicken in the contact-exposed groups with an average of $10^{4.1}$ and $10^{4.4}$ GCN log₁₀, and identified by PCR-RFLP as TCO and CEO vaccine viruses, respectively (data not shown). From all samples collected post-challenge, viral DNA was detected and virus was isolated only in samples from the SE-NVx-Ch group. Viral DNA was detected from day 6 to 12 post-challenge in the eye conjunctiva and from day 7 to 12 in the trachea (Figure 4.1). The peak of viral DNA was observed for eye conjunctiva and trachea at day 9 post-challenge with $10^{5.8}$ and $10^{5.3}$ GCN log₁₀,

respectively. The challenge virus was isolated from eye conjunctiva and trachea at days 8, 9, and 10 post-challenge from all samples collected from the SE-NVx-Ch group, and identified by PCR-RFLP as group VI viral genotype (data not shown). Samples with GCN equal to or higher than $10^{4.27}$ were positive for virus isolation. Samples from SE-TCOVx-Ch, SE-CEOVx-Ch and NVx-NCh groups were all negative. The challenge virus was isolated from CT-CEOVx-Ch and CT-TCOVx-Ch groups and identified by PCR-RFLP as group VI genotype (data not shown).

Clinical signs. Total clinical signs scores per day for CEOVx-Ch, CT-CEOVx-Ch, TCOVx-Ch, CT-TCOVx-Ch, NVx-Ch, and NVx-NCh groups are represented in Figure 4.2. Chickens of CEOVx-Ch, TCOVx-Ch, and NVx-NCh groups showed mild clinical signs characterized by mild breathing and depression with no mortality. The total clinical signs scores among these groups were not significantly different. On the other hand, chickens of CT-CEOVx-Ch, CT-TCOVx-Ch, and NVx-Ch groups showed open mouth breathing, gasping with an extended neck, mild and severe conjunctivitis with closed and watery eyes, different levels of depression, and mortality. Clinical signs were observed in all five chickens from day 2 to 12 in NVx-Ch, from day 4 to 12 in CT-CEOVx-Ch (Figure 4.2a) and CT-TCOVx-Ch group (Figure 4.2b). The total clinical signs scores for CT-CEOVx-Ch ($P \leq 0.002$), CT-TCOVx-Ch ($P \leq 0.001$), and NVx-Ch ($P \leq 0.001$) groups were significantly different when compared with scores from the CEOVx-Ch, TCOVx-Ch, and NVx-NCh groups, respectively.

Mortality. The percentage of mortality per group after 12 days post-challenge is shown in Table 4.1. No mortality occurred in CEOVx-Ch, SE-CEOVx-Ch, TCOVx-Ch, SE-TCOVx-Ch, and NVx-NCh groups. However, mortality was observed in CT-CEOVx-Ch, CT-TCOVx-

Ch, NVx-Ch, and SE-NVx.Ch groups between days 8 and 12 post-challenge. These groups presented mortalities ranging from 25 to 40%. Percentages of mortality recorded for the CT-CEOVx-Ch ($P \leq 0.021$), CT-TCOVx-Ch ($P \leq 0.012$), NVx-Ch ($P \leq 0.014$), and SE-NVx-Ch ($P \leq 0.005$) groups were significantly different from the NVx-NCh group.

Percentage body weight gained. The percentage of body weight gained for each group from four- to eight-weeks of age pre-challenge, and after 12 days post-challenge is presented in Figure 4.3. The percentage of body weight gained among groups from four- to eight-weeks of age pre-challenge was not significantly different. The percentage of body weight gained 12 days post-challenge for CEOVx-Ch, SE-CEOVx-Ch, TCOVx-Ch, SE-TCOVx-Ch, and NVx-NCh groups was not significantly different. However, the percentage of body weight gained for CT-CEOVx-Ch ($P \leq 0.002$), CT-TCOVx-Ch ($P \leq 0.002$), NVx-Ch ($P \leq 0.001$), and SE-NVx-Ch ($P \leq 0.001$) was significantly different from that of the NVx-NCh group. As compared to the 21.5% body weight gained by the NVx-NCh group, non-vaccinated-challenged chickens gained 1.2%, sentinel chickens from the non-vaccinated-challenged group lost 2.1%, contact-exposed chickens to TCOVx lost 1.2%, and contact-exposed chickens to CEOVx gained only 3.2% of their body weight (Figure 4.3a and 4.3b).

Serology. The results of ELISA for samples collected before vaccination (four-weeks of age), pre-challenge (eight-weeks of age), and 12 days post-challenge are presented in Table 4.2. All samples collected before vaccinations were negative by the ELISA test. Samples collected pre-challenge from CEOVx, CT-CEOVx, TCOVx, and CT-TCOVx groups were positive by ELISA, while samples collected from the NVx-NCh group were negative. Samples collected 12

days post-challenge from CEOVx-Ch, CT-CEOVx-Ch, TCOVx-Ch, CT-TCOVx-Ch, NVx-Ch, and SE-NVx-Ch groups were positive, while samples collected from SE-CEOVx-Ch, SE-TCOVx-Ch, and NVx-NCh groups were negative.

DISCUSSION

The objective of this study was to evaluate the protection induced by the CEO and TCO vaccines against a current group VI genotype ILTV isolate. In this challenge experiment contact-exposed chickens were used to evaluate transmission of the ILTV live attenuated vaccines four weeks after vaccination, and sentinel chickens were used to assess the shedding of the challenge virus up to 12 days post-challenge. As previously reported, the CEO and TCO vaccines can be transmitted from vaccinated to contact-exposed chickens (Gelenczei & Marty, 1964; Hilbink *et al.*, 1987; Rodriguez-Avila *et al.*, 2007). The transmission of the CEO and TCO vaccine viruses was demonstrated by the presence of ILTV antibodies four weeks after vaccination (pre-challenge) and by the detection of viral DNA 9 days after vaccination in the contact-exposed chickens. The contact-exposed groups presented a similar curve of total clinical signs scores as the non-vaccinated-challenged group typified by acute conjunctivitis, breathing with extended neck, severe depression and significant percentage of mortality.

Kirkpatrick *et al.* (2006) found that together with clinical signs and mortality, body weight gain was a consistent parameter to evaluate pathogenicity. Body weight gain was utilized in this study to reinforce the clinical signs and mortality findings. This parameter was compared at three points during the experiment, the day of vaccination, pre-challenge, and post-challenge. No significant body weight gain was observed in either contact-exposed group post-challenge

but compared to the non-vaccinated-non-challenged group there was a significant difference in body weight gain.

In previous studies performed by Gelenczei & Marty (1964) and Hilbink *et al.* (1987), chickens exposed as contacts and chickens used as sentinels to vaccinates have been utilized to assess vaccine spread and protection by seroconversion and the presence of neutralizing antibodies. Even though in this study, seroconversion and viral DNA detection during four weeks after vaccination demonstrated that vaccine viruses were shed to contact-exposed chickens, significant evidence was obtained 12 days post-challenge from clinical signs, mortality, and body weight gain in both contact-exposed groups to indicate that chickens in these groups were not protected against challenge. It can be speculated that after four weeks of exposure the lack of protection in contact-exposed chickens might be due to insufficient vaccine virus replication in these chickens to generate the cell mediated immunity necessary to protect against challenge.

The protection induced by CEO and TCO vaccines in vaccinated-challenged chickens was demonstrated by clinical signs, mortality, body weight, and shedding of the challenge virus to sentinel chickens. Clinical signs and mortality were scored daily from vaccinated-challenged, non-vaccinated-challenged, and non-vaccinated-non-challenged groups. No significant differences were observed between vaccinated-challenged and non-vaccinated-non-challenged groups for either clinical signs or mortality; however, a significant difference was found between non-vaccinated-challenged and vaccinated-challenged groups. Furthermore, total mortality recorded among contact-exposed, non-vaccinated-challenged, and sentinel chickens from the non-vaccinated-challenged groups as compared to the non-vaccinated-non-challenged group was significant.

Different to other challenge studies (Hilbink *et al.*, 1987; Fulton *et al.*, 2000; Han & Kim 2003) clinical signs and mortality were scored every day from day 2 to 12 post-challenge in order to monitor the length of infection. Viral replication and shedding to sentinel chickens was examined by real time PCR and virus isolation. Neither viral DNA was detected, or virus isolated in samples collected from sentinel chickens from the CEO or TCO vaccinated-challenged and non-vaccinated-non-challenged groups. Nevertheless, shedding of the challenge virus was confirmed in samples collected from sentinel chickens from the non-vaccinated-challenged group. It appears that both CEO and TCO vaccination of chickens significantly reduces shed of challenge virus when immunized chickens are challenged.

As previously reported by our research group, there was a correlation in samples with genome copy number equal to or higher than $10^{4.3}$ and successful virus isolation (Rodriguez-Avila *et al.*, 2007). The peak of viral DNA detection, positive virus isolation, and highest clinical signs scores coincided from day 8 to 10 post-challenge in non-vaccinated-challenged and sentinel chickens within the same group (Figures 4.1 and 4.2).

Similar to contact-exposed chickens, body weight was a significant parameter to determine protection in vaccinated chickens. No significant differences were found among groups the day of vaccination and pre-challenge, indicating that neither bird husbandry nor eye drop vaccination influenced body weight gain. Similarly, no significant differences were found post-challenge among vaccinated-challenged, sentinel chickens from vaccinated-challenged, and non-vaccinated-non-challenged groups. On the other hand, significant differences were observed among non-vaccinated-challenged, sentinel chickens from the non-vaccinated-challenged group, and the non-vaccinated-non-challenged group. As reported by Kirkpatrick *et al.* (2006) in the pathogenicity study, for this study, body weight was a determinant parameter to evaluate vaccine

protection. Different from contact-exposed, CEO and TCO eye drop vaccinated chickens were protected against challenge as elucidated by clinical signs, mortality and body weight gain. The results obtained from sentinel chickens suggest that the CEO and TCO vaccines generate sufficient cell mediated immunity in vaccinated chickens to avoid this group VI challenge virus replication; therefore, these chickens did not shed the challenge virus up to 12 days post-challenge.

The ILTV enzyme link immunosorbent assay (ELISA) demonstrated antibody production after vaccination and post-challenge in vaccinated-challenged, contact-exposed, and non-vaccinated-challenged chickens. Antibody production in contact-exposed chickens suggests vaccine virus transmission in these groups; however, as previously shown (Fahey *et al.*, 1983; Fahey & York, 1990) and despite the detection of antibodies, these chickens were not protected against challenge as demonstrated by the presence of clinical signs, mortality and percentage of body weight gained. This study provides further evidence that levels of humoral immunity do not correlate to resistance to challenge. Sentinel chickens from vaccinated groups were negative for ILTV antibodies, and together with the lack of viral DNA detection and virus isolation, further indicated that no viral replication or shedding occurred in either CEO or TCO eye drop vaccinated chickens after 12 days post-challenge. In contrast, sentinel chickens, from the non-vaccinated-challenged group, were positive for ILTV antibodies and together with the presence of viral DNA and positive virus isolation, proved that challenge virus shedding occurred.

In conclusion, based on clinical signs, mortality, body weight gain, virus isolation, and viral DNA detection, protection induced by CEO and TCO eye drop vaccination against ILTV group VI genotype virus was demonstrated. Even though this group of viruses is genetically different to the live attenuated vaccines (Oldoni & García, 2007), antigenically they appear to be

closely related. The transmission of the vaccine virus was confirmed, and founded on the parameters utilized to define protection; both groups of contact-exposed chickens were not protected against challenge. In particular, this result emphasizes the importance of a uniform vaccination to obtain adequate protection, to avoid the presence of susceptible chickens, and to prevent recrudescence of live attenuated vaccines.

Overall, the use of contact-exposed and sentinel chickens was useful to assess transmission and shedding of the vaccines and challenge virus. Together with clinical signs, mortality, and body weight gain this challenge model was a reliable tool to evaluate the protection induced by these infectious laryngotracheitis virus live attenuated vaccines. In addition, this challenge model can be applied to evaluate the safety and efficacy of the newly developed ILTV vaccines.

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Table 4.1. *Percentage of mortality per group after 12 days post-challenge*

Group ^a	Death/Total Chickens	% Mortality ^b
CEOV _x -Ch	0/20	0 ^A
SE-CEOV _x -Ch	0/20	0 ^A
CT-CEOV _x -Ch	5/20	25 ^B
TCOV _x -Ch	0/20	0 ^A
SE-TCOV _x -Ch	0/20	0 ^A
CT-TCOV _x -Ch	6/20	30 ^B
NV _x -Ch	3/10	30 ^B
SE-NV _x -Ch	4/10	40 ^B
NV _x -NCh	0/20	0 ^A

^a Chicken embryo origin vaccinated-challenge (CEOV_x-Ch), sentinels-chicken embryo origin vaccinated-challenge (SE-CEOV_x-Ch), contact-exposed-chicken embryo origin vaccinated-challenge (CT-CEOV_x-Ch), tissue culture origin vaccinated-challenge (TCOV_x-Ch), sentinels-tissue culture vaccinated-challenge (SE-TCOV_x-Ch), contact-exposed-tissue culture vaccinated-challenge (CT-TCOV_x-Ch), non-vaccinated-challenge (NV_x-Ch), sentinels of non-vaccinated-challenge (SE-NV_x-Ch), and non-vaccinated-non-challenge (NV_x-NCh) groups.

^b Significantly different mortality percentages ($P < 0.05$) are shown by different superscript letters.

Table 4.2. *ELISA results for sera samples collected before vaccination (four-weeks of age), pre-challenge (eight-weeks of age), and twelve days post-challenge*

Group ^a	Before Vaccination		Pre-challenge		Post-challenge	
	Mean ^b	CV (%) ^c	Mean	CV (%)	Mean	CV (%)
CEOV _x -Ch	0	0	1004	88.9	1997	81.33
SE-CEOV _x -Ch	0	0	0	0	0	0
CT-CEOV _x -Ch	0	0	2126	53.7	1400	70.84
TCOV _x -Ch	0	0	716	71.4	927	90.02
SE-TCOV _x -Ch	0	0	0	0	0	0
CT-TCOV _x -Ch	0	0	407	167.9	342	90
NV _x -Ch	0	0	0	0	319	90.04
SE-NV _x -Ch	0	0	0	0	449	118.45
NV _x -NCh	0	0	0	0	0	0

^a Chicken embryo origin vaccinated-challenge (CEOV_x-Ch), sentinels-chicken embryo origin vaccinated-challenge (SE-CEOV_x-Ch), contact-exposed-chicken embryo origin vaccinated-challenge (CT-CEOV_x-Ch), tissue culture origin vaccinated-challenge (TCOV_x-Ch), sentinels-tissue culture vaccinated-challenge (SE-TCOV_x-Ch), contact-exposed-tissue culture vaccinated-challenge (CT-TCOV_x-Ch), non-vaccinated-challenge (NV_x-Ch), sentinels of non-vaccinated-challenge (SE-NV_x-Ch), and non-vaccinated-non-challenge (NV_x-NCh) groups.

^b Mean titers

^c Coefficient of variation (percentage)

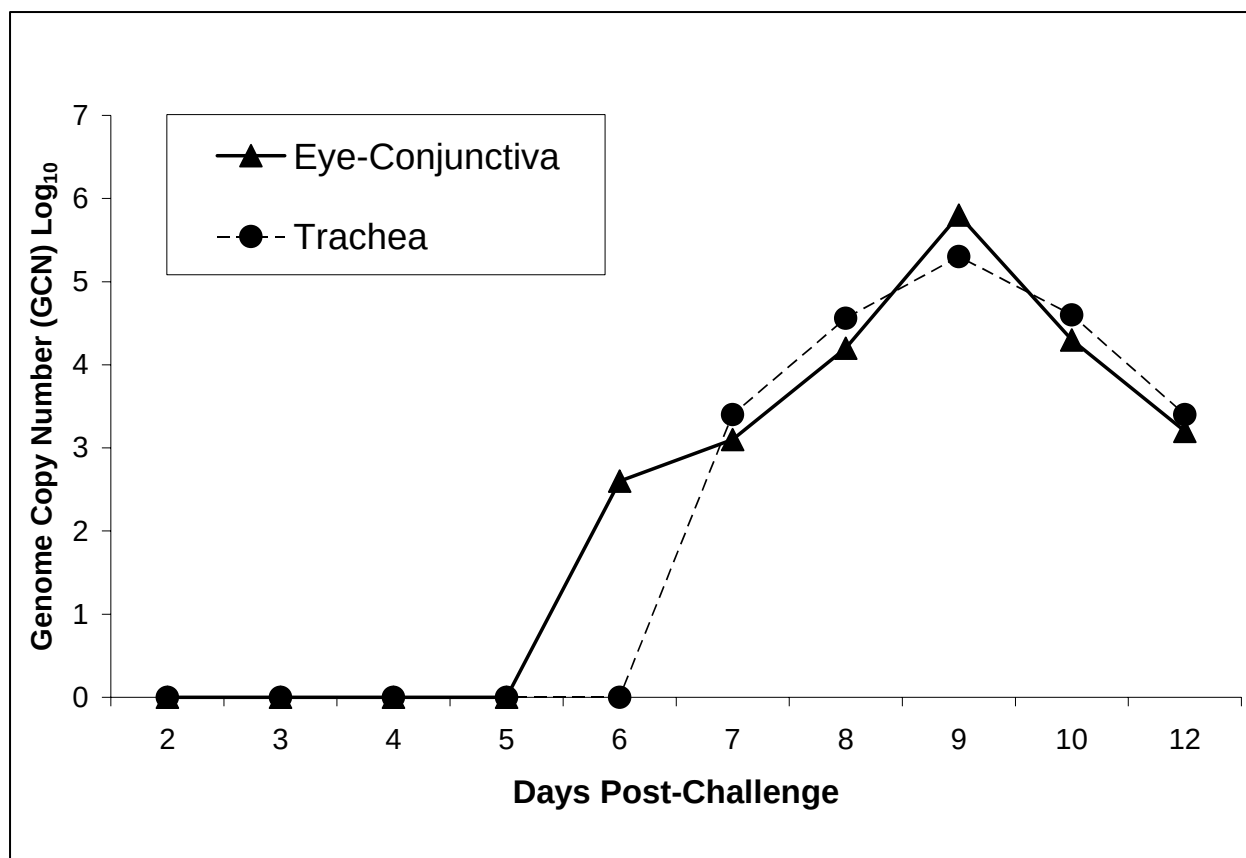


Figure 4.1. Viral genome copy number Log_{10} detected in the eye conjunctiva and trachea by Real Time PCR Taqman Assay (ReTi-PCR) from sentinel chickens (SE-NV_x-Ch) of the non-vaccinated-challenged group.

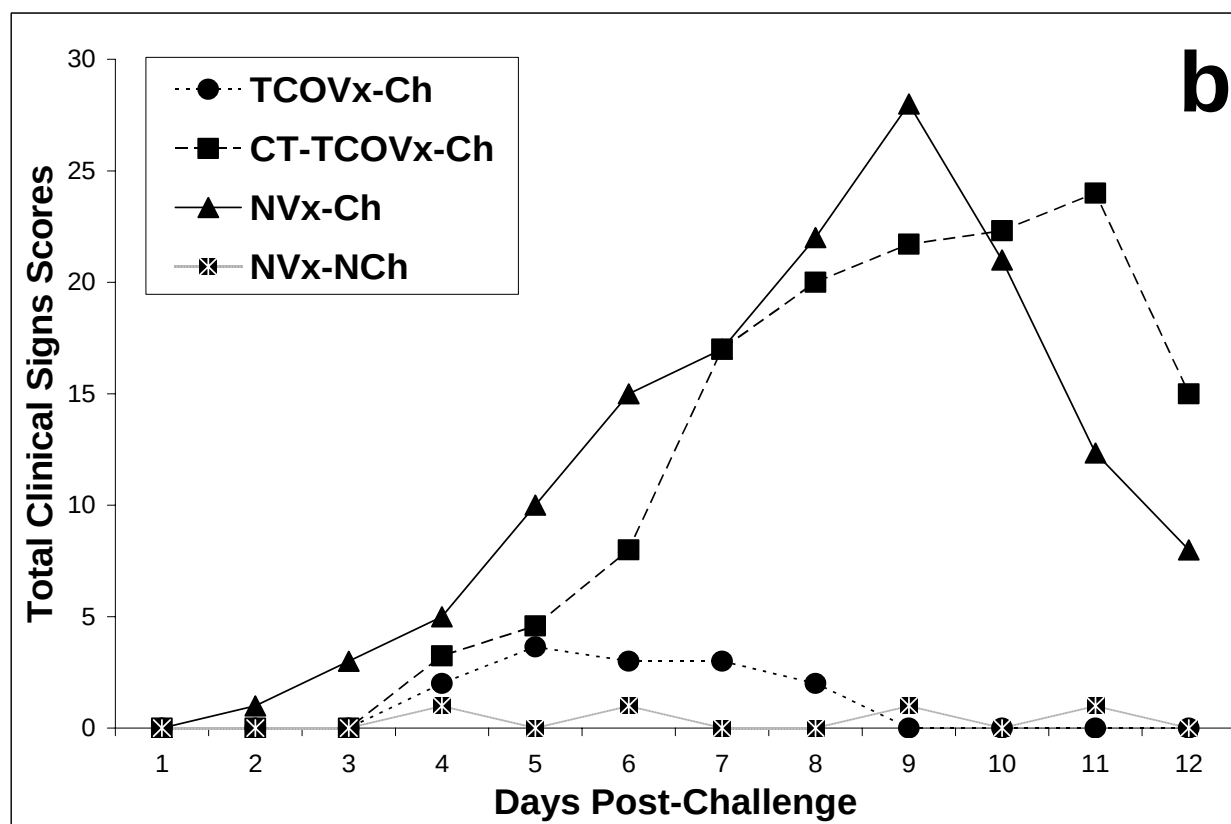
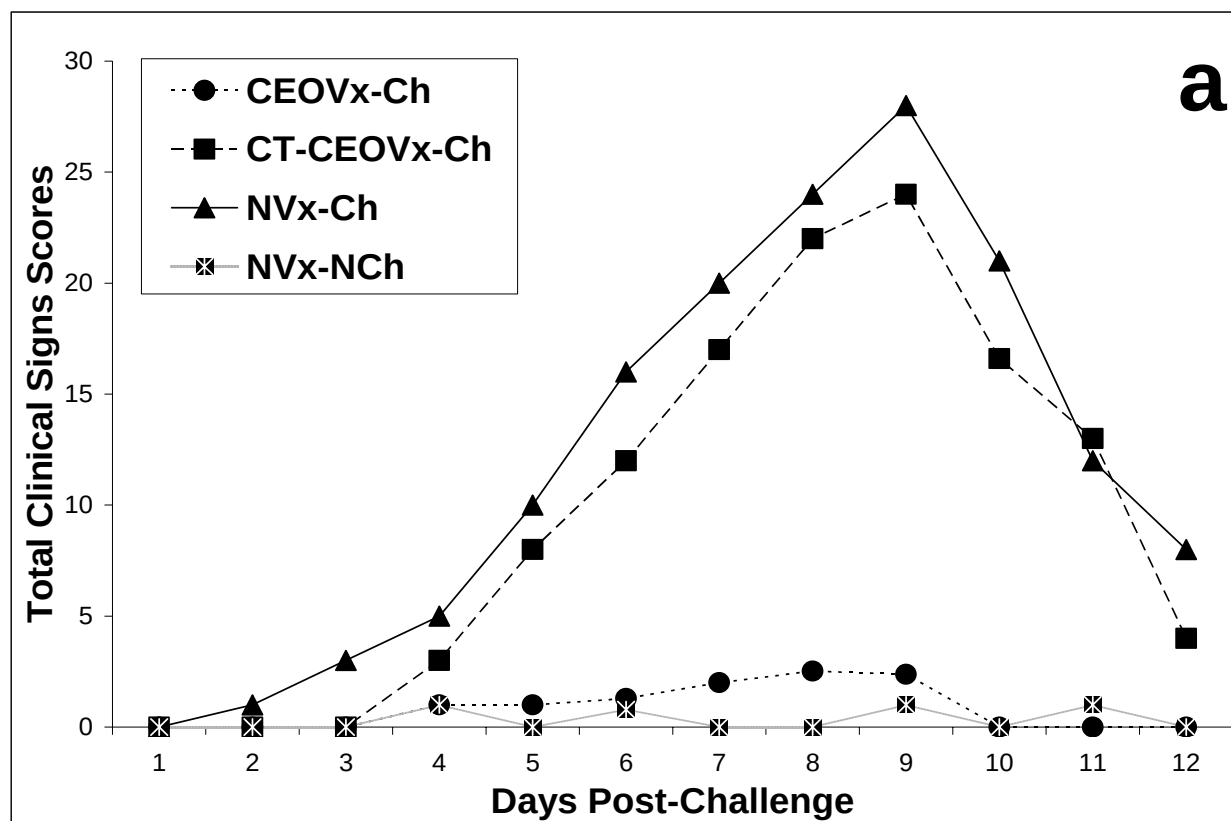


Figure 4.2. *Total clinical signs scores recorded per day in eight-weeks of age chickens from days 2 to 12 post-challenge. Chicken embryo origin vaccinated-challenge (CEOVx-Ch), contact-exposed-chicken embryo origin vaccinated-challenge (SE-CEOVx-Ch) (a), tissue culture origin vaccinated-challenge (TCOVx-Ch), and contact-exposed to tissue culture vaccinated-challenge (CT-TCOVx-Ch) (b) groups were significantly different ($P < 0.05$) from non-vaccinated-challenge (NVx-Ch) and non-vaccinated-non-challenge (NVx-NCh) chickens groups (a and b).*

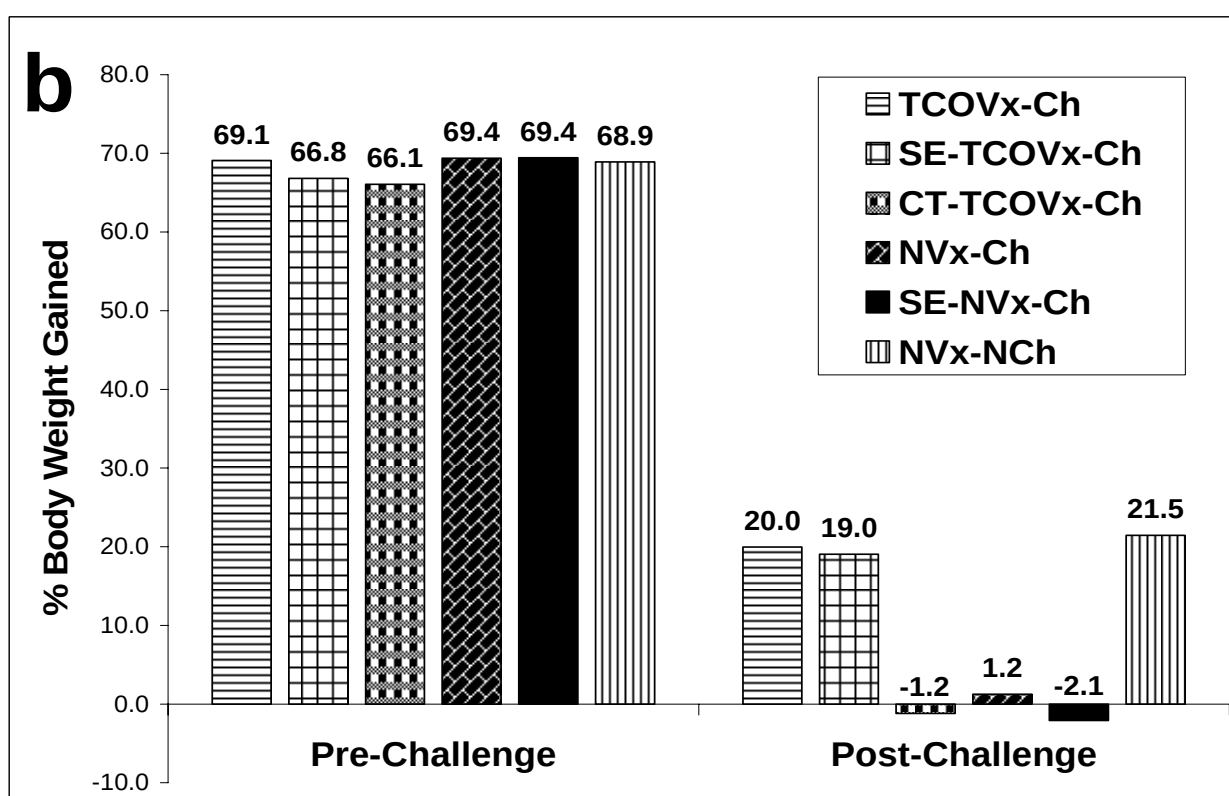
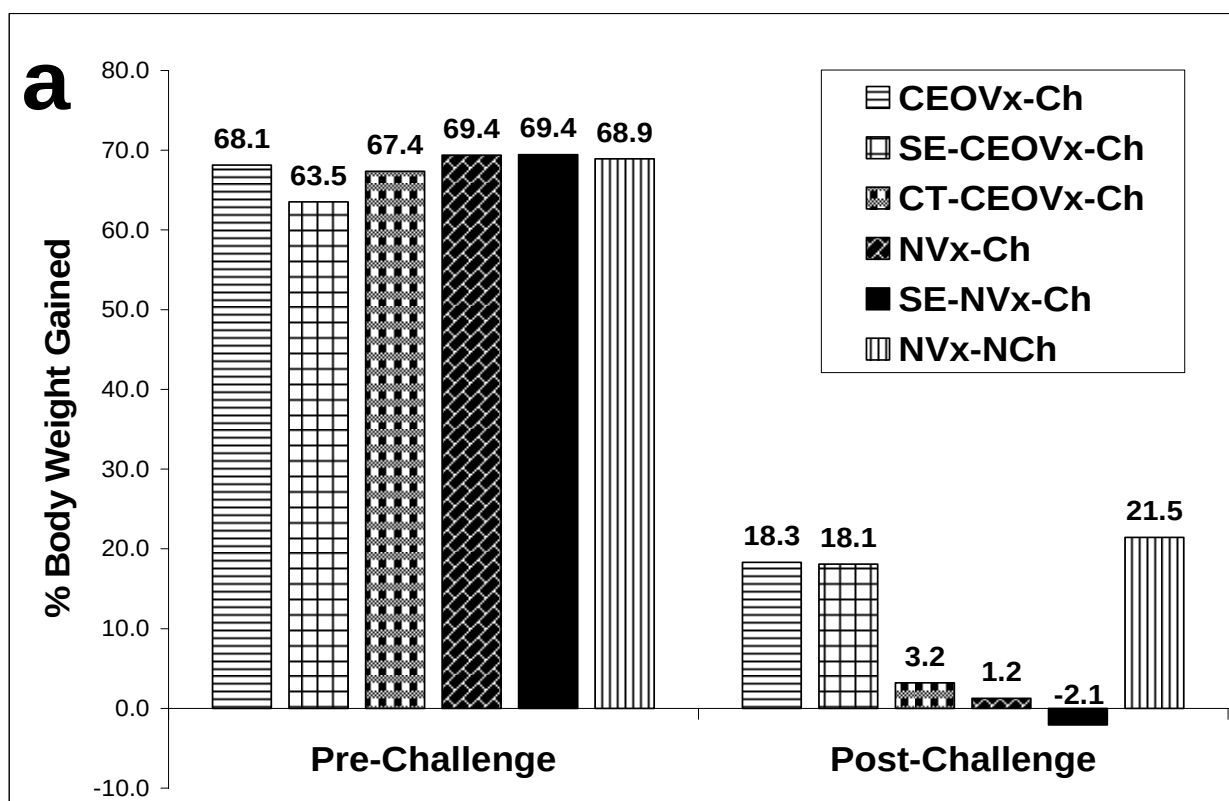


Figure 4.3. *Percentage of body weight gained for each group from four-weeks to eight-weeks of age pre-challenge and 12 days post-challenge. Groups contact-exposed-chicken embryo origin vaccinated-challenge (CT-CEOVx-Ch) (a), contact-exposed-tissue culture vaccinated-challenge (CT-TCOVx-Ch) (b), and non-vaccinated-non-challenge (NVx-NCh) (a and b) were significantly different ($P < 0.05$) from chicken embryo origin vaccinated-challenge (CEOVx-Ch) (a), tissue culture origin vaccinated-challenge (TCOVx-Ch) (b), and non-vaccinated-challenge (NVx-Ch) groups (a and b), respectively.*

CHAPTER 5

DISCUSSION

Infectious Laryngotracheitis (ILT) is a viral upper respiratory disease of chickens responsible for serious economic losses in the poultry industry due to moderate morbidity and mortality, decreased egg production, and predisposition to other poultry pathogens. Infectious laryngotracheitis virus (ILTV) or Gallid Herpesvirus 1 (GHV-1) is a highly contagious avian pathogen that belongs to family *herpesviridae*, subfamily *alphaherpesvirinae*.

Currently, there are two main types of ILTV live attenuated vaccines commercially available, those attenuated by sequential passages in chicken embryos (chicken embryo origin-CEO) or attenuated by sequential passages in tissue culture (tissue culture origin-TCO). Experimental studies and field observations have allowed a wide evaluation of both live-attenuated vaccines (CEO and TCO). These attenuated vaccines induce protection, preventing clinical signs and mortality. Both can persist in apparently healthy birds and can spread from bird to bird. Gain of virulence after bird - to - bird passage has been reported, in the case of CEO causing severe respiratory disease and mortality, and in the case of TCO causing a milder respiratory response at the same back passage level. The route of vaccination is extremely important since some of the available live-attenuated vaccines provide different grades of protection, particularly when applied by coarse spray or in the drinking water. Eye-drop vaccination has been demonstrated to provide a more uniform protection, and less severe reactions as compared to spray vaccination.

Recent molecular epidemiology studies indicated that outbreaks of the disease in the USA are caused by strains closely related to the CEO vaccines, and strains genetically different to the vaccines, while outbreaks with TCO type isolates are rare. Despite the available field and experimental evidence indicating that live-attenuated vaccines CEO and TCO behave differently, the replication and transmission of both live attenuated vaccines have not been comprehensively compared using new and more sensitive methods of viral detection. The protection efficacy of live-attenuated vaccines has been evaluated against a variety of historical ILTV strains (USDA and CSW challenge strains) by clinical signs, mortality, viral recovery and viral spread. However, assessment of ILTV vaccine protection efficacy against currently circulating viral strains from the USA has not been performed.

The objectives of this work were: 1) to compare the replication and transmission of the CEO and TCO vaccines at different time points post-inoculation using virus isolation and quantitative ReTi-PCR; 2) to evaluate the protection induced by the CEO and TCO vaccines against a current ILTV genotype circulating in the USA by using contact-exposed and sentinel chickens.

In the first study, the replication and transmission of the TCO and CEO vaccines were evaluated in specific pathogen free chickens after the administration of the vaccines via eye-drop exposure. Virus isolation and real time PCR were used to detect the presence of live virus and viral DNA, respectively, in the trachea, trigeminal ganglia, eye conjunctiva, cecal tonsils, and cloaca, from eye drop inoculated and contact exposed birds, at days 2, 4 to 10, 14, 18, 21, 24, and 28 post-inoculation. No differences were observed in the ability of the TCO and CEO vaccines to replicate in the examined tissues. Both vaccines presented a localized replication in the eye conjunctiva and the trachea. Both vaccines were capable of transmitting to contact-

exposed birds, attaining peaks of viral DNA as elevated as those observed in inoculated birds. The CEO vaccine replicated faster and reached higher viral genome copy number (GCN) than the TCO vaccine in the conjunctiva and trachea of eye drop inoculated and contact exposed chickens. The DNA of both vaccine viruses migrated to the trigeminal ganglia during early stages of infection. Although the CEO and TCO vaccines were not recovered from the cecal tonsils and the cloaca, low levels of viral DNA were detected in these sites during the peak of viral replication in the upper respiratory tract. In this study, the absence of virus isolation from the trigeminal ganglia may reflect the lack of sensitivity of the chicken kidney cells to detect the vaccine viruses in the ganglia during early stages of infection. On the other hand, the ReTi-PCR assay was capable of detecting viral DNA in the ganglia during the early stages of infection, but not during late stages of infection. The lack of virus isolation, and the low levels of viral DNA detected in the cecal tonsils and cloaca, suggests that neither vaccine virus actively replicates in these sites during early stages of infection. Overall in this study, it was demonstrated that ILTV vaccines have a similar replication, both presented a localized replication in the eye conjunctiva and the trachea, and both were capable of transmitting to contact-exposed chickens. The earlier recovery, frequency of isolation, and higher viral GCNs detected in inoculated and contact-exposed chickens proved that the CEO vaccine replicates and spreads faster than the TCO vaccine.

It is believed that most of the outbreaks in the USA are caused by vaccine related isolates that persist in the field. In a recent study, current USA field isolates from poultry were characterized into six genotype groups. Group VI virus was characterized as genotypically and biologically different than the vaccine viruses. In the second study, the protection induced by the CEO and TCO vaccines against a current group VI genotype ILTV isolate was evaluated.

Contact-exposed and sentinel chickens were utilized to assess the shedding of vaccines and challenge viruses, respectively. Protection was assessed scoring clinical signs and mortality, quantifying weight gain, and viral shedding to sentinel chickens. In this study, significant evidence was obtained from the evaluated parameters in chickens vaccinated via eye drop with the CEO and TCO vaccines. Vaccinated chickens were protected while chickens contact-exposed to vaccine chickens were not protected against challenge with the group VI genotype virus. The use of sentinel chickens verified that the CEO and TCO vaccinated chickens did not shed the challenge virus. In this study, the transmission of the vaccine virus was confirmed, and based on the parameters utilized to define protection; both groups of contact-exposed chickens were not protected against challenge. Therefore, it is extremely important, in order to achieve good protection, that a flock receives a uniform vaccination to avoid the presence of susceptible chickens in the house. The proposed challenge model should be applied to evaluate protection produce by current and newly developed ILTV vaccines.

All the objectives projected for this study were achieved utilizing the experimental designs proposed and the latest available laboratory tools. This work was intended to evaluate the replication, transmission, and protection of current live attenuated infectious laryngotracheitis virus vaccines. In summary, new evidence was obtained related to replication of CEO and TCO vaccines. The CEO vaccine was confirmed to replicate more aggressively than the TCO vaccine, however, the vaccines induced equal protection. The CEO vaccine transmission was more frequent and faster than the transmission of the TCO vaccine; although contact-exposed chickens were positive by virus isolation, real-time PCR and serology, they were not protected against challenge. This specific finding provides evidence to understand the increased number of outbreaks since mass application methods are utilized in the field in the USA. The CEO and

TCO eye drop vaccinated challenged chickens did not shed the challenge virus as proved by using sentinel chickens and they were protected against group VI genotype challenge virus. Further studies must be conducted using the proposed challenge model to evaluate the efficiency of mass application methods for the live attenuated vaccines, and the protection induced by the newly developed ILTV vaccines.