STUDIES WITH INFECTIOUS LARYNGOTRACHEITIS (ILTV): IMMUNITY IN LAYERS, SEROLOGY, AND ATTENUATION IN TISSUE CULTURE

by

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(Under the Direction of Guillermo Zavala)

ABSTRACT

Infectious Laryngotracheitis (ILT) is an economically relevant upper-respiratory disease of chickens caused by an alphaherpesvirus taxonomically identified as *Gallid herpesvirus 1* (GaHV-1) also known as ILTV. In densely populated poultry areas in which ILT is present, the disease is controlled by biosecurity and vaccination. Currently, two kinds of commercially available vaccines are being used for ILTV control, live-modified vaccines (Chicken Embryo Origin [CEO], and Tissue Culture Origin [TCO]) and ILT recombinant vaccines (Herpes Virus of Turkey-LT [HVT-LT] and Fowlpox-LT [FPV-LT]). Furthermore, the spread of ILTV to non-enzootic areas has encouraged the manufacture and license of oil-based inactivated ILTV vaccines in zones where the use of live-modified vaccines is prohibited and only the use of recombinant vaccines is allowed. The present work evaluated the protection conferred by these three types of vaccines, individually and in combination, in layers challenged with a virulent ILTV strain; established a serological baseline for the implementation of ELISA in surveillance

programs; and attenuated a virulent strain of ILTV in cell culture for future vaccine development.

INDEX WORDS: Infectious Laryngotracheitis, Layers, CEO, TCO, HVT-LT, POX-LT, Immunity, Inactivated vaccine, qPCR, Cell culture, ELISA, Attenuation.

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DEDICATION

To the memory of my grandfather, who taught me the value of being kind with people. To my family and especially to my parents, Victor Antonio and Asuncion Felicita, and my sister, Joana, for their continuous support and love, and for those dazzling Sunday morning walks by the beach in which I learn that even withered oyster shells can tell beautiful stories.

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CHAPTER 1

INTRODUCTION

Infectious Laryngotracheitis (ILT) is an economically relevant acute respiratory disease of chickens, caused by an alphaherpesvirus, genus *Iltovirus*, species *Gallid Herpesvirus 1* (GaHv-1) also known as ILTV. The disease can cause a wide range of clinical signs and mortality, (7) and frequently accounts for relevant economic losses in densely poultry populated areas.

Two classes of live-modified vaccines, Chicken Embryo Origen (CEO) and Tissue Culture Origin (TCO), have been used for decades as part of ILT control programs in the poultry industry. These two vaccines can stimulate a strong and prolonged immune response, but such protection is not life-long. CEO vaccines are preferred in many operations due to their ready availability, since they are commercialized by various vaccine manufacturing companies. They are also preferred because it is possible to vaccinate chickens with CEO vaccines using mass-delivery systems (i.e. drinking water and/or spray vaccination). In contrast, there is only one TCO vaccine available in the western world and is commercialized by a single company and can only produce adequate immunity when applied by individual-bird vaccination methods such as the conjunctival route (eye-drop). CEO vaccines have proved to be controversial, due to their capacity to regain virulence when passaged in naive birds (6). Moreover, research done in the USA (1, 10), United Kingdom (9), and Australia (3, 8) have shown that CEO-related strains seem to predominate in field outbreaks, while field TCO-related strains are rare (11, 15). Thus,

outbreaks caused by CEO-related strains are conventionally called "Vaccinal Laryngotracheitis" (VLT) and constitute a major problem in the USA and UK (4).

The use of live-modified vaccines implies several drawbacks in the vaccinated flock such as significant vaccine reactions, increased feed conversion, delayed growth, and increased mortality that put together, represent an important economic cost to the poultry industry. In addition, the potential of establishing latency in infected birds (due to vaccination or challenge) gives the virus a remarkable evolutionary advantage and reduces the effectiveness of the control programs, particularly in long-lived birds, in which immunity against ILT wanes after several months post infection, allowing the latent vaccine virus to be shed by carrier birds and infect naive birds. The disadvantages aforementioned for live-modified vaccine issues led to the research and development of recombinant vaccines against ILT (i.e. Fowlpox-LT [FP-LT] and Herpes Virus of Turkey-LT [HVT-LT]). These novel vaccines can be mass delivered (in-ovo); do not produce ILTV latency (HVT vector does establish latency, albeit without detrimental consequences); and lack the ability to generate vaccine reactions or potential outbreaks due to "vaccinal ILT".

As demonstrated through previous research at the University of Georgia, a significant disadvantage of recombinant vaccines is that HVT-LT or POX-LT recombinant-vaccinated birds can shed as much virus as naive (non-vaccinated) birds if challenged with virulent field ILTV, even when there is a decrease in clinical signs in the vaccinated-challenged birds. Much is yet to be known about the use of recombinant vaccines, especially in commercial egg layers, for which studies evaluating the onset and duration of immunity conferred by recombinant vaccines and combinations of those vaccines with live-modified vaccines have not been studied.

Active immunity against ILT is primarily cell-dependent and not humoral (12, 13). Clinical trials with chickens vaccinated with killed vaccines have shown inconsistent results (5, 14) (2). The recent spread of ILT into previously non-enzootic areas of South America, together with some local regulatory policies allowing the use of exclusively recombinant and/or inactivated vaccines and prohibiting the use of live-modified vaccines has stimulated some local pharmaceutical companies to manufacture, register, and commercialize inactivated oil-emulsified ILT vaccines that are usually applied alone or combined with recombinant vaccines. There are no published reports documenting the effectiveness of such vaccine associations against a virulent challenge.

An important part of a successful surveillance and control program includes the diagnosis of ILT in the field. A survey performed in six major state poultry laboratories in the USA revealed that ILT diagnosis was mainly based on at least two rapid tests such as histopathology examination of formalin-fixed paraffin embedded tracheal and eyelid tissues; a direct fluorescent antibody test (DFAT), Immunohistochemistry (IHC), or molecular detection by PCR (4). Serology tests, including enzyme-linked immunosorbent assay (ELISA), have proven to be unreliable tools for ILT diagnosis. In addition, ILT ELISA serology has a low predictive value relative to protection induced by vaccines. This is partially due to the brief period of time that elapses between field challenge and processing of broiler flocks, preventing the development of a strong humoral response that can be detectable by serologyThe primary objective of the proposed research was to assess the protection induced by live-modified, recombinant, inactivated vaccines and combinations of such against a virulent strain of ILTV in commercial layers.

A secondary objective was the attenuation of a field CEO-related strain of ILTV isolated from a case of VLT in broilers and identified as “63140”. Attenuation was attempted by serial passages in primary cultures of chicken embryo kidney cells (CEK), chicken embryo liver cells (CEL), chicken embryo fibroblasts (CEF), and immortalized continuous cell lines such as VERO, E6VERO, LMH and DF-1.

The third objective was to examine by ELISA the serological responses of commercial layers vaccinated against ILT using different immunization approaches that included modified live, recombinant, inactivated vaccines, and their combinations.

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CHAPTER 2

LITERATURE REVIEW

The 90-year history of Infectious Laryngotracheitis (ILT) research can be divided into three main periods, namely: first period, from 1923 to 1935; second period, from 1935 to 1991; and a third period from 1991 to date.

HISTORY OF THE DISEASE

First Period: From 1923 to 1935. The first detailed description of ILT virus (ILTV) was done in 1925 by May and Tittsler (85), who reported an ILTV outbreak in a Rhode Island poultry farm, and identified the disease as a separate entity from other diseases hitherto known. Laryngotracheitis was probably present as early as 1920, according to some early reports, but misdiagnosed as avian diphtheria (Fowl Pox) (58) or as other respiratory diseases (13).

ILTV outbreaks across the United States and in other parts of the world were reported in poultry with a high percentage of morbidity and mortality in affected flocks (13, 27). The early research done with ILT is confusing, due to the various names used to identify the novel disease such as infectious bronchitis (12, 65), tracheo-laryngitis (85), and others; while severe gasping with the presence of hemorrhagic exudate inside the trachea was a common denominator among the disease descriptions. For this reason, the name “Infectious Laryngotracheitis” was appointed by the special committee on Poultry Diseases of the American Veterinary Medical Association in

1931 (27). Beach (1930) demonstrated that the cause of the disease was not a bacteria (10) but a filterable virus (11, 13); furthermore, some of the challenged and vaccinated birds were shown to be chronic carriers of ILTV (49).

The end of this first period was defined by two main events, including the successful propagation of field isolates in chicken embryonated eggs (20), and the development of a novel cloacal vaccination method named “vent brush vaccination” (65). Both events allowed the early poultry industry, for the first time in history, to effectively protect chickens from a viral infection while not developing significant clinical signs.

Second Period: From 1935 to 1991. This period began with Seddon’s research (1936), who identified chickens, pheasants and hybrids of them as the only susceptible species, although now it is known that other species such as turkeys, peafowl (131), and ducks (132) can be experimentally infected. Although several different routes of ILT vaccine delivery were assessed, vent vaccination in chickens for ILTV control persisted as the preferred method of vaccination for disease control until the late 60s (71). Despite being the method of choice, vent brush vaccination had two major setbacks: first, the vaccinal virus used had to be a fully virulent strain, since it had been found that strains with reduced virulence gave poor protection, allowing the introduction of virulent virus in vast areas (14, 65); and second, the duration of the immunity was only about 8 weeks (71). In consequence, low virulent strains were isolated from different parts of the world, further attenuated by propagation in the chorioallantoic membrane of embryonated eggs (Chicken Embryo Origin – CEO), and delivered into birds through a variety of vaccination routes, such as cloacal (98), oral via the drinking water (60, 62, 106, 109), intranasal (28, 29), coarse spray (26, 63, 101), and conjunctival (104, 114, 117).

A Tissue-Culture Origin (TCO) strain was developed from a virulent strain named “L6” by serial passages in chicken and turkey kidney and liver cells (48). Unlike CEO vaccines that can be delivered by mass-application routes such as drinking water or spray, the TCO vaccine can only provide appropriate protection when delivered by the conjunctival (eye drop) route (47).

Finally, this period concludes with studies demonstrating that field viruses in North Carolina were CEO-like viruses (4, 54) and that commercial CEO vaccines may revert to virulence when allowed to back-passage in susceptible birds (55).

Third Period: From 1991 to date. Research done in the USA (4, 91), United Kingdom (89), Australia (17, 78), and Egypt (112), showed that CEO-related strains seem to predominate in field outbreaks, while field TCO-related strains are rare (92, 119). Furthermore, high throughput sequencing allowed field and vaccine ILTV strain genomes to be sequenced (119, 121) and compared (119) for identification of virulence genes. The pursuit of safer vaccines that are able to provide good protection against ILTV and that can be mass applied without generating latency or reversion to virulence in the field resulted in the development of novel recombinant vaccines (i.e. Fowlpox-LT [FP-LT] and Herpes Virus of Turkey-LT [HVT-LT]).

Although recombinant vaccines do diminish clinical signs in flocks infected with field viruses, several reports have documented lack of or insufficient protection in broiler flocks vaccinated with FP-LT administered in ovo (33). The development of these recombinant vaccines is relatively recent (FP-LT has been used by the poultry industry since April 2002 in layers and 2005 in broilers using the in ovo route while HVT-LT was licensed in September 2007). There is a knowledge gap about protection against the challenge and extent of such immunity in commercial egg layers.

ETIOLOGICAL AGENT

Classification

Infectious Laryngotracheitis Virus has been taxonomically identified as Gallid Herpesvirus 1 (GaHV-1), a member of the family Herpesviridae, subfamily Alphaherpesvirinae, genus Iltovirus. The virus has been classified in a different family than other herpesviruses of fowl, i.e. Marek's disease serotypes 1, 2, and 3 (GaHV-2, GaHV-3, and HVT, respectively) because of its phylogenetic distance (69). For many years GaHV-1 was considered the "type species" virus and the only member of this family (57); later, Psittacid herpesvirus 1 (PsHV-1), the causative agent of a highly contagious upper respiratory and systemic disease of psittacines named "Pacheco's Disease" was also included within this family (32).

Finally, other viruses of recent discovery have been proposed to be included in the "Iltovirus" family, such as: Psittacid herpesvirus 2 (PsHV-2) isolated from African green parrots (120); Passerid herpesvirus 1 (PasHV-1) isolated from Gouldian Finches (128), Psittacid herpesvirus 3 (PsHV-3) isolated from Bourke's Parrots (116), and Gaviid herpesvirus 1 (GavHV-1) isolated from Common Loons (99).

Morphology

An infectious ILTV particle displays an irregular envelope in which several viral glycoproteins are embedded as spikes. The irregular membrane, with a diameter of 195-250 nm, contains an icosahedral nucleocapsid with an approximate diameter of 80-100 nm constituted by 162 elongated hollow capsomers as described by Cruickshank et al. in 1963 (31). ILTV is similar in structure to other herpesvirus virions, such as Herpes Simplex Virus 1 (HSV-1).

Genome Structure, Gene Content and Surface glycoproteins

The ILTV genome is composed by a double stranded linear DNA genome with a total size of 150 to 155 kb (57, 119), with a guanine plus cytosine ratio of 45 to 48% (45, 95). The genome consists of two unique regions designated as US (Unique Short), and UL (Unique Long); and two inverted repeat sequences flanking the US region. Such structure allows the formation of two isomers, each of them with a differently oriented US region. This genome alignment is not uncommon between herpesviruses and has been classified as a type D herpesvirus genome.

A total of 80 predicted open reading frames (ORFs) have been identified and described using high-throughput sequencing technologies (79, 81, 119). Previous reports cited only 77 using the Sanger sequencing technology (57, 121). Sixty-five ORFs are located in the UL region; nine in the US region; and three within each of the two repeat regions of the ILTV genome. Since multiple genes are conserved among herpesviruses, the homologous gene and protein designations of Herpes Simplex Virus 1 (HSV-1) are used for ILTV, given that HSV-1 has been more widely studied than ILTV (57). The ILTV genes having a homolog in HSV-1 have been designated as UL1 to UL54, US2 to US8 and US10. ILTV is different from HSV-1 in various ways. For instance, ILTV possesses a UL3.5 ORF that is found in most alphaherpesviruses but is absent in HSV-1 (43); and lacks the UL16 ORF, which is conserved through most members of the Herpesviridae family (44). Moreover, the unique genomic features of the only two recognized members of the Iltovirus family, GaHV-1 and PsHV-1, consist of a cluster of 5 ORFs denominated ORF A through E, located the UL genome region, and the presence of a UL (-1) gene (136), while the UL0 gene is unique for ILTV.

Other unique features that distinguish the ILTV genome from other similar viruses include the translocation of the UL47 gene from the UL to the US region and the internal inversion of the UL22 to UL44 genes due to a partial inversion of the UL region (126). These features reveal an important phylogenetic distance between ILTV and other avian herpesviruses such as GaHV-2 and GaHV-3 viruses also known as the serotypes 1 and 2 of Marek's Disease virus (MDV).

ILTV viral glycoproteins are encoded by 11 ORFs with a homolog in HSV-1 and are known as glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gN, and gM. These viral glycoproteins control virus attachment and entry, cell fusion, and virus egress from the target cell, albeit the exact function and interactions of each of the glycoproteins has not been fully discerned. The major immunogenic glycoprotein is gJ, which can be expressed as multiple proteins of 85, 115, 160, and 200 kD sizes (46). A more detailed view of the functions of each of these glycoproteins is addressed in the viral replication section of the present review.

Replication

ILTV replication initiates with the attachment of viral surface glycoproteins to the cell receptor and the subsequent fusion of the viral and cell membranes. Although the cell receptor for ILTV has not been recognized, ILTV glycoproteins involved in viral attachment have been identified as gB (97) and gC (68). Furthermore, it is believed that gC, mediates the initial attachment, although through a mechanism dissimilar to that of other alphaherpesviruses such as HSV-1.

HSV-1 gC is able to interact with cellular heparan sulfate proteoglycans, which constitute the primary host cell surface receptor (73). In contrast, ILTV gC has been found to be smaller than the HSV-1 gC and other common alphaherpesviruses, lacking the conserved heparin binding domain; consequently, heparan sulfate proteoglycans are not likely ILTV cell receptors (74). Furthermore, single ΔgI and ΔgE deleted mutants have been found to produce reduced plaques in cell culture when compared to the parental virus, whereas a dual deleted ΔgI/gE mutant was able to infect single cells with no plaque formation, suggesting that gI and gE have a significant role in cell-to-cell spread as in other alphaherpesviruses (68).

Following the attachment and fusion steps, the nucleocapsid is released into the cytoplasm and then transported to the nuclear membrane. Thereafter, viral DNA is released from the nucleocapsid and migrates into the nucleus through the nuclear pores. Transcription and replication take place in the nucleus of the host cell in a tightly regulated "cascade" fashion, characteristic of herpesviruses. Gene transcripts are categorized according to the time of their expression as: a) immediate early (alpha): b) early (beta); and c) late transcripts (gamma).

Alpha genes such as ICP4 and UL48 (homologous to the HSV-1 VP16 transcription factor) are responsible for regulation and expression of the beta and gamma gene transcripts (45, 61). Thereafter, beta gene transcripts encoding proteins needed for viral DNA replication are produced and translated. Lastly, the gamma genes, which encode viral structural proteins are transcribed and translated. Capsid proteins are assembled in the nucleus and incorporate a newly cleaved monomeric viral DNA before being transported into the cytoplasm by successive envelopment and de-envelopment at the inner and outer lamellae of the nuclear membrane (52). Thereafter, the nucleocapsid acquires a final envelope structure with embedded ILTV

glycoproteins in a second budding event, this time at the trans-Golgi region. Finally, mature viral particles are released to the extracellular space by exocytosis or cytolysis (51).

Strain Classification

ILTV isolates are considered to belong to one antigenic type based on virus-neutralization (105, 113), cross-protection studies and immunofluorescence tests (28, 113). However, some strains were found to be more readily neutralized by homologous antisera rather than by heterologous antisera, indicating minor antigenic variations among ILTV strains (19). Because of the minor antigenic changes among ILTV strains and the complexity of the serological procedures, molecular classification systems are preferred to study epidemiological relationships between strains.

Molecular methods for the classification of ILTV strains include: Restriction endonuclease analysis of viral DNA (54, 77), DNA hybridization assays (76), polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) analysis of amplified DNA (PCR-RFLP) (4, 23, 24, 50, 54, 82, 91), PCR followed by gene sequencing (59, 87, 90), and whole genome sequencing (79, 81, 119, 121). Although the PCR-RFLP technique has been extensively used for ILTV strain classification due to its low cost and availability of reagents, high throughput sequence techniques have now made whole genome sequencing more accessible and reliable for future epidemiological studies.

PATHOGENESIS AND EPIDEMIOLOGY

Infectious laryngotracheitis has been described in numerous countries since its first description in 1923 (USA) and thus ILT is considered a disease with global distribution (57, 85). Restrictions in international trade can be imposed for areas affected with ILT.

Natural and Experimental Hosts

The primary host species for ILTV is the chicken, although pheasant and pheasant-chicken hybrids are also susceptible to ILT (107), while susceptibility in other avian species such as turkeys, peafowl (131), and ducks (132) has only been described experimentally. Recently, the cause of a mild respiratory disease in commercial turkeys in Brazil was identified as ILTV (96). Natural transmission in chickens occurs by direct contact with infected poultry shedding infectious virus. The virus initially infects the upper respiratory tract and/or the conjunctiva via aerosols from respiratory exudates produced by clinically affected birds, or via contaminated fomites. No evidence of vertical transmission has been found (8).

Clinical Signs

Depending on the virulence of the ILTV strain, a mild or a severe form of the disease can be seen in the field (7, 53, 84). The clinical signs seen in mild forms of ILT include nasal discharge, conjunctivitis, sneezing, coughing and a mild tracheitis with low mortality (0.1-2.0%); whereas clinical signs associated with severe ILT include gasping, coughing, depression, severe dyspnea, and expectoration of bloody stain mucus with the presence of fibrino-hemorrhagic exudate occluding the lumen of the trachea and mortality can reach 20% or more (7). Feed intake

and water consumption are also affected in infected birds. Clinical signs appear between 6 to 12 days after natural infection, whereas clinical signs in experimentally infected birds via the intra-tracheal route may appear as early as 48 hours post-challenge; peak at 5 days post challenge (DPC); and wane beginning at approximately at 7 DPC (70, 72, 123).

Pathogenesis

The first replication sites in naturally infected chickens are the conjunctiva and the mucosae of the upper respiratory tract, such as the nasal epithelium (11, 13, 15). Although birds can also acquire infection by the oral route, due to the connection of the oral cavity with the nasal mucosa by way of the cleft palate (104). After this first replication, the contiguous larynx and tracheal tissues are also infected. Regardless of ILTV infection route (nasal, conjunctival, intranasal, or other) the most active viral replication occurs in the epithelium of the trachea and consequently, the trachea is considered the ILTV target organ (6).

Histological tracheal lesions are consistent with macroscopic clinical signs. Early microscopic changes in the tracheal epithelium include the loss of goblets cells, formation of multinucleated syncytial cells, and infiltration of the mucosa with inflammatory cells such as lymphocytes, histiocytes and plasma cells as early as 2 days after challenge. Thereafter, affected epithelia desquamate, exposing the lamina propria and the blood vessels embedded in the lamina propria.

Severe destruction of the tracheal epithelium and damage to the local blood vessels causes hemorrhage and mucosal cell sloughing, which compounded with fibrin and inflammatory cell infiltration, results in the production of fibrino-hemorrhagic exudate in the tracheal lumen, which is in part responsible for the clinical signs observed during the acute

infection (8, 57). Intranuclear inclusion bodies and syncytia can be found in the trachea between 1 and 5 DPC. Evidence of regeneration of the affected tissues can be observed beginning at 6-7 DPC (56, 125). Although no solid evidence of a viremic phase has been found, it is known that ILTV can naturally infect cells and tissues other than epithelial cells as is the case in macrophages (22), and replicate in or infect extra-tracheal organs such as the lung, air sacs (64), and trigeminal ganglia (5).

Latency

Virus latency has been defined as the ability of a pathogenic virus to lie dormant in an infected cell, without the production of viral particles. Because the virus is not eliminated, during reactivation it can migrate to the trachea and produce large amounts of viral progeny (37). ILTV is believed to establish an "episomal latency" in sensory neurons of the trigeminal ganglia, as it occurs with other alphaherpesviruses such as HSV-1 and HHV-3 (Varicella Zoster virus), by leaving linear and/or lariat extra-chromosomal genetic material floating in the cytoplasm or nuclei (86), and is tightly regulated by the expression of viral Latency Associated Transcripts (LAT) which regulate the natural cell death mechanisms. By maintaining latency in immunological privileged cells, a reservoir of the virus is preserved which allows a subsequent reactivation of the virus. Such reactivation is commonly known as "recrudescence" while recovered birds actively shedding infectious virus are named "carriers".

Early descriptions of both phenomena, determined that approximately 2% of an infected flock remain as silent "carriers" of the disease for periods up to 16 months after the outbreak (49, 75). Moreover, infection of the trigeminal ganglion was determined by Bagust et al. (5) to occur between 3 and 7 days post infection with wild and vaccine strains alike, while Williams et al.

(129) confirmed the trigeminal ganglion as the latent site for ILTV by PCR. Activation of the latent virus and re-excretion of fully infectious ILTV occur after stressful events in the life of the latently infected bird, such as re-housing, onset of reproduction (66), aging and transportation to the processing plant (18).

IMMUNITY TO ILTV

The host immunological response against ILTV comprises both arms of the immune system, the humoral immunity and the cell-mediated immunity. Virus-neutralizing antibody becomes detectable in serum within 5-7 days post infection, peaks at 21 days and wanes over the following months after persisting for a year or more (5, 134). Under experimental conditions, the number of cells producing IgA and IgG in the trachea increases between days 3 and 7 post infection (135). Maternal antibodies to ILTV present in the egg are transferred to the progeny, but generally without providing protection and without interfering with vaccination (41). There is poor correlation between the level of serum ILTV antibodies and protection against challenge with virulent virus, which indicates that protective immunity is not primarily humoral (41, 42).

Protective immunity against ILT has been shown to be primarily cell-mediated. Fahey and York (41) bursectomized chickens and showed that humoral immunity is not essential for ILT protection. Robertson (102) noted that while bursectomized and cyclophosphamide-treated birds were unable to mount a humoral response, they were still resistant to challenge after live-modified vaccine immunization. Later, it was shown that resistance against ILTV could be

transferred by transplanting spleen cells and peripheral blood leukocytes from congenic immunized donors (42).

Other factors that can affect the protection against ILTV are age and gender; susceptibility of chickens to field ILTV has been shown to decline with age (12), and meat-type male chickens are more susceptible than meat-type female chickens under high temperature conditions (35ºC) (57).

DIAGNOSIS

A strong preliminary diagnosis can be obtained in severe ILT cases with high mortality and expectoration of blood. However, the milder forms of ILT should be differentiated from other agents with similar clinical signs such as Newcastle Disease, Infectious Bronchitis or Fowl pox and other diseases with viral and/or Mycoplasma etiologies. Diagnosis of mild to moderate ILT cases should be done on the basis of clinical signs and two or more confirmatory laboratory tests, including histopathology, virus isolation in CAM or tissue culture, detection of viral antigens in tracheal tissues or secretions, or detection of viral DNA. A survey performed over six state diagnostic laboratories within the USA determined that all laboratories based their ILT diagnosis on at least two tests, including one rapid test followed by a virus isolation method. Five out of six laboratories performed a confirmatory test over the virus isolation either on CAM or chicken embryonic cell culture accompanied by either histopathology, PCR, direct fluorescent antibodies (DFA), or immunohistochemistry (IHC) (34).

Histopathology

The presence of syncytia along with eosinophilic intranuclear inclusion bodies in epithelial cells from the conjunctiva and the trachea is considered pathognomonic for ILTV infection (8). Inclusion bodies can be identified in tissues stained with Giemsa or hematoxylin and eosin (HE) stain (94, 108). In addition to the characteristic intranuclear inclusion bodies (INIB) and the syncytial cells and hyperplastic epithelia in the trachea, an invasion of heterophils and macrophages can be detected since the second day after infection. Thereafter, edema is observed along with hemorrhage stemming from the lamina propria. Crespo et al (30) compared several diagnostic tests during an epornitic of ILT and established that a positive diagnosis using histopathology examination or FA over tracheal and conjunctival sections was successful only if the viral concentration by qPCR was ≥ 4 $\log_{10}$ viral particles.

The main advantage of using histopathology as a diagnostic tool is that it allows for a definitive diagnosis within 24 hours. Moreover, rapid methods of tissue processing for histopathology are available and the time required can be reduced to only three hours (94, 111). Conversely, the main disadvantages of histopathology are that a trained pathologist is needed to evaluate the tissues accurately, and that a positive diagnosis is highly dependent of how early in the infection the samples were collected (intranuclear inclusion bodies and syncytia are present primarily during the early stages of the disease).

Isolation and Identification of ILTV

Virus isolation (VI) for detection of ILTV is performed by a majority of laboratories and is considered as the gold standard of diagnostic methods. VI can be done in CAM or in chicken embryonic (CE) cell cultures, of which the former is more frequently used. Briefly, swabs or

suspensions of respiratory or conjunctival exudate and/ or tissue homogenates of larynx, trachea, lung or conjunctiva are inoculated on the CAM of 9-12 day-old SPF embryos, SPF embryonated chicken eggs or onto susceptible CE cultures. ILTV positive CAMs will develop macroscopic plaque lesions (pocks) and membrane swelling by 5 days post-inoculation, whereas a microscopic cytopathic effect (CPE) consisting of multinucleated syncytial cells with areas of cell necrosis and detachment can be seen in inoculated CE cultures. Not all viral isolation systems (embryonated CAM and CE cultures) share the same sensitivity. Hugues and Jones (67) compared different isolation methods in 11 suspect field outbreaks and found similar sensitivities in chicken embryo liver (CEL) cells and chicken kidney (CK) cells by passage 1. In contrast, CAM, chicken embryo kidney (CEK) and chicken embryo lung (CELu) cell cultures were less sensitive, detecting 10 of the 11 samples at passage 1. Chicken embryo fibroblasts (CEF) and Vero cells were unsuitable for field ILTV isolation. A definitive ILT diagnosis can be accomplished using infected CAM or CE material complemented with virus identification by PCR or EM. Other techniques such as histopathology, FA, and IHC can be used over CAM after 5 days of infection, whereas EM, FA, and IHC can be utilized for virus identification in CE. Despite a lower sensitivity for the CAM isolation method relative to CE cultures, CAM inoculation is preferred, because replication in primary cell cultures is often overgrown by other viruses, masking the presence of ILT in the sample and also because of ease of inoculation in the CAM method without the need of preparing primary or secondary CE cultures (34).

The main advantage of VI is that it provides a definitive diagnosis. However, not all strains of ILTV are easily propagated in CAM or CE (30, 67). In addition, ILTV can be overgrown by other viruses that can mask ILTV CPE, also, isolation of ILTV may take up to 3 or 4 consecutive passages (33, 34), and successful isolation depends on the virus load present in the

sample at the time of sample collection (57). Samples should be collected early in the course of infection because ILTV may not be easily detected by VI after 6 days post infection (6).

Several molecular techniques for ILTV detection have been described in the literature, such as conventional PCR (1, 3), which targets highly conserved regions of the ILTV genome; a multiplex PCR (93), intended to detect multiple avian pathogens including ILTV; and the highly sensitive quantitative real-time PCR (qPCR) (21), able to quantify the amount of virus or virus genome copy numbers present in a sample. These techniques are performed using total DNA extracted from clinical samples (such as trachea, conjunctiva, lung) or from inoculated CAM or CE used for VI. Molecular detection techniques, particularly conventional PCR and qPCR are considered to be more sensitive than histopathology, FA, and VI (30). PCR and qPCR techniques make possible detecting ILTV in the presence of other viruses that can interfere with ILT in the laboratory, such as adenovirus or reovirus (100).

Fluorescent antibody (FA) and immunohistochemistry (IHC) are techniques that can be used to detect ILTV antigens expressed in a tissue section or in infected cells in culture. Both techniques depend on specific antibody binding to their target antigens and, since the antibodies can be tagged with a fluorochrome (FA) or conjugated with an enzyme, such as peroxidase (IHC), it is possible to visualize infected cells bearing viral antigens. Although both techniques are highly sensitive and specific, research has shown that IHC is more sensitive than FA in ILTV-positive tissues (56), and that FA sensitivity is comparable to histopathology examination of the trachea and conjunctiva (30).

The use of electron microscopy (EM) for diagnosis of ILT depends on the visualization and identification of virions with morphology and dimensions consistent with those of herpesvirus; thus, it success depends on the amount of viral particles present in the sample to be

analyzed and on proper sample preparation. Hughes and Jones (67) established that the threshold concentration for ILTV detection by EM was $10^{3.5}$ particles per each 100 uL of sample. Usually, these amounts of virus are more consistently found in infected CAM or CE. Therefore, EM has been more commonly used to support virus isolation rather than to perform direct examination of field samples. EM is expensive, cumbersome, requires special skills in the laboratory as well as expensive laboratory equipment and thus, it is no longer used for diagnosis of ILT.

Detection of Antibodies

Since immunity against ILTV is mainly cellular rather than humoral (41, 42), high antibody levels are not indicative of protection prior to challenge, and flock sero-conversion after vaccination does not correlate with protection. Virus neutralization (VN) was one of the first serological tests used to assess ILTV-specific antibodies, first by means of embryonating egg inoculation via the CAM route (19), and thereafter in cell culture systems (103). Commercial ELISA systems were developed during the 1980s and used by the industry to measure antibody levels after challenge and vaccination. ELISA can be automated and has proven to be more sensitive and less cumbersome than VN methods (9), although a significant number of false positives may be detected due to non-specific reactions related to age, breed and the ELISA systems proper (2, 118, 134).

Automated ELISA systems are able to handle a large number of samples in a short period of time. The main drawbacks of the ELISA include the inability of the test to discern between live-modified vaccinated and challenged birds; and the need for acute and convalescent serum samples obtained 2-3 weeks apart for identification of seroconversion. Thus, the use of serology

to assess outbreaks in broilers in the field can be futile because most of the outbreaks occur around 40 days of age, or near processing age, therefore with little time for seroconversion (110).

In consequence, the use of serology in broilers has been considered by many of no diagnostic usefulness (30, 83). However, it has been used as a tool for mass screening of sera in surveillance programs intended to detect seroconversion in areas where ILT vaccination is not practiced and where outbreaks have not yet been recorded.

PREVENTION AND CONTROL

ILTV epornitics are caused by either back-passaged live-modified vaccine viruses or by virulent field viruses. Most of the countries around the globe have recognized CEO vaccine-related viruses as the source of outbreaks in commercial poultry, in which cases the term “vaccinal laryngotracheitis” (VLT) is used. In the case of layers and breeders in densely populated areas disease prevention is based on good biosecurity practices and vaccination. For broilers, strict biosecurity without vaccination is the most effective disease prevention approach due to the short life of these chickens. Consequently, vaccination with live-modified or recombinant ILT vaccines in broilers is usually not performed unless the flock is located in a high-risk area (33, 34).

No proven treatment that mitigates the clinical signs or lesions produced by the disease has been described. In the event of an outbreak in layers or breeders, CEO vaccination by mass application methods can successfully reduce the spread of the disease if the outbreak is at an early stage. In contrast, vaccination of broiler flocks amidst an outbreak has shown mixed results, with success in some instances, and failure in some other instances in which therapeutic

vaccination increased the mortality instead of reducing it (34). It is of paramount importance to avoid mixing vaccinated or recovered birds with susceptible chickens, which is many times done inadvertently during routine management practices such as commingling of male and female breeders or spiking (male replacement or complementation).

Good biosecurity practices (127), quarantine, hygiene, increased downtime (25) and the use of live-haul routes that minimize exposure of litter or birds contaminated with ILT to susceptible farms are critical factors in a comprehensive plan to control ILT (18, 39). Furthermore, virus shedding associated with transportation to processing plants in CEO-vaccinated broilers has been studied by Brinson et al (2011) (18), who sampled 9 CEO-vaccinated flocks in the field and at the processing plant and found that 8 to 40% (average 23% per flock) were positive for ILTV per flock by qPCR at processing age. For this reason, the use of live-modified vaccines that can establish latency in vaccinated birds is not recommended in areas in which ILT is considered exotic (7).

Live-modified vaccines

ILTV is the first poultry disease for which a successful commercial vaccine was developed (27). Although this early vaccine delivered by vent-brush route was effective in preventing the high mortality and severe clinical signs associated with infection with virulent ILTV, it had several drawbacks. First, the vent-brush vaccination route was cumbersome and time-consuming; second, the immunity waned rapidly and re-vaccinations were required in long-lived birds (71); and third, only virulent strains could provide adequate protection through this route, and thus virulent virus could spread through large and highly dense poultry production areas, thereby perpetuating the disease. In response, several mild strains, such as Cover 146 (16,

29), and SA2 (104) were selected, passaged in chicken embryos for attenuation, and tested for mass-delivery systems such as drinking water and coarse spray. The CEO strains thus developed have been effective in inducing immunity against ILT through various routes, such as conjunctival, drinking water, and coarse spray in 10 day-old birds and older.

Gelenczei (47, 48) developed a tissue-culture adapted strain, capable of stimulating immunity when delivered by eye drop but not through mass-vaccination methods. Vaccination of birds before 10 days of age by either TCO or CEO is regarded as ineffective since only a fraction of the flock will be properly immunized (27). Both CEO and TCO establish latency (5), can be spread horizontally, and may lose attenuation when back-passaged in naive birds, although TCO is less prone than CEO to revert to virulence due to its high passage and method of attenuation (55).

Recently, natural recombination events between CEO vaccines were documented in Australia (78, 80). Such finding occurred after a government authorization to use a European CEO, in the midst of a major Australian ILT epornitic and because of the shortage of local CEO vaccine supply. Although such recombination events have been controversial, recombination events may actually result in increased virulence and their effects on the epidemiology of the disease are worth studying.

Recombinant vaccines

Two classes of genetically-modified vaccines against ILT have been developed using recombinant-DNA technology, namely virus-vectored vaccines and deletion mutant vaccines. Virus-vectored vaccines have been developed to express ILTV proteins capable of inducing an immune response against ILTV challenge. Attenuated deletion mutant vaccines have been

developed by removing selected non-essential genes responsible for virulence but not for virus infection and replication, with the objective of eliciting immunity against ILT without inducing disease or adverse reactions.

Several vectors have been used for virus-vectored vaccines e.g. Fowlpox virus (FPV-LT) (35, 122) and Herpes virus of turkeys (HVT-LT) (70). These recombinant virus-vectored vaccines are widely used currently by the poultry industry worldwide. Three such recombinant vaccines have been used in the American market, namely an FPV-LT vaccine containing ILTV gB and UL-34 genes, licensed on April 2002; an HVT-LT vaccine containing ILTV gD and gI genes launched in September 2007; and most recently, a second HVT-LT vaccine containing the gB gene and licensed in 2012.

In many instances, the use of recombinant vaccines is preferred over the use of live-modified vaccines due to the lack of adverse reactions, no impact on feed conversion and the suitability for mass-delivery at hatchery by in-ovo inoculation or subcutaneous vaccination at day old. The FPV-LT vaccine was originally intended for wing web stab application at 6 weeks of age or more, but a modified claim for in ovo application was granted in 2007 for in-ovo delivery so it could be used routinely in broilers.

FPV-LT application through the in-ovo route needs to be performed at days 18.5 to 19 of embryonation (d.o.e.) and not between 17.5 to 18 due to the risk of developing vaccine-induced granulomatous pneumonia in approximately 5% of the vaccinated broilers (130). Although there is much to learn from these products, it is known that the FPV-LT and the first HVT-LT vaccines offer only partial protection against field challenge by reducing clinical signs and mortality but having no effect in viral replication and shedding (similar degree of viral shedding in recombinant vaccinated birds as in unprotected chickens) (70, 124). Furthermore, severe

outbreaks have been reported in recombinant-vaccinated chickens, suggesting that these vaccines perform better under low challenge situations or as a complement in an ILTV eradication program (7, 25, 30, 34, 38). To date, there is no related published information in commercial layers.

ILTV deletion mutants have been produced by altering, deleting, or replacing ILTV genes. Some single deletion mutants (i.e. ΔgG(36), ΔgJ(88), ΔUL0(126), and ΔUL47(61)) are capable of inducing protective immunity without causing disease, while they have retained their suitability for mass-application and their ability to propagate in CE and CAM systems. Furthermore, ΔgG and ΔgJ are of particular importance because of the possibility to discriminate challenged from immunized birds through a DIVA (Differentiating infected from vaccinated animals) ELISA strategy (115), since the host serological response against either gG or gJ would indicate field challenge.

Other mutants have been obtained by replacing non-essential genes of ILTV with immunogenic proteins from other avian pathogens, such as avian influenza, infectious bronchitis virus, infectious bursal disease (45). ILTV deleted mutants are so far experimental and have not been developed into commercial products.

Inactivated vaccines

Inactivated vaccines produced with whole ILT virions (40, 41) or affinity-purify glycoproteins (133) elicit a strong humoral response in vaccinated chickens, although with varying levels of protection. Since immunity against ILT is cell-mediated and not humoral, a successful inactivated vaccine would need to be capable of stimulating not only humoral immunity but also cell-mediated immunity. The recent spread of ILT into previously non-

enzootic areas of South America along with local prohibition to use live attenuated vaccines has resulted in the development of commercially-produced ILTV inactivated vaccines that are administered either alone or combination with recombinant vaccines. There is no experimental data documenting the efficacy of such vaccinal associations against a virulent challenge.

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CHAPTER 3

PROTECTION AGAINST A VIRULENT FIELD ISOLATE OF ILTV INDUCED BY INACTIVATED, RECOMBINANT, AND MODIFIED LIVE VIRUS VACCINES IN COMMERCIAL LAYERS[1]

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Keywords

Infectious Laryngotracheitis, Layers, HVT-LT, POX-LT, Immunity, Inactivated vaccine, qPCR

Abbreviations

CAM = chorioallantoic membrane; CEO = chicken embryo origin; CK = chicken kidney cells; DPC = Days post Challenge; DPE = Days post-exposure; FPV-LT= Fowlpox Virus vectored ILT; GaHV-1= Gallid Herpesvirus 1; GNC = Genome copy number; HVT-LT= Herpes Virus of Turkey-vectored ILT; ILT = infectious laryngotracheitis; ILTV = infectious laryngotracheitis virus; NVx/Ch = Non-vaccinated Challenged; NVx/NCh = Non-Vaccinated Non-Challenged; PCR = polymerase chain reaction; qPCR = Real-Time PCR; TCO = tissue culture origin; USA = United States of America; USDA = United States Department of Agriculture; VLT = Vaccinal Laryngotracheitis; WCH= Week Old Challenge; WPV= Weeks post vaccination

ABSTRACT

Infectious Laryngotracheitis (ILT) is an infectious respiratory disease of chickens causing important economic losses worldwide and is preventable through vaccination with live-modified vaccines (CEO and TCO). Recently, novel recombinant vaccines have been developed (HVT-LT and FPV-LT) for protection against ILT. The objective of this research was to study the immunity against ILT induced by various vaccines: a) recombinant; b) live-modified attenuated; and c) inactivated virus vaccines. Commercial layer pullets were vaccinated using one or more vaccines and challenged at 4, 9, 35 or 74 weeks of age. Protection was assessed by scoring clinical signs; and by determining the challenge viral loads in tracheal swabs at five days post challenge.

The FPV-LT vaccinated birds showed no significant protection when challenged at 4, 9, or 35 weeks of age ; the HVT-LT and TCO vaccines in combination provided protection similar to the groups of chickens vaccinated with HVT-LT or TCO at 4 and 35 weeks of age, whereas protection induced by HVT-LT and TCO co-administered was higher at 74 weeks of age. The FPV-LT+TCO group showed less protection than the HVT-LT and TCO vaccinated group at 9 weeks of age but was similar in the birds challenged at 35 weeks of age (74 week challenge was not done). Birds given the inactivated ILT vaccine had fewer clinical signs and/or viral shedding at 74 weeks of age when combined with TCO or HVT-LT, but not when given alone. Finally, the CEO vaccinated birds had the greatest reduction of clinical signs and viral shedding when challenged at 9 and 35 weeks of age.

INTRODUCTION

Infectious Laryngotracheitis (ILT) is a highly infectious upper respiratory disease of fowl that causes weight loss, drop in egg production, increased mortality, and predisposition to bacterial infections by secondary pathogens (16). The disease is caused by a virus taxonomically classified as Gallid Herpesvirus 1 (GaHV-1), member of the *Iltovirus* genus within the *Alphaherpesvirinae* subfamily of the *Herpesviridae* family (18). Although many ILT control strategies have been attempted since the 1930s including vaccination (17), the disease has persisted worldwide and spread into areas where it had never been reported, causing important economic losses to the poultry industry. Failure to control ILT in the field is strongly influenced by at least three factors: first, the ability of GaHV-1 to establish latency in the trigeminal ganglia of vaccinated or naturally infected birds allowing the virus to be shed once immunity has waned, a phenomenon known as recrudescence; lapses in biosecurity; and the use of live-modified vaccines that are not fully attenuated which are capable of regaining virulence when back-passaged in naïve birds (15).

Two classes of live-modified vaccines are available: Chicken Embryo Origin (CEO) and Tissue-culture Origin (TCO); both named after the method used for virus attenuation and propagation. CEO is less attenuated than TCO, but provides strong immunity and may be administered by mass application via the drinking water or coarse spray routes in addition to individual eye drop application, while TCO is limited to the eye-drop route. Due to the less attenuated nature of CEO vaccines, they may cause severe reactions in vaccinated broiler flocks which may express higher-than-normal mortality and feed conversion when compared to

unvaccinated and not challenged flocks (8). Moreover, CEO is prone to regaining virulence and becoming the prevalent circulating strain in the field causing vaccinal ILT (VLT) in poultry operations. Viruses obtained from numerous field outbreaks have failed to show differences in their restriction enzyme cleavage patterns (Restriction Fragment Length Polymorphism, or PCR-RFLP) compared to CEO vaccine (5, 6, 13, 14, 21, 24). Furthermore, under special conditions, ILTVs isolated from the field can be originated from recombination events between vaccine strains (23)

The search for safer vaccines providing adequate protection against ILTV, and that can be mass-applied without establishing latency or undergoing reversion to virulence lead to the development of recombinant ILT vaccines, Fowlpox virus vectored ILT (FPV-LT) and Herpes virus of turkey-vectored ILT (HVT-LT). Recent research has found that recombinant vaccines offer partial protection against clinical signs but not against viral shedding after challenge in broilers (9, 20), a phenomenon that has not been described in layers so far. Also, there are reports documenting several “vaccine breaks” in broiler flocks vaccinated with FPV-LT administered *in ovo* (7). Recombinant vaccines have been in use for only a few years, as FPV-LT was introduced for use in layers in April 2002 and in 2005 in broilers whereas the first HVT-LT was licensed in September 2007. These vaccines have been used off-label for periods of time. For instance, the first FPV-LT vaccine was originally licensed for transcutaneous application but has been used extensively *in ovo* in broiler chickens; and the first HVT-LT vaccine was originally licensed for use in long-lived chickens to be vaccinated subcutaneously at hatch, whereas the vaccine has been used largely *in ovo* in broiler chickens. Licensing for various application routes has been modified ever since and *in ovo* application clearance has been obtained for both types of vaccine.

In addition to live-modified and recombinant vaccines, a third class of ILT vaccines has been used by commercial poultry. The recent spread of ILT into non-enzootic areas of South America, together with local regulatory policies prohibiting the use of live-modified vaccines and authorizing the use only recombinant vaccines, has stimulated local use of inactivated oil-emulsified ILT vaccines, which are typically applied alone or in combination with recombinant vaccines. There is no recent experimental data documenting the efficacy of such vaccine associations against a virulent challenge. Previous clinical trials with killed vaccines have shown variable results (10, 33) (2) possibly because the active immunity against ILT is primary cell-dependent and not humoral (27, 28). Efficacy of inactivated vaccines likely relies on triggering or enhancing cell-mediated responses against ILTV.

The objective of the present study was to examine the immunity provided by live-modified, recombinant vectored, and inactivated vaccines, and combinations of those vaccines in commercial table egg layers by means of a long-term study designed to include challenges at various times between 4 and 74 weeks of age with one or more classes of ILT vaccines.

MATERIAL AND METHODS

Experimental Design

One-day-old commercial table egg layer pullets (Hy-Line, variety W36) all vaccinated with a full dose of the CVI988 strain of Marek's disease virus (Pfizer Animal Health [Zoetis], Durham, NC), were acquired from a commercial hatchery, wing-tagged for identification, and divided into several groups with different vaccination schemes (Table 3.2). Appropriate groups

of vaccinated and non-vaccinated chickens were included in four different challenge studies performed at 4, 9, 35, and 74 weeks of age. A summary of the vaccines used in all studies is shown in Table 3.1, and a list of the vaccine groups included in each challenge is presented in Table 3.2.

The various vaccination schemes consisted of single or multiple vaccinations with live-modified, recombinant, and inactivated vaccines (Table 3.2). The recombinant vaccines HVT-LT (Innovax® ILT, [Merck Animal Health, Summit, NJ]), and FPV-LT (Vectormune® FPV-LT [CEVA Biomune, Lenexa, KS]) were applied as a full dose at hatch via the subcutaneous route. The live-modified vaccines TCO (LT-IVAX® [Merck Animal Health, Summit, NJ]) and CEO (LT-BLEN [Merial Select Inc., Gainesville, GA]) were delivered by eye-drop at different ages depending on each particular study.

The TCO vaccine was delivered at 14 days of age for the 4 WCH; at 6 weeks of age for 9 WCH; and at 6 weeks with a revaccination at 13 weeks of age for the 35 and 74 WCH, whereas the CEO vaccine was applied at 6 weeks of age for the 9 WCH and 35 WCH studies. A non-commercial oil-based inactivated vaccine (KILLED, Lohmann Animal Health, Winslow, ME) was applied in the breast by intramuscular injection in a volume of 0.5 ml per bird at 13 weeks of age for the 35 and 74 week old challenge studies. One group was kept without vaccination (NVx) to be used as a non-vaccinated challenged (positive control) group (NVxCh), or a non-vaccinated non-challenged (negative control) group (NVxNCh) in each challenge study. Once vaccinated, each group was placed in isolated colony houses at the Poultry Diagnostic and Research Center, Department of Population Health, University of Georgia (PDRC-UGA). Experimental groups were maintained separate to prevent horizontal spread of live-modified vaccines between groups.

Challenge studies were performed at 4, 9, 35 and 74 weeks of age. For the 4, 9 and 74 WCH 5 birds from each of the groups shown in Table 3.2 were transferred to filtered-air negative pressure (FANP) isolators located at PDRC-UGA with one replicate (10 birds per group in total) and challenged. While all other challenge studies were done in FANP isolators, the 35 WCH was performed on the floor in isolation rooms (Table 3.2). To comply with the Animal Welfare Act (35), a maximum of 4 birds per replicate were used for the 74 WCH, instead of the usual 5, with a total of 9 birds per group with exception of the HVT-LT group, which had a total of only 7 birds.

The level of protection conferred by each treatment was assessed by clinical sign scoring and challenge ILTV quantitation on tracheal swab samples collected at 5 and 7 days post challenge (DPC) for the 4, 9 and 35WCH studies; whereas clinical signs and tracheal swabs were taken at 5 and 6 DPC for the 74WCH.

All animals were treated as established in the applicable portions of the Animal Welfare Act and the DHSS “Guide for the Care and Use of Laboratory Animals”.

Contact-exposed SPF Birds

Possible horizontal transmission from vaccinated-challenged chickens to contact-exposed specific pathogen free (SPF) chickens was examined at 9 WCH. Five 28-day-old SPF birds were placed as contact-exposed naïve birds per isolator (10 per treatment group in total) to remain in contact with the infected birds for 24 hours. After the exposure period, each contact group was moved and placed in a different isolator for 9 days. Tracheal swabs were collected at 5, 7 and 9 days post-exposure (DPE), pooled by group (2 pools of 5 samples each per treatment), inoculated

into CK cells and then into Chicken Embryo Liver (CEL) cells for 2 passages and tested by indirect Immunofluorescence (IFA).

ILTV Challenge Strain

The genotype V (CEO-related) 63140 strain of ILTV, isolated from a VLT outbreak with severe respiratory illness and mortality in a 45 day-old broiler was used in all challenges (20, 36, 37). The virus was propagated in Chicken Kidney (CK) cells and titration was done as previously described (30). Titer calculations were done using the Reed and Muench method and expressed as 50% tissue culture infective dose ($TCID_{50}$). The challenge virus titer used was $10^{3.5}$ $TCID_{50}$ per bird/dose delivered in a total of 200 uL (100 uL intratracheally and 50 uL into each eye).

Preparation of Hyperimmune Serum and Indirect Immunofluorescent Antibody Test

An indirect immunofluorescent antibody test (IFA) was used to determine whether ILTV was present in chicken embryo cultures inoculated with pooled samples obtained from SPF contact birds in the 9 WCH. Hyperimmune serum against 63140 ILT was prepared in SPF chickens and used as primary antibody; while a secondary fluorescein-labeled anti-chicken antiserum was obtained from a commercial source. The hyperimmune antiserum was prepared by inoculating three times, with two-week intervals, 4-week-old SPF chickens, each time with one ml of a 63140 solution containing $10^{3.5}$ $TCID_{50}$ per ml titrated in chicken kidney cells (26).

The delivery routes used in the first inoculation were three: the conjunctival route (50 ul each eye), the intranasal route (50 ul into each nostril), and the intramuscular route (800 ul) in the breast, for a total of 1 ml of solution; in contrast, the second and third inoculations were only given by the intramuscular route.

HVT-LT and FPV-LT Detection

Successful delivery of the recombinant vaccines (HVT-LT and FPV-LT) was assessed by PCR. Both vaccines were applied by subcutaneous injection in the neck at hatch. FPV-LT delivery was tested by collecting connective tissue samples from the necks of 15 FPV-LT-vaccinated chickens at 7 days post vaccination, which were analyzed by conventional PCR using previously published primers and procedures (12, 38). For HVT-vaccinates, spleen samples were taken from 15 HVT-LT birds for analysis by qPCR as previously described (19, 20, 37).

Furthermore, persistence of HVT-LT in vaccinated birds was assessed by detection of HVT-LT DNA in feather pulp samples collected at 3, 5, and 10 weeks post-vaccination; and in spleens collected at 3, 5, 10, 18, 37, 39, and 75 weeks post vaccination.

DNA Extraction from Tracheal swabs, Spleens, Feather Pulp and Connective Tissue

Tracheal swab samples were obtained at 5 and 7 DPC in all the experimental groups in the 4, 9, and 35 WCH experiments. For the 74 WCH experiment the tracheal swab samples were collected at 5 and 6 DPC. At collection, each swab was shaken into a 1.8 ml microcentrifuge tube containing 1 ml sterile PBS with 2% antibiotic-antimycotic 100X solution (Gibco, Grand Island, NY) and 2% calf serum (CS) for 20 seconds and stored at -80 C until processing. DNA extraction from tracheal swab samples was performed using the MagaZorb® DNA mini-prep 96-well kit (Promega, Madison, WI) following the manufacturer's recommendations with modifications. Briefly, 70 ml of sample (swab suspension) was incubated with 7 ml of proteinase K and 50 ml of lysis buffer at 56 C for 10 min in a 96-well plate, and 10 ul of magnetic beads were added along with 125 ml of binding buffer to each well and incubated for 10 min at room temperature (RT). The supernatant was separated and the beads washed twice with washing

buffer. Finally, the DNA was eluted from the beads with 100 ml of elution buffer and frozen at -80C until further use.

Connective tissue obtained from the neck and feather pulp samples were collected in 1.8 ml microcentrifuge tubes and stored at -80C until further processing, while spleen samples were collected into 2 ml homogenizing tubes (Lysing Matrix D Tubes, MP Biomedicals LLC, Solon, OH) previously loaded with 0.75 ml of PBS with 2% calf serum, and homogenized in two 40-second rounds using a FastPrep FP120 instrument (Bio 101, Thermo Electron Corporation, Milford, MA). In the case of neck connective tissue and feather pulp, the totality of the sample was used for DNA extraction, while in the case of the spleen samples a 200 uL aliquot was used for the extraction. DNA from these samples (neck connective tissue, feather pulp, and spleen) was extracted using a commercial DNA extraction and purification kit (High pure PCR Template Preparation Kit, Roche Diagnostics, Indianapolis, IN) following the manufacturer's instructions.

Quantitative real-time PCR (qPCR) for ILTV and HVT-LT genome detection

Genome copy numbers of ILT viral DNA present in tracheal swabs and of HVT-LT recombinant vaccine DNA present in spleen samples were quantified by qPCR in a duplex assay normalized for the host DNA. The assays were carried out on a PTC-200 thermal cycler (MJ Research/Bio-Rad, Hercules, CA) and real-time PCR read by a Chromo 4 optical detector (MJ Research/Bio-Rad, Hercules, CA). Primers and probe were designed to amplify a 103 bp region of the UL44 gene (glycoprotein C, or gC) of ILTV (4, 20, 36, 37) and a 76-bp region of the SORF1 gene of HVT (19, 37).

A 76-bp region of α-collagen gene from chicken (*Gallus gallus domesticus*) was used as a control for DNA extraction as described (Islam, et al 2004). Each multiplex qPCR assay was performed with a final reaction volume of 26.5 uL that consisted in: 12.5 uL of 2X iQ Multiplex Powermix® (Bio-Rad Laboratories, Inc). Primers combined at a final concentration of 1 uM and probes at a final concentration of 0.1 uM, 1.0 uL of thermo labile uracil N-glycosilase at 0.05 U/ml (HK-UNC Epicentre Biotechnologies, Madison, WI) and 5 uL of DNA template. The thermal cycling profile used was as follows: 50 C for 2 min; 95 C for 15 min; 40 cycles of 94 C for 15 sec; and 60 C for 45 sec. In the case of HVT-LT and ILTV a sample is considered as positive when its CT value is less or equal as 38, as previously described (4, 19).

Clinical sign score evaluation

Clinical signs were recorded at 5 and 7 DPC for the 4, 9 and 35 WCH experiments; or at 5, 6 and 7 DPC for the 74 WCH experiment, according to previously reported methods (20, 36). Briefly, respiratory signs, conjunctivitis, and depression were scored on a scale of 0 to 3, with normal (0), mild (1), moderate (2), and severe (3) scores. Any mortality was given a total score of 9, as it is the highest sum value in each category. The median clinical score was calculated and the median values were statistically analyzed.

Serology

Serum samples from all experimental groups and controls were taken prior the challenge and post-challenge (Figure 3.5). Sera obtained post-challenge were taken at 7 DPC for the 4 and 9 week old challenges, while for the 35 and 74 week old challenges, samples were taken at 13 and 9 DPC respectively. All serum samples collected were processed at the Georgia Poultry

Laboratory Network (GPLN, Oakwood, GA 30566) using a commercial ELISA kit for detection of ILTV antibodies [ILT ELISA CK124 (BioChek, Middlesex, United Kingdom)]. The ELISA procedure, data processing, sample to positive value (s/p value) and titer calculations were done according to the manufacturers' instructions (s/p value of 0.5 or a titer of 1070 as cut-off value).

The following s/p equation was used:

$$\frac{\text{(Mean Sample Absorbance)} - \text{(Mean Absorbance of Negative control)}}{\text{(Mean Absorbance of positive control)} - \text{(Mean Absorbance of negative control)}} = \text{S/P}$$

The formula used to transform s/p values into titers was:

LOG_{10} Titer = (1.1 * LOG_{10} (s/p)) + 3.361

The titer was obtained from the log_{10} titer using the following formula:

Titer = Antilog of Log_{10} Titer

Statistical analysis

Data were analyzed using the GraphPad Prism 6.01 statistical analysis package (GraphPad Software, La Jolla, CA). qPCR data was analyzed using one-way ANOVA with Bonferroni's method for multiple pair-wise comparisons, while the Kruskal-Wallis test was independently used to compare median clinical scores for each group followed by a multiple pair-wise comparison performed for *post hoc* comparisons.

RESULTS

Detection of FPV-LT and HVT-LT genomes by PCR analysis at 7 days post vaccination

In order to verify that the vaccine delivery was effective, a subset of 15 hatch mates vaccinated with the FPV-LT or HVT-LT vaccines were sampled for detection of vaccine vector DNA. The FPV-LT genomes were detected by conventional PCR using two sets of primers in 9 out of 15 neck connective tissues collected from chickens vaccinated with FPV-LT at hatch. HVT-LT genomes were detected in all the spleen samples collected (15 out of 15). No FPV-LT or HVT-LT genomes were detected in unvaccinated groups by qPCR.

Clinical sign score assessment

The mean clinical sign scores were higher at 5 DPC (Figure 3.1) than at 7DPC in the 4, 9, and 35 WCH experiments; and at 6 DPC and 7 DPC in the 74 WCH experiment (Figure 3.2). The NVx/Ch group (positive control) had the highest mean clinical sign score in all challenge experiments, whereas the group with the lowest clinical score mean was the NVx/NCh (negative control) at both 5 and 7 DPC; and at 5, 6 and 7 DPC for the 74 WCH experiment. The predominant clinical signs observed were depression and respiratory signs such as snicks and gasping due to the presence of bloody/caseous exudate in the trachea. Conjunctivitis was rarely seen in the 4, 9, and 35 WCH experiments; and was undetectable in the 74 WCH experiment (data not shown). At 5 DPC in the 4 WCH experiment (Figure 3.1A), the HVT-LT+TCO and TCO groups were statistically different from the NVx/Ch group ($p<0.001$), while the FPV-LT+TCO and HVT-LT groups were also statistically different but with a higher p value ($p<0.01$). At 7 DPC in the 4 WCH experiment (Figure 3.2a), only the TCO ($p<0.01$) and HVT-

LT groups showed a significant reduction of clinical signs ($p<0.05$). The 5 DPC results in the 9 WCH experiment (Figure 3.1B) were similar to the results of the 4 WCH experiment; that is, the CEO, TCO and HVT-LT+TCO groups were statistically different from the positive control group ($p<0.001$); the HVT-LT group was also different from the positive control group ($p<0.01$). The FPV-LT+TCO group was statistically different from the positive control group, albeit at a lower significance ($p<0.05$). For the 9 WCH experiment, the 7 DPC data (Figure 3.2b) indicated that all groups with the exception of the FPV-LT group were significantly different from the positive (NVx/Ch) control group ($p<0.001$). In the 35 WCH experiment, at 5 DPC only the CEO and HVT-LT+TCO (both at $p<0.001$); HVT-LT+KILLED and TCO groups (both at $p<0.05$) were statistically different from the NVx/Ch positive control group; whereas in the 35 WCH experiment, at 7DPC, the CEO ($p<0.001$); HVT-LT+TCO; HVT-LT+KILLED; FPV-LT+KILLED; KILLED (all four at $p<0.01$); and HVT-LT and TCO (both at $p<0.05$) showed a significant reduction in mean clinical sign scores when compared with the positive control.

The HVT-LT+TCO group ($p<0.001$); TCO+KILLED; TCO; and HVT-LT+KILLED ($p<0.01$) were statistically different from the positive control, while the HVT-LT, and KILLED groups were statistically similar to the positive control. In contrast, in the 74 WCH experiment the HVT-LT+TCO and TCO+KILLED groups ($p<0.001$); HVT-LT+KILLED ($p<0.01$); and HVT-LT ($p<0.05$) groups were statistically different from the positive control at 6 DPC. Finally, in the 74 WCH only the HVT-LT+TCO ($p<0.05$) group was different from the positive control at 7DPC.

Tracheal challenge virus loads

Tracheal viral loads were assessed by qPCR on tracheal swabs at 5 and 7 DPC for most experiments (or at 5 and 6 DPC but not at 7 DPC for the 74 WCH experiment) and were expressed in Log_{10} ($2^{-\Delta\Delta ct}$) genome copy numbers (GCN) as shown in Figures 3.1 and 3.2. As was the case for clinical signs, GCN were higher overall at 5 DPC than at 7 DPC and thus GCN were assessed at 5 or 6 days post-challenge.

At 5DPC in the 4 WCH experiment, genome copy numbers were statistically different from the positive control (Figure 3.1a) for the HVT-LT+TCO, TCO, HVT-LT ($p<0.001$) and FPV-LT+TCO groups ($p<0.01$). The 9 WCH experiment data showed that at 5 DPC the CEO, HVT-LT+TCO ($p<0.001$), and TCO groups were statistically different ($p<0.01$) from the positive control. The 35 WCH data showed that at 5 DPC all the groups vaccinated with a live-modified vaccine, alone or in combination with other vaccines ($p<0.001$); and the HVT-LT and HVT-LT+KILLED groups ($p<0.01$) had a significant reduction in the GCNs when compared to the NVx/Ch (positive control) group (Figure 3.1c). GCNs from CEO, TCO+KILLED ($p<0.01$), TCO, and HVT-LT groups ($p<0.05$) were statistically different from NVx/Ch group at 7DPC (Figure 3.2c).

By 5 DPC in the 74 WCH experiment, the GCN of groups HVT-LT+TCO, TCO+KILLED ($p<0.001$) and HVT-LT+KILLED ($p<0.05$) were statistically different from the positive control. The GCNs at 6 DPC in this experiment show a similar pattern than at 5 DPC; that is, groups TCO+KILLED ($p<0.001$) and HVT-LT+TCO were statistically different ($p<0.01$) from the positive control.

Contact-exposed SPF Birds

Results for virus isolation and IFA from pooled tracheal samples collected from the 9 week challenged contact-exposed SPF birds are presented in Table 3.3. At 5 DPE, both pools from the SPF chickens in contact with FPV-LT-vaccinated-challenged birds, and TCO+FPV-LT-vaccinated-challenged birds along with the SPF chickens exposed to the positive controls (NVx/Ch) were positive to the presence of ILTV by virus isolation in cell culture and confirmed by indirect immunofluorescence using polyclonal antibodies. Only one pooled sample in the TCO-vaccinated-challenged SPF contact group was positive while all other groups remained negative. At 7 and 9 DPE, only the NVx/Ch, FPV-LT and positive control-exposed SPF contact chickens were positive to ILTV by immunofluorescence.

HVT-LT detection

Spleen samples from HVT-LT-vaccinated birds taken at sequential ages between 1 and 75 weeks of age to assess viral HVT GCN and percent HVT-positive chickens were found to vary during the lifetime of the vaccinated birds. That is, early in life (1, 3 and 5 WPV) all spleens were found to be positive for viral HVT and contained a higher HVT GCN; whereas a decrease in percentage of positives (from 100% to 80% positives) and in GCN was observed at 10 WPV. As HVT-LT vaccinated birds aged (18, 37 and 39 WPV), the percentage of HVT-positive birds remained at 50%, which was the lowest HVT positive rate recorded in all samplings, and was statistically different from the HVT positive rate obtained at 1 to 5 WPV. Finally, at the last sampling (75 WPV), 100% of spleens were positive to HVT, similarly to the early samplings at 1, 3 and 5 WPV, albeit with a lower GCN (Figure 3.3a). Spleens from non-vaccinated birds contained no detectable HVT.

Spleens from groups receiving HVT-LT vaccines alone or in combination with other vaccines (HVT-LT; HVT-LT+TCO; and HVT-LT+KILLED) were tested using qPCR at 75 weeks of age for detection of HVT DNA. Interestingly, the percentage of HVT positives and the HVT GCN were dissimilar among groups (Figure 3.3b). The group vaccinated with HVT-LT only had the highest positive percentage of positives (100%) and also the highest HVT GNC; this group (HVT-LT) was statistically different ($p<0.01$) form the non-vaccinated group, while the other two groups of chickens receiving HVT-LT vaccine, HVT-LT+TCO and HVT-LT+KILLED, had 33.3% positives and 44.4% positives, respectively, and both had a lower GNC value and were statistically similar to the negative control.

HVT-LT GCN was also assessed in feather pulps of vaccinated birds at 5 and 10 WPV, and the findings are presented in Figure 3.4a. Briefly, a decrease in the HVT GCN and percentage of HVT positives was observed, as was seen in the spleen samples.

Serology

ILTV antibody titers obtained by ELISA technique in the two sets of sera taken from all experimental and control groups prior the challenge and post-challenge (7 DPC for the 4 and 9 week old challenges; 13 DPC for the 35 week old challenge; and 9 DPC for the 74 week old challenge) were graphed and are shown in Figure 3.5. The lowest titers obtained before challenge were found in the non-vaccinated and FPV-LT groups, whereas the group with the lowest titers after challenge was the non-vaccinated non-challenged group. The groups with the highest titer levels prior and after challenge were the KILLED group and their combinations (HVT-LT+KILLED, FPV-LT+KILLED, and TCO+KILLED). Non-specific (false positive) reactions in the non-vaccinated non-challenged sera samples prior and after challenge were absent at 4 or at 9

WCH but were present at 35 WCH (2 false positives over 30 negative control tested) and 74 WCH (12 birds over 27 negative control tested). No seroconversion was detected at 4WCH in any challenged group (Figure 3.5a) while only some groups (HVT-LT and HVT-LT+TCO) seroconverted after the challenge when samples were analyzed at 7 DPC. Seroconversion was seen at 35WCH at 13 DPC and at 74WCH at 9 DPC in all non-vaccinated challenged groups.

DISCUSSION

The objective of the present study was to assess the protection against ILT by several vaccines and vaccine combinations, including live-modified, recombinant, and inactivated vaccines in commercial table egg layers. ILTV challenge studies were done at various ages (4, 9, 35, and 74 weeks of age) and protection was assessed on the basis of prevention or reduction of clinical signs and total viral loads in the trachea after challenge with a virulent strain of the virus. Other aspects related to ILT immunity in recombinant-vaccinated chickens were also examined, including the serological response against ILT post-vaccination and post-challenge by ILTV antibody ELISA, the persistence of HVT-LT recombinant virus in the spleens of vaccinated birds and, the spread of the challenge ILT virus from vaccinated-challenged to contact chickens by using qPCR technique.

Since the peak of clinical signs and viral shedding occurred at 5 DPC, consistent with previous similar research (20, 25) in the four challenge studies at 4, 9, 35, and 74 weeks of age, our conclusions were based on data collected at 5 DPC rather than at 6 or 7DPC. As shown in previous research performed in broilers (20, 25, 29, 37), vaccination with modified live vaccines

(CEO or TCO) alone induced better protection than vaccination with recombinant vaccines at 5 DPC in all ages studied (4, 9, 35 and 74 weeks of age), as shown in previous research performed in In contrast, vaccination with FPV-LT, KILLED (inactivated vaccine at 13 weeks old) and the combination of FPV-LT+KILLED resulted in clinical sign scores and viral shedding statistically not different to clinical signs or viral shedding recorded for the non-vaccinated challenged group, indicating that FPV-LT alone or combined with the inactivated vaccine; and the inactivated vaccine alone did not induce significant protection against the ILTV challenge. The absence of protection against the virulent challenge in the birds vaccinated with the FPV-LT vaccine assessed in the present work disagree with previous research performed in broilers (20, 37), in which FPV-LT-vaccinated broilers either by in-ovo or subcutaneous route were at least partially protected against clinical signs and viral shedding induced by the same ILTV challenge virus (63140) inoculated at similar doses. We speculate that such difference in FPV-LT protection may be due to intrinsic differences between the immune systems of meat type and egg type chickens (22), in addition to differences in the challenge ages used. The results in the case of the KILLED vaccine group are consistent with common knowledge that ILT immunity is independent from humoral immunity (10, 11).

The level of protection observed in groups of chickens receiving the HVT-LT or TCO vaccines waned as the birds aged. For instance, vaccination with HVT-LT resulted in reduction in clinical signs and viral shedding at 4 and 9 WCH, partial protection as shown by reduced viral shedding at 35 WCH, and no significant protection at 74 WCH. However, TCO vaccination resulted in reduced clinical signs and viral shedding at 4, 9, and 35 WCH, but conferred only partial protection at 74 WCH, as expressed by reduced clinical signs. Dually vaccinated groups

like HVT-LT+TCO, HVT-LT+KILLED and TCO+KILLED were partially protected as expressed by a reduction of both clinical signs and viral shedding at 74 weeks and better protected in comparison to groups vaccinated with a single vaccine. The exact mechanism by which these combinations of vaccines increased protection at 74 WCH is unknown.

Based on the serological results of the present study, it was concluded that ELISA systems may be inefficient in the identification of infection at 4 and 9 WCH when blood is collected within 7 days post-challenge due to insufficient time for adequate and detectable seroconversion (31). In contrast, antibody ELISA was able to detect seroconversion 13 days post-challenge when the infection occurred at 35 WCH or at 9 days post-challenge for 75-week-old chickens. However, the proportion of reactors present in non-vaccinated birds increased with age, an observation that had already been described (1, 32).

Detection of HVT vector DNA in the spleens of singly or dually vaccinated chickens was pursued with the intention of demonstrating persistence of the vector over a period of time post-vaccination. Interestingly, as compared to groups vaccinated with HVT-LT+KILLED; and HVT-LT+TCO, a higher proportion of HVT-positive chickens were detected in the group vaccinated with HVT-LT alone by 75 weeks of age. Fifty percent of chickens vaccinated with HVT-LT+KILLED or HVT-LT+TCO had HVT-positive spleens, whereas the group receiving the HVT-LT vaccine alone had 100% positive spleens. It is possible that either the TCO and/or the KILLED vaccine may have stimulated an immune response resulting in a lower HVT-LT concentration in the spleens of chickens receiving the dual vaccines.

As previously observed in broilers (34) in the 9 WCH experiment the vaccine combination FPV-LT administered at day of age followed by TCO at 6 weeks resulted in reduced viral shedding but did not in reduced clinical signs, while the group that received TCO alone showed a reduction of both clinical signs and viral shedding.

In the 9 WCH study, spread of the challenge virus occurred from FPV-LT+TCO, FPV-LT, and TCO vaccinated/challenged groups of chickens to susceptible SPF chickens, indicating that vaccinated birds with little or no clinical signs of infection, can transmit pathogenic field virus to susceptible birds.

Data obtained at 5 DPC in the 4, 9, 35 and 74 WCH experiments indicated that clinical sign scores diminished in severity as the age of chickens progressed. At early ages, 4 and 9 WCH, clinical sign scores are clearly higher than at older ages 35 and 74 WCH, albeit loads of viral shedding post-challenge were not reduced in older chickens. Confirming previous work, this pattern suggested a natural age-dependent resistance against the disease (3), suggesting that a reduction of viral loads provide a better indication of the degree of protection than clinical sign scores in long-lived birds. Consequently, differences in mean clinical sign scores between vaccinated/challenged groups and the non-vaccinated/challenged group were not as significant as age progressed (35 and 74 weeks). Such differences in older birds were subtle and less significant.

The results of these studies are important because they demonstrate that vaccinated birds with little or no clinical signs of infection, can transmit pathogenic filed viruses to susceptible birds and that susceptible old birds exhibit less clinical signs than younger birds challenged, albeit with similar amount of viral shedding.

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The authors gratefully acknowledge Drs. Rodrigo Espinoza, Peter O'Kane, Denise Brinson and Yun Ting Wang for their colaboration during the execution of this project as well as Dr. Roy D. Berghaus for his valuable statistical insights.

Table 3.1: Vaccines used in the experiment

Type of Vaccine	Trade Name	Manufacturer	Titer Dose / Bird	Route[a]	Vaccination Age[b]
HVT-LT	Innovax ILT®	Merck Animal Health, Summit, NJ	7700 pfu in 200 uL	SC	1 DO
FPV-LT	Vectormune FP-LT	Ceva Biomune, Lenexa, KS	3860 pfu in 200 uL	SC	1 DO
TCO	LT-IVAX	Merck Animal Health, Summit, NJ	$10^{3.83}$ TCID in 100 uL	ED	2 WO[c] 6 WO[d] 6 WO & 13 WO[e]
CEO	LT BLEN	Merial Select, Inc Gainesville, GA	$10^{4.38}$ TCID in 100 uL	ED	6 WO
KILLED	Experimental Oil – Based Inactivated	Lohmann Animal Health, Winslow, ME	$10^{6.9}$ EID in 0.5 ml	IM	13 WO

[a]Route: SC, Subcutaneous; ED, Eye Drop; IM, Intramuscular. [b]Vaccination Age : DO, Day Old; WO, Weeks Old. [c]TCO vaccinated groups at 4 week Challenge. [d]TCO vaccinated groups at 9 week challenge. [e]TCO vaccinated groups at 35 and 74 week challenge

Table 3.2: Treatment groups for each of four challenge studies.

Treatments	Challenge Age			
	4 Week	9 Week	35 Week	74 Week
NVx/NCh[a]	---	---	---	---
CEO	ND	IT/ED[c]	IT/ED[c]	ND
HVT-LT+TCO	IT/ED[c]	IT/EDc	IT/ED[c]	IT/ED[c]
FPV-LT+TCO	IT/ED[c]	IT/ED[c]	IT/ED[c]	ND
TCO+KILLED	ND	ND	IT/ED[c]	IT/ED[c]
TCO	IT/ED[c]	IT/ED[c]	IT/ED[c]	IT/ED[c]
HVT-LT+KILLED	ND	ND	IT/ED[c]	IT/ED[c]
HVT-LT	IT/ED[c]	IT/ED[c]	IT/ED[c]	IT/ED[c]
FPV-LT+KILLED	ND	ND	IT/ED[c]	ND
FPV-LT	IT/ED[c]	IT/ED[c]	IT/ED[c]	ND
KILLED	ND	ND	IT/ED[c]	IT/ED[c]
NVx/Ch[b]	IT/ED[c]	IT/ED[c]	IT/ED[c]	IT/ED[c]

[a] NVx/NCh, Non vaccinated non challenged group; [b] NVx/Ch, Non vaccinated challenged group; [c] IT/ED, Intratracheally/eye drop challenge at dose $10^{3.5}$ $TCID_{50}$ /chicken

Table 3.3: Virus isolation and immunofluorescence on pooled tracheal swabs from contact SPF sentinel chickens exposed to vaccinated-challenged birds at the 9 Week Old Challenge sampled at 5, 7, and 9 days post-exposure (DPE).

DPE	NVx/Ch	FPV-LT	HVT-LT	TCO	TCO+ FPV-LT	TCO+ HVT-LT	CEO	NVx/ NCh	63140 Control	Cell Control
5	2/2[a]	2/2	0/2	1/2	2/2	0/2	0/2	0/2	2/2	0/2
7	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	0/2
9	2/2	2/2	0/2	0/2	0/2	0/2	0/2	0/2	2/2	0/2

[a] Number of positive pooled samples over total number of samples per group treatment.

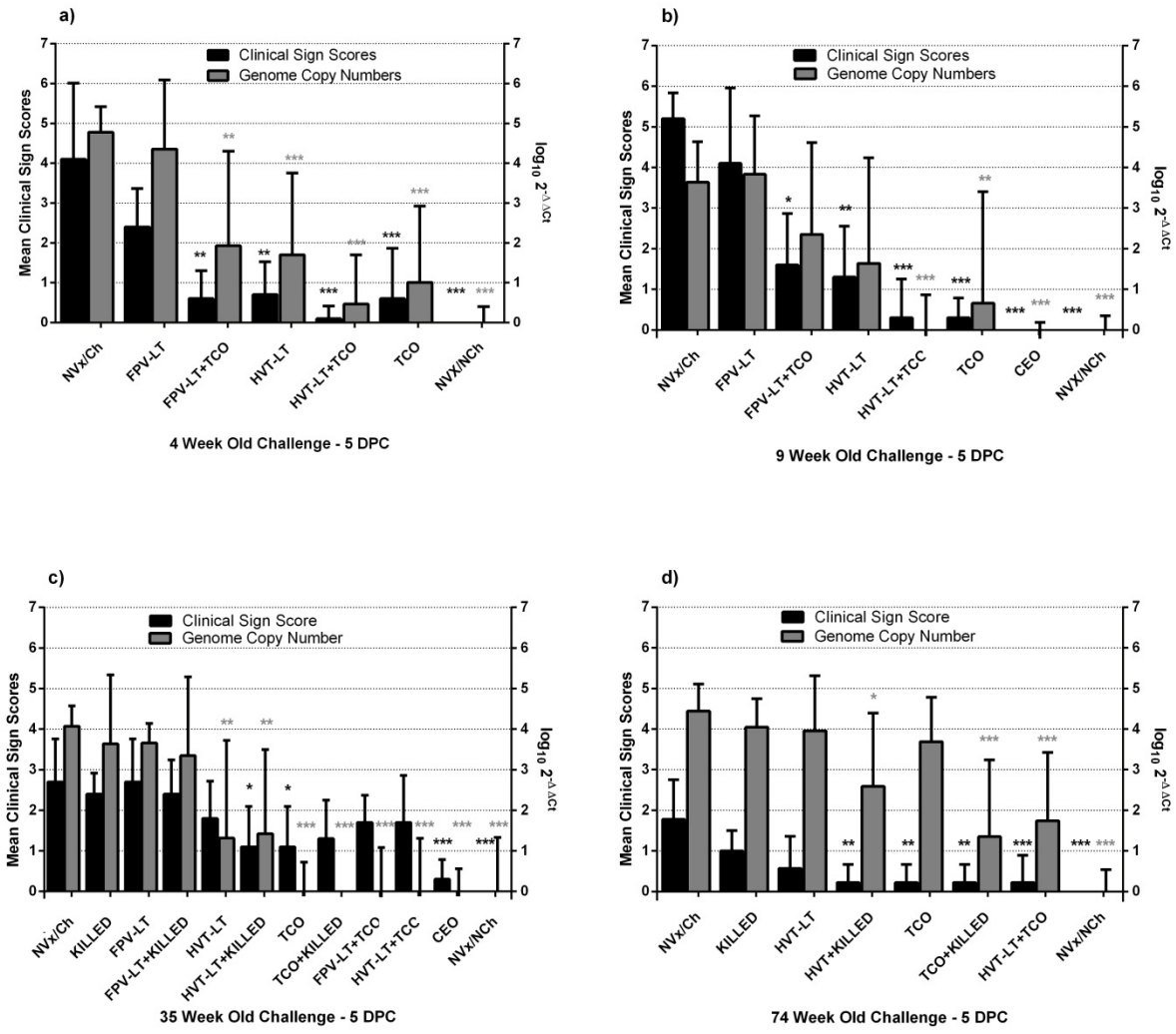
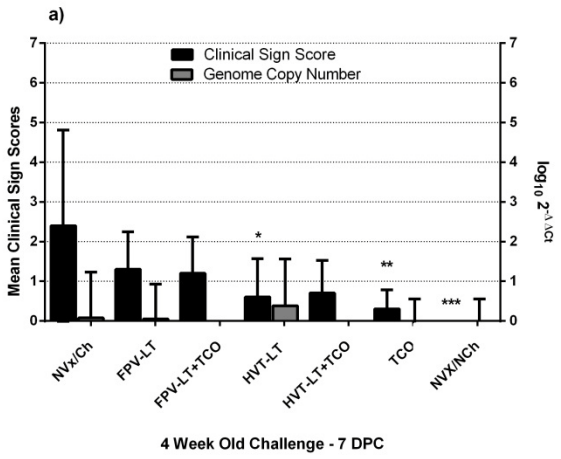
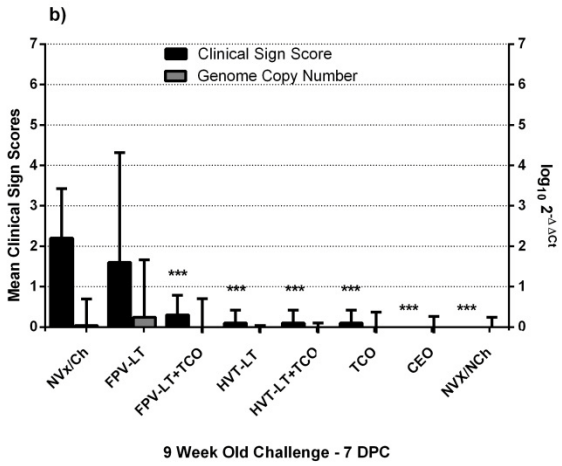
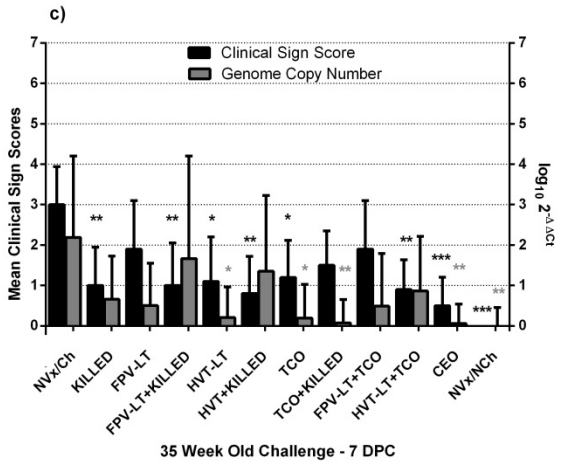
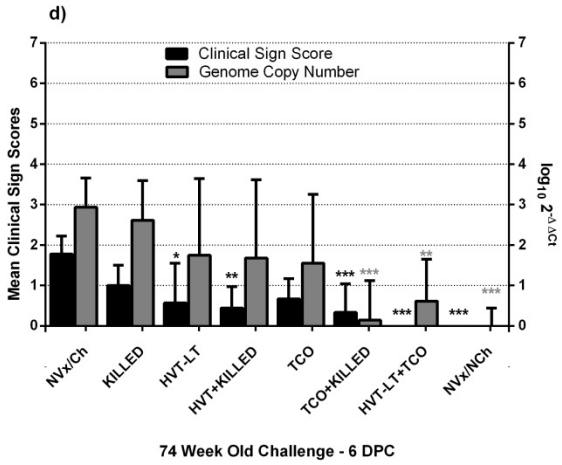


Figure 3.1: Mean clinical sign scores (black bars) and tracheal viral loads (grey bars) were assessed at 5 DPC after virulent ILTV challenge at 4 Weeks (a), 9 Weeks (b), 35 Weeks (c) and 74 Weeks (d) old Asterisks indicate a statistically significant difference relative to the positive control (NVx/Ch). Black and grey asterisks correspond to clinical signs and genome copy numbers, respectively. Data are presented as mean ± SD (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).


a)
Clinical Sign Score
Genome Copy Number
Mean Clinical Sign Scores
$\log_{10} 2^{-\Delta\Delta Ct}$
NVx/Ch
FPV-LT
FPV-LT+TCO
HVT-LT
HVT-LT+TCO
TCO
NVX/NCh
4 Week Old Challenge - 7 DPC


b)
Clinical Sign Score
Genome Copy Number
Mean Clinical Sign Scores
$\log_{10} 2^{-\Delta\Delta Ct}$
NVx/Ch
FPV-LT
FPV-LT+TCO
HVT-LT
HVT-LT+TCO
TCO
CEO
NVX/NCh
9 Week Old Challenge - 7 DPC


c)
Clinical Sign Score
Genome Copy Number
Mean Clinical Sign Scores
$\log_{10} 2^{-\Delta\Delta Ct}$
NVx/Ch
KILLED
FPV-LT
FPV-LT+KILLED
HVT-LT
HVT+KILLED
TCO
TCO+KILLED
FPV-LT+TCO
HVT-LT+TCO
CEO
NVx/NCh
35 Week Old Challenge - 7 DPC


d)
Clinical Sign Score
Genome Copy Number
Mean Clinical Sign Scores
$\log_{10} 2^{-\Delta\Delta Ct}$
NVx/Ch
KILLED
HVT-LT
HVT+KILLED
TCO
TCO+KILLED
HVT-LT+TCO
NVx/NCh
74 Week Old Challenge - 6 DPC

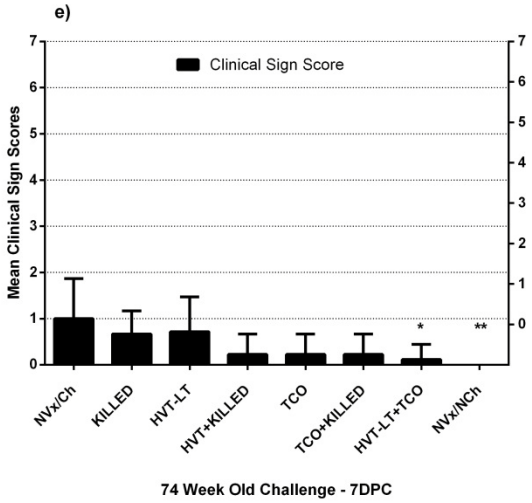

e)
Clinical Sign Score
Mean Clinical Sign Scores
NVx/Ch
KILLED
HVT-LT
HVT+KILLED
TCO
TCO+KILLED
HVT-LT+TCO
NVx/NCh
74 Week Old Challenge - 7DPC

Figure 3.2: Mean clinical signs scores (black bars) and tracheal viral loads (grey bars) were assessed at 7 DPC after virulent ILTV challenge at 4 Week (a), 9 Week (b), 35 Week (c), and 74 Week old (e) challenges; and at 6DPC (d) for the 74 Week old challenge experiment. Asterisks indicate a statistically significant difference relative to the positive control (NVx/Ch). Black and grey asterisks correspond to clinical signs and genome copy numbers, respectively. Data are presented as mean ± SD (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

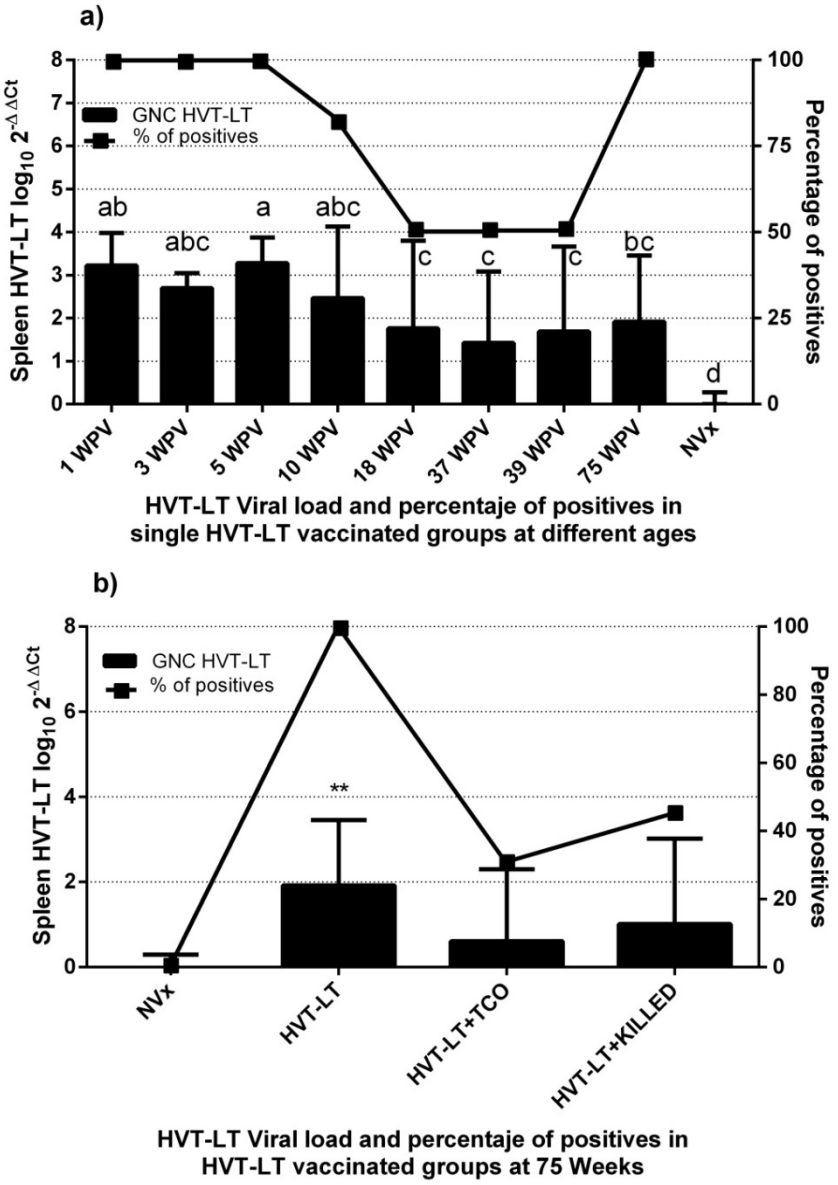


Figure 3.3: HVT-LT viral loads in the spleen (bars) and HVT-LT percentage of positive chickens at sequential ages (lines) assessed by qPCR at 1, 3, 5, 10, 18, 37, 39 and 75 weeks post-vaccination (a). HVT-LT Spleen Viral loads (black bars) and HVT-LT percentage of positives within each vaccination group (black line with black square) assessed by qPCR at 75 Weeks after vaccination (b). Asterisks indicate statistically significant difference when compared to the

non-vaccinated group (NVx). Data is presented as mean ± SD (** $p<0.01$). A CT value of 38 or less was considered as positive, while above 38 as negative, as previously described (19).

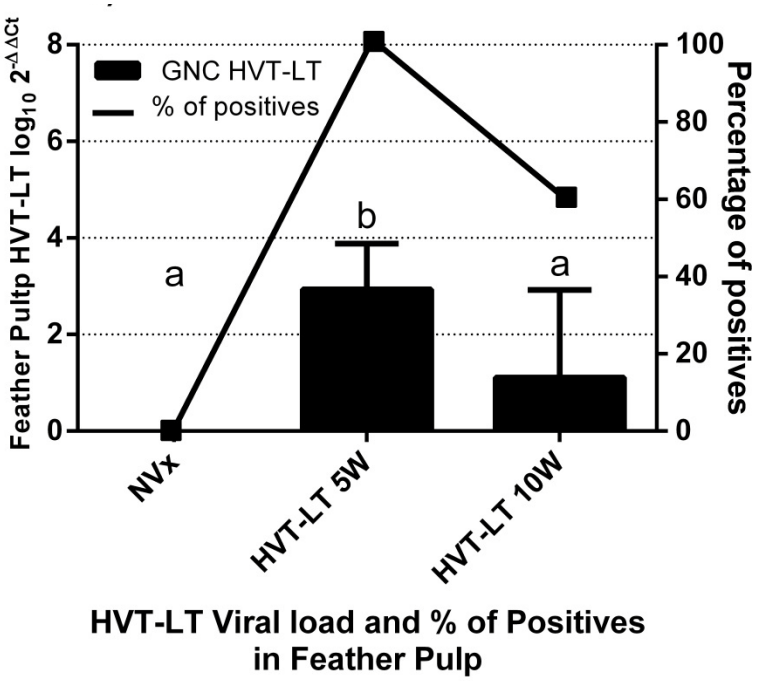


Figure 3.4: HVT-LT viral loads in feather pulp at 5 and 10 weeks post-vaccination. Different superscripts indicate different statistical groups. Data are presented as the mean (bars) ± one standard deviation (SD) ($p<0.01$). A CT value of less or equal to 38 was considered positive, while above 38 was negative, as previously described (19).

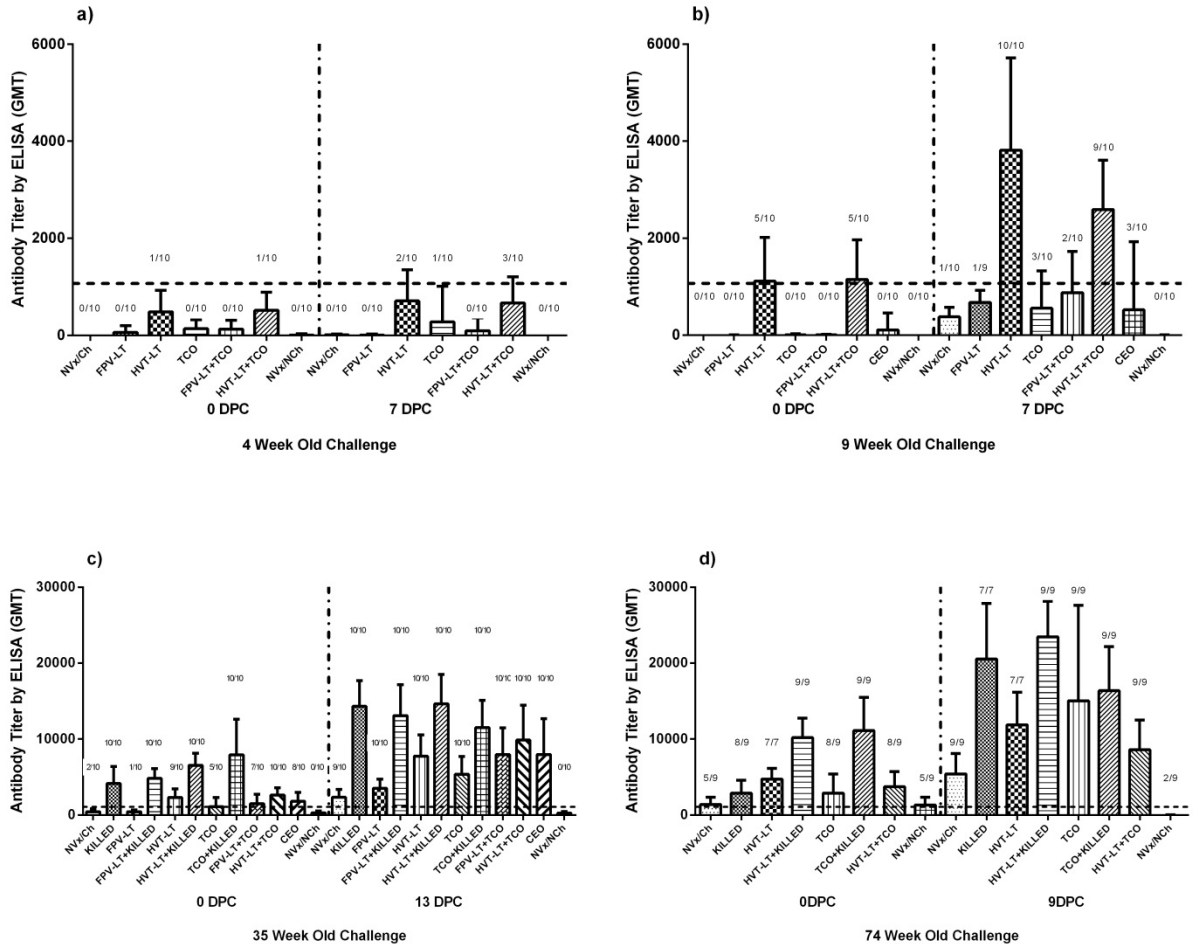


Figure. 3.5: ELISA titers pre and post challenge at 4 week (a), 9 week (b), 35 week (c), and 74 week (d) old challenges obtained with BioChek ELISA Kit. Post challenge sampling was done at 7 DPC for 4 and 9WCH, 13 DPC at 35WCH, and 9DPC at 74WCH. Bars represent antibody titers in Geometric Mean Titer (GMT), the positive/total sample ratio is found above each bar while the horizontal discontinuous line present in each graph represent the cut-off value, corresponding to a titer of ≥1070.

CHAPTER 4

EVALUATION OF COMMERCIAL ELISAs IN RECOMBINANT, INACTIVATED, AND LIVE ATTENUATED INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) VACCINATED AND INFECTED LAYERS[1]

[1] Victor Palomino, Guillermo Zavala, Sunny Cheng and Maricarmen Garcia. To be submitted to *Avian Diseases*

Keywords

ILTV-ELISA, ELISA, Serology, Infectious Laryngotracheitis, BioChek, Synbiotics, TropBio, recombinant vaccines.

Abbreviations

ABTS= 2,2-azino-bis (3-ethylbenzothiazolin sulphonic acid)-di-ammoniumsalt; CEO= Chicken Embryo Origin; DPC = Days post challenge; DO = Days old; ED = Eye drop; ELISA = Enzyme-linked immunosorbent assay; FANP = Filtered-air negative pressure isolation units; FPV-LT = Fowlpox Virus vectored ILT; GPLN = Georgia Poultry Laboratory Network; HRPO = 5 horseradish peroxidase; HVT-LT = Herpesvirus of turkey vectored ILT vaccine ; ILT = Infectious Laryngotracheitis; ILTV = Infectious Laryngotracheitis Virus; IM = Intramuscular ; KILLED = Inactivated oil-based vaccine against ILT; NON VX= Non-vaccinated group; NON VX/CH= Non-vaccinated challenged group; NON VX/NON CH= Non Vaccinated non-challenged group; OD = Optical density; PDRC-UGA= Poultry diagnostic and Research Center of the University of Georgia; PNPP = p-Nitrophenyl Phosphate; SC = Subcutaneous; SD = Standard Deviation; s/p = sample/positive ratio; TCO = Tissue Culture Origin; WO= Weeks old.

ABSTRACT

The objective of the present study was to assess serological responses against infectious laryngotracheitis virus (ILTV) using three commercial ELISA (Enzyme-linked immunosorbent assay) systems, namely: BioChek (United Kingdom); Synbiotics Corporation (United States); and TropBio (Australia). Serum samples tested were collected from chickens vaccinated with ILT live-modified, recombinant, or inactivated vaccines and their combinations, with or without a challenge with a virulent virus. Sample to positive (s/p) values in duplicate samples varied similarly, between 92% and 95% among the kits, particularly in samples with high s/p values. The sensitivity and specificity percentages for each ELISA kit were calculated to be 86.71% and 94.00%, respectively for BioChek; 96.68% and 28.00% for Synbiotics, and 86.10% and 92.00% for TropBio. For the FPV-LT-vaccinated group and the non-vaccinated non-challenged group ELISA titers were statistically similar in all three kits, indicating low seroconversion in birds vaccinated with only FPV-LT vaccine at hatch, whereas birds vaccinated with an oil-based inactivated (killed) vaccine alone or combined with other vaccines showed the highest antibody titers. Based on the present experiment the BioChek and TropBio ELISA kits detected a higher proportion of vaccinated and challenged birds with an acceptable level of specificity.

INTRODUCTION

Infectious Laryngotracheitis virus (ILTV) is an alphaherpesvirus that causes an economically relevant upper-respiratory disease in chickens and is characterized by tracheal rales, conjunctivitis and fibrino-hemorrhagic tracheitis (13). All ILTV strains hitherto studied had been

shown to be antigenically similar based on serological assays such as virus-neutralization, indirect immunofluorescence and cross-protection studies (17). In consequence, serological differentiation between vaccinated and challenged birds in the field is not possible (1), although molecular differentiation between vaccine and field strains could be done by restriction endonuclease analysis (2, 12) and sequencing (18). Although ILT has a worldwide distribution, there are areas in which commercial poultry operations are free from ILT and areas in which outbreaks have not been reported in several years. Trade restrictions can be imposed on poultry goods from ILT-prevalent zones to protect local poultry industry free from ILT. The control of this disease in commercial operations relies on proper biosecurity practices and vaccination strategies, some of which include live-modified vaccines (i.e. Chicken Embryo Origen [CEO] and Tissue Culture Origin [TCO] vaccines); and the recombinant vectored vaccines Herpes Virus of turkey vector (HVT-LT) and Fowlpox vector (FPV-LT). Recently, in South America, the spread of ILT into previously non-enzootic areas has stimulated the use of inactivated oil-emulsified ILT vaccines, mainly due to local regulatory policies in some countries that prohibit the use of live-modified vaccines with the authorization of only recombinant vaccines. Inactivated vaccines are usually applied alone or in combination with recombinant vaccines.

Serological surveillance using ELISA for detection of antibodies against ILTV is usually employed to monitor vaccinated and non-vaccinated flocks in ILTV-free areas as part of disease control programs (e.g. Brazil). However, there is scarce data documenting serological responses of flocks vaccinated with recombinant vaccines applied alone or in combination with live-modified or inactivated vaccines in layer flocks. Differences among ELISA kit manufacturers regarding antigen level of purity, cut-off value, and reagents may have an impact on the results

and interpretation of the various ILT-antibody ELISA kits. The objective of this research was to examine serological responses of commercial layers vaccinated with live-modified, recombinant, and oil-based inactivated vaccines and their combinations against ILTV, using three commercial ELISA kits for the detection of antibodies against ILT.

MATERIAL AND METHODS

Origen of the sera

One-day-old commercial table egg layer pullets (Hy-Line, variety W36) all vaccinated with a full dose of the CVI988 strain of Marek's disease virus (Pfizer Animal Health, Durham, NC), were acquired from a commercial hatchery, wing-tagged for identification, and divided into several groups with different vaccination schemes (Table 4.2). A summary of the vaccines used in the present study is shown in Tables 4.1 and 4.2, including a description of groups that were challenged with virulent ILTV at 35 weeks of age. The recombinant vaccines HVT-LT (Innovax® ILT, Merck Sharpe and Dohme Animal Health, Summit, NJ), and FPV-LT (Vectormune® FPV-LT, CEVA Biomune, Lenexa, KS) were applied as a full dose at hatch via the subcutaneous route. The live-modified vaccines TCO (LT-IVAX®, Merck Sharpe and Dohme Animal Health, Summit, NJ) and CEO (LT-BLEN®, Merial Select Inc., Gainesville, GA) were applied by eye-drop. The TCO-vaccinated groups received the vaccine at 6 weeks of age with a revaccination at 13 weeks of age, whereas the CEO vaccine was applied once at 6 weeks of age. A non-commercial oil-based inactivated vaccine (KILLED, Lohmann Animal Health, Winslow, ME) was applied in the breast by intramuscular injection in a volume of 0.5 ml per bird at 13 weeks of age. One group of birds was not vaccinated and later subdivided into a non-

vaccinated non-challenged (negative control) group, and a non-vaccinated challenged (positive control) group. After vaccination, live-modified, recombinant vaccinated and non-vaccinated groups were placed in separated isolation pens at the Poultry Diagnostic and Research Center, University of Georgia (PDRC-UGA) to avoid horizontal cross-contamination between groups.

A challenge study was conducted on the floor of isolation rooms at 35 weeks of age; 5 birds from each treatment were transferred to filtered-air negative pressure (FANP) isolation rooms located at PDRC-UGA with one replicate (5 birds per treatment per room; 10 birds per treatment in total) while the negative controls where placed in a separate room in FANP isolation units. Serum samples were taken from all treatment groups at 19 weeks of age; immediately before the challenge at 35 weeks of age (0 DPC); and finally at 13 days post challenge (13 DPC, or 37 weeks of age).

Commercial Elisa Kits

All serum samples collected at 19, 35 and 37 weeks of age were processed in duplicate at the Georgia Poultry Laboratory Network (GPLN, Oakwood, GA 30566) using three different commercial ELISA kits for detection of ILTV antibodies [Trop-ELISA (TropBio, Townsville, Australia); Pro-FLOK (Synbiotics Corporation, Kansas City, MO, USA); and ILT ELISA CK124 (BioChek, Middlesex, United Kingdom)]. General technical data on the three ELISA Kits is shown in Table 4.3. The ELISA procedure, data processing, sample to positive value (s/p value) and titer calculations were done according to the manufacturers' instructions for the BioChek and Synbiotics kits (s/p value of 0.5 or a titer of 1070; and s/p value of 0.15 or a titer of 343 as cut-off value for BioChek and Synbiotics kits, respectively). The TropBio's protocol and data analysis were modified to fit automated laboratory processing as described in Table 4.3.

Briefly, a two-step serum dilution was prepared to reach the 1:100 serum dilution required. An automatic washing machine was used instead of the hand-wash steps suggested by the manufacturer. The reading of the absorbance of each sample, or optical density (OD) was done with a 405 nm filter. Seven different standards, designated as standard 1 through 7, were provided with the kit corresponding to titers 0, 2, 8, 16, 32, 64 and 256, respectively. These standards were run in each ELISA plate and finally, 14 values of each standard were obtained. Standard 1 and Standard 7 were considered as a negative and positive control for each ELISA plate, respectively, and were used to establish an s/p value. The following s/p equation was used for all commercial kits:

$$\frac{\text{(Mean Sample Absorbance)} - \text{(Mean Absorbance of Negative control)}}{\text{(Mean Absorbance of positive control)} - \text{(Mean Absorbance of negative control)}} = \text{s/p}$$

TropBio standard #5 was used as a cut-off value as established by Bauer et al. (3) and, for such purpose, it was run 14 times to calculate a mean s/p value of 0.53, which corresponds to a cut-off value titer of 1140.1 (1140.1 ± 237 SD) that was used for all TropBio plates.

Since there is a lack of information regarding ELISA titers present in recombinant vaccinated flocks; each ELISA kit sensitivity was determined based on successful detection of live modified and oil-based inactivated vaccinated birds and challenged birds as positives to ILTV, whereas non-vaccinated birds were used for assessing specificity on the three ELISA kits.

Repeatability of the tests

Repeatability was estimated by analyzing each sample in duplicate using two different ELISA plates from the same batch and expressed as the percent of mean differences within a

range determined by the mean difference of the s/p values ± 2 standard deviation (SD) (4). This method was used instead of the usual method to determine repeatability in ELISA kits, due to the reduced number of readings performed on each sample (only 2 observations) (5, 6). Briefly, the s/p data was obtained as previously described and entered into a spreadsheet where the mean and standard deviation (SD) differences between the S/P values from each sample were obtained and plotted. The plot shows each mean difference obtained after subtracting the second s/p from the first s/p value in the Y_1 axis, whereas the mean s/p mean values corresponding to each difference are plotted in the X axis, thus evaluating the difference against the mean s/p for each sera. Also, the range of mean s/p differences ± 2 SD is shown in the Y_2 axis along with the percent of mean differences included within that range for each kit.

Statistical Analysis

The GraphPad Prism 6.01 statistical analysis package (GraphPad Software, La Jolla, CA) was used to analyze the antibody titer data for each of the treatment groups, utilizing the one-way ANOVA with Bonferroni's method for multiple pairwise comparisons. For repeatability, sensitivity and specificity assessments, data was entered into a Microsoft Office Excel 2007 spreadsheet (Redmond, WA) further analyzed with Excel functions and graphed in GraphPad Prism 6.01.

RESULTS

Repeatability of s/p measurements

Repeatability of the assays was evaluated as each sample was tested in duplicate with each commercial ELISA results and results are shown in Figure 4.1. The mean of the differences among s/p values and the percent of mean differences on range was 0.015 and 92.93% for BioChek (Figure 4.1a), 0.17 and 92.69% for Synbiotics (Figure 4.1b), and 0.02 and 95.48% for TropBio (Figure 4.1c), respectively.

Percent positive sera and ELISA titers

Table 4.4 and 4.5 show the ratio of positives over the total number of samples along with the arithmetical mean ELISA titers obtained for each of the treatment groups. Superscripts in the graph denote statistical grouping for each kit at 19 weeks of age (Table 4.4); 35 weeks of age (prior to challenge) (Table 4.5); and 37 weeks of age (13 days post challenge) (Table 4.5). Figure 4.2 illustrates the percent positives per treatment as obtained using three different ELISA systems.

Statistically, for all ELISA kits, the non-vaccinated (NVx) and FPV-LT groups produced the lowest antibody titers, while the highest titers were observed in groups vaccinated with CEO and those in which the inactivated (KILLED) vaccine was used alone or in combination with another vaccine (KILLED; FPV-LT+KILLED; TCO+KILLED; and HVT-LT+KILLED) at all ages tested (pre-challenge at 19 and 35 weeks of age; and post-challenge at 37 weeks of age). FPV-LT-vaccinated birds did not seroconvert to produce significant antibody levels as assessed by all three kits (12 birds were detected out of 42; 33 out of 42; and 12 out of 42 for BioChek,

Synbiotics and TropBio, respectively). A higher number of birds vaccinated with a different recombinant vaccine (HVT-LT) showed detectable seroconversion measurable with all three kits (41 out of 42; 42 out of 42; and 27 out of 42 for BioChek, Synbiotics and TropBio, respectively).

Additional treatment groups with a low number of detections were TCO (24/42; 34/42; and 29/42), and FPV-LT+TCO (20/42; 39/42; and 30/42) for BioChek, Synbiotics and TropBio, respectively. All HVT-LT+TCO vaccinated birds (100%) were detected by BioChek and Synbiotics; however, the TropBio kit only detected 22 out of 42 samples (Table 4.4).

Sensitivity and Specificity among three ELISA kits

A summary of the sensitivity and specificity per ELISA is presented in Table 4.6. Sensitivity and specificity percentages were 86.71% and 94.00% for BioChek; 96.68% and 28.00% for Synbiotics; and 86.10% and 92.00% for TropBio.

DISCUSSION

The present study compared the performance of three different commercial ELISA kits for ILTV antibody detection, one from The Netherlands and manufactured in the United Kingdom (BioChek); a second one from United States (Synbiotics); and a third one from Australia (TropBio). The serum samples tested were obtained from commercial layer chickens receiving one of various ILT vaccines. The serum samples were obtained at 19 and 35 weeks of age prior to infection with virulent ILTV; and thereafter at 37 weeks of age, or 13 days post-challenge with ILTV. S/p repeatability percentages were obtained based on only two measurements from each sample and ranged between 92% and 95% among the commercial

ELISAs. Likely, should a larger number of replications be used, a higher repeatability would have been observed for each one of the commercial ELISAs.

Although, the results indicated that sensitivity among commercial ILTV ELISAs was above 85% (86.7%, 96.7% and 86.1% for BioChek, Synbiotics and TropBio, respectively), the most significant difference between commercial ELISAs was their specificities (BioChek 94%, TropBio 92% and Synbiotics 28%). As compared to virus neutralization, low specificity of the Synbiotics ELISA has been previously described (3) in a study with sera from various broiler breeder flocks of various ages with different ILTV vaccination programs. Low specificity of ILTV ELISA have been previously addressed (1, 15, 16, 19, 22, 23); and found to be dependent on age and breed of the chickens sampled (16).

Most of the vaccinated birds not detected serologically by the ELISA kits were from the TCO and FPV-LT+TCO vaccinated groups, whereas all HVT-LT+TCO vaccinated birds were detected by both BioChek and Syntiobics but only approximately half of them (22/42) were detected as positives by TropBio. We speculate that the results observed stem from the high cell-associated nature of the TCO vaccine, which elicits primarily cell mediated immunity and not a robust humoral immune response detectable by ELISA.

Serological responses on egg layer chickens receiving recombinant vaccines have not been documented before. According to the results of the present work, FPV-LT vaccinated birds seroconvert poorly to this recombinant vaccine. FPV stimulates primarily a cellular response instead of a humoral response (20), and thus serological responses induced by FPV-ILT-vectored vaccines may be modest and difficult to detect by ELISA. In addition, after the replication of the

vector, there is no further stimulation of the immune system as FPV does not induce latency, and there is no anamnestic response to further enhance antibody titers. Furthermore, immunity stimulated by FPV-LT has been reported to be less robust than the immunity induced by other ILT vaccines such as HVT-LT, TCO or CEO (7, 8, 14, 21). In the present study, HVT-LT vaccinated birds elicited a detectable antibody response and most of the vaccinated birds were detected by BioChek and Synbiotics tests while only few birds were detected by TropBio.

Increased convalescent ELISA antibody titers were observed for all challenged groups from 35 weeks of age (pre-challenge) to 37 weeks of age (13 days post-challenge) using BioChek or Synbiotics (Table 4.4). For the TropBio ELISA kit, all samples from challenged birds were ILT ELISA positive, but no differences were seen between pre-challenge and convalescent titers for the KILLED, FPV-LT+KILLED, HVT-LT+KILLED, TCO+KILLED, and CEO groups. However, a significant increase in antibody titers and was seen in the FPV-LT, HVT-LT, HVT-LT+TCO and TCO groups. Such findings are probably the result of using a high OD value standard as positive control in the TropBio ELISA plate utilized for s/p ratio calculations, which may also explain the narrow distribution of the s/p differences between measurements, as seen in Figure 4.1c (Standard 7 provided with the kit).

Actual protection against ILTV cannot be predicted by antibody levels, since protective immunity against ILT is primarily based on the cellular immune response (9-11). However, ELISA antibody detection may be useful for disease surveillance in areas where ILT is thought to be absent and where vaccination is not practiced. Thus, ELISA can be used to indirectly detect field challenge and also to monitor vaccinated flocks.

The results of the present study are important because it assess that the amount of reactors among non-vaccinated non-challenged birds can differ between kits, also, that live-modified and inactivated vaccinated birds do seroconvert, and that FPV-LT vaccinated birds seroconvert poorly to vaccination whereas HVT-LT vaccinated birds elicit an antibody response detectable in most vaccinated birds.

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Table 4.1: Vaccines used to determine serological responses against ILTV in layers

Type of Vaccine	Trade Name	Manufacturer	Titer Dose / Bird	Route[a]	Vaccination Age[b]
HVT-LT	Innovax ILT®	MSD Animal Health, Summit, NJ	7700 pfu in 200 uL	SC	1 DO
FPV-LT	Vectormune® FP-LT	Ceva Biomune, Lenexa, KS	3860 pfu in 200 uL	SC	1 DO
TCO	LT-IVAX	MSD Animal Health, Summit, NJ	$10^{3.83}$ TCID in 100 uL	ED	6 WO & 13 WO
CEO	LT BLEN®	Merial Select, Inc Gainesville, GA	$10^{4.38}$ TCID in 100 uL	ED	6 WO
KILLED	Experimental Oil – Based Inactivated	Lohmann Animal Health, Winslow, ME	$10^{6.9}$ EID in 0.5 ml	IM	13 WO

[a]Route: SC, Subcutaneous; ED, Eye Drop; IM, Intramuscular. [b]Vaccination Age: DO, Day Old; WO, Weeks Old.

Table 4.2: Experimental design for determination of serological responses in layers

Age / Treatment	1 Day	6 Weeks	13 Weeks	19 Weeks	35 Weeks	35 weeks + 13 Days
FPV-LT	FPV-LT					
HVT-LT	HVT-LT					
TCO		TCO	TCO			
FPV-LT+TCO	FPV-LT	TCO	TCO	S		S
HVT-LT+TCO	HVT-LT	TCO	TCO	A	SAMPLING AND CHALLENGE	A
CEO		CEO		M		M
FPV-LT+KILLED	FPV-LT		KILLED	P		P
HVT-LT+KILLED	HVT-LT		KILLED	L		L
TCO+KILLED		TCO	TCO KILLED	I N		I N
KILLED			KILLED	G		G
NON VX/CH NON VX/NON CH					SAMPLING	

Table 4.3. Technical data of the ILT-ELISA detection kits. (modified from Bauer et al. (3))

Technical Data	CK 213	ProFLOK-ELISA	Trop-ELISA
Manufacturer	BioChek	Synbiotics	Trop-Bio Australia
Antigen	Sinkovic	Not Stated	SA 2
Substrate	PNPP[a]	ABTS[b]	ABTS[b]
Conjugate	Sheep-anti-chicken-HRPO[c]	Goat-anti-chicken-HRPO[c]	Anti-Chicken-HRPO[c]
Stop-solution	Sodium Hydroxide	None	Sulphuric Acid
Serum dilution	1:500	1:100	1:100
Serum reaction	30 min at RT	30 min at RT	1 hour at RT
Conjugate reaction	30 min at RT	30 min at RT	1 hour at RT
Substrate Reaction and color	15 min at RT / yellow	15 min at RT / Green-blue	1 hour at RT / Dark-blue
Wave length	405 nm	405-410 nm	405 nm
Cut-off Value	s/p = 0.5 Titer=1070	s/p = 0.15 Titer=343	s/p = 0.526[d] Titer=1140.1[d]

[a]PNPP = p-Nitrophenyl Phosphate
[b]ABTS = 2,2-azino-bis (3-ethylbenzothiazolin sulphonic acid)-di-ammonium salt.
[c]HRPO = 5 horseradish peroxidase.
[d]S/p and Titer formula were adapted for TropBio since manufacturer does not use s/p or Titer values.

Table 4.4: Antibody titers against ILTV determined by three different commercial ELISA in experimental groups tested at 19 weeks of age.

	19 WEEK OLD[1]		
	BIOCHEK (cut-off 1071)	SYNBIOTICS (cut-off 343)	TROPBIO (cut-off 1140.1)
NVx	1/20 (457±168.6) [a]	18/20 (929.3±204.7) [a]	3/20 (642.3±253) [ab]
KILLED	22/22 (5279±715) [d]	22/22 (31094±4152) [cd]	22/22 (2987±47) [d]
FPV-LT	1/22 (520.4±121.9) [ab]	21/22 (1362±287) [a]	2/22 (627±140.9) [a]
FPV-LT+KILLED	22/22 (6929±756) [de]	22/22 (24748±1550) [bc]	22/22 (3021±68) [d]
HVT-LT	22/22 (2839±358) [bc]	22/22 (1075±2410) [a]	16/22 (1300±130) [c]
HVT-LT+KILLED	22/22 (7470±718) [ef]	22/22 (19352±1581) [b]	22/22 (3253±44) [d]
TCO	9/22(1114±349.8) [abc]	16/22 (3621±1829) [a]	13/22 (1417±381) [c]
TCO+KILLED	21/21(11182±2114) [g]	21/21 (37885±7055) [d]	21/21 (2874±273) [d]
FPV-LT+TCO	3/22 (726±190.4) [ab]	19/22 (2802±1473) [a]	14/22 (1306±340.4) [c]
HVT-LT+TCO	22/22 (3007±547) [c]	22/22 (1800±692) [a]	7/22 (1128±244.7) [bc]
CEO	7/8 (7487±4701) [f]	8/8 (31107±15626) [cd]	8/8 (2941±113) [d]

[1] The information in each cell is as follows: the fraction indicates the ratio of samples detected by the test as positives for ILTV antibodies, the number in parenthesis the mean arithmetical titer ± SD, and the letter besides the parenthesis indicates the statistical grouping of the given set of data within each ELISA kit

Table 4.5: Antibody titers against ILTV determined by three different commercial ELISA in experimental groups tested at 35 weeks of age (pre-challenge with virulent ILTV); and at 37 weeks of age (13 days post-challenge).

	35 WCH – 0 DPC[1]			35 WCH – 13 DPC[1]		
	BioCheck	Synbiotics	TropBio	BioCheck	Synbiotics	TropBio
NVx/Ch	2/10 (615±401.1)[a]	6/10 (407±229.6) [a]	0/10 (102±103)[a]	9/10 (2614±910) [ab]	10/10 (5063±3112)[ab]	10/10 (2727±263) [b]
KILLED	10/10 (4777±1720)[bcd]	10/10 (16350±5900)[b]	10/10 (2735±157) [e]	10/10 (14862±2927)[f]	10/10 (26761±7981)[ef]	10/10 (3089±132) [bc]
FPV-LT	1/10 (508.1±208.8)[a]	2/10 (366.8±309)[a]	0/10 (200±96)[a]	10/10 (3815±1222)[abc]	10/10 (9282±3708)[abc]	10/10 (2756±153) [bc]
FPV-LT+KILLED	10/10 (5065±1215)[cd]	10/10 (23165±6300)[bc]	10/10 (2785±96)[ef]	10/10 (13923±3521)[ef]	10/10 (28405±7615)[f]	10/10 (3073±133) [bc]
HVT-LT	9/10 (2609±874)[abc]	10/10 (2076±1168.6)[a]	1/10 (788±294)[ab]	10/10 (8381±2367)[bcde]	10/10 (12127±3570)[abcd]	10/10 (2762±200) [bc]
HVT-LT+KILLED	10/10 (6846±1629)[de]	10/10 (19870±3753)[bc]	10/10 (2971±208)[f]	10/10 (15300±3140)[f]	10/10 (25614±4719)[ef]	10/10 (3170±141) [c]
TCO	5/10 (1724±1111.7)[a]	8/10 (4553±3625.8) [a]	6/10 (1550±787) [cd]	10/10 (6030±2144)[abcd]	10/10 (7463±2718)[abc]	10/10 (3035±178) [bc]
TCO+KILLED	10/10 (9138±2829)[e]	10/10 (27889±8782)[c]	10/10 (2872±156) [ef]	10/10 (12266±3316)[def]	10/10 (23978±5691)[def]	10/10 (3016±118) [bc]
FPV-LT+TCO	7/10 (1971±944)[a]	10/10 (4753±3607)[a]	6/10 (1570±650 [cd]	10/10 (8843±2758)[bcdef]	10/10 (9496±3931)[abc]	10/10 (3025±279) [bc]
HVT-LT+TCO	10/10 (2855±840)[abc]	10/10 (3115±2316.1)[a]	5/10 (1182±396 [bc]	10/10 (11188±4156)[def]	10/10 (15637±9181)[bcde]	10/10 (2920±215) [bc]
CEO	8/10 (2233±1043)[ab]	10/10 (6613±3145)[a]	9/10 (2208±381) [de]	10/10 (9841±5428)[cdef]	10/10 (19035±9174)[cde]	10/10 (3044±146) [bc]
NVx/NCh	0/10 (387.4±170.2)[a]	7/10 (546.5±304.9)[a]	0/10 (183±146) [a]	0/10 (320.3±120.5)[a]	5/10 (312.4±119)[a]	1/10 (536.1±314) [a]

[1] The information in each cell is as follows: the fraction indicates the ratio of samples detected by the test as positives for ILTV antibodies, the number in parenthesis the mean arithmetical titer ± SD, and the letter besides the parenthesis indicates the statistical grouping of the given set of data within each ELISA kit.

Table 4.6: Sensitivity and specificity of three commercial ILT-ELISA kits

	SENSITIVITY % [a]	SPECIFICITY % [b]
BioChek	86.71%	94.00%
Synbiotics	96.68%	28.00%
TropBio	86.10%	92.00%

[a] Sensitivity was obtained by detecting live-modified vaccinated, inactivated vaccinated, and challenged birds.

[b] Specificity was obtained by detecting non-vaccinated non-challenged birds.

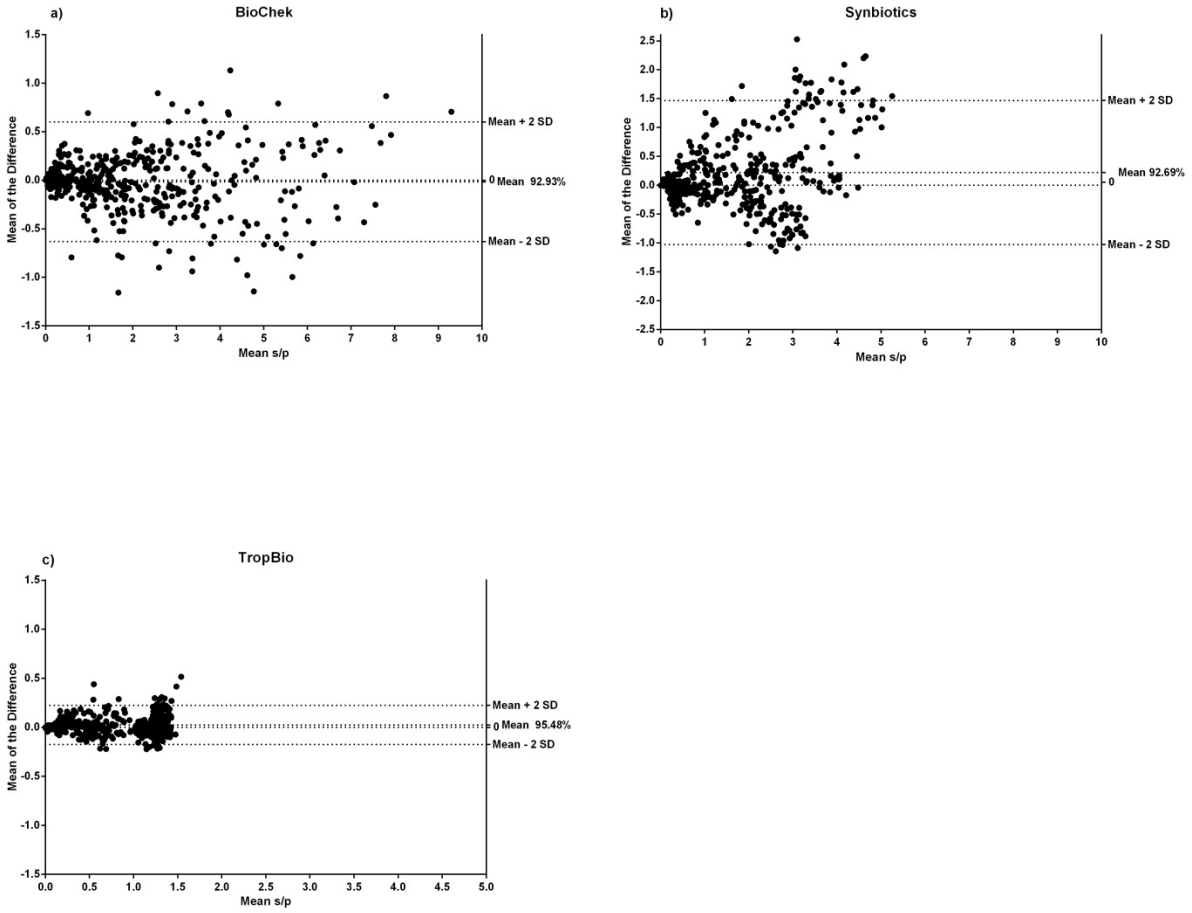

Figure 4.1: Difference against mean for s/p data according to the results assessed by a) BioChek; b) Synbiotics, and c) TropBio.

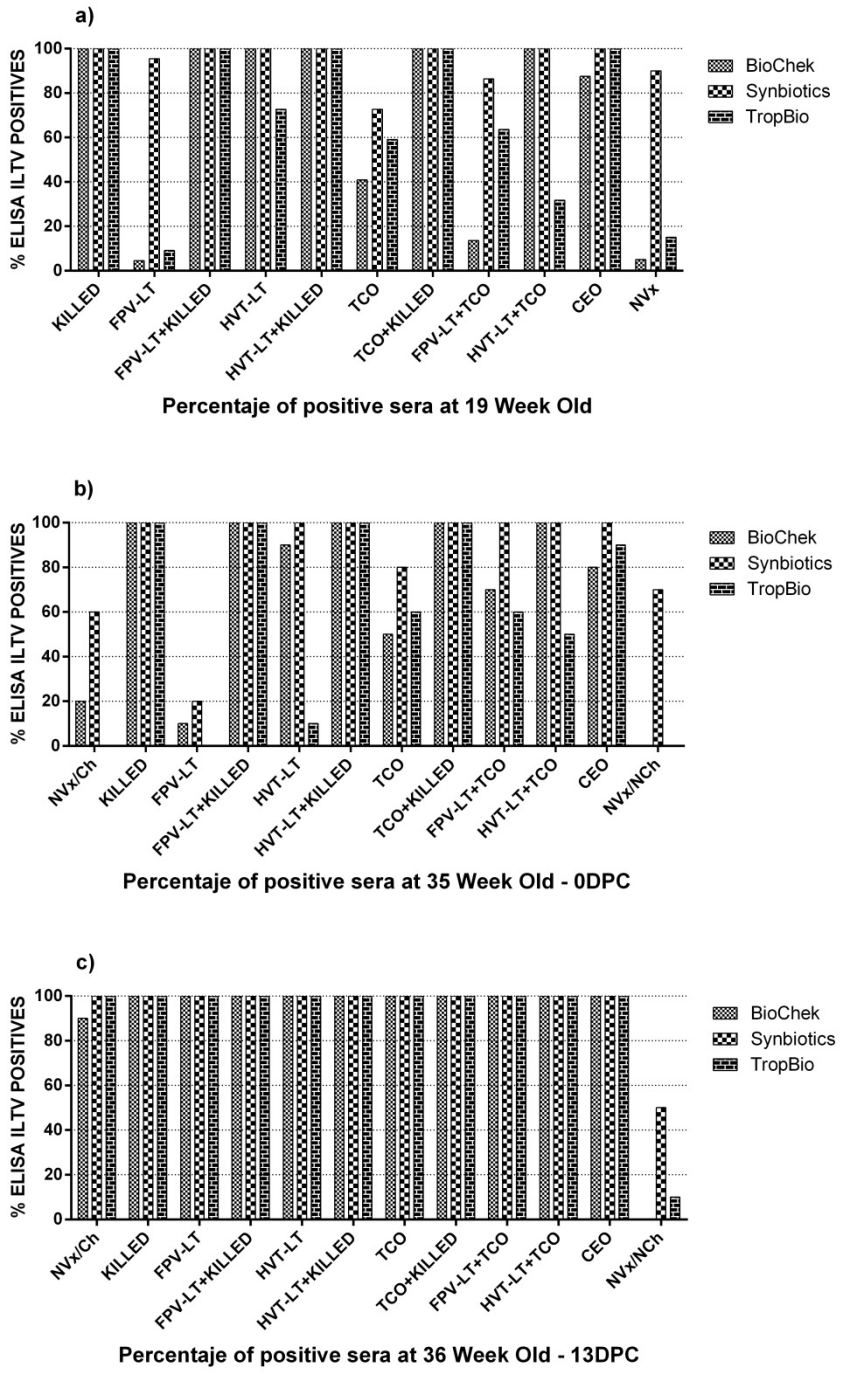

Figure 4.2: Percent positives per experimental group at 19 weeks of age (a) 35 weeks of age prior to infection with virulent ILTV (b); and 37 weeks of age (13 days post challenge) (c). Positive reactions were determined according to cut-off values provided by each ELISA kit manufacturing company or as previously described (Table 4.3)

CHAPTER 5

ATTENUATION OF A FIELD STRAIN OF INFECTIOUS LARYNGOTRACHEITIS VIRUS IN PRIMARY CELL CULTURES AND ADAPTATION TO CHICKEN EMBRYO FIBROBLASTS[1]

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Keywords

Infectious Laryngotracheitis, TCO, CEO, attenuation, cell culture, serial passage, chicken embryo fibroblasts, CEF

Abbreviations

ATCC= American Type Culture Collection; CEO = chicken embryo origin; CEF = Chicken Embryo Fibroblasts; CEK = Chicken Embryo Kidney, CEL = Chicken Embryo Liver; Ch = Challenged group; CK = chicken kidney cells; CPE = Cytophatic effect; DABCO = 1,4-diazabicyclo[2.2.2]octane; DF-1= Spontaneous immortalized chicken embryo fibroblast; DMEM= Dulbecco's Modified Eagle Medium; DPI = Days post infection; GM= Growth media IF= Immunofluorescence; ILT = Infectious laryngotracheitis; ILTV = Infectious laryngotracheitis virus; LMH = Leghorn male hepathoma; MM= Maintenance media; NCh = Non challenged group; PBS= Phosphate buffered saline; PCR = Polymerase chain reaction; PP= Plaque purification; RT-PCR= Reverse transcriptase PCR; $TCID_{50}$ = Tissue culture infectious dose 50%; TCO = tissue culture origin; USA = United States of America; VERO= "Verda reno", immortalized green monkey kidney cell line ; VERO E6 = VERO E6 clone; VLT= Vaccinal Laryngotracheitis; WCH= Week Challenge

ABSTRACT

Attenuation of a CEO-related ILTV field strain designated “63140” was attempted by serial passage in avian immortalized cell lines (i.e. LMH, and DF-1), mammalian immortalized cell lines (i.e. VERO and VERO E6, a clone of VERO adapted for long incubation periods) and primary cell cultures from chicken origin, (i.e. chicken embryo kidney cells [CEK]; chicken embryo liver cells [CEL]; and chicken embryo fibroblasts [CEF]). VERO E6 and DF-1 cells were negative by PCR at the 2nd passage, while VERO cells were positive by PCR up to 4 passages without exhibiting CPE. In contrast, LMH cells were permissive to virus propagation, although virus yield was low. Primary cell cultures, CEK and CEL, were permissive and produced considerably high titers when compared to LMH cells. A parental 63140 inoculum was passaged twice in LMH cells and then serially passaged in CEL. This strain was named “CEL(L)” and was selected to assess virulence in live birds after each 10 passages. A subculture capable of growing in chicken embryo fibroblasts [“CEF(4.1)”] was selected after 52 passages in CEL, plaque purified (4.1), passaged further in CEFs and used for in-vivo experiments to assess its virulence. Two safety trials were conducted in 28-day-old broilers by means of intra-tracheal and conjunctival inoculation of various virus passages tested. Clinical signs were evaluated at 5 days post inoculation (DPI).

Partial attenuation was observed for the CEL(L) virus at passages 60, 80, 90 and 100; and for passages CEF(4.1) virus at passages 6, P10 and P20, which were initially passaged 52 times in CEL and plaque purified. Since none of the groups of inoculated chickens was completely free of clinical signs we concluded that further serial passages are necessary to achieve full attenuation of ILTV strain 63140.

INTRODUCTION

Infectious Laryngotracheitis (ILT) is an important respiratory disease of chickens caused by an Alphaherpesvirus, genus *Iltovirus*. The disease can cause a wide range of clinical signs and mortality (8), and frequently accounts for relevant losses in densely populated poultry producing areas. Modified live vaccines of chicken embryo origin (CEO) and tissue culture origin (TCO), have been used for decades as part of ILT control programs. These two types of vaccine can induce strong and lengthy immune responses, although immunity is not life-long. CEO vaccines are usually preferred over TCO for their availability (CEOs are commercialized by several vaccine companies while only one company produces TCO (4)), and ease of application (CEOs can be mass-applied via drinking water or spray while TCO must be delivered by eye-drop).

Despite these advantages, the use of CEO vaccines became controversial due to its potential to regain virulence (7). Moreover, research done in the USA (2), United Kingdom (16), and Australia (15), has revealed the isolation of CEO-related ILTV strains from outbreaks in commercial poultry flocks. In contrast, TCO has been recognized as a safer vaccine due to its lesser reactivity when compared to CEO. The objective of the present research was to attenuate a field strain of ILTV in primary cell cultures and in continuous cell lines.

MATERIAL AND METHODS

Virus Strain

The 63140 ILTV field strain used in the present experiment was isolated in chicken kidney (CK) cells from an outbreak in unvaccinated broiler flocks (27, 28). Later, 63140 was

typed as a virulent CEO-related genotype group V strain of ILTV, responsible for severe clinical signs and mortality in naïve broiler chickens (27). An eight passage of 63140 in CK cells was used as a parental virus and inoculated in various continuous cell lines and embryonic cell cultures in the present research.

Tissue Culture and Cell Lines

Continuous cell lines derived from chicken embryo fibroblasts (DF-1) (9), chicken hepatocellular-carcinoma (LMH) (13) and African green monkey kidney cells (Vero) (29) a clone of Vero cells, named E6 (Vero E6) selected for long incubation viruses (5); together with chicken primary cells such as chicken embryonic liver (CEL), and chicken embryonic kidney (CEK) cells; and a chicken secondary cells such as chicken embryonic fibroblast (CEF) were used to attempt attenuation of the 63140 ILT strain.

Primary CEL, CEK, and secondary CEF cells were prepared using 10-15; 17-20; and 7-10 day old SPF embryos, respectively. Growth media (GM) and maintenance media (MM) were used to perform the cell cultures. GM consisted of DMEM (Mediatech Inc., Manassas VA, 20109) with 10% Fetal Bovine Serum (FBS) and 2% Antibiotic-Antimycotic (Ab) solution 100X; while MM consisted of DMEM with 1% Calf Serum (CS) + 2% Ab. Methodologies previously described (1) with modifications, were used for primary and secondary cell culture.

Briefly, in the case of primary cell cultures such as CEL and CEK, organs were harvested, washed in PBS three times, minced and washed again for clearing red blood cells. Cells were then placed in a 50 ml trypsinizing flask with pre-warmed Trypsin with EDTA (Mediatech Inc. Manassas VA, 20109) for a single 10-minute trypsinization step. Thereafter, the mix was combined with chilled GM, filtered and centrifuged at 410 g for 10 minutes. After

obtaining the pellet, cells were re-suspended in pre-warmed GM at an approximate density of 5 x 10^5 cells per mL and plated in T25 tissue culture flasks incubated at 37 C in an atmosphere enriched with 5% CO_2 until 85-95% confluence. The same procedure was used for CEF, except that the cells were cultured in a T75 culture flask and trypsinized when the monolayer reached 100% confluency prior to subculturing in T25 flasks to obtain subsequent passages.

The immortalized cell lines, VERO, VERO E6 and DF-1, were originally obtained from the American Type culture Collection (ATCC), while a subset of LMH originally acquired from ATCC and adapted to multiply at 39 C was kindly provided by Dr. Garcia's laboratory at the University of Georgia. Subcultures were performed according to ATCC recommendations in all cases. Similar GM and MM were used for embryonic cell cultures, VERO, VERO E6, and DF-1; whereas for LMH HyClone® DME/F-12 media (HyClone Laboratories Inc., South Logan, UT, 84321) was used instead of DMEM for GM and MM.

Serial Passage

Two T25 flasks with a 85-95% confluent monolayer were used to perform each passage. GM from both flasks was collected and discarded. For each passage, one flask was inoculated with a given passage of ILTV using various adsorption times and incubation temperatures (Table 5.1), while the other remained uninfected as a negative control. After adsorption, 5 ml of pre-warmed MM was placed into each flask and after 5 days incubation, both flasks were frozen and thawed three times. The supernatants were stored in 2-ml cryogenic vials at -80ºC; and 200 uL of the supernatant was inoculated into a fresh cell culture monolayer with 85-90% confluence for a subsequent passage. Infected cells were incubated at 37ºC with 5% CO_2 for 5 days. LMH,

VERO, and VERO E6, were incubated at 39ºC with 5% CO2. The incubation period was reduced from 5 to 2 days of incubation for cultures over 60 passages.

Each 10^{th} passage, ILTV was cultured in a T75 flask which was frozen and thawed 3 times at 5 days post infection. The culture material was harvested, centrifuged and the supernatant aliquoted and titrated in 96-well plates containing CK cells as previously described (12) for inoculations in chickens in *in-vivo* studies.

The 63140 CK8 parental strain was initially inoculated into CEL and CEK; thus, such serial passages were labeled CEL(K) and CEK(K) respectively. Furthermore, two additional inoculums with a previous cell culture step, were continuously passaged in CEL and CEK and were labeled as CEL(L) and CEK(L). For CEL(L), the parental strain was previously passaged 2 times in LMH and then, continue passaged in CEL, while in the case of CEK(L), the parental strain was previously passaged 8 times in CEL and after continue passaged in CEK. A summary of labels used in the experiment is shown in Table 5.5.

Conventional PCR and RT-PCR

Purity of the cultures was assessed by detection of ILTV in the absence of contaminant viruses (such as Reovirus and Adenovirus) each 20 passages by RT-PCR or PCR. DNA and RNA were extracted from the infected cells using a commercial DNA extraction and purification kit (High Pure Template DNA Purification Kit, Roche Diagnostics, Indianapolis, IN); and RNA purification kit (High Pure Template RNA Purification Kit, Roche Diagnostics, Indianapolis, IN) following the manufacturer's instructions. A conserved region of ILTV glycoprotein B (gB) of approximately 2.7 kb was amplified in the case of ILTV (PCR primers unpublished); while a conserved section of the Sigma C gene was amplified in the case of Reovirus (25) and the L1

Loop of the Hexon gene in the case of Fowl Adenovirus (21). While RT-PCR and PCR techniques for Reovirus and Adenovirus were done according to the publications mentioned, in the case of ILT the reaction was performed as follows: Each PCR assay was performed with a final reaction of 20 uL that consisted in 18 uL of Platinum PCR SuperMix High Fidelity, (Invitrogen, Carlsbad, CA), primers combined to a final concentration of 1uM and 1uL of DNA template. The thermal profiling used was as follows: 94 C for 2 min, 35 cycles of 94 C for 30 sec, 55 C for 45 sec, and 68 C for 3 min, and a final cycle of 68 C for 10 min.

Possible Mycoplasma contamination in the biological material was examined by culture. A summary of the primers used in each instance is included in Table 5.2.

Preparation of Hyperimmune Sera and Indirect Immunofluorescent Antibody Test

Polyclonal hyperimmune serum against 63140 ILT was produced in SPF chickens for indirect immunofluorescence assays with the purpose of demonstrating ILTV replication and protein expression in infected cells. Briefly, a 63140 previously passaged ten times in CEL was harvested, frozen and thawed three times and centrifuged at 410 g for 10 minutes. Thereafter, the supernatant was harvested, aliquoted, and titrated using the Reed and Muench method (22) in CK cells cultured in 96-well plates. Virus titers were expressed in $TCID_{50}$ units per ml.

Four-week-old SPF chickens were inoculated in three occasions with two week intervals and through different delivery routes with 1 ml of a 63140 virus solution containing a titer of $10^{3.5}$ $TCID_{50}$ per ml. Three delivery routes were used for the first inoculation: conjunctival route (50 uL each eye), the intranasal route (50 uL each nostril) and intramuscular route (400 uL per each breast). In contrast, the second and third inoculations were only given by intramuscular

route in the breast (0.5 ml per each breast). Fifteen days after the final inoculation, the chickens were bled and the serum was harvested and frozen at -20 C until further use.

Indirect immunofluorescence was done in ILTV-infected cells plated in 96 well plates. The MM was withdrawn from the plates; the cells were washed with warm PBS and fixed with 100% cold ethanol at -20C for 20 minutes. The cells were then dried at room temperature (RT) and 5% skim milk in PBS was added into each well as a blocking solution for an incubation step at 37C for one hour. After three washes with PBS, a dilution of 1:10 of the anti-ILTV antiserum was applied as primary antibody with an incubation time of one hour at 37C followed by a three-wash step with PBS. Thereafter, a dilution of 1:200 of FITC-labeled mouse anti-chicken IgG (Sigma, Saint Louis, MS) was used as secondary with the same incubation time and wash procedure as the previous step. Finally, 100 uL of a mounting solution, consisting of DABCO-glycerol with PBS (1:1) were added to each well to prevent photobleaching. DABCO-Glycerol was obtained by dissolving 2.5 g of DABCO (1,4-diazabicyclo[2.2.2] octane) in 90 ml glycerol by stirring for 3.5 hours at 37 C on a warm plate. After DABCO was dissolved, 10 ml of PBS was added and 10% HCl was used to adjust the pH level to 8.6. Finally, the cells were observed under green fluorescent light with an immunofluorescence (IF) microscope.

Plaque purification

Plaque purification was done only for CEF P4. CEF cells were plated and grown in 6-well plates. Serial dilutions of a 63140 CEF P4 sample were prepared and wells were infected with one replicate. After adsorption, cells were covered by MM with 0.5% SeaPlaque agarose from Lonza (Rockland, ME). A plaque was selected 7 days post infection for continued passages in CEFs.

Experimental Design of Safety studies

Two safety studies were done to test *in vivo* the level of attenuation of serially passaged ILTV 63140. The experimental designs for the first and second experiments are summarized in Tables 5.3 and 5.4, respectively. Briefly, unvaccinated broiler chickens were acquired from a commercial source at hatch and reared in isolation pens at the Poultry Diagnostic and Research Center (PDRC). For the first safety trial, 90 broilers were randomly distributed into 9 groups of 5 birds each with one replicate. At 28 days of age, all chickens were placed in filtered-air isolation units; selected groups were inoculated with 200 uL (100 uL intratracheal and 50 uL in each eye) of a solution containing $\log_{10}$ 3.5 of passages CEL(L)P10, 20, 30, 40, 50, 60, and CEF(4.1)P6. One group was inoculated with the parental (unattenuated) 63140 strain and was considered as a positive control, while another group received no inoculum and was considered as a negative control.

For the second experiment, 80 broiler chickens were randomly distributed into 8 groups of 5 birds with one replicate. Twenty eight-day-old broilers from the same origin and breed as in the first trial were placed in isolators for inoculations with passages CEL(L)P70, 80, 90, 100 and CEF(4.1)P10, and CEF(4.1)P20. A 63140 parental virus-challenged group and a non-challenged group were included as positive and negative controls, respectively.

Clinical Sign Scoring

At 5 Days post inoculation (DPI), clinical sign scores were recorded as previously described (12, 27, 28). Clinical sign scores consisted of scores from 0 to 3, with 0 being normal, 1 = mild, 2 = moderate; and 3 = severe for each of the following: respiratory signs,

conjunctivitis, and depression. In case of mortality, the bird involved was automatically scored with a value of 9.

Statistical analysis

The GraphPad Prism version 6.01 statistical package was used to analyze clinical sign score data obtained from the two trials. The Kruskall-Wallis test was independently used to compare median clinical sign scores for each group against the group inoculated with the parental 63140 strain (positive control), followed by multiple pair-wise comparisons to search for statistical differences.

RESULTS

No cytopathic effect (CPE) was observed in CEF, DF-1, VERO, or VERO E6 after inoculation of the 63140 parental strain. VERO E6 passages were negative to ILTV by PCR, while VERO passages were positive up to the fourth passage. LMH cells supported replication of 63140 for eight passages exhibiting CPE, although titer yield in this cell line was much lower when compared to 63140 virus yield in primary cell cultures (results not shown).

The CEL and CEK primary cell cultures were permissive to the parental ILTV 63140, with CEL tolerating ILTV infection for a longer period of time without the cells detaching (5-7 days in CEL instead of 2-4 for CEK). CPE in both cell cultures was characterized by formation of multinucleated giant cells, cell degeneration and necrosis as shown in Figure 5.1a for CEL and 5.1c for CEK.

ILTV was lost from CEK(K) at the ninth passage, while 63140 CEK(L) adapted better to CEK and higher passages were possible (Table 5.6). CEL(L) and CEL(K) were able to replicate efficiently. Beginning on CEL(L) P48, fibroblasts did not cover the empty space left by necrotic ILTV-infected liver cells. At higher passages, CEL(K) and CEK(L) also showed similar characteristics but to a lesser extent. A CEL(L)52 sample was inoculated into a secondary CEF monolayer (CEFP1) producing CPE by 48 hours post infection. Such CPE consisted mainly in rounding of CEFs with multiple vacuoles in the cytoplasm, usually forming part of a multicellular plain aggregate that did not detach (Figure 5.2a). Several tests to rule out possible viral and bacterial contaminants such as fowl adenovirus, reovirus, and mycoplasma were performed and all resulted in negative detection of contamination by PCR or RT-PCR (results not shown). ILTV confirmation in CEF infected cells was assessed by PCR, IFA (Figure 5.3) and electron microscopy (EM) (Figure 5.4).

No contamination by reovirus, or adenovirus was detected at the passages described in the material and methods sections, by PCR or RT-PCR. Samples were placed in culture for Mycoplasma isolation and no organism was isolated.

Virus titration

A summary of stock titrations expressed in $TCID_{50}$ per mL for CEK(L), CEL(L) and CEL(K) up to is shown in Table 5.6. Only CEL(L) passages were continued beyond the 60^{th} passage unlike CEK(L) and CEL(K), due to CEL capability to tolerate ILTV infection longer than CEK(L) and because it generated higher virus yields than the other CEL passage, CEL(K).

Titers for CEF(4.1) passages 6, 10, and 20 (previously passaged 52 times in CEL) are also shown in Table 5.6.

In vivo Safety Trials

Two *in vivo* safety trials were performed and their respective results are summarized in Figures 5.5 and 5.6. Since these trials were performed using both CEL(L) and CEF(4.1) passages, statistics were performed individually for each of these tissue culture attenuation methods. Median clinical sign scores from passages CEL(L) 60 and CEF(4.1) 6 were statistically different from the 63140 parental-challenged group (positive control) in trial #1, as shown in figures 5.5a and 5.6a; while median clinical sign scores from passages CEL(L)80, 90, and 100 as well as CEF(4.1)10 and CEF(4.1)20 were statistically different from the scores obtained in the 63140 parental-challenged group (positive control) in trial #2, as shown in figures 5.5b and 5.6b.

DISCUSSION

Multiplication of ILTV in cell cultures has been well documented, e.g. Vero (10, 19), LMH (23), QT-35 (24), CEF (14), CEL and CEK (10). In contrast, reports describing *in vitro* attenuation of a virulent virus in cell culture are scarce (6, 11, 20); consequently, there is only one TCO vaccine in the western world.

That TCO vaccine was obtained after 150 serial passages in CEL and CEK from chicken and turkey origin (6) of an American ILTV parental strain named as “L-6”, genetically different from American CEO vaccines (26). The present research shows, for the first time, the attenuation

in tissue culture of a virulent field CEO-related ILTV strain classified within the RFLP Group V (17) and identified as 63140 (3, 18).

Partial attenuation of ILTV 63140 in primary cells occurred only after 60 passages in CEL in the case of CEL(L)60, and 6 passages for CEF(4.1) which has been previously passaged 52 times in CEL, with one plaque purification round. Despite the decrease in clinical sign scores with higher passages of virus (CEF 90, 100; and CEF 10 and 20), mild to moderate clinical signs were still detectable in some birds inoculated with these passages. Thus, further research efforts are needed to achieve a total attenuation of the 63140 strain in these two attenuation systems, namely CEL and CEF.

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Table 5.1: Absorption time and temperature used per culture.

Cell Culture	Adsorption Time	Incubation Temperature ºC
CEL	1-2 Hours	37
CEK	1-2 Hours	37
CEF	1-24 Hours	37
DF-1	1-24 Hours	37
LMH	1-6 Hours	37-39
VERO	1-24 Hours	37-39
E6VERO	1-24 Hours	37-39

Table 5.2: Summary of primers used for detection of contaminants by conventional PCR

Virus	Primer Identity	Sequences
Fowl Adenovirus	FADV H1a	5’-tggacatgggggcgaccta-3’
	FADV H2a	5’-aagggattgacgttgtcca-3’
Reovirus	ReoV-P1b	5’-agtatttgtgagtacgattg-3’
	ReoV-P2b	5’-gatactgtcattgacttcga-3’
ILTV	gB-BNCU158c	5’-caatcctacatcgccgtgaac-3’
	gB-BNCL2854c	5’-cgcaaatgatggcagactga-3’

[a] Raue and Hess 1998; [b] Shapouri et al 1995; [c] Unpublished.

Table 5.3: Treatment groups used for Safety Trial #1.

Group Name \ Age	1 Day old	27 Day old	28 Day old	35 Day Old (5 DPI)
Non Challenged (NCh)			----------	
CEF(4.1)P6	BROILERS	BROILERS	CEF(4.1)P6[b]	
CEL(L)P10[a]		PLACED IN	CEL(L)P10[b]	Clinical
CEL(L)P20[a]	PLACED IN	ISOLATION	CEL(L)P20[b]	
CEL(L)P30[a]		UNITS (5@ PER	CEL(L)P30[b]	Sign
CEL(L)P40[a]	ISOLATED	GROUP/ UNIT	CEL(L)P40[b]	
CEL(L)P50[a]		WITH ONE	CEL(L)P50[b]	Scoring
CEL(L)P60[a]	PENS	REPLICATE)	CEL(L)P60[b]	
Challenged (Ch)[c]			CHALLENGED (Ch)[c]	

[a]CEL(L) P10 to P60 correspond to continuous passages of a 63140 sample in CEL cells as previously described.

[b]200 uL of a DMEM solution containing LOG_{10} 3.50 $TCID_{50}$ of each passage were inoculated into the birds : 100 uL intratracheally; 50 uL into each eye.

[c]63140 parent virulent strain (CKP5) is used as positive control / challenge strain.

Table 5.4: Treatment groups used for Safety Trial #2

Age / Group Name	1 Day old	27 Day old	28 Day old	35 Day Old(5 DPI)
Non Challenged (NCH)	BROILERS	BROILERS	----------	
CEF(4.1)P10		PLACED IN	CEF(4.1)P10[b]	Clinical
CEF(4.1)P20	PLACED IN	ISOLATION	CEF(4.1)P20[b]	
CEL(L)P70[a]		UNITS (5@ PER	CEL(L)P70[b]	Sign
CEL(L)P80 [a]	ISOLATED	GROUP/ UNIT	CEL(L)P80[b]	
CEL(L)P90 [a]		WITH ONE	CEL(L)P90[b]	Scoring
CEL(L)P100 [a]	PENS	REPLICATE)	CEL(L)P100[b]	
Challenged (Ch) [c]			CHALLENGED (Ch)[c]	

[a]CEL(L) P10 to P60 correspond to continuous passages of a 63140 sample in CEL cells as previously described.

[b]200 uL of a DMEM solution containing LOG_{10} 3.50 $TCID_{50}$ of each passage were inoculated into the birds : 100 uL intratracheally; 50 uL into each eye.

[c]63140 parent virulent strain (CKP5) is used as positive control / challenge strain.

Table 5.5: Codes of passages in primary tissue culture

Name	Treatment
CEL(K)	Parental Strain + CEL passages
CEL(L)	Parent Strain + 2 LMH passages + CEL passages
CEK(K)	Parental Strain + CEK passages
CEK(L)	Parent Strain + 8 CEL passages + CEK passages
CEF	CEL(L) P48 + CEF passages
CEF(4.1)	CEL(L) P48 + CEF passages (plaque purified)

Table 5.6. Stock titrations in CK cells expressed in $TCID_{50}$ per mL.

PASSAGE	CEK(L)	CEL(L)	CEL(K)	CEF(4.1)[a]
06	ND[b]	ND[b]	ND[b]	$\log_{10}$ 4.38
10	$\log_{10}$ 6.50	$\log_{10}$ 6.38	$\log_{10}$ 5.63	$\log_{10}$ 5.50
20	$\log_{10}$ 4.83	$\log_{10}$ 6.63	$\log_{10}$ 6.00	$\log_{10}$ 4.50
30	$\log_{10}$ 3.17	$\log_{10}$ 5.83	$\log_{10}$ 5.63	ND[b]
40	$\log_{10}$ 6.63	$\log_{10}$ 6.50	$\log_{10}$ 5.50	ND[b]
50	$\log_{10}$ 5.83	$\log_{10}$ 5.63	$\log_{10}$ 5.68	ND[b]
60	$\log_{10}$ 5.17	$\log_{10}$ 5.68	$\log_{10}$ 5.00	ND[b]
70	ND[b]	$\log_{10}$ 5.38	ND[b]	ND[b]
80	ND[b]	$\log_{10}$ 5.63	ND[b]	ND[b]
90	ND[b]	$\log_{10}$ 6.63	ND[b]	ND[b]
100	ND[b]	$\log_{10}$ 6.50	ND[b]	ND[b]

[a]CEF(4.1) was selected after 52 passages in CEL.

[b] ND = Not Done.

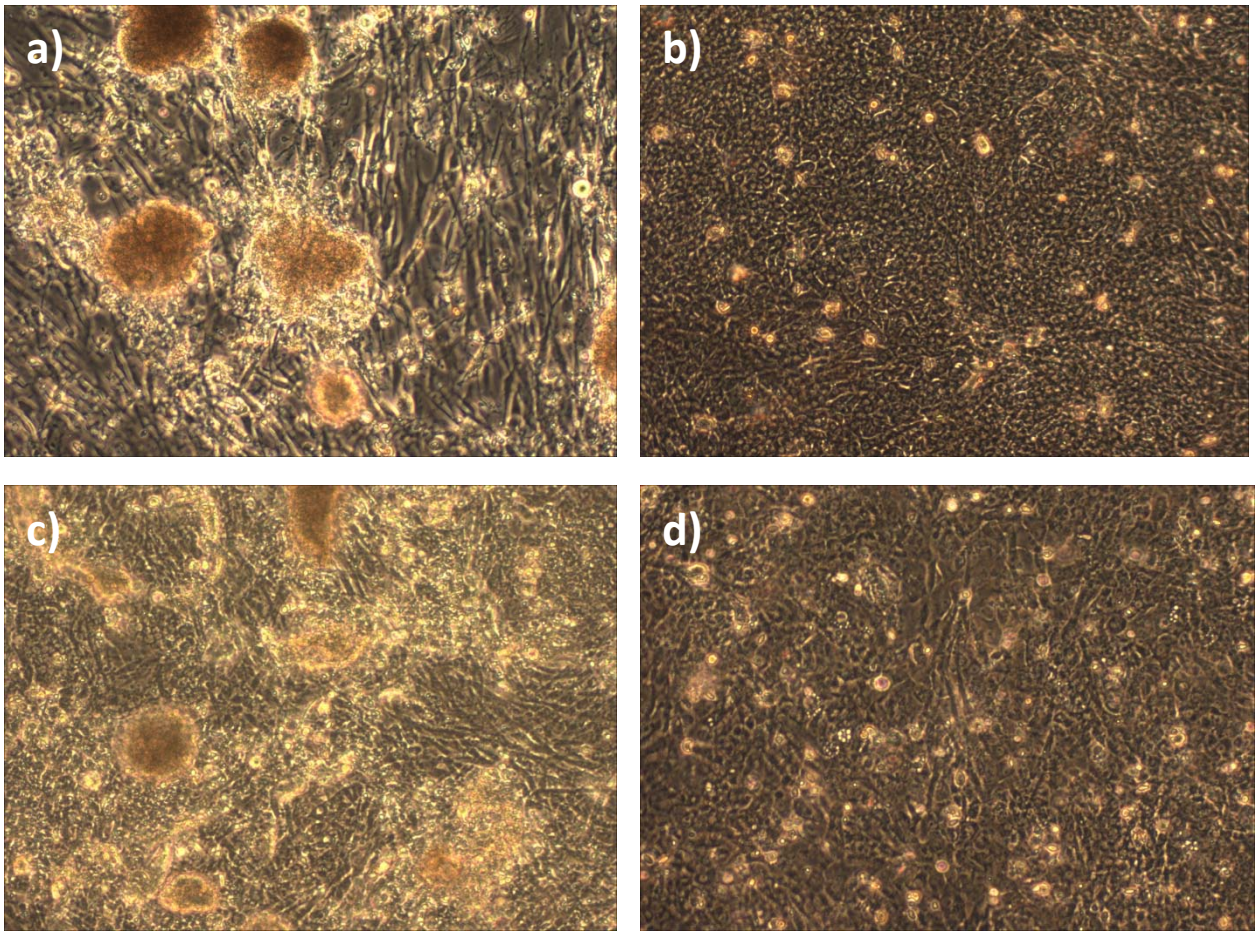

Figure 5.1: CPE produced by 63140 inoculation in CEL and CEK cell cultures observed at 100X. a) CEL cells at 5 Days post infection (DPI) with 63140 CEL(L)P20 inoculum. b) CEL control cells. C) CEK cells at 2 DPI with 63140 CEK(L)P22 inoculum. d) CEK control cells.

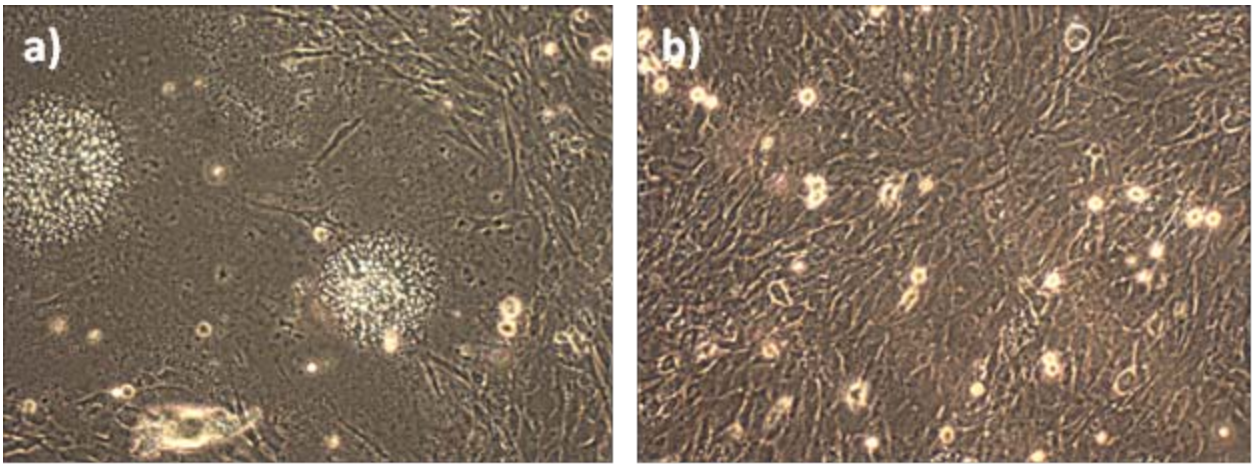

Figure 5.2: CPE produced by 63140 in CEF cell cultures observed at 100X. a) CEF infected with 63140 CEFP4 at 1.5 DPI with CPE consisting in multiple fibroblasts with multiple vacuoles usually forming part of a multicellular plain aggregate. b) CEF control cells.

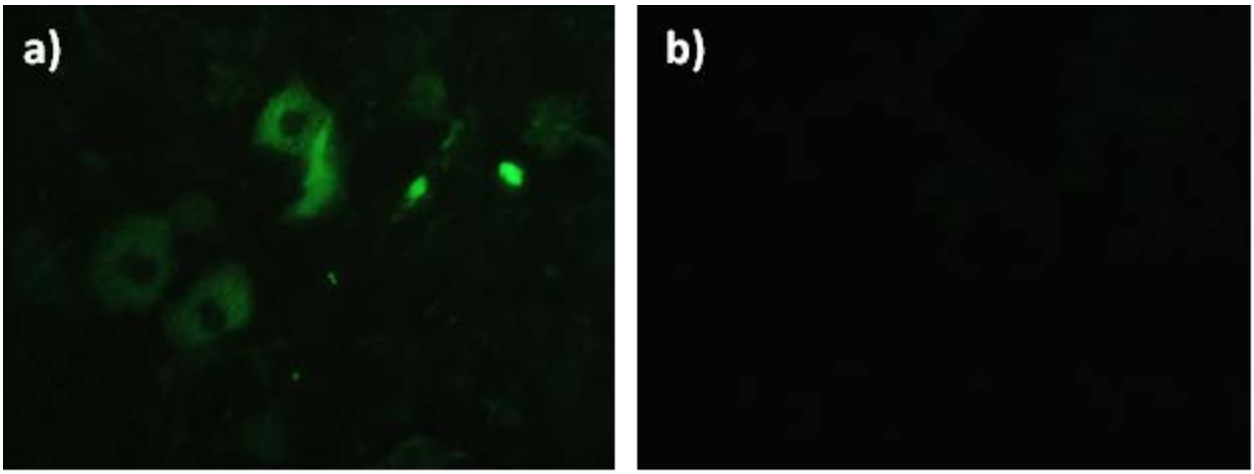

Figure 5.3: Indirect Immunofluorescence assay (IFA) observed at 400X. a) 63140 CEF P5 at 5 DPI; b) Non-infected negative control CEFs at 5 DPI.

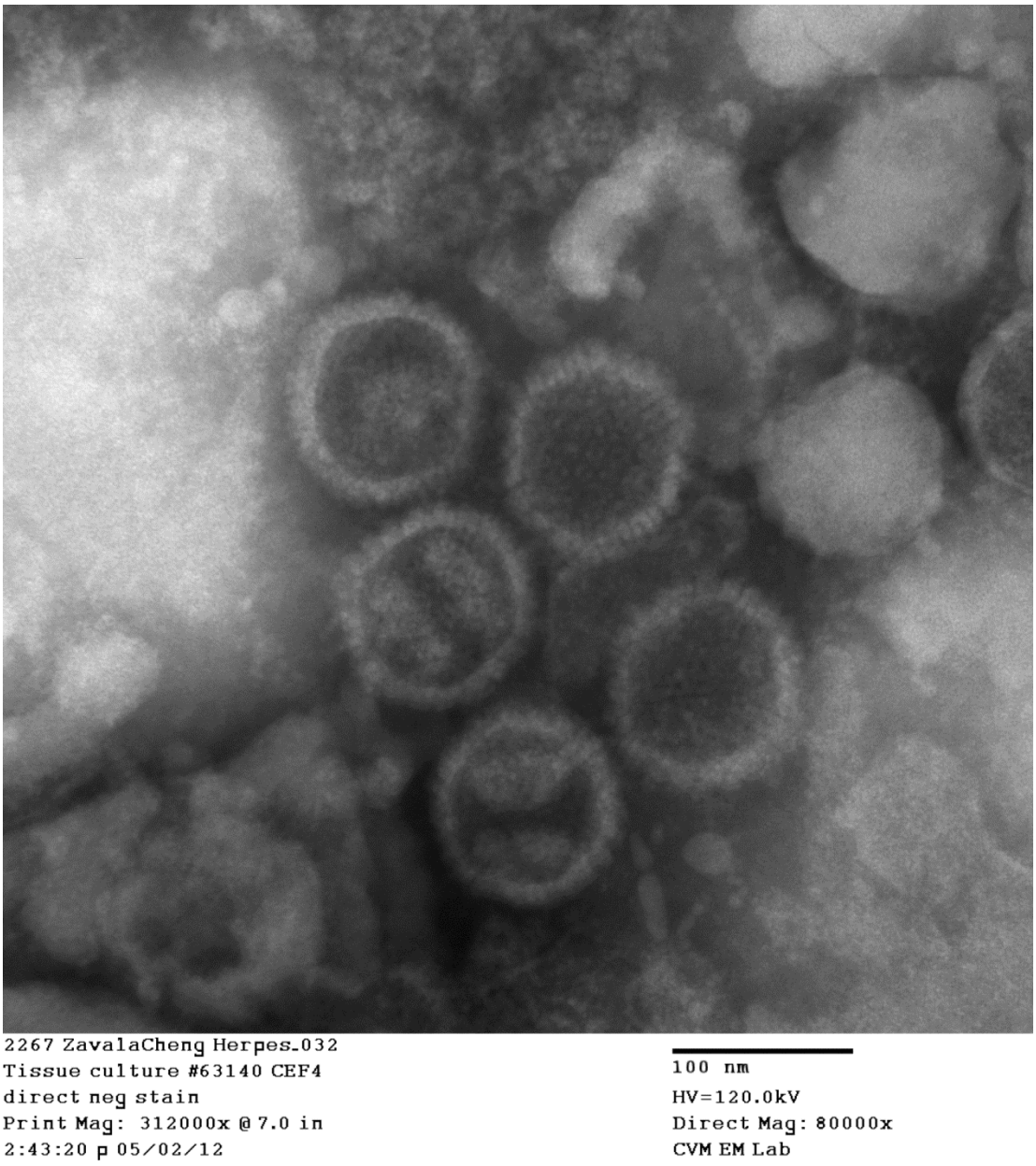

Figure 5.4: Transmission electron microscopy of negatively stained CEF cells infected with the 63140 isolate of ILTV (amplification = 80,000X) previously passaged 52 times in CEL cells and 3 times in CEF cells. Reference bar = 100 nm.

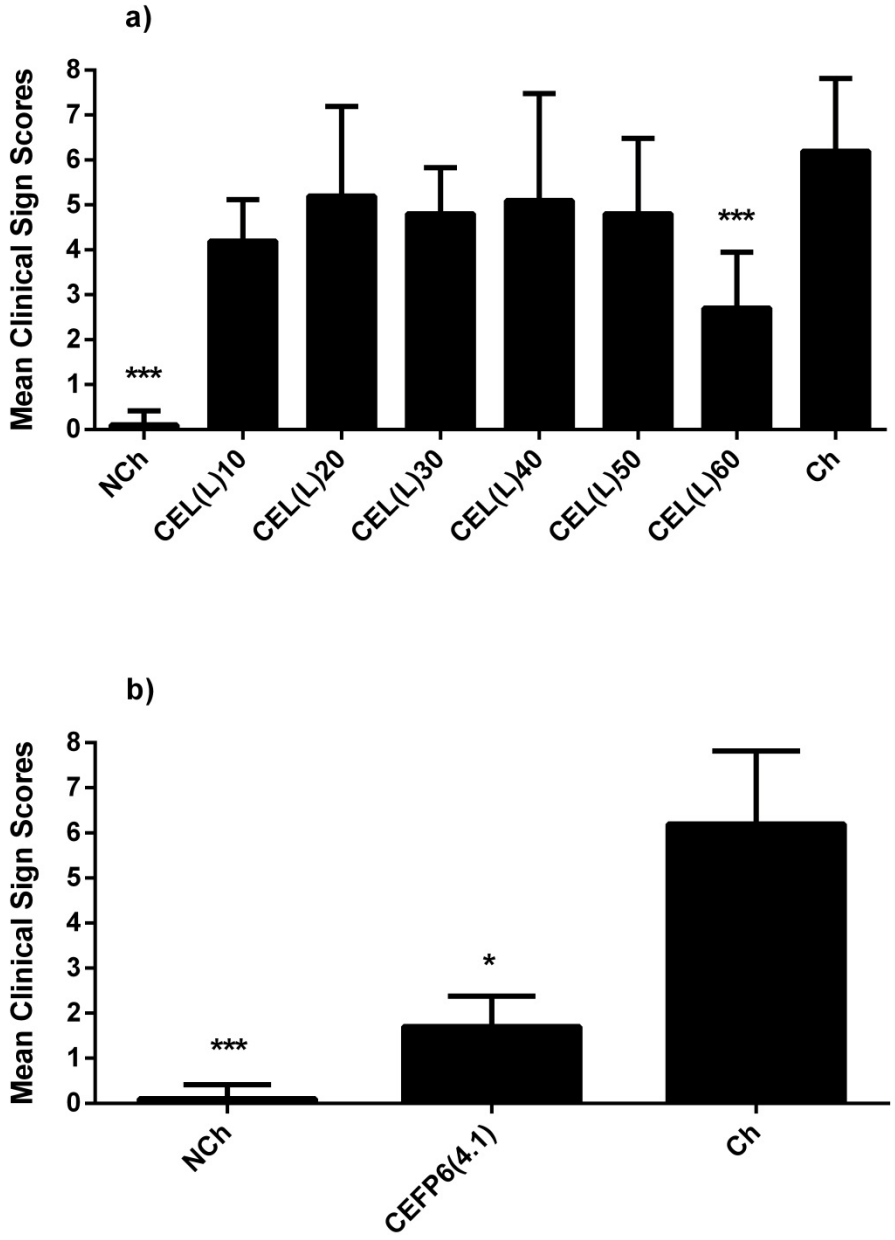

Figure 5.5: Clinical Sign Scores at 5 Days Post Challenge of Safety Trial #1. a) CEL(L) passages; b) CEF(4.1) passages (63140 CEF was selected after 52 passages in CEL, (Table 5) . Asterisks indicate a statistically significant difference relative to the positive control (Ch). Data are presented as mean ± SD (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

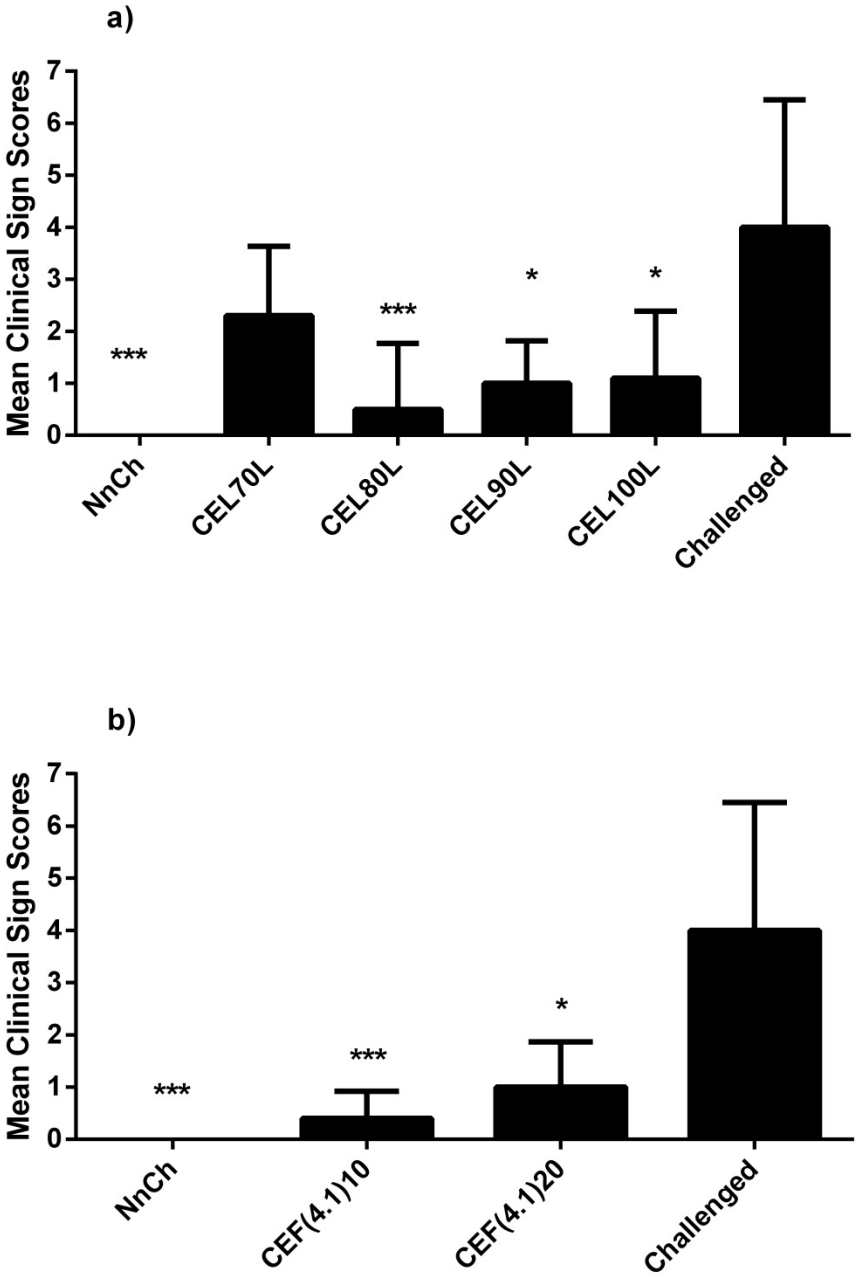

Figure 5.6: Clinical Sign Scores at 5 Days Post Challenge of Safety Trial #2. a) CEL(L) passages; b) CEF(4.1) passages (63140 CEF was selected after 52 passages in CEL, Table 5). Asterisks indicate a statistically significant difference relative to the positive control (Ch). Data are presented as mean ± SD (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).

CHAPTER 6

DISCUSSION

The objectives of this research were: 1) to assess the protection induced by live-modified, recombinant, inactivated vaccines and combinations of such against a virulent strain of ILTV in commercial layers; 2) to examine by ELISA the serological responses of commercial layers vaccinated against ILT using different immunization approaches that included modified live, recombinant, inactivated vaccines, and their combinations; and 3) to attenuate a field CEO-related strain of ILTV identified as “63140” by serial passages in primary cultures of chicken embryo kidney cells (CEK), chicken embryo liver cells (CEL), chicken embryo fibroblasts (CEF), and immortalized continuous cell lines such as VERO, E6VERO, LMH and DF-1.

In the first study, the protection against ILT by several vaccines and vaccine combinations, including live-modified (CEO and TCO), recombinant (FPV-LT and HVT-LT), and inactivated (KILLED) vaccines was assessed in commercial table egg layers by a series of challenge studies performed at 4, 9, 35, and 74 weeks of age. Protection was measured on the basis of prevention or reduction of clinical signs and viral loads in the trachea after inoculation with virulent virus. Other aspects related to ILT immunity in recombinant-vaccinated chickens were also examined, including the persistence of HVT-LT recombinant virus in the spleens of vaccinated birds; the spread of virulent virus from vaccinated-challenged birds to contact

chickens; and serological responses against ILT vaccines and/or virulent ILTV evaluated using ELISA.

Data post-challenge were collected between 5 and 7 days after inoculations with virulent virus. In agreement with previous research performed in broilers (8, 16), vaccination with only modified live vaccines (CEO or TCO) induced better protection than vaccination with only recombinant vaccines (FPV-LT or HVT-LT). This pattern was consistently observed in all ages studied (4, 9, 35 and 74 weeks of age). The FPV-LT alone or combined with the KILLED vaccine; and the KILLED vaccine alone did not induce significant protection against the ILTV challenge, contrasting with previous findings in broilers in which partial protection was documented using the same challenge strain (63140) and delivery route. Such difference in FPV-LT protection may be due to intrinsic differences between the immune systems of meat type and egg type chickens (9) along with differences in challenge ages.

The level of protection observed in the groups of chickens receiving the HVT-LT or TCO vaccines waned as the birds aged; the HVT-LT group provided significant protection against clinical signs and viral shedding in the 4 WCH and 9 WCH studies; only partial protection in the 35 WCH and no significant protection at 74 weeks of age. The TCO-vaccinated group exhibited a reduction in clinical signs and viral shedding in the 4 WCH, 9 WCH and 35 WCH experiments, but only reduced clinical signs and not viral shedding in the 74 WCH study.

Birds vaccinated with two different vaccines such as HVT-LT+TCO, HVT-LT+KILLED and TCO+KILLED showed enhanced protection seen as reduced clinical signs and viral shedding at 74 weeks of age. The exact mechanism by which dually vaccinated groups exhibited increased protection is unknown. Data obtained in the various experiments herein described indicated that clinical sign scores diminished in severity as the age of chickens progressed.

While, viral shedding post-challenge was similar at any age, independently of the clinical signs severity. Confirming previous work, these results suggested a natural age-dependent resistance against the disease (2).

Finally, based on the serological results for young birds, it was concluded that ELISA systems might be inadequate for the identification of infection at 4 and 9 weeks of age if blood is collected within 7 days post-challenge, which may be insufficient time for adequate and detectable seroconversion (12). In contrast, antibody ELISA was able to detect seroconversion 13 days post-challenge when the infection occurred at 35 WCH or at 9 days post-challenge for 75-week-old chickens. However, the proportion of non-specific (false positive) reactions in ILTV-negative birds increased with age (1, 13).

The performance of three ILT antibody ELISA kits was examined, including one kit from The Netherlands and manufactured in the United Kingdom (BioChek); a second one from United States (Synbiotics); and a third one from Australia (TropBio). Sensitivity and specificity were assessed on sera from commercial layers vaccinated with live-modified vaccines; an oil-based inactivated vaccine; and from non-vaccinated challenged birds. Sensitivity in all kits was above 85%, while specificity was variable among kits. Non-specific ELISA reactions in the antibody ELISA system have been previously described (1, 10, 11, 14, 17, 18); moreover, such non-specific reactions were found to be dependent on age and breed of the chickens sampled (11). FPV-LT vaccinated birds seroconvert poorly to the vaccination, probably due to the vector biology or the nature of the ILTV proteins expressed by the vector. That is, FPV stimulates primarily a cellular response instead of a humoral response (15); second, after the replication of

the vector in the vaccinated chicken there is no further stimulation of the immune system (FPV does not induce latency), and thus there is no anamnestic response and antibody titers do not rise any further; in addition, immunity conferred by FPV-LT vaccines in the field is known not to be as robust as that observed with other ILT vaccines (3, 4, 8, 16). In contrast, the HVT-LT vaccine elicited an antibody response that was detectable in most of the vaccinated birds using the BioChek and Synbiotics ELISA kits, whereas only few seropositive birds were detected using the TropBio ELISA.

Since protection against ILTV cannot be predicted by antibody levels (5-7), ELISA antibody detection systems may be useful for disease surveillance in areas where ILT is thought to be absent and where vaccination is not practiced. Thus, ELISA can be used to indirectly detect field challenge and also to monitor vaccinated flocks. The present serological establishes reference guidelines for commercial layer flocks vaccinated against ILT using various vaccines or vaccine combinations.

As a third objective, attenuation of a CEO-related ILTV field strain designated “63140” was pursued by first: serial passage in avian immortalized cell lines (i.e. LMH, and DF-1), mammalian immortalized cell lines (i.e. VERO and VERO E6, a clone of VERO adapted for long incubation periods) and primary cell cultures from chicken origin, (i.e. chicken embryo kidney cells [CEK]; chicken embryo liver cells [CEL]; and chicken embryo fibroblasts [CEF]); and second: by assessing such attenuation in 28-day old broilers. Partial attenuation of ILTV was achieved only in CEK and CEL cultures, whereas LMH, DF-1, VERO, and its clone VERO E6 were unsuitable for such purpose. 63140 in primary cells occurred only after 60 passages in CEL in the case of CEL(L)60; and after 52 passages in CEL and 6 passages in CEF with one round of

plaque purification in the case of CEF(4.1)P6, based on the reduced clinical sign scores observed at 5 days post-challenge in inoculated 28-day old birds, compared to the control inoculated with the parental (virulent) virus. Despite the decrease in clinical sign scores with higher passages of virus (CEF 90, 100; and CEF 10 and 20), mild to moderate clinical signs were still detectable in some birds inoculated with these passages, indicating that further efforts are needed to achieve total attenuation of the 63140 strain in CEL and CEF.

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