# BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) FROM THE UNITED STATES

by

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(Under the Direction of Maricarmen Garcia)

## ABSTRACT

Infectious laryngotracheitis (ILT) is an upper-respiratory disease of poultry of worldwide distribution. The disease is caused by infectious laryngotracheitis virus (ILTV) a member of family Herpesviridae, is characterized by acute respiratory signs, and is common in areas of intense poultry production. The disease is controlled by vaccination with live attenuated vaccines. Once vaccine strains have been introduced in the field the differentiation of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses. In this study, polymerase chain reaction and restriction fragment polymorphism (PCR-RFLP) of genome regions was utilized to characterize ILTV isolates from commercial poultry and backyard flocks from the US. Combinations of PCR-RFLP patterns classified the infectious laryngotracheitis virus (ILTV) isolates into different groups, as well as backyard flock isolates. Some of the isolates were closely related to the CEO vaccine strains. However isolates different to the vaccine strains were also identified in commercial poultry. Sequencing analysis of multiple genome regions were used to validate genotype groups obtained by PCR-RFLP analyses. This study presents the first comparative sequencing analysis for a wide variety of ITLV isolates from

the US where specific genome differences were identified for commercial poultry and backyard flock isolates from the US. In addition, the evaluation of clinical signs, viral tissue distribution in chickens, viral replication in cell culture, and plaque formation ability of several ILTV isolates categorized into different PCR-RFLP or sequencing groups showed biological differences among US ILTV isolates.

INDEX WORDS: Infectious laryngotracheitis virus; ILTV; ILT; chicken embryo origin vaccine; tissue culture origin vaccine; polymerase chain reaction; restriction fragment length polymorphism; sequencing analyses; in vitro characterization; viral pathogenicity.

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# DEDICATION

This dissertation is dedicated to my parents, without whom I could never have come so far and specially to my wife, who always gives me support during the good and bad times.

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# CHAPTER 1

# INTRODUCTION

Infectious laryngotracheitis (ILT) is a highly contagious acute respiratory disease of chickens, of worldwide distribution. The disease causes great economic losses during severe outbreaks due to a decrease in egg production, high mortality rates and implementation of depopulation procedures (11). Infectious laryngotracheitis virus (ILTV) is a member of the genus Iltovirus, within the family *Herpesviridae*, subfamily *Alphaherpesvirinae* (7). ILTV establishes a primary lytic infection in the trachea followed by periods of latency in the trigeminal ganglia with episodes of reactivation where viral replication resumes in the trachea (11).

Modified-live ILT vaccines have been the principal tool utilized to control the spread of the disease (11). Traditionally, in the United States (US), two types of modified-live ILT vaccines have been used to control ILTV; vaccines attenuated by multiple passages in embryonated eggs (chicken embryo origin [CEO]) (25); and the vaccine generated by multiple passages in tissues culture (tissue culture origin [TCO]) (9). There are currently several CEO vaccines, one TCO vaccine commercially available. The CEO vaccines are labeled for administration by water and spray in addition to the eyedrop method. The TCO vaccine is labeled for eyedrop administration only. In addition to modified-live ILT vaccines, there is also a fowl pox-vector ILT vaccine (FP-LT) commercially available for administration only by wing web stab inoculation at about 8 weeks of age (8).

ILT vaccines have been associated with a variety of adverse effects including spread of vaccine virus to non-vaccinated birds (1, 17, 24), production of latently infected carriers (3), and increased virulence as a result of consecutive passages *in vivo* (14), or after reactivation from latency (18). Moreover, modified-live ILT vaccine viruses are involved in field outbreaks of the disease (12-14). Once vaccine strains have been introduced in the field the identification of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses (11).

Initial attempts to differentiate among ILTV strains in the US were achieved by restriction fragment length polymorphism (RFLP) analysis of the viral genome (2, 13, 19, 20, 22). Although these studies provided the first evidence that US ILTV field isolates were closely related to the CEO vaccine strains, they displayed low discriminatory power and lack of reproducibility, and the routine use of RFLP analysis for epidemiological purposes was limited due to the difficulties in obtaining high yields of pure viral DNA. The use of the polymerase chain reaction restriction fragment length polymorphism of PCR products (PCR-RFLP) facilitated the differentiation of ILTV strains. PCR-RFLP analysis of single and multiple viral genes and genome regions has permitted the differentiation of ILTV isolates from different parts of the world (4-6, 10, 15, 16, 21, 23). In general, molecular epidemiology studies have suggested that most of ILT outbreaks in broilers in the US are caused by viruses closely related to the CEO vaccines, while outbreaks with TCO type isolates are rare (13, 19, 20). However, viral genome regions of genetic diversity useful for epidemiological studies have not been clearly identified for US ILTV isolates, this has limited our knowledge about the epidemiology of the disease. The first aim of this study was to utilize multiple PCR-RFLP assays to identify regions of genomic diversity, and to genetically categorize ILTV isolates from the US. Consequently, the types of

virus that are circulating in the field and are involved with ILT outbreaks in commercial poultry were identified. The second aim of this study was to sequence ILTV genes that presented genetic variability by multiple PCR-RFLP analysis to identify specific genomic differences among vaccines, commercial poultry, and backyard flock isolates from the US. The third aim of this study was to evaluate the *in vivo* pathogenicity, and *in vitro* replication of ILTV isolates from commercial poultry, representative of the different genotype groups previously identified by PCR-RFLP and sequencing analysis, as compared to the CEO vaccine.

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# CHAPTER 2

# LITERATURE REVIEW

Infectious laryngotracheitis (ILT) is an acute viral respiratory disease that affects primarily chickens, and is of great economical importance due to severe production losses resulting from mortality and/or decreased in egg production (51). The disease is caused by infectious laryngotracheitis virus (ILTV), a member of the family *Herpesviridae* subfamily *Alphaherpesvirinae*, genus Iltovirus (28). Severe epizootic forms of infection are characterized by signs of respiratory depression, gasping, expectoration of bloody mucus, and high mortality. Mild enzootic forms of infection are encountered increasingly in developed poultry industries and manifested as mucoid tracheitis, sinusitis, conjunctivitis, general depression, and low mortality (51).

First described in 1925 (90), ILT has been reported in most countries and remains a serious disease wherever large concentrations of susceptible poultry populations exist (51). Vaccination programs using modified-live vaccines have been applied to control ILT in areas of intensive production such as in the United States, Europe, China, Southeast Asia, and Australia. However, modified-live vaccines also have been associated with a variety of adverse effects including spread of vaccine virus to non vaccinated birds (4, 62, 107), production of latently infected carriers (5), and increased virulence as a result of consecutive passages *in vivo* (53), or after reactivation from latency (67). Despite vaccination, ILT continues to cause outbreaks of

respiratory diseases worldwide, and in the United States epidemics of the disease are an emerging problem.

## ETIOLOGICAL AGENT

### Classification

Infectious laryngotracheitis virus is classified as a member of the family Herpesviridae, subfamily Alphaherpesvirinae, and because of its phylogenetic distance from all previously characterized alphaherpesviruses, is classified as a member of the genus Iltovirus (28, 70, 92). The virus is taxonomically identified as Gallid herpesvirus 1 (28, 108).

### Morphology

The morphology of ILTV is similar to the herpes simplex virus, consisting of a DNA-containing core within an icosahedral capsid which is surrounded by a tegument layer, formed by proteins, and an outer envelope with integrated viral glycoproteins spikes on its surface (26, 110). The viral capsid is composed by 162 elongated hollow capsomeres (26), resulting in a diameter of 100 nm. Particle size depends on the amount of incorporated tegument protein. It can vary between 200 and 350 nm in size (49).

### Genome Structure, Gene Content and Phylogenetic Considerations

Infectious laryngotracheitis virus has a linear double-stranded DNA genome with 76 predicted protein open reading frames (ORFs) (121). Similar to other avian herpesviruses, the G+C content is 46% and similar to its host DNA it has a buoyant density of 1.705 ± 0.001 g/ml

(99). The ILTV genome has been classified as type D genome (110, 121) of approximately 150 kbp (121). The ILTV genome consists of long (UL) and short (US) unique regions, with inverted repeat (internal repeat [IR], terminal repeat [TR]) sequences flanking the latter (69, 85). The ILTV genes are similar to those found in other alphaherpesviruses and the designations for ORFs and proteins have been widely adopted from the homologues genes and proteins of the herpes simplex virus type 1 (HSV-1) genome (91). As for HSV-1, ILTV ORFs designated UL1 to UL54 are located in the UL, US2 to US8 located in the US region, and 11 ORF encoding homologous HSV-1 glycoproteins (gB, gC, gD, gE, Gg, gH, gI, gJ, gK, gM) has been identified in both the UL and US regions of the genome. Different to the HSV-1 genome, five genes designated ORF A to ORF E (Ziemann et al., 1998a; Veits et al., 2003a), and two genes designated UL0 and UL-1 (141) have been identified as unique to ILTV. Also the gene arrangement of the ILTV genome differ from the HSV-1 genome, ILTV has a translocation of the UL47 gene from the UL to US region (130), and a large internal inversion of a conserved gene cluster within the UL region (from UL22 to UL44) similar to an inversion observed in the pseudorabies virus genome (121). The presence of several genes unique to ILTV indicated that this virus might represent a different branch within the herpesviruses evolution. This assumption was confirmed by comparative analysis of amino acid sequences of conserved gene products that showed that ILTV ancestors probably separated from those of the mammalian alphaherpesviruses earlier than the ancestors of Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) (70, 92). Therefore, ILTV has been assigned as the only member to the genus Iltovirus of the *Alphaherpesvirinae* subfamily of the *Herpesviridae* (28). Recently analysis of the psittacid herpesvirus 1 (PsHV-1) genome revealed that this virus shares the specific genes and

genome characteristics of ILTV (121), suggesting that this avian herpesvirus should also be grouped into the same genus.

**Viral Glycoproteins**

Viral envelope glycoproteins of herpesviruses mediate virus entry, cell fusion and virus egress (98, 120). In addition, they are important immunogens and potential targets of cell-mediated and humoral immune responses. Previous studies identified five major envelope glycoproteins with molecular weights of 205, 160, 115, 90, and 60 kD, to be the major immunogens of ILTV (139, 140). Subsequently, characterization of ILTV glycoproteins utilizing monospecific antisera or monoclonal antibodies has been undertaken in several laboratories. Several glycoproteins that are homologous to those of human herpes simplex virus (HSV) have been characterized; these are designated glycoprotein B (gB) (100), gC (77, 127), gN (40), gM (39, 40), gG (81), and gJ (127).

**Virus Replication**

The replication cycle of ILTV appears to be similar to that of other alphaherpesviruses like HSV-1(109). The ILTV infection initiates by the viral attachment to cell receptors. The glycoproteins involved in viral attachment for herpesviruses in general are glycoproteins B (gB) and C (gC), which interact with heparan sulfate at cell membrane surface (110). However, ILTV does not interact with heparan sulfate (78) and the cellular receptor for this virus is still not known. Co-receptor binding then occurs between glycoprotein D (gD) and one of the several proteins from cellular membranes (109). The fusion between viral envelope and cell membrane follows receptor binding. Viral glycoproteins gB, gD and also the heterodimer formed by

glycoprotein H (gH) and L (gL) are required during the fusion process (38, 87, 110). After viral membrane fusion, the capsid is released into the cytoplasm and, along with some remaining tegument, is transported to the nuclear membrane through the microtubular network and becomes associated with a nuclear pore complex in the nuclear membrane. Viral DNA is released from the capsid and migrates into the nucleus through nuclear pores, leaving an empty capsid on the cytoplasm side of the complex (109). Once inside of nucleus, ILTV DNA replication and transcription occur in a highly regulated and ordered cascade as is typical for herpesviruses (101). Transfection experiments with plasmids expressing the ILTV ICP4 and UL48 proteins revealed that these two proteins were functional homologous to the major HSV-1 transcription activator VP16 (43, 60). Newly synthesized viral DNA is processed and packaged into preformed immature capsids, which then becomes associated with altered regions of inner layer of the nuclear envelop and bud through into the cytoplasm. It is currently unknown whether the capsids obtain their final envelope from the nuclear envelope or are released from the outer nuclear membrane as free capsids and re-enveloped at the membranes of the trans-Golgi network (49, 109). Mature virions are released from host cells by exocytosis or cytolysis (109) . Uncommon features frequently observed in ILTV infected cells are the formation of tubular structures of unknown function, large cytoplasmic vacuoles containing numerous virions (50), and the formation of many light (L) particles, which consist of tegument and envelope, but lack the nucleocapsid (49).

**Viral Strains Differentiation**

Earlier studies demonstrated that ILTV strains differ on their virulence for chicken embryos (68), plaque size and morphology in cell culture (111), pock size on chorioallantoic

membrane (CAM) of embryonated eggs (102). Assessment of mortality patterns in embryonated chicken eggs was proposed as a biological system for differentiating ILTV strains (68) as it was found that embryo mortality patterns correlated closely to viral strain virulence. Nowadays differentiation of ILTV strains based on their virulence remains an important practical problem, mainly to differentiate virulence differences between field strains and modified live-vaccine viruses. Recently the pathogenicity of ILTV strains has been evaluated based on mortality, weight loss, and severity of clinical signs induced by the different strains in experimentally infected chickens (79).

ILTV strains are antigenically homogeneous based on virus neutralization (VN), immunofluorescence (IF) and cross-protection tests (22, 119). However, minor antigenic variation among strains has been suggested by findings that some strains are neutralized poorly by heterologous antisera (102, 111, 119). Since antigenic differences are not sufficient to differentiate ILTV strains, molecular methods have been widely used instead. The methods utilized for differentiation of ILTV strains include restriction endonuclease analyses of viral DNA (52, 57, 83, 84), DNA hybridization assays (82), polymerase chain reaction (PCR) procedures combined with restriction fragment length polymorphism (RFLP) analyses of amplified DNA (PCR-RFLP) (19, 21, 25, 45, 48, 58, 80), PCR-RFLP combined with gene sequencing (55), and gene sequencing (96). The use of PCR-RFLP and sequencing analysis has been very useful tool in epidemiological studies of field to differentiate modified-live vaccine viruses from non-vaccine (wild-type) ILTV strains (19, 21, 25, 48, 56, 80, 96).

## EPIDEMIOLOGY AND PATHOGENESIS

Infectious laryngotracheitis virus has been detected in most countries around the world and remains a serious disease wherever susceptible high-density poultry populations exist (14, 51). In areas of intensive production and large concentrations of poultry such as in the United States, Europe, China, Southeast Asia, and Australia, ILTV is usually well controlled in layers by the use of modified-live virus vaccines. For intensive broiler production, the short growth cycle and high level of quarantine on sites can reduce the need for prophylactic vaccination. Seasonal incidence varies between states and outbreaks; however, since the development of intensive broiler production in the US, ILTV tends to occur through the year. Persistence of cases into the summer months and after cessation of broiler vaccination schemes also seems to be an emerging problem. Usually, during ILTV epornitics one poultry production area has been involved; however, the distribution of cases throughout different geographic areas seems to be increasing (29).

### Infection and transmission

Chickens are the primary host of ILTV, although the disease can also affect pheasants, partridges, and peafowl (24, 64). Chickens are susceptible to infection at all ages; however most of characteristic signs are seen in adult birds (51). Starlings, sparrows, crows, doves, ducks, pigeons, and guinea fowl appear to be refractory to ILTV (10, 15, 115); however sub-clinical infection and seroconversion in ducks has been reported (136). Turkeys have been found to be susceptible to infection under experimental conditions and the virus can be propagated in turkey embryos (135).

The main route of infection is the upper respiratory tract and conjunctiva (12), and less commonly by ingestion with exposure of the nasal epithelium (106). Infected birds with clinical disease are the major source of virus spread as compared to the clinically recovered carrier birds. Moreover, the virus has been shown to survive for several weeks out of the host, fomites and mechanical carriers (12, 34, 47, 76). Vertical transmission of ILT has not been demonstrated. Transmission between flocks has primarily been associated with flock proximity, breakdowns in biosecurity, and mixing of vaccinated and non-vaccinated birds. Research has clearly shown that after backpassage in the field the chicken embryo origin (CEO) vaccine virus, and rarely the tissue culture origin (TCO) vaccine virus may revert to pathogenicity causing outbreaks of the disease (53). Direct or indirect contact with backyard fowls or game fowls has not been proved a common risk factor. Live haul trucks carrying flocks with active disease to the processing plant, and moving of litter from infected houses are considered sources of epornitics (29).

**Clinical signs**

Clinical signs of ILT include conjunctivitis, nasal discharge, depression, sneezing, coughing, gasping and usually appear within 6 -12 days after natural infection (74, 116). However, birds intratracheally experimentally infected showed clinical signs within 48 hours post inoculation, which persisted for about five days with abrupt disappearance of virus on the seventh day after inoculation (13, 71, 104, 116). Marked dyspnea, expectoration of blood stained mucous and an elevated mortality rate characterizes the severe form of the disease. In addition, hemorrhages can be found on closer examination of the trachea and larynx and occasionally mucous plugs can block the trachea and cause asphyxiation. A drop in egg

production has also been associated with ILTV infection. Decrease in feed consumption, simultaneously a decline in egg production and eggshell thickness has been also observed (105).

**Pathology**

During ILTV infection, gross lesions are most consistently observed in the larynx and trachea but may be found throughout the respiratory tract and in the conjunctiva, varying in severity. In severe forms of the disease clinical signs generally appear after six to twelve days from natural exposure (74, 116). Characteristic clinical signs include conjunctivitis, nasal discharge, depression, sneezing, and gasping (11, 74). Marked dyspnea, expectoration of blood stained mucous, and mortality are characteristics of severe epizootic forms of the disease. During severe forms of ILTV, there is catarrhal inflammation in the trachea early in the infection followed by degeneration, necrosis, and hemorrhage in later stages. Diphtheritic changes are common and may be seen as mucoid casts that extend the entire length of the trachea. In some cases, severe hemorrhage into the tracheal lumen may result in blood casts, or blood may be mixed with mucus and necrotic tissue. Inflammation during severe cases also may extend down the bronchi into the lungs and air sacs. In mild forms of ILT, gross lesions may consist only of conjunctivitis, sinusitis, and catarrhal tracheitis. The severe forms were commonly described in early years. Nevertheless, in recent years mild forms have been more frequently observed in high density populated poultry producing areas (32, 88, 117). In mild forms of ILT, gross lesions may consist only of conjunctivitis, sinusitis, swelling of the infraorbital sinus and catarrhal tracheitis. Other signs related with mild enzootic forms include decreased egg production, weight losses, and persistent watery eyes. The curve of infection varies with the severity of lesions; usually, most chickens recover in 10 to 14 days (51). In some cases, edema and congestion of

the epithelium of the conjunctiva and infraorbital sinuses may be the only detectable gross lesion observed for mild forms of ILT (117).

As the gross lesions, microscopic changes also vary with the severity and stage of the disease. The histopathological observation reveals inflammation and necrosis of the respiratory mucosa with or without hemorrhage. The earliest microscopic changes in tracheal mucosa detectable are the loss of goblet cells and infiltration of mucosa with inflammatory cells. Multinucleated cells (syncytia) also are observed, and lymphocytes, histiocytes, and plasma cells migrate into the mucosa and submucosa after 2 - 3 days. Eosinophilic intranuclear inclusion bodies develop in the respiratory tract and conjunctival epithelia are pathognomonic for ILTV in chickens. The inclusion bodies are present in the early stages of infection (1-5 days) (103, 118) and then disappear during the process of epithelial necrosis and desquamation or regeneration (54, 126). Epithelial necrosis and desquamation, results in a mucosal surface either covered by a thin layer of basal cells or lacking any epithelial covering; blood vessels within the lamina propria may protrude into the tracheal lumen. Hemorrhage may occur in cases of severe epithelial destruction and desquamation with the exposure and rupture of blood capillaries.

**Morbidity and mortality**

During severe outbreaks of the disease, the morbidity can reach 80-100%, while mortality in some cases can be 80%, although it is more common to remain between 10 and 20% (11, 116). During outbreaks caused by less pathogenic ILTV strains the morbidity can still be the same but with lower mortality (2-5%). Some mild enzootic forms of the disease results in morbidity as low as 5% and very low mortality (0.1 to 2%) (22, 88, 89, 105, 117, 129), with most

birds appearing clinically normal and only a few displaying mild pathological changes at post-mortem examination (6).

## VIRAL LATENCY

ILTV is able to establish a carrier state in recovered birds (5), and this is characterized by periods of latency interspersed with episodes of virus shedding detectable by tracheal swabbing (65, 66). During the acute phase the main site of virus replication is the trachea, whether birds are experimentally inoculated via the respiratory tract, the eye or a variety of other routes (63, 65, 104). Like other alphaherpesviruses, ILTV migrates to the central nervous systems, and the trigeminal ganglion (TRG) is the main site of ILTV latency (132), which provides the main sensory innervations to the tissues of the upper respiratory tract, mouth and eyes (16). Stress of rehousing and the onset of reproduction can trigger the re-excretion of ILTV from latently infected birds to the trachea (65).

## HOST IMMUNOLOGIC RESPONSE

The response to ILTV infection from the specific immune system involves both humoral and cell-mediated immunity (CMI). In immunologically mature birds (5 wk of age), antibodies can be detected 5 days post-inoculation (PI) with peak levels of IgM detected 5 days PI, and levels of IgG and IgA peaking 10 days PI (27). However, maternal IgG antibody does not confer resistance to infection with ILTV (23). It has been shown that passive transfer of anti-ILTV serum has no effect on reducing the susceptibility of 2-day-old or 4-wk-old chickens to infection

(35). Humoral immune responses to ILTV, although associated with infection, are not the primary mechanism of protection, and a poor correlation generally has been found between serum antibody titers and immune status of flocks (72). Bursectomized chickens, which showed no detectable levels of ILTV-specific serum antibodies, could be protected from viral challenge after vaccination (35). The absence of mucosal antibody (IgA) does not impair the ability of vaccinated-bursectomized chickens to resist challenge. The principal mediator of ILTV resistance is the local cell mediated immunity (CMI) response produced in the trachea (36). Protection from viral challenge can also be transferred between inbred chickens with immune spleen cells or peripheral blood lymphocytes (37). The mechanism by which the CMI response confers protection has been poorly studied. The humoral and CMI responses elicited by ILTV are ineffective against the virus during latent stages of infection.

## DIAGNOSIS

In cases of severe acute disease with high mortality and expectoration of blood, ILT can be diagnosed based on clinical signs (51). However, ILT diagnosis requires laboratory assistance since other respiratory pathogens of poultry can cause similar clinical signs and lesions. Diagnosis of ILT is based on one or more confirmatory laboratory diagnostic procedures including the detection of intranuclear inclusion bodie, by histopathology, virus isolation, the detection of ILTV antigens in tracheal tissues or respiratory mucus, the detection of ILTV specific DNA, or serology (124).

Usually, ILTV is diagnosed by histopathologic examination of tracheal lesions for the presence of intranuclear bodies. Histopathology examination remains the standard method for the

rapid diagnosis of ILTV (51). In addition, viral particles with typical herpesvirus morphology can be detected by electron microscopy. The agent can be isolated in chorioallantoic membrane (CAM) of 9-12-day-old embryonated eggs, where the lesions become visible after 5-7 days post-inoculation. The disadvantages of histopathology are that a trained pathologist is needed to provide an accurate diagnosis; the inclusion bodies are present only at an early stage of infection, and other avian viruses produce inclusion bodies (30). Other substrates, as chicken embryo kidney (CEK) cells, chicken embryo liver (CELi) or chicken kidney (CK) cells from adult birds, are also used for virus isolations, where the characteristic ILTV cytopathic effect is observed during viral replication. Besides histopathologic examination or electron microscopy to confirm etiology, other antigen detection tests, such as fluorescent antibody (FA) (127, 131), immunoperoxidase (IPX) (54, 117, 122) and agar-gel immunodifusion (AGID) can also be used to detect ILTV specific antigens (124).

Molecular identification of the agent is usually performed by polymerase chain reaction (PCR). The main applications of PCR are the detection of small amounts of viral nucleic acid in clinical samples and as a method to trace viral infection. Conventional PCR based assays have been used to successfully detect ILTV DNA from the trachea of experimentally (1), or naturally infected chickens (133), and from extra-tracheal sites such as the conjunctiva (3), and the trigeminal ganglia (58, 132). Conventional PCR has proven to be useful to detect ILTV infected birds during both severe (133) and mild forms (117) of the disease. Additionally, PCR procedures allowed detection of ILTV in samples contaminated with other microorganisms, such as adenoviruses, that may prevent ILTV isolation due to overgrowth in culture (134). Recently, a real time PCR assay was developed. This assay was capable of detecting and

quantifying viral DNA from tracheal and conjunctival swabs of naturally and experimentally infected birds (17).

Other techniques, such as dot-blot hybridization assays (1, 73, 75), and *in situ* hybridization for detection of ILTV DNA in tissues ((94), have been described to identify ILTV specific DNA but their utility in the diagnostic laboratory has been limited due to the lack of speed of these assays.

Although serology is not a primary ILTV diagnostic tool, since immunity to ILTV infection is primarily cellular rather than humoral (72, 106), several serological tests, such as agar gel immunodiffusion (AGID), indirect fluorescent antibody (IFA) test, enzyme linked immunoabsorbent assay (ELISA) have been developed. Serological monitoring will not differentiate infected, asymptomatic or uninfected flocks, and it has been mostly utilized to evaluate the uniformity of vaccinated flocks (86). Enzyme linked immunosorbent assay systems have been developed for detection and quantitation of ILTV specific antibodies using whole virus preparations as antigen (93, 95, 138). Direct comparison of the AGID, IFA, and ELISA demonstrated that all were valid systems for detecting and quantifying ILTV-specific antibodies (2). Both ELISA and IFA have the advantages of speed and sensitivity; however, ELISA lacks the subjectivity inherent in the IFA test, and is more suitable for testing large numbers of sera (9). An ELISA for detection of ILTV specific antibodies was developed utilizing recombinant *Escherichia coli*-expressed ILTV glycoproteins, gE and gp60 (18). It was shown that this recombinant protein based ELISA differentiated between ILTV vaccinated and unvaccinated/unexposed chickens, but the assay sensitivity and specificity were not reported. The use of recent development of deletion mutants lacking immunogenic glycoproteins as vaccines (33, 61, 128), and the availability of ILTV glycoprotein specific antibodies (127) opens

the possibility to develop ELISA technology capable of differentiating vaccinated from infected animals (DIVA).

### Differential Diagnosis

Even if the classical clinical signs for ILTV are presented, many other respiratory diseases can cause similar signs, therefore a clinical diagnosis of the disease is not reliable. Among other infections that can appear clinically similar to ILTV are the diphtheritic form of avian poxvirus and infections caused by Newcastle disease virus, avian influenza virus, infectious bronchitis virus, fowl adenovirus, mycoplamosis and *Aspergillus* spp (51, 124).

## PREVENTION AND CONTROL

To control ILTV outbreaks, the most effective approach is a coordinated effort to obtain a rapid diagnosis, institute a vaccination program, and prevent further virus spread. Vaccination in the face of an outbreak effectively limits virus spread and shortens the duration of disease. Spread of ILTV between sites can be prevented by appropriate biosecurity measures (7). In areas of intensive poultry production, ILT is usually controlled by a combination of biosecutity measures and vaccination using modified-live vaccine. This is particularly true for layers flocks and broiler breeder flocks; however, for intensive broiler production, a combination of strict biosecurity and short growth cycle can obviate the need for prophylactic vaccination. Also, it is important to avoid mixing vaccinated or recovered birds with susceptible chickens. Quarantine and hygiene also are very important, as well as preventing the movement of potentially contaminated personnel, feed, equipment, and birds (51).

## Vaccination control

### Modified live vaccines

Modified live (ML) vaccine strains of ILTV were originated by sequential passages of wild type viruses in cell cultures or embryonated eggs (46, 113). ILT vaccines are generally administrated to chickens by eye drop, orally (through drinking water), or by spray (6). However, problems have been associated with both drinking water and spray routes of vaccination. Successful vaccination via drinking water requires that the vaccine virus contact susceptible nasal epithelial cells as a result of aspiration of the virus through the external nares or choanae. When the procedure is not performed properly it may result in a large proportion of the flock failing to develop protective immunity (106). In case of the spray route, application of ILT vaccines may result in adverse reaction as a result of insufficient attenuation of vaccine virus, deep penetration of the respiratory tract due to small droplet size of spray, excessive dosage, or insufficient coverage (20). The route of vaccination for the prevention of ILTV is extremely important because some of the available live-attenuated vaccines provide different grades of protection when applied by different routes, particularly when applied by coarse spray or the drinking water (44, 62). Eye-drop vaccination has been demonstrated to be the optimal delivery method to ensure uniform and effective protection (44). Uses of ML ILTV vaccines produce latent infected carriers, potential for spread to non-vaccinated flocks, and are characterized by insufficient attenuation (5, 53). The two commercially available ML ILT vaccines, CEO and TCO, have been widely evaluated experimentally and in the field. Both attenuated vaccines induced protection (4), preventing against clinical signs and mortality (57, 62). The TCO and CEO vaccines both can persist in apparently healthy birds (4, 65) and can spread from bird to

bird (4, 62). Both vaccines can lose attenuation after bird to bird passage in the case of CEO causing severe respiratory disease and mortality, while the TCO vaccine caused a milder respiratory disease at the same back passage level (53).

**Inactivated vaccines**

The advantages of inactivated vaccines are to avoid the problems of residual virulence, reversion to virulence, spread of infection and establishment of latent infection, which happens with modified live vaccines. Such vaccines have been made from inactivated whole ILTV (8) and from affinity-purified glycoproteins (137). Inactivated vaccines are capable of stimulating immune responses in chickens, resulting in different levels of protection against ILTV challenge; however practical field use of these types of vaccines is unlikely due to the high cost of preparation and delivery (51).

**Recombinant vaccines**

During the past 40 years, classically attenuated live vaccines have been utilized for the prevention of ILTV in chickens (51). The development of recombinant live virus vaccine based on recombinant DNA technology appears more promising because their optimal attenuation and safety as compared to the currently utilize modified-live vaccines (6). Genes that code for antigens capable of inducing protective immunity can be inserted into recombinant vectors such as fowlpox virus, fowl andenovirus, or .Marek's disease virus (MDV). Construction of a fowlpox virus containing ILTV glycoprotein B (gB) produced protection against ILTV challenge similar to that induced by modified-live vaccines (123). Currently, a fowlpox virus recombinant containing ILTV gB and UL-32 genes is commercially available in US (31). A recombinant

herpesvirus of turkeys (HVT) containing immunogenic viral envelope proteins also has been reported to produce protection against ILTV challenge similar to that induced by modified live-virus vaccines (112). However, unlike conventional ILTV live-virus vaccines, these recombinant viruses require individual administration, and are not suitable for mass application.

Alternatively, stably attenuated ILTV vaccines can be generated by the directed deletion of genes that code for viral virulence factors. Recombinant ILTV viruses lacking thymidine kinase, which has been associated with herpesvirus virulence (59), was generated by insertion of *Lac-Z* marker genes into the thymidine kinase gene of ILTV (97, 114). The UL-50 gene, which codes for a viral dUTPase enzyme and also is a virulence factor, was replaced by the gene that codes for the green fluorescent protein (GFP), which has been utilized as a marker (43). Laryngotracheitis virus mutants lacking the gJ (42), gG (33), and the UL0 genes (128), UL47 (61) also showed an attenuated phenotype in chickens while maintaining their immunogenicity. Deletion mutant vaccines comprised of gene deletions in the thymidine kinase, UL0, gG and gJ gene are considered to be suitable candidates for vaccine use. In addition ILTV recombinants with deletions of immunogenic glycoproteins, like gG and gJ, could be used as marker vaccines, which permit serological differentiation of field-virus infected from vaccinated animals (DIVA strategy) (125) on the basis of the presence or absence of antibodies against the respective viral gene product (42). ILTV mutants have also been shown to be useful viral vectors for the expression of immunogenic proteins of other chicken pathogens like avian influenza, infectious bronchitis virus, bursal disease virus or fowlpox virus, and also of bacteria or parasites (41), with the advantage of suitability for mass application by aerosol or drinking water (51).

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# CHAPTER 3

## CHARACTERIZATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) ISOLATES FROM UNITED STATES

# PART I

## CHARACTERIZATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) ISOLATES FROM UNITED STATES BY POLYMERASE CHAIN REACTION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP) OF MULTIPLE GENOME REGIONS[1]

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**Abstract**

Infectious laryngotracheitis (ILT) is an acute viral respiratory disease primarily of chickens. Economic losses attributable to ILT affect many poultry producing areas throughout United States and the world. Despite efforts to control the disease by vaccination, prolonged epidemics of ILT remain a threat to the poultry industry. Earlier epidemiological and molecular evidence indicated that outbreaks in the US are caused by vaccine related strains. In this study, polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of four genome regions was utilized to characterize 25 isolates from commercial poultry and backyard flocks from the US. Combinations of PCR-RFLP patterns classified the infectious laryngotracheitis virus (ILTV) isolates into nine groups. Backyard flock isolates were categorized in three separate groups. The ILTV USDA reference strain and the tissue culture origin (TCO) vaccine strain were categorized in two separate groups. Twenty-two isolates from commercial poultry were categorized in four groups: one group, of six isolates, showed patterns identical to the chicken embryo origin (CEO) vaccines; a second group, of nine isolates, differed in only one pattern from the CEO vaccines; a third group, of two isolates, differed in only one pattern from the TCO vaccine; a fourth group, of five isolates, differed in six and nine patterns from the CEO and TCO vaccines, respectively. Results obtained from this study clearly demonstrated that most of the commercial poultry isolates (17 of 22 isolates) were closely related to the CEO vaccine strains. However isolates different to the vaccine strains were also identified in commercial poultry.

**Introduction**

Infectious laryngotracheitis (ILT) is a highly contagious, acute respiratory disease of chickens of worldwide distribution that affects growth and egg production and leads to significant economic losses during periodic outbreaks of the diseases (Guy & Bagust, 2003). The causative agent is infectious laryngotracheitis virus (ILTV or Gallid herpesvirus I), a member of the *Herpesviridae* family, *Alphaherpesvirinae* subfamily (Roizman, 1996). Vaccination with live-attenuated vaccines has been the principal tool used to control the spread of the disease (Guy & Bagust, 2003). Traditionally in the United States (US) two types of live-attenuated vaccines have been widely utilized; the vaccines attenuated by multiple passages in embryonated eggs (chicken embryo origin [CEO]) (Samberg *et al.* 1971); and the vaccine generated by multiple passages in tissues culture (tissue culture origin [TCO]) (Gelenczei & Marty, 1965). There are currently several CEO vaccines, one TCO vaccine commercially available. Several CEO vaccines are labeled for administration by water and spray in addition to the preferred eyedrop method. The TCO vaccine is labeled for eyedrop administration only. Most recently a fowl pox-vector ILT vaccine (FP-LT) is commercially available for administration only by wing web stab inoculation at about 8 weeks of age (Davison *et al*., 2006). Vaccination programs vary widely in the US between states and between different companies within a state. Commercial layers, layer breeders, and broiler breeders are usually vaccinated twice with CEO and/or TCO vaccines. Broilers are vaccinated only in case of outbreaks with the CEO vaccines. The increased frequency of outbreaks in broilers has been associated with denser poultry populations, mixing of different type of birds (breeders, leghorns, and broilers) in the same geographical area, shorter down times, and lax biosecurity. It is believe that most of the outbreaks in the US are caused by

CEO vaccine isolates that persist in long-lived bird operations and spill - over broiler populations (Davison *et al*., 2005). Earlier experimental evidence demonstrated that live attenuated vaccine strains, particularly the CEO vaccines, could easily revert to virulence after bird-to-bird passage (Guy *et al.*, 1991), or after reactivation from latency (Hughes *et al.*, 1991).

Once vaccine strains have been introduced in the field the identification of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses (Guy & Bagust, 2003). Initial attempts to differentiate among ILTV strains in the US were achieved by restriction fragment length polymorphism (RFLP) analysis of the viral genome (Leib *et al.*, 1986; Guy *et al.*, 1989; Andreasen *et al.*, 1990; Keller *et al.*, 1992; Keeler *et al.*, 1993). These studies provided the first evidence indicating that US ILTV field isolates were closely related to the CEO vaccine strains. However, routine use of RFLP analysis of the viral genome for epidemiological purposes was limited due to the difficulties in obtaining high yields of pure viral DNA. With the advent of the polymerase chain reaction restriction fragment length polymorphism of PCR products (PCR-RFLP) has greatly facilitated the differentiation of ILTV strains. PCR-RFLP analysis of single and multiple viral genes and genome regions has permitted the differentiation of ILTV isolates from different parts of the world (Chang *et al.*, 1997; Clavijo & Nagy, 1997; Graham *et al.*, 2000; Han & Kim, 2001; Han & Kim, 2003; Kirkpatrick *et al.*, 2006; Creelan *et al*., 2006, Ojkic *et al.*, 2006). Analysis of the ICP4 gene by PCR-RFLP was utilized to differentiate vaccine strains from field isolates in Taiwan (Chang *et al.*, 1997), Northern Ireland (Graham *et al.*, 2000) and to detect concurrent infections of “wild type” and vaccine strain in the United Kingdom (Creelan *et al*., 2006). Analysis of multiple genome regions by PCR-RFLP was utilized to gain a more precise identification of geographically and historical distinct Australian ILTV isolates (Kirkpatrick *et al.*, 2006). Sequencing analysis of

multiple viral genes has also been utilized to differentiate ILTV isolates. Sequencing of the thymidine kinase (TK) and glycoprotein G (gG) genes allowed the differentiation of vaccine strains and field isolates from Korea (Han & Kim, 2001), while sequencing analysis of the gG and UL47 genes allowed the differentiation of outbreak related isolates from Canada (Ojkic *et al.,* 2006). Isolates from the US has been identified as viral subpopulations derived from the CEO vaccines by PCR-RFLP analysis of the glycoprotein E (gE) (García & Riblet, 2001). However PCR-RFLP analysis of the gE gene by itself lacks the discriminatory potential to accurately differentiate among closely related vaccines and field isolates (Kirkpatrick *et al.,* 2006). Moreover viral genome regions of genetic diversity have not been clearly identified for US ILTV isolates. The objective of the present study was to utilize multiple PCR-RFLP assays to identify regions of diversity and to genetically categorize ILTV isolates from the US. To accomplish this objective 22 field isolates from commercial poultry, and three isolates from backyard flocks collected from diverse poultry production regions of the US were analyzed by PCR-RFLP of four viral genome regions.

## Material and Methods

**Strains, isolates and viral propagation.** Seven ILTV vaccine strains were used in this study, six CEO vaccines Biotrach (Intervet Inc., Millsboro, DE), Broilertrake-M (American Scientific Laboratories, Inc., Millsboro, DE), BioTrach (TrioBio Laboratories, State College, PA), Trachivax (Schering-Plough Animal Health, Kenilworth, NJ), Laryngo-Vac (Fort Dodge Animal Health, Fort Dodge, IA), and Fowl Laryngotracheitis Vaccine (Lohmann, Animal Health, Winslow, ME), and the TCO vaccine LT-IVAX (Schering-Plough Animal Health,

Kenilworth, NJ). A total of 25 field isolates collected between 1988 and 2005 from nine states were analyzed in this study, 22 isolates were obtained from commercial poultry and three isolates from backyard flocks (BCY) (Table 3.1). Of the 22 commercial poultry isolates, 19 were recovered from broiler (BR), and three from broiler breeder (BBR) flocks. Isolates were identified by: sample number, followed by a letter, each letter represents a different state of origin (A to I), year of isolation, and type of bird the isolate was collected from.

All viruses were isolated from the upper respiratory tract of birds and propagated as previously described (Garcia & Riblet, 2001). Briefly, ILTV isolates were propagated in the chorioallantoic membrane (CAM) of 9 to 11 day old specific pathogen free (SPF) chicken embryonated eggs. Embryos were incubated for five days at 37° C and monitored daily. After incubation, CAMs were examined for the presence of plaques characteristic of ILTV replication. Isolated plaques were minced in 100ul of phosphate-buffered saline (PBS), and freeze/thawed three times. Single plaques were utilized for DNA extraction and were maintained as the viral stock at -80° C. The USDA ILTV reference strain (ATCC #N-71851) was propagated in chicken embryo kidney (CEK) cells prepared as previously described (Tripathy, 1998) and infected cultures were maintained at -80° C for DNA extraction. Virus isolation from experimentally vaccinated birds was performed in chicken kidney (CK) cells from adult birds (Hughes & Jones, 1988).

**Vaccination experiments**. Twenty-six leghorn type specific pathogen free chickens were divided in two groups of 13 birds and placed in two isolation units with filtered-air and positive-pressure, and fed a standard diet and water *ad libitum*. At four weeks of age, one group of 13 birds was vaccinated with the CEO vaccine Trachivax (Schering-Plough Animal Health,

Kenilworth, NJ), and the remaining 13 birds were vaccinated with the TCO vaccine LT-IVAX (Schering-Plough Animal Health, Kenilworth, NJ). Both vaccines were diluted as recommended by the manufacturer and applied via eye drop. All birds were vaccinated in the left eye. Ten days post vaccination all the vaccinated birds were euthanized and the trachea, including the larynx, was collected from each bird. Te trachea was cut longitudinally and the epithelium was scraped. Each larynx-trachea scraping was re-suspended in 2 ml of phosphate buffered saline with sterile phosphate buffered saline solution containing 2% antibiotic-antimycotic solution (Invitrogen, Carlsbad, CA) and 2% of calf serum. The tracheal scrapings were first tested for the presence of viral DNA using a quantitative Real-Time PCR method previously described by Callison *et al*., 2006. Only those samples with more than $10^5$ viral genome copies were further tested for virus isolation and for multiple PCR-RFLP analysis.

**Extraction of viral DNA.** DNA from field isolates was extracted from individual CAM plaques, while DNA from the commercial vaccines was extracted directly from vaccine preparations, or from infected CEK supernatants using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The DNA was diluted in 50µl of elution buffer and stored at -20° C.

**Primers and polymerase chain reaction.** Some of the primers utilized in this study were selected from previously published work and others were designed using the ILTV genome sequence (GenBank accession number U28832) with Primer Select software (DNASTAR, Madison, WI) (Table 3.2). All amplifications were performed using high fidelity Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Each amplification reaction was performed in a

50µl volume, containing 200µM of dNTPs, 2mM of MgSO4, 250µM of each primer, 1U Taq polymerase, 5µl of buffer, and 5µl of template DNA. All amplification reactions used an initial denaturing step at 94° C for 1 min, followed by 35 amplification cycles of 94° C for 1 min, annealing temperatures ranging from 54° to 60° C (Table 3.2), for 30 sec (gM/UL9), 45 sec (ORF B-TK, ICP4, TK and gD/gI/gE), or 1 min (UL47gG and UL0/UL-1). Extension was performed at 68° C with extension times that varied accordingly to the size of the target region amplified (UL0/UL-1, TK and UL47/gG for 3 min; gM/UL9 for 2 min; ICP4, gD/gI/gE and ORF B-TK for 7 min), and a final extension at 68° C for 7 min (UL0/UL-1, TK, UL47/gG and gM/UL9), or 10 min (ICP4, gD/gI/gE and ORF B-TK). The PCR products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and exposed to ultraviolet light for visualization.

**Restriction fragment length polymorphism (RFLP) analysis**. A total of 36 restriction endonucleases (REs) were initially selected for RFLP analysis of PCR products from representative ILTV strains (Table 3.2). Digestion of the ICP4 gene with REs *HinP1I*, *HaeIII*, *MspI* (Chang *et al.,* 1997), the ORF-BTK with *FokI* (Kirkpatrick *et al.,* 2006), and the TK gene with *HaeIII* and *MspI* (Chang *et al*., 1997) were performed as previously described. Digestion of the UL47/gG genome region was performed using REs *AflII*, *NlaIV*, and *FspI*. These enzymes target single nucleotide polymorphisms (SNPs) previously described by Ojkic *et al.* (2006) found in Canadian isolates. The remaining REs utilized in the study were chosen based on the USDA strain (GenBank access number U28832) restriction sites using the MapDraw software (DNASTAR, Madison, WI) (Table 3.2). Amplification products (10µl) were digested separately with 10U of RE (New England Biolabs, Beverly, MA) at adequate temperature for three hours.

After digestion, DNA fragments were separated in 15% polyacrylamide mini gels of 10 x 8 cm or 10 x 10.5 cm. Fragments were visualized by silver staining using the DNA Silver Stain Kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's recommendations. Gels were analyzed under a light box and pattern differences were classified for each enzyme. Once the different patterns were identified the absence or presence of a given fragment in the RFLP pattern were assigned 0 or 1, respectively, this data was imported into Free Tree software for cluster analysis (Pavlicek *et al.*, 1999). Similarity coefficients were calculated using the method Nei and Li (Nei and Li, 1979) and DICE. An unrooted dendrogram was constructed using the un-weighted pair group method and statistical support for the dendrogram was obtained by bootstrapping using 500 resamplings.

## Results

**Selection of genome regions and restriction endonucleases (REs).** Of the initial seven genome regions and 36 REs tested, four genome regions and ten REs were selected for their ability to differentiate among representative US ILTV strains and isolates. The selected genome regions were ORFB-TK digested with RE *BstF5I*; ICP4 digested with REs *HaeIII* and *HinP1I* (Chang *et al.*, 1997), *AlwI* and *AvaI***;** UL47/gG digested with REs *MspI*, *AflII*, *NlaIV*, *FspI* and *HaeIII*; gM/UL9 region digested with RE *MwoI*. The UL0/UL-1, gD/E/I and TK genome regions did not show RFLP pattern differences with any of the RE tested (data not shown).

**PCR amplification of the ORFB-TK, gM/UL9, ICP4, UL47/gG.** With the exception of the ICP4 amplification products obtained for the USDA strain and the TCO vaccine, all

amplification reactions produced the expected PCR product size (Table 3.2). The USDA strain and the TCO vaccine produced two ICP4 amplification products one of the expected size (4,980 bp), and a larger fragment of approximately 5,800 bp (data not shown). The presence of two amplification products may reflect size differences between the two ICP4 gene copies present in the genomes of the USDA and TCO strains. Further sequence analysis of both ICP4 PCR products will further prove or refute this conjecture.

**RFLP patterns for ICP4 region.** Digestion of the ICP4 genome region of the US isolates with *HaeIII* and *Hinp1I* generated different patterns as previously described by Chang *et al.* (1997) and Kirkpatrick *et al.* (2006). However, no pattern differences were observed between the US isolates when digested with the *MspI* enzyme as previously reported for isolates from Taiwan (Chang *et al.*, 1997), Northern Ireland (Graham *et al.*, 2000), and United Kingdom (Creelan *et al.*, 2006). Digestion of the ICP4 region with *Hae III* generated seven different patterns (Figure 3.1a, Table 3.3). Pattern A corresponds to the USDA strain, patterns $B^0$ and B were both characteristic of the TCO vaccine obtained directly from vaccine vial or from experimentally vaccinated birds, respectively. As pattern A, pattern $B^0$ has an additional 500 bp fragment, while pattern B lacks this fragment. Both $B^0$ and B patterns have an additional fragment of approximately 80 bp. Patterns C to F also lack a 500 bp fragment present in patterns A and $B^0$. Patterns C to F lack a 170 bp fragment present in patterns A and B, while patterns D and E shows unique 360 and 330 bp fragments, respectively. Pattern B was characteristic of 17 commercial poultry isolates, the TCO and CEO vaccine strains. Pattern C was characteristic of five commercial poultry isolates. Patterns D, E and F were characteristic of the three backyard flock isolates (Table 3.3). Restriction endonuclease digestion of the ICP4 region with the *HinP1I*

enzyme generated four different patterns (Figure 3.1b, Table 3.3). Pattern A was characteristic of the USDA strain, the TCO vaccine, and two commercial poultry isolates. As compared to pattern A, pattern B lacks a 150 and 350 bp fragments and has a fragment of approximately 500 bp. Pattern B was characteristic of the 20 commercial poultry isolates and of the CEO vaccines. Pattern C lacks a 110 bp fragment and has a fragment of approximately 400 bp. Pattern C was characteristic of the two backyard flock isolates. Pattern D has an additional fragment of approximately 400 bp and was characteristic of one backyard flock isolate. Restriction endonuclease digestion of the ICP4 region with the *AlwI* enzyme generated three different patterns (Figure 3.1c, Table 3.3). Pattern A was characteristic of the USDA strain and the TCO vaccine. As compared to pattern A, pattern B lacks a 1200 bp and has an additional band of approximately 800 bp. Pattern B was characteristic of the two commercial poultry isolates and the three backyard flock isolates. Pattern C lacks a 1200 bp fragment and has a fragment of approximately 600 bp. Pattern C was characteristic of CEO vaccine strains and 20 commercial poultry isolates. Restriction endonuclease digestion of the ICP4 region with the *AvaI* enzyme generated two different patterns (Figure 3.1d, Table 3.3). Pattern A was characteristic of the USDA strain, the TCO vaccine, two commercial poultry isolates and one backyard flock isolate. As compared to pattern A, pattern B lacks a 750 bp and has an additional band of approximately 2000 bp. Pattern B was characteristic of the CEO vaccine, 20 commercial poultry isolates and two backyard flock isolates.

**RFLP patterns for ORFB-TK and gM/UL9 regions.** Restriction endonuclease digestion of the ORFB-TK region with the *BstUI* enzyme produced identical patterns with all the US ILTV strains and isolates tested (data not shown), however digestion with the *BstF51*

enzyme produced two different patterns (Figure 3.2a, Table 3.3). Pattern A consisted of fragment sizes of approximately 2422, 931, 513, 352, 232, 156 and 65 bp. This pattern was characteristic of the USDA strain, the vaccine strains (TCO and CEO), and all the commercial poultry isolates. Pattern B lacks the 930 bp fragment and has a 400 bp fragment. Pattern B was characteristic of the three backyard flock isolates. Restriction endonuclease digestion of the gM/UL9 region with the *MwoI* enzyme generated three different patterns (Figure 3.2b, Table 3.3). Pattern A consisted of fragment sizes of approximately 429, 312, 180, 107, 97, 86, 71, 61 and 48 bp. This pattern was characteristic of the USDA strain, the TCO vaccine, 11 commercial poultry isolates, and three backyard flock isolates. Pattern B lacks the 71 and 61 bp fragments and has a 130 bp fragment. This pattern was characteristic of the CEO vaccines and six commercial poultry isolates. Pattern C lacks the 86 bp fragment and has a 55 bp fragment. Pattern C is characteristic of five commercial poultry isolates.

**RFLP patterns for UL47/gG region.** Restriction endonuclease digestion of the UL47/gG region with the *AflII* enzyme produced two patterns (Figure 3.2c, Table 3.3). Pattern A consisted of fragments sizes of 1675 and 1257 bp. This pattern was characteristic of the USDA strain, the vaccine strains (TCO and CEO), and 17 commercial poultry isolates. Pattern B produced a single fragment of 2932 bp indicating the lack of this site in five commercial poultry isolates and the backyard flock isolates. Digestion with the *NlaIV* enzyme produced three patterns (Figure 3.2d, Table 3.3). Pattern A consisted of 12 fragments of 710, 500, 437, 280, 197, 174/170, 151, 93, 79 and 65/63 bp. This pattern was characteristic of the USDA strain, the vaccine strains (TCO and CEO), and 17 commercial poultry isolates. Pattern B lacks the 197 and 79 bp fragments producing an uncut 276 bp fragment that cannot be visualized in the gel because

migrates together with the 280 bp fragment. This pattern is characteristic of five commercial poultry isolates and one backyard flock isolate. Pattern C lacks the 437 bp fragment and has a fragment of approximately 350 bp. Pattern C is characteristic of two backyard flock isolates. Digestion with *FspI* enzyme produced two patterns (Figure 3.2e, Table 3.3). Pattern A consisted of four fragments 835, 826, 646 and 624 bp, however the fragments of 646 and 624 bp were not separated on the 15% polyacrylamide gel, allowing the visualization of only three bands. This pattern was characteristic of the USDA strain, the vaccine strains (CEO and TCO), and 17 commercial poultry isolates. Pattern B lacks the 835 and 826 bp fragments and has a 1700 bp fragment. This pattern was characteristic of five commercial poultry isolates and three backyard flock isolates. Digestion of the UL47/gG region with *HaeIII* enzyme produced three patterns (Figure 3.2f, Table 3.3). Pattern A consisted of 11 fragments 1051, 377, 285, 276, 221, 190, 181, 159, 84 and 49/41 bp. This pattern was characteristic of the USDA strain, the vaccine strains (CEO and TCO) and 22 commercial poultry isolates. Pattern B lacks the 1051 bp fragment and shows two fragments of approximately 650 and 400 bp. This pattern is characteristic of two backyard flock isolates. Pattern C lacks the 1051 bp fragment and shows a 650, 400 and 70 bp fragments. This pattern was characteristic of one backyard flock isolate. Digestion with *MspI* enzyme produced two patterns (Figure 3.2g, Table 3.3). Pattern A consisted of 13 fragments of 563, 363/352, 330, 280, 263, 228,128, 98, 84, 69, 54, 45 bp. This pattern was characteristic of the USDA strain, the vaccine strains (CEO and TCO), 17 commercial poultry isolates, and three backyard flock isolates. Pattern B lacks the 69 and a 45 bp fragments and has a fragment of approximately 114 bp. This pattern is characteristic of five commercial poultry isolates.

**Stability of PCR-RFLP patterns.** The stability of the PCR-RFLP patterns was evaluated for five CEO, and six TCO viruses recovered from birds 10 days post-vaccination. The PCR-RFLP patterns obtained for the five CEO vaccine viruses, recovered from experimentally vaccinated birds, were identical to the patterns obtained from the DNA extracted directly from the vaccine vial (data not shown). For the TCO vaccine, ten of the eleven PCR-RFLP patterns generated for the six viruses recovered from experimentally vaccinated birds were identical to patterns generated for the TCO vaccine preparation. However, PCR-RFLP pattern for the ICP4 region digested with the *HaeIII* enzyme for the TCO vaccine preparation showed an additional 500 bp fragment (Figure 3.1a, patterns $B^0$). This 500 bp fragment was not detected in viruses retrieved from birds 10 days post vaccination with the TCO vaccine. This ICP4/ *HaeIII* pattern was designated B (Figure 3.1a).

**Multiple PCR-RFLP analysis.** Analysis of combined RFLP patterns generated a total of nine groups (Table 3.3). The USDA strain and the TCO vaccine differed in only one pattern and were categorized as groups I and II. Two ILTV isolates from commercial breeders had ten identical PCR-RFLP patterns to commercial TCO vaccine RFLP patterns, and were categorized into group III. Six ILTV isolates from commercial poultry had identical PCR-RFLP patterns to the commercial CEO vaccines, differed in five patterns from the USDA strain and in four patterns from the TCO vaccine, and were categorized as group IV. Nine ILTV isolates had ten identical patterns to CEO vaccines. These isolates differed in only one pattern from the CEO vaccines and shared this pattern with the USDA strain and the TCO vaccine and were identified as group V. Five isolates from commercial poultry shared two pattern with the USDA strain and the TCO vaccine, five patterns with the CEO vaccines, four patterns with the one backyard flock

isolate, and had three unique patterns; these isolates were categorized as group VI. Backyard flock isolates differed in at least seven patterns when compared to USDA strain and the vaccine strains (CEO and TCO) and were categorized separately as groups VII, VIII and IX.

**Cluster analyses of ILTV PCR-RFLP groups**. The nine groups generated by combining RFLP patterns were segregated in three main clusters as determined by cluster and boostraping analysis (Figure 3.3). The first cluster was composed by groups I (USDA reference strain), II (TCO vaccine) and III (two commercial poultry isolates). The second cluster was composed by closely related group IV (CEO vaccines and commercial poultry isolates) and group V (commercial poultry isolates). A third cluster was composed by group VI from commercial poultry and groups VII, VIII and IX from backyard flock. Bootstrapping analysis demonstrated that group VI isolates from commercial poultry was more closely related to groups VII, VIII and IX from backyard flocks than to other commercial poultry isolates and vaccine strains.

## Discussion

This study presents the genetic characterization of ILTV isolates collected between 1988 and 2005 from different regions of dense poultry production in the US. Twenty-two isolates were collected from commercial poultry and three isolates from backyard flocks. The eleven PCR-RFLP pattern combinations generated by the digestion of four genome regions with ten REs categorized the US isolates in nine groups (Table 3.3). ILTV isolates from commercial poultry were categorized into four groups (III, IV, V and VI), and the backyard flock isolates

were categorized into three separated groups (groups VII, VIII and IX). The stability of the eleven PCR-RFLP patterns was tested for the vaccine strains after replication in chickens. The PCR-RFLP patterns for one CEO vaccine showed to be stable after vaccine strain replication in chickens. For the TCO vaccine ten of the eleven patterns showed to be stable after vaccine strain replication in chickens. However the ICP4/*HaeIII* digestion pattern of the TCO vaccine, pattern $B^0$ (Figure 3.1a), was characterized by an additional 500 bp fragment that was not detected after the TCO vaccine strain replicated in chickens (pattern B, Figure 3.1a), indicating that this site is not stable and cannot be considered as marker to identify TCO related strains.

Previous studies using PCR-RFLP of the ICP4 gene had demonstrated the importance of this genome region to differentiate vaccine strains from ILTV isolates from Taiwan with REs *MspI* and *HaeIII* (Chang *et al.,* 1997), from Northern Ireland with *HaeIII* and *MspI* (Graham *et al.,* 2000), from United Kingdom with *MspI* (Creelan *et al.,* 2006), from Australia (Kirkpatrick *et al.,* 2006) and from Canada (Ojkic *et al*., 2006) with *HaeIII*. As in other countries the PCR-RFLP analysis of the ICP4 was valuable to separate ILTV isolates from the US. In this analysis digestion of the ICP4 region with *HaeIII*, *HinP1l*, and *AlwI* allowed the differentiation of vaccine strains from commercial poultry and backyard flock isolates. Partial sequence of the ICP4 region, corresponding to nucleotide positions 2039 to 2950 of the ICP4 gene (Accession number L32139) was analyzed for all the 25 isolates (data not shown). This region of the ICP4 gene was selected for sequencing analysis because enclosed two *AlwI* sites that have proven informative by PCR-RFLP analysis (Table 3.3). However sequence analysis of this fragment did not allowed the differentiation of vaccine strains from field isolates (groups III and VI) as PCR-RFLP analysis of the complete ICP4 region did. Therefore sequence analysis of the complete ICP4 region is needed to identify all the informative ICP4 SNPs found among US isolates.

Analysis of Australian isolates showed that PCR-RFLP analysis of the ORF B-TK genome region with *FokI* allowed the differentiation of ILTV vaccine strains from outbreak isolates (Kirkpatrick *et al.,* 2006), nevertheless analysis of the ORF B-TK genome region of US isolates with *FokI* did not discriminated between vaccine strains, commercial poultry, and backyard flock isolates (data not shown). Digestion of the ORF B-TK region with *BstF5I* allowed the differentiation of backyard flock isolates from commercial poultry isolates and vaccine strains utilized in the US. Earlier reports indicated that the thymidine kinase (TK) gene was valuable to differentiate field isolates from vaccine strains utilized in Korea (Han & Kim, 2001), and Australia (Kirkpatrick *et al.,* 2006), however PCR-RFLP analysis of the TK gene with REs *HaeIII* and *MspI* did not differentiated between vaccine strains and commercial poultry isolates from the US (data not shown). Differentiation of "wild type" from vaccinal type isolates from United Kingdom was possible by PCR-RFLP of a 222 base pair ICP4 gene fragment digested with the *MspI* enzyme (Creelan *et al*., 2006). Digestion of the complete ICP4 region (4,980 bp) with the *MspI* enzyme did not differentiated between US ILTV isolates from commercial poultry and backyard flocks. Consequently, PCR-RFLP assays need to be tailored for each country in order to precisely characterize the ILTV isolates circulating in a particular region at different time points.

PCR-RFLP analysis of the UL47/gG region with enzymes *AflII, FspI* and *NlaIV* produced different patterns among commercial poultry isolates (group VI), and backyard flock isolates (groups VII, VIII and IX). The construction of ILTV gG deletion mutant has provided evidence indicating that the glycoprotein G may contribute to the viral virulence (Devlin *et al*., 2006). Therefore SNPs of the gG gene are relevant as they may influence viral virulence.

Genetic diversity was also detected in the gM/UL9 region where a SNP in the gM recognized by the *MwoI* enzyme separated commercial poultry isolates (group V) from the CEO vaccines.

PCR-RFLP analysis of multiple genome regions was required to precisely differentiate among closely related US ILTV isolates. PCR-RFLP pattern combinations categorized the 22 commercial poultry isolates in four distinct groups. Six of the 22 isolates showed PCR-RFLP patterns identical to the CEO vaccine (group IV), one of these isolates was recovered from a CEO vaccinated broiler flock. Other nine isolates differed in only one pattern from the CEO vaccine (group V). Isolates from groups IV and V identified as closely related to the CEO vaccine strains, were collected from seven of the nine states represented in this study indicating that these types of isolates are the most prevalent in ILT outbreaks in the US. These findings confirm previous reports that isolates closely related to the vaccine strains are involved in outbreaks of the disease in different regions of the US (Guy *et al.,* 1989; Andreasen *et al.,* 1990; Keller *et al.,* 1992; Keeler *et al.,* 1993). Whether the origin of these isolates is vaccine strains that lost attenuation and consequently persists in the field is not completely understood. To confirm that the isolates categorize in groups III, IV, and V are derived from vaccine strains complete genome sequencing of representative isolates and vaccine strains will be necessary. Although, most of the commercial poultry isolates were closely related to the commercial vaccines, five commercial poultry isolates (group VI) differed in six and nine patterns when compared to the CEO and TCO vaccine strains, respectively. All five isolates were recovered during the same outbreak of the disease in 2004. These isolates share similar patterns with the backyard flock isolate 24/H/91/BCK and Canadian wild type isolates (Ojkic *et al*., 2006).

Overall, using multiple PCR-RFLP analysis of four genome regions digested with ten restrictions revealed genetic diversity of the ICP4, gM/UL9, and gG/UL47 genome regions of

US isolates from commercial poultry and backyard flocks. PCR-RFLP analysis of gM/UL9 with *MwoI* and the ICP4 region with *HaeIII* and *AlwI* was sufficient to differentiate vaccine strains, commercial poultry and backyard flock isolates in nine genetic groups. PCR-RFLP analysis of the UL47-gG region further confirmed that backyard flock isolates (groups VII, VIII and IX), and commercial poultry isolates from group VI were genetically different to the vaccine strains and the remaining commercial poultry isolates from the US. In addition with this study it was confirmed that most of the US ILTV isolates from commercial poultry were similar, or closely related to the commercial vaccine strains, in particular to the CEO vaccines. This result was not unexpected, previous ILTV genotyping studies from Northern Ireland (Graham *et al*., 2000) and Taiwan (Chang *et al*., 1997) concluded that vaccine viruses once established in the field displace wild-type virus and are responsible for many of the outbreaks. In the US the intermittent use of CEO vaccines in regions of diverse poultry populations, combined with lax biosecurity, has permitted the emergence of vaccine-related viruses that are well adapted to linger in the field, and incite outbreaks when introduced into naïve broiler flocks.

Although the PCR-RFLP categories generated in this study may be modified when more intense sequencing analysis of these isolates is performed, this report identifies genome regions of genetic diversity among US ILTV isolates, presents the first comprehensive genotyping characterization of a diverse group of isolates from the US, and provides the framework for further sequencing analysis. In addition to sequencing analysis, future work will incorporate pathotyping studies of representative isolates from the different PCR-RFLP groups identified in this study.

Table 3.1: Infectious laryngotracheitis virus field isolates used for PCR-RFLP.

| Isolate[a] | Species | Age[b] | Vaccination |
|---|---|---|---|
| 25/H/88/BCY | chicken | 28 to 56 | NV[d] |
| 24/H/91/BCY | chicken | 183 to 548 | NV |
| 9/C/97BR | chicken | 56 | NV |
| 10/C/97/BR | chicken | 53 | CEO |
| 6/B/99/BR | chicken | U[c] | NV |
| 7/B/99/BR | chicken | U | NV |
| 8/B/99/BR | chicken | U | NV |
| 23/H/01/BBR | chicken | 392 | NV |
| 12/D/02/BCY | peafowl | U | NV |
| 13/E/03/BBR | chicken | 441 | TCO |
| 14/E/03/BBR | chicken | U | TCO |
| 15/E/03/BR | chicken | 35 | NV |
| 16/F/05/BR | chicken | 55 | NV |
| 26/I/03/BR | chicken | 40 | NV |
| 1/A/04/BR | chicken | U | NV |
| 2/A/04/BR | chicken | U | NV |
| 3/A/04/BR | chicken | 35 | TCO |
| 4/A/04/BR | chicken | U | NV |
| 5/A/04/BR | chicken | U | NV |
| 11/C/05/BR | chicken | 42 | NV |
| 17/F/05/BR | chicken | U | NV |
| 18/F/05/BR | chicken | 55 | NV |
| 19/F/05/BR | chicken | 60 | NV |
| 21/G/05/BR | chicken | 42 | NV |
| 22/G/05/BR | chicken | 42 | NV |

[a] Sample number/State/year of isolation/bird type; States of isolation are indicate by letters (A to I). Each sample with same letter is originated in the same state; bird type: from commercial poultry broiler (BR), broiler breeder (BBR), from back yard flock (BCY)
[b] age in days;
[c] unknown
[d] non vaccinated

Table 3.2: Primers and genome regions utilized in PCR-RFLP analysis.

| Primer name | Target genome regions | Sequence (5' - 3') | Expected product size (bp) | Annealing temperature PCR (°C) | Restriction endonucleases |
|---|---|---|---|---|---|
| ORFB-TK F[a] | ORF B-TK[a] | TCTGCGATCTTCGCAGTGGTCAG | 4675 | 58 | BstF5I, FokI, MlyI, XmnI |
| ORFB-TK R | | TGACGAGGAGAGCGAACTTTAATCC | | | |
| ICP4 F[b] | ICP4[b] | AACCTGTAGAGACAGTACCGTGACCC | 4980 | 57 | HaeIII, HinP1I, MspI, AlwI, AvaI |
| ICP4 R | | CCATTACTACGTGACCTACATTGAGCC | | | |
| UL47gG F[c] | UL47/gG[c] | TCTTGAATGACCTTGCCCCAT | 2931 | 58 | MspI, BstUI NlaIV, Afl II, FspI , AluI, HaeIII, Hinf I, TspRI |
| UL47gG R | | ACTCTCGGGTGGCTACTGCTG | | | |
| gM F[c] | gM/UL9[c] | GCTGAGATCGCATCCGTACA | 1424 | 58 | MwoI, RsaI, HpyCH4III, MspI, Hinf I |
| gM R | | CTTCTAGCAGCCACTGGCTC | | | |
| UL0 UL-1 F[c] | UL0/UL-1[c] | TGCCAGGTATATCGACACTTGAAC | 1924 | 60 | MspI, FokI, HaeIII, ScrFI, AluI, NlaIII |
| UL0 UL-1 R | | GACGGACCTGATTTATAGACTGACAA | | | |
| gDIE F[c] | gD/gI/gE[c] | TCTCCGGAACCTTACTGTCTTTT | 4784 | 54 | BccI, BsmFI, BsmAI,MlyI, HpyCH4III, Hinf I |
| gDIE R | | GCACGCGCCCATACTCA | | | |
| TK forward[b] | TK[b] | CTGGGCTAAATCATCCAAGACATCA | 2237 | 58 | HaeIII, MspI |
| TK reverse | | GCTCTCTCGAGTAAGAATGAGTACA | | | |

[a] Genome regions previously amplified by Kirkpatrick *et al.*, 2006.
[b] Genome regions previously amplified by Chang *et al.*, 1997.
[c] Genome regions amplified in this study.

Table 3.3: Comparison of patterns generated by PCR-RFLP of selected regions from different ILTV isolates and strains.

| Genome region | ORFB-TK | gM/UL9 | ICP4 | ICP4 | ICP4 | ICP4 | UL47/ gG | UL47/ gG | UL47/ gG | UL47/ gG | UL47/ gG | Pattern combination | Group |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Endonucleases | BstF5 I | Mwo I | Hae III | HinP1 I | AlwI | AvaI | AflII | NlaIV | FspI | Hae III | Msp I | | |
| **Isolate ID/Strain** | Types of patterns | | | | | | | | | | | | |
| USDA | A | A | A | A | A | A | A | A | A | A | A | AAAAAAAAAAA | I |
| TCO vaccine | A | A | B | A | A | A | A | A | A | A | A | AABAAAAAAAA | II |
| 13/E/03/BBR | A | A | B | A | B | A | A | A | A | A | A | AABABAAAAAA | III |
| 14/E/03/BBR | A | A | B | A | B | A | A | A | A | A | A | AABABAAAAAA | III |
| CEO vaccine 1 | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| CEO vaccine 2 | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| CEO vaccine 3 | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| CEO vaccine 4 | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| CEO vaccine 5 | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| CEO vaccine 6 | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 9/C/97BR | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 10/C/97/BR | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 11/C/05/BR | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 23/H/01/BBR | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 21/G/05/BR | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 22/G/05/BR | A | B | B | B | C | B | A | A | A | A | A | ABBBCBAAAAA | IV |
| 15/E/03/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 16/F/05/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 17/F/05/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 18/F/05/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 6/B/99/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 19/F/05/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 7/B/99/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 8/B/99/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 26/I/03/BR | A | A | B | B | C | B | A | A | A | A | A | AABBCBAAAAA | V |
| 1/A/04/BR | A | C | C | B | C | B | B | B | B | A | B | ACCBCBBBBAB | VI |
| 2/A/04/BR | A | C | C | B | C | B | B | B | B | A | B | ACCBCBBBBAB | VI |
| 3/A/04/BR | A | C | C | B | C | B | B | B | B | A | B | ACCBCBBBBAB | VI |
| 4/A/04/BR | A | C | C | B | C | B | B | B | B | A | B | ACCBCBBBBAB | VI |
| 5/A/04/BR | A | C | C | B | C | B | B | B | B | A | B | ACCBCBBBBAB | VI |
| 12/D/02/BCK | B | A | D | C | B | A | B | C | B | C | A | BADCBABCBCA | VII |
| 24/H/91/BCK | B | A | E | D | B | B | B | B | B | B | A | BAEDBBBBBBA | VIII |
| 25/H/88/BCK | B | A | F | C | B | B | B | C | B | B | A | BAFCBBBCBBA | IX |

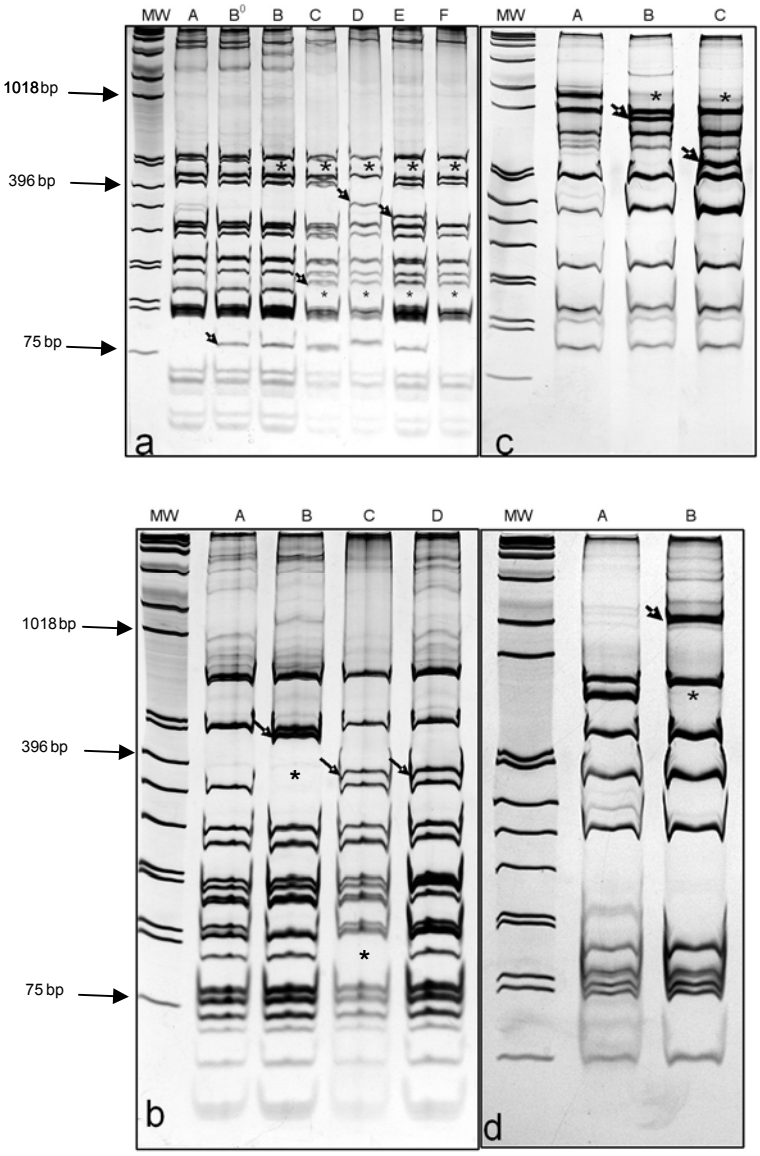


Figure 3.1: Polyacrylamide gel electrophoresis of DNA fragments generated by restriction endonuclease digestion of ICP4 genome region with enzyme HaeIII (a), HinP1I (b), AlwI (c) and AvaI (d); Letters indicate different patterns as compared to the USDA reference strain. Additional bands are indicated by arrows and missing bands are indicated by stars in comparison with reference strain. $B^0$ and B patterns are expected from ICP4 of TCO like strains digested by HaeIII, by lack of stability of the site that generates the 500 bp fragment.

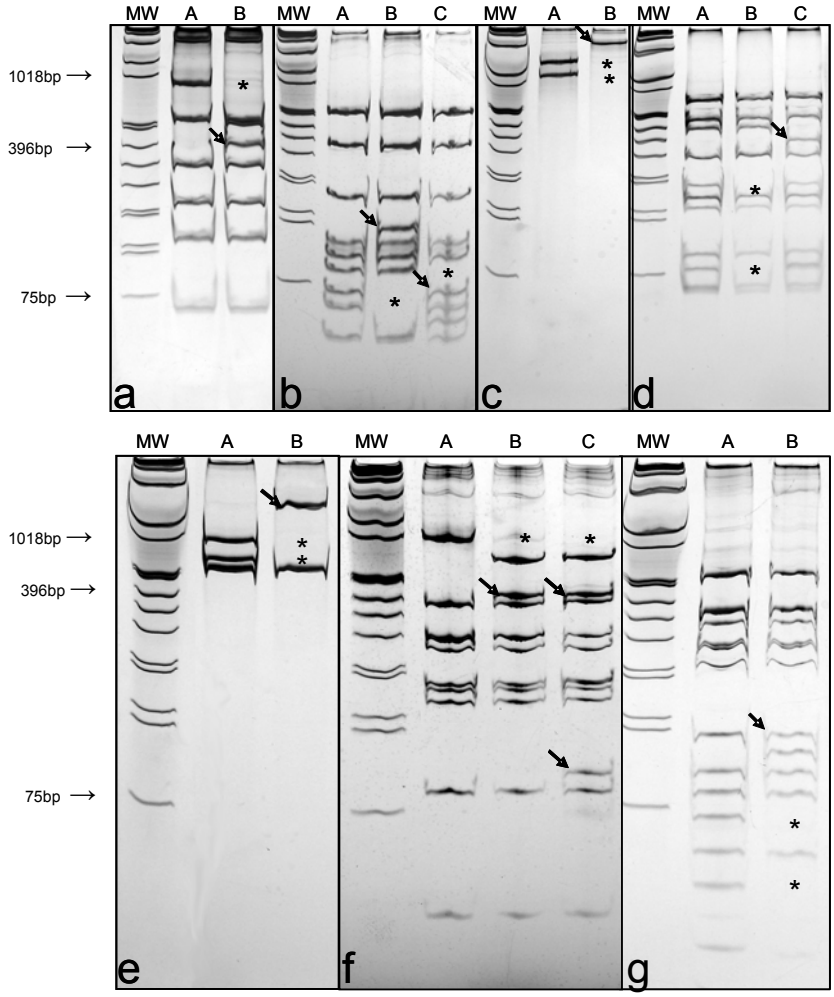


Figure 3.2: Polyacrylamide gel electrophoresis of DNA fragments generated by restriction endonuclease digestion; a: ORF B-TK digested with enzyme BstF5I; b: gM/UL9 digested with enzyme MwoI; c: UL47/gG digested with enzyme AflII; d: UL47/gG digested with enzyme NlaIV; e: UL47/gG digested with enzyme FspI; f: UL47/gG digested with enzyme HaeIII; g: UL47/gG digested with enzyme MspI. Letters indicates different patterns as compared with USDA reference strain. Additional bands are indicated by arrows and missing bands are indicated by stars in comparison with reference strain.

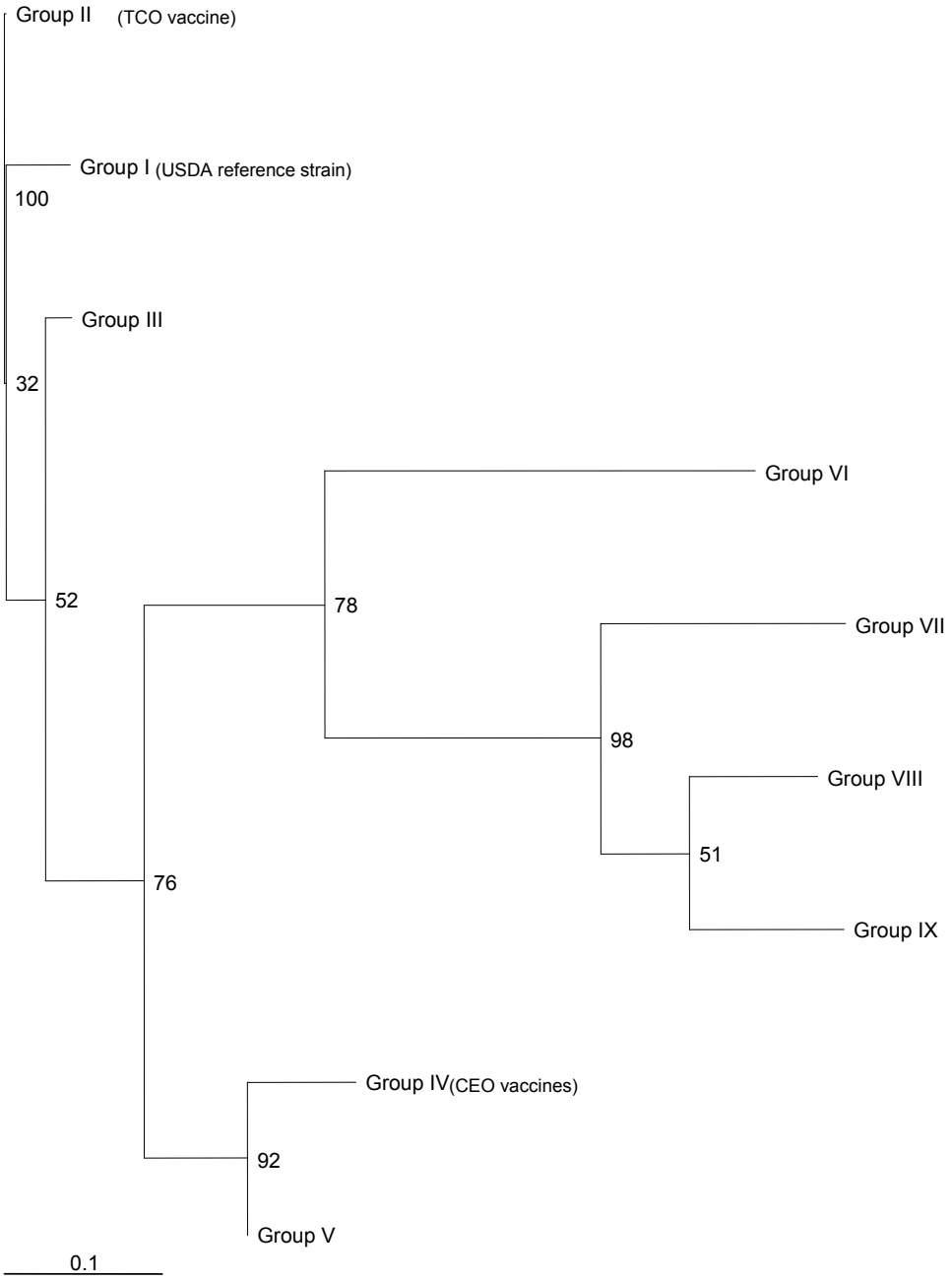


Figure 3.3: Dendogram based on cluster analysis of PCR-RFLP pattern combination of nine groups of ILTV isolates and strains. Similarity coefficients were calculated according the method of Nei & Li (1978). The branch lengths represent the genetic distance between the groups, and numbers on the branches are bootstrap values as a percentage at internal nodes (500 resampling). Group I: USDA Vaccine Strain; Group II: TCO vaccine strain; Group III: two commercial poultry isolates; Group IV: CEO vaccine strains and six commercial poultry isolates; Group V: nine commercial poultry isolates; Group VI: Five commercial poultry isolates; Groups VII, VIII and IX: Three backyard flock isolates.

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# PART II

# CHARACTERIZATION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) ISOLATES FROM COMMERCIAL POULTRY BY POLYMERASE CHAIN REACTION AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM (PCR-RFLP)[1]

[1] Oldoni, I., Rodríguez-Avila, A., Riblet, S., and García, M. Submitted to *Avian Diseases*, 07/09/2007.

**Keywords**

Infectious laryngotracheitis virus; chicken embryo origin vaccine; tissue culture origin vaccine; polymerase chain reaction, restriction fragment length polymorphism; PCR-RFLP.

**Abbreviations**

ILT = infectious laryngotracheitis; ILTV = infectious laryngotracheitis virus; US = United States; CEO = chicken embryo origin; TCO = tissue culture origin; RFLP = restriction fragment length polymorphism; PCR-RFLP = polymerase chain reaction and restriction fragment length polymorphism; USDA = United Sates Department of Agriculture; CAM = chorioallantoic membrane; CEK = chicken embryo kidney; BR = broiler; BBR = broiler breeder; REs = restriction endonucleases;

**Summary**

Infectious laryngotracheitis (ILT) is a highly contagious, acute respiratory disease of chickens, of worldwide distribution, that affects growth and egg production and leads to significant economic losses during periodic outbreaks of the disease. Live attenuated vaccines (chicken embryo origin [CEO] and tissue culture origin [TCO]) have been widely used to control the disease in the United States (US). It is believed that most of the outbreaks in the US are caused by vaccine related isolates that persist in the field and spill over into naïve poultry populations. The objective of this study was to utilize multiple polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) analysis to genotype recent ILTV isolates from commercial poultry. Forty-six samples were collected during January 2006 to April 2007 from five poultry production regions of the US and were characterized within PCR-RFLP groups III, IV, V and VI. Sixty three percent of the samples analyzed were categorized as closely related to the vaccine strains (Groups III, IV, V), while 33% were categorized as group VI viruses that differed in six and nine PCR-RFLP patterns from the CEO and TCO vaccines, respectively. A mixture of group IV and V viruses was detected in two samples (4%). In general, groups V and VI viruses were found in 52% and 33% of the samples tested respectively. Both types of viruses were detected in vaccinated and non-vaccinated flocks, although genetically different, both viruses produced moderate to severe disease in the field. Viruses categorized into group V were the most frequent detected genotype followed by groups VI and IV viral genotypes.

## Introduction

The causative agent of infectious laryngotracheitis (ILT) is the infectious laryngotracheitis virus (ILTV or Gallid herpesvirus I), a member of the family *Herpesviridae*, and subfamily *Alphaherpesvirinae* (21). ILT is a highly contagious, acute respiratory disease of chickens, of worldwide distribution, that affects growth and egg production and leads to significant economic losses during periodic outbreaks of the disease (8). Two types of ILTV live attenuated vaccines have been widely used to control the disease in the United States (US), those attenuated by sequential passages in embryonated eggs (chicken embryo origin [CEO]) (22); and the vaccine generated by sequential passages in tissue culture (tissue culture origin [TCO]) (6). Attenuated vaccines can spread from bird to bird in close contact (6, 12, 20). Experimental evidence has demonstrated that live attenuated vaccine strains, particularly the CEO vaccines, could easily revert to virulence after bird-to-bird passage (10) or after reactivation from latency (13). It is believed that most of the outbreaks in the US are caused by vaccine related isolates that persist in long-lived bird operations and spill-over to a dense ILTV-naïve broiler population (4). Once vaccines have been introduced in the field, the differentiation of ILTV strains is difficult because of the antigenic and genetic homogeneity of the circulating viruses with the vaccine strains (8).

The first attempt to genetically differentiate ILTV strains in the US was conducted by restriction fragment length polymorphism (RFLP) analysis of the viral genome (1, 9, 14, 15, 17). These studies provided the first evidence indicating that most of ILTV circulating in commercial poultry in the US were closely related to the CEO vaccine strains. Recently, the use of polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of

multiple genome regions have proven to be a very useful method to search for regions of genetic diversity within the ILTV genome, and to genetically differentiate vaccine and field strains. This approach has been utilized successfully in several countries to characterize circulating field strains (2, 3, 7, 11, 16, 18). In the US, utilizing multiple PCR-RFLP analysis, ILTV vaccine strains and isolates from commercial poultry (1997 through 2005) were categorized into six different groups (19). Group I and II included the United Sates Department of Agriculture (USDA) reference strain and the TCO vaccine strain, respectively. Group III included isolates that differ in one PCR-RFLP pattern to the TCO vaccine. Group IV and V included CEO related isolates, where group IV isolates have identical patterns to the CEO vaccines, and group V isolates vary in one pattern from the group IV isolates. Group VI included isolates that differed in six and nine PCR-RFLP patterns from CEO and TCO vaccines, respectively. The objective of this study was to utilize PCR-RFLP analysis to genotype recent ILTV isolates from commercial poultry. To accomplish this objective 46 samples from commercial poultry collected during January of 2006 and April of 2007, from different poultry production regions of the US, were analyzed and categorized within PCR-RFLP groups III, IV, V and VI.

## Material and Methods

**Strains, samples and viral propagation.** Two ILTV vaccine strains were used in this study, one CEO vaccine (Trachivax; Schering-Plough Animal Health, Kenilworth, NJ) and the TCO vaccine (LT-IVAX; Schering-Plough Animal Health, Kenilworth, NJ). A total of 46 field samples were collected between January of 2006 and April 2007. From the 46 samples analyzed, 27 were collected in 2006 and 19 in 2007. Samples were identified by: sample number, followed

by letters representing the state of origin (A, B, C, J, and K), year the sample was collected (2006 or 2007), and from the type of bird the sample was collected (broiler, broiler breeder) (Table 3.4). All samples were obtained from different commercial poultry operations during outbreaks of the disease. Samples were received either directly from the field, in that case tracheal scraping were prepared in our laboratory, or samples were submitted already propagated in the chorioallantoic membrane (CAM) of chicken embryos or chicken embryo kidney (CEK) cells for genotyping analysis. Of the 46 samples, thirty-seven were originated from broiler (BR) flocks of the five states, and nine were from broiler breeder (BBR) flocks, from states A and C. The USDA ILTV reference strain (NVSL, Ames, IA) was propagated in CEK cells prepared as previously described (23) and infected cultures were maintained at -80° C for DNA extraction.

**Extraction of viral DNA.** DNA was extracted directly from CEO and TCO vaccine preparations and USDA infected CEK cells. For propagated viruses DNA was extracted from individual CAM plaques or CEK cells, from non-propagated viruses DNA was extracted directly from tracheal scrapings. DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The DNA was diluted in 50µl of elution buffer and stored at -20° C.

**Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis**. Amplification of the genome regions ORFB-TK, ICP4, UL47/gG, and gM/UL9 was performed as previously described by Oldoni and García (19) using high fidelity Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), following the manufacturer's recommendations. Restriction fragment length polymorphism analysis of four PCR products was performed using

10 restriction endonucleases (REs). The ORFB-TK region was digested with RE *BstF5I*; ICP4 digested with REs *HaeIII* and *HinP1I* (2), *AlwI* and *AvaI*; UL47/gG digested with REs *MspI*, *AflII*, *NlaIV*, *Fsp*I and *HaeIII*; and gM/UL9 digested with RE *MwoI* (19). After digestion, DNA fragments were separated in 15% polyacrylamide mini gels of 10 x 8 cm or 10 x 10.5 cm in size. Fragments were visualized by silver staining using the DNA Silver Stain Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) following the manufacturer's recommendations. Gels were analyzed under a light box and pattern differences were classified for each enzyme according to the patterns previously described by Oldoni and García (19). The USDA reference strain PCR-RFLP patterns were identified by letter A and any PCR-RFLP pattern which differed from the USDA was identified by letters B to F. Pattern combinations generated for the four genome regions digested with 10 REs were used to categorize ILTV samples and strains (19).

**Polymerase chain reactions of glycoprotein M gene fragment:** A PCR assay was developed to amplify a 138 bp gM gene fragment that flanks the *Mwo*I restriction site located in nucleotide position 122 of the gM open reading frame, and is present in group V but not in group IV samples. Primers gM138UP (5'-GCATGTGGCTGATTGAAGTAATC-3') and gM138DOWN (5'-TTGCTGGCTTAAGGTGTCCAC-3'), located at positions 94566 to 94586, and 94681 to 94703, of the ILTV genome sequence (GenBank accession number NC_006623), respectively, were designed with Primer Select software (DNASTAR, Madison, WI). Amplification of the gM fragment was performed using Go *Taq* DNA polymerase (Promega, Madison, WI). The amplification reaction used an initial denaturing step at 94° C for 1 min, followed by 35 amplification cycles of 94° C for 30 sec, annealing temperature of 58° C, for 30

sec, extension at 72° C for 45 sec, and a final extension at 72° C for 2 min. The *MwoI* digestion reaction and gel separation was performed as described above. The *Mwo*I digestion of the 138 bp fragment was performed for all in order to identify mixed populations of viruses within samples identified as group IV and V viruses.

**Results**

Using multiple PCR-RFLP analysis of four PCR products (ORFB-TK, ICP4, UL47/gG, and gM/UL9) digested with 10 restriction enzymes generated patterns identical to those previously described by Oldoni and Garcia (19) and it was possible to categorize 44 of the 46 ILTV samples into groups III, IV, V, and VI (Table 3.4). Fifteen samples were categorized into group VI; twenty-two samples were categorized into group V; six samples were categorized into group IV; and one sample was categorized into group III. The remaining two samples (317/K/07/BR and 105/B/07/BR) were tentatively categorized as a combination of groups IV and V patterns (data not show) as additional bands for the gM/UL9 PCR product were observed when digested with the *MwoI* enzyme. Although not an equal number of samples were tested for each state, the distribution of the different viral genotypes was examined (Figure 3.4). Eleven samples were analyzed from state A. One was categorized into group III, 3 into group V, and 7 into group VI. A total of 7 samples were analyzed from state B; 3 samples were categorized into group IV, and 4 samples were categorized into group V. Eleven samples were analyzed from state C; 10 were categorized into group V and one sample was identified as a mixture of viral groups IV/V. Seven samples were analyzed from state J; two were categorized into group IV, and 5 were categorized into group V. Eleven samples were analyzed from state K; 2 samples

were categorized into group IV, 8 samples were categorized into group VI, and one sample was categorized as a mixture of viral groups IV/V.

Samples categorized as group IV, V, 317/K/07/BR and 105/B/07/BR categorized as IV/V, and the CEO vaccine were further analyzed by PCR-RFLP analysis of the 138 bp gM fragment encompassing the *MwoI* site. This analysis showed that samples categorized into group IV were not digested with the *MwoI* restriction enzyme as expected. On the other hand, digestion of the 138 bp amplicon from group V samples generated a 62 and 76 bp fragments when digested with *MwoI*, confirming the presence of this site for samples categorized into group V (Fig. 3.5). Digestion of the 138 bp gM amplicon for samples 105/B/07/BR and 317/K/07/BR generated fragments of 138, 76 and 62 bp (Fig. 3.5, lanes 7 and 9 respectively) indicating that viruses with and without the *MwoI* site, were present in these samples.

**Discussion**

This study presents the genetic characterization of 46 ILTV samples collected between January 2006 and April 2007 from five states in the US. Similar to previously characterized ILTV isolates, collected between 1997 to 2005, current US isolates from commercial poultry were categorized into four genotype groups (III, IV, V, VI) by PCR-RFLP analysis (19). Overall, 60% of the samples analyzed were categorized as closely related to the vaccine strains (Groups III, IV, V), while 33% were categorized as group VI viruses, which differed in six and nine PCR-RFLP patterns from the CEO and TCO vaccines, respectively. We first identified Group VI viruses in 2004 from state A (19); during the 2006 and 2007 outbreaks group VI viruses were identified in states A and K. In state A, in addition to group VI viruses, group V were found in

non-vaccinated flocks. In state K, group VI viruses, as well as groups IV and V were identified from either non-vaccinated, CEO vaccinated, or flocks vaccinated with the fowl LT pox-vector vaccine. Group VI viruses share single nucleotide polymorphisms (SNPs) in the gG/UL47 genes, with isolates from 2004-2005 outbreaks reported in the Niagara Peninsula from Canada (18), and with US backyard flock isolate 24/H/91/BCK (19). Although group VI viruses are genetically different from the CEO and TCO vaccines, the source of these viruses is not known, and further sequencing will be necessary to better define their origin.

One sample (401/A/06/BBR) was categorized into group III isolates. This sample differed in only one pattern from the TCO vaccine (19). The history of this sample indicated that it came from a non-vaccinated broiler-breeder flock; however this flock was located in very close proximity to a TCO vaccinated farm from the same owner. Recent experimental evidence indicated that the spread of TCO from vaccinated to non-vaccinated birds in close contact is possible (20). However, there is not enough information around the case to conclude that the TCO vaccinated flock was the sole source of the virus.

Viruses categorized into group V were the most frequent genotype detected among the five states; group V viruses were present in 52% of the samples analyzed. Particularly in state C, group V viruses were identified in non-vaccinated broiler flocks, and in broiler-breeder flocks vaccinated with the LT fowl pox vector vaccine, and the live-attenuated TCO vaccine. In four of the five states tested, eight of the samples showed patterns identical to the CEO vaccine and were categorized as group IV. Evidence of CEO vaccination was documented for two of the eight flocks where group IV viruses were identified.

Group V isolates differed in only one pattern from CEO vaccines recognized by the *Mwo*I enzyme in the gM gene (19). A PCR was developed to target this site. All samples

categorized within PCR-RFLP groups IV and V were further tested by the gM/*MwoI* PCR-RFLP, and confirmed that the CEO vaccine and group IV samples lack the *MwoI* site in the gM gene. However, two samples showed gM patterns characteristic of a mixture of groups IV and V viruses. One of the samples (317/K/07/BR) was collected from a CEO vaccinated flock, while the second sample (105/B/07/BR) was collected from an LT fowl pox-vector vaccinated flock. One possibility is that group V viruses originated from a subpopulation of the CEO vaccine (group IV) after back passage in birds. Viral subpopulations in the CEO vaccine preparations have been previously identified for the glycoprotein E gene (5). Sequencing and PCR-RFLP analysis of the different CEO vaccines did not reveal the presence of viral subpopulations for the gM *Mwo*I site (data not shown). Therefore, group V virus does not appear to be present as a viral subpopulation in the CEO vaccine preparations. The high prevalence of group V viruses in different regions of the US, together with the increase use of CEO vaccination may explain the co-circulation of these two types of viruses. Recently, Creelan *et al*. (3) reported the co-circulation of a vaccine and a field isolate in a backyard flock. Further sequencing analysis is necessary to determine with more accuracy how closely related are group IV and V viruses to the CEO vaccines.

In general, groups V and VI viruses were the most prevalent viruses identified among the samples tested. Both types of viruses were detected in vaccinated and non-vaccinated flocks, although genetically different, both viruses produced severe disease in the field. Group V viruses were more widely spread than group VI viruses.

Table 3.4: Infectious laryngotracheitis virus (ILTV) field isolates and ILTV strains used for PCR-RFLP, and categorization into RFLP groups

| Samples ID[a]/Strain | Age (days) | Vaccination | Type of sample | Pattern combination[b] | RFLP Group[c] |
|---|---|---|---|---|---|
| USDA[d] | | | | AAAAAAAAAAA | I |
| TCO[e] Vaccine | | | | AABAAAAAAAA | II |
| CEO[f] vaccine | | | | ABBBCBAAAAA | IV |
| | | | | | |
| 401/A/06/BBR | 280 | Not vaccinated | Tracheal scraping | AABABAAAAAA | III |
| 402/A/06/BR | 54 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 403/A/06/BR | 42 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 404/A/06/BR | 26 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 405/A/06/BR | 60 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 407/A/06/BBR | 441 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 414/A/06/BR | 56 | Pox Vector[g] | Tracheal scraping | ACCBCBBBBAB | VI |
| 416/A/06/BBR | 182 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 417/A/06/BR | 60 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 418/A/07/BR | Not available | Not available | Tracheal scraping | ACCBCBBBBAB | VI |
| 513/A/07/BBR | 399 | Not available | Tracheal scraping | ACCBCBBBBAB | VI |
| | | | | | |
| 106/B/07/BR | Not available | CEO vaccine | Tracheal scraping | ABBBCBAAAAA | IV |
| 107/B/07/BR | Not available | Not vaccinated | Tracheal scraping | ABBBCBAAAAA | IV |
| 102/B/06/BR | 35 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 105/B/07/BR | Not available | Pox Vector | Chorioallantoic membrane | AB/ABBCBAAAAA | IV/V |
| 103/B/06/BR | 34 | Not available | Chorioallantoic membrane | AABBCBAAAAA | V |
| 104/B/06/BR | 41 | Not available | Chorioallantoic membrane | AABBCBAAAAA | V |
| | | | | | |
| 501/C/06/BBR | 203 | Pox Vector & TCO | Tracheal scraping | AABBCBAAAAA | V |
| 502/C/06/BR | 31 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 503/C/06/BR | 34 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 504/C/06/BR | Not Available | Not available | Tracheal scraping | AABBCBAAAAA | V |
| 505/C/07/BBR | 357 | Pox Vector & TCO | Tracheal scraping | AABBCBAAAAA | V |
| 506/C/07/BBR | 70 | Not available | Tracheal scraping | AABBCBAAAAA | V |
| 507/C/07/BR | 44 | Not available | Tracheal scraping | AABBCBAAAAA | V |
| 509/C/07/BBR | 238 | Pox Vector & TCO | Tracheal scraping | AABBCBAAAAA | V |
| 510/C/07/BBR | 196 | Pox Vector & TCO | Tracheal scraping | AABBCBAAAAA | V |
| 511/C/07/BR | 39 | Not vaccinated | Tracheal scraping | AABBCBAAAAA | V |
| 512/C/07/BR | 44 | Not available | Tracheal scraping | AABBCBAAAAA | V |
| | | | | | |
| 209/J/07/BR | Not available | Not available | Tracheal scraping | ABBBCBAAAAA | IV |
| 210/J/07/BR | Not available | Not available | Tracheal scraping | ABBBCBAAAAA | IV |
| 204/J/06/BR | 46 | Pox Vector | Chicken embryo kidney cells | AABBCBAAAAA | V |
| 205/J/06/BR | 52 | Not vaccinated | Chicken embryo kidney cells | AABBCBAAAAA | V |
| 206/J/06/BR | 53 | Not vaccinated | Chicken embryo kidney cells | AABBCBAAAAA | V |
| 207/J/06/BR | 39 | Pox Vector | Chicken embryo kidney cells | AABBCBAAAAA | V |
| 208/J/06/BR | 30 | Not vaccinated | Chicken embryo kidney cells | AABBCBAAAAA | V |

| | | | | | |
|---|---|---|---|---|---|
| 301/K/06/BR | Not available | Not available | Chorioallantoic membrane | ABBBCBAAAAA | IV |
| 316/K/07/BR | 42 | CEO | Tracheal scraping | ABBBCBAAAAA | IV |
| 317/K/07/BR | 46 | CEO | Tracheal scraping | AB/ABBCBAAAAA | IV/V |
| 304/K/06/BR | 30 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 310/K/06/BR | Not available | Pox Vector | Tracheal scraping | ACCBCBBBBAB | VI |
| 311/K/06/BR | 29 | Pox Vector | Tracheal scraping | ACCBCBBBBAB | VI |
| 312/K/06/BR | 33 | Pox Vector | Tracheal scraping | ACCBCBBBBAB | VI |
| 313/K/06/BR | 42 | Pox Vector | Tracheal scraping | ACCBCBBBBAB | VI |
| 314/K/07/BR | 36 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 519/K/07/BR | Not available | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |
| 520/K/07/BR | 56 | Not vaccinated | Tracheal scraping | ACCBCBBBBAB | VI |

[a] Samples identified by: sample number, letters, representing different state of origin, year of collection, and type of bird the isolate was collected from (BR: Broiler; BBR: Broiler Breeder).
[b] Pattern combination that resulted from digestion of four genome regions with ten restriction endonucleases (19).
[c] RFLP groups categorization according to Oldoni and Garcia (19).
[d] United States Department of Agriculture ILTV reference strain.
[e] Tissue culture origin vaccine.
[f] Chicken embryo origin vaccine.
[g] Vectormune® FP-LT+AE vaccine (Biomune Co., Lenexa, KS).

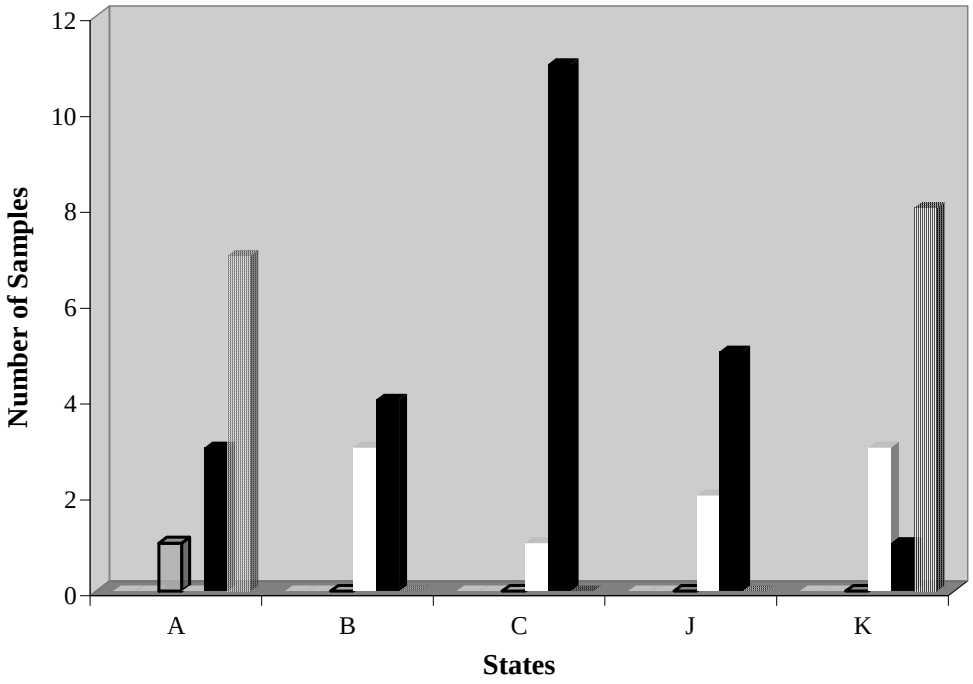


Figure 3.4: Distribution of ILTV PCR-RFLP groups by state of origin identified during recent outbreaks of the disease (2006 to April 2007). Samples were categorized as group III (column with borders), group IV (white color column), group V (black color column), and group VI (gray color column).

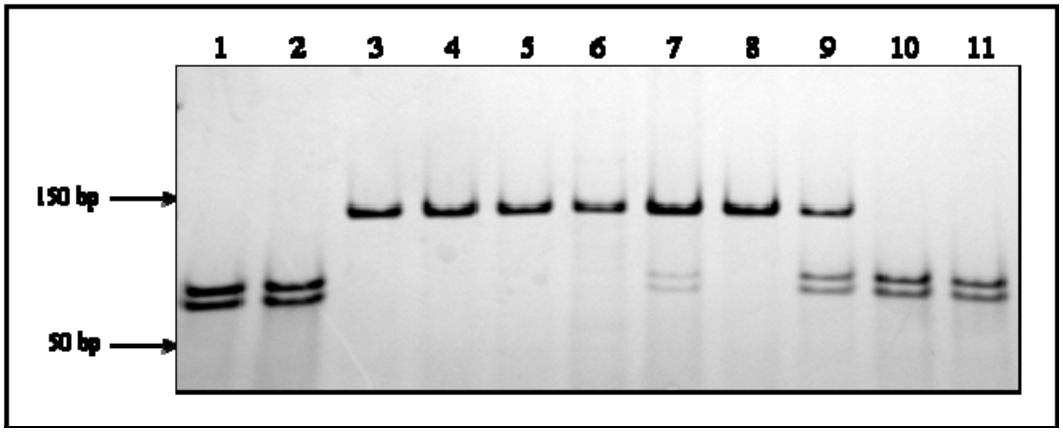


Figure 3.5: Polyacrylamide gel electrophoresis of DNA fragments generated by restriction endonuclease digestion of a fragment of 138 bp amplified from gM gene digested with enzyme *MwoI*. Lanes 1, 2, 10 and 11 show fragments of 76 and 62 bp, and samples were categorized into group V; Lanes 3, 4, 5, 6 and 8 show a fragment of 138, and samples were categorized into group IV; Lanes 7 and 9 show fragments of 138, 76 and 62 bp, and samples were categorized into groups IV/V.

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# CHAPTER 4

# SEQUENCING ANALYSES OF MULTIPLE GLYCOPROTEINS AND REGULATORY GENES OF INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV)[1]

[1] Oldoni, I. and García, M. To be submitted to *Veterinary Microbiology*.

**Keywords**

Infectious laryngotracheitis virus; chicken embryo origin vaccine; tissue culture origin vaccine; polymerase chain reaction; restriction fragment length polymorphism; sequencing analyses.

**Abstract**

Infectious laryngotracheitis (ILT) is an upper respiratory disease of poultry with a worldwide distribution. ILT is caused by infectious laryngotracheitis virus (ILTV), a member of family *Herpesviridae*, and is characterized by acute respiratory signs and is common in areas of intense poultry production. The disease is controlled by vaccination with live attenuated vaccines. Once vaccine strains have been introduced in the field the differentiation of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses. The objective of this study was to sequence ILTV genes that have previously presented genetic variability by multiple PCR-RFLP analysis in order to identify specific genomic differences among a diverse group of US isolates. In this study the open reading frames (ORF) of the viral glycoprotein genes B (gB), G (gG), M (gM), UL32, the major tegument protein gene UL47, and the immediate early regulatory gene ICP4 were sequenced for vaccine strains, commercial poultry and back yard flock isolates. Sequencing analysis validated previous genotype groups (I to IX) obtained by PCR-RFLP analyses. The backyard flock isolates showed more genetic variability than commercial poultry isolates, and within commercial poultry isolates, group VI isolates showed the greatest genetic variability. The UL32 and gM genes were most conserved and ICP4 and gB genes showed more variability among isolates. This study presents the first comparative sequencing analysis for a wide variety of ITLV isolates from the US where specific genome differences were identified for commercial poultry and backyard flock isolates from the US.

## Introduction

Infectious laryngotracheitis (ILT) is an upper-respiratory disease of poultry with a worldwide distribution (Guy and Bagust, 2003). The disease was first described in 1925 (May and Tisttsler, 1925) and is caused by infectious laryngotracheitis virus (ILTV), a member of the genus *Iltovirus,* within the family *Herpesviridae*, subfamily *Alphaherpesvirinae* (Davison et al., 2005). ILT is characterized by acute respiratory signs and is common in areas of intense poultry production. During severe outbreaks it causes great economic losses due to moderate to severe morbidity, reduction of egg production and moderate mortality. Modified-live ILT vaccines have traditionally been utilized to control ILT; vaccines attenuated by multiple passages in embryonated eggs (chicken embryo origin [CEO]) (Samberg et al., 1971); or generated by multiple passages in tissues culture (tissue culture origin [TCO]) (Gelenczei and Marty, 1965). ILTV vaccines have been associated with a variety of adverse effects including spread of vaccine virus to non-vaccinated birds (Hilbink et al., 1987; Andreasen et al., 1989; Rodríguez-Avila et al., 2007), production of latently infected carriers (Bagust et al., 1986), and increased virulence as a result of consecutive passages *in vivo* (Guy *et al.,* 1991) or after reactivation from latency (Hughes et al., 1991). Moreover, modified-live LT vaccine viruses are involved in field outbreaks of the disease (Guy et al., 1989; Guy et al., 1990; Guy et al., 1991); Once vaccine strains have been introduced in the field the differentiation of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses (Guy and Bagust, 2003). In the US, restriction endonuclease analyses of the viral genome (Guy et al., 1989; Andreasen et al., 1990; Keller et al., 1992; Keeler et al., 1993) and polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) of single and multiple viral genes

(Garcia and Riblet, 2001; Oldoni and Garcia, 2007) have been used to genetically differentiate ILTV strains. Multiple PCR-RFLP analysis of US ILTV vaccine strains, and isolates from commercial poultry and backyard flocks (1997 through 2005) categorized ILTV viruses into nine different groups (Oldoni and Garcia, 2007). Groups I and II included the United States Department of Agriculture (USDA) reference strain and the TCO vaccine strain, respectively. Group III included isolates that differ in one PCR-RFLP pattern from the TCO vaccine. Group IV and V included CEO related isolates, group IV isolates have identical patterns to the CEO vaccines, while group V isolates varied in one pattern from the group IV isolates. Group VI included isolates that differed in six and nine PCR-RFLP patterns from CEO and TCO vaccines, respectively. Three backyard flock isolates were categorized separately into groups VII, VIII and IX. In addition to PCR-RFLP, differentiation of ILTV by sequencing analyses has also been used in other countries for ILTV strains differentiation (Han and Kim, 2001; Ojkic et al., 2006) Although US ILTV isolates were separated into different PCR-RFLP groups specific genome differences characteristics of the different groups of isolates has not been identified. The objective of this study was to sequence ILTV genes that have previously presented genetic variability by multiple PCR-RFLP analysis in order to identify specific genomic differences among vaccines, commercial poultry and backyard flock ILTV isolates from the US. In this study the open reading frames (ORF) of the viral glycoprotein genes B (gB), G (gG), M (gM), UL32, a major tegument protein gene UL47, and the immediate early regulatory gene ICP4 were sequenced for ILTV isolates from the US including vaccine strains, commercial poultry and back yard flock isolates.

## Materials and methods

**Viral isolates.** Nineteen viruses were selected for this study representing the nine groups previously identified by PCR-RFLP (Oldoni and Garcia, 2007). All isolates originated from outbreaks of the disease that occurred between 1988 and 2006 in different regions of the US. The viruses were isolated from tracheal swabs or scrapings in the chorioallantoic membrane (CAM) of 9 to 11 day old specific pathogen free (SPF) chicken embryonated eggs as previously described (Garcia and Riblet, 2001). After incubation individual viral plaques were removed and maintained at -80° C for DNA extraction. The identification and the origin of viruses utilized in this study are summarized in table 4.1.

**Virus strains.** The USDA ILTV reference strain (NVSL, Ames, IA) was propagated in chicken embryo kidney (CEK) cells as previously described (Tripathy, 1998), and infected cultures were maintained at -80° C for DNA extraction. Two ILTV vaccine strains were used in this study, one CEO vaccine (Trachivax; Schering-Plough Animal Health, Kenilworth, NJ) and the TCO vaccine (LT-IVAX; Schering-Plough Animal Health, Kenilworth, NJ).

**Extraction of viral DNA.** DNA from field isolates was extracted from individual CAM plaques, while DNA from the commercial vaccines was extracted directly from vaccine preparations. DNA from USDA reference strain was extracted from infected CEK supernatants. Viral DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The DNA was diluted in 50µl of elution buffer and stored at -20° C.

**Primers.** Primers used for amplification and sequencing of glycoprotein B (gB), UL32, glycoprotein M (gM), ICP4, UL47 and glycoprotein G (gG) genes were designed from the ILTV genome sequence (GenBank accession number NC006623) using the Primer Select software of LASERGENE software package (DNASTAR Inc., Madison, WI, USA) and are presented in table 4.2. The gB and UL32 were amplified with single sets of primers (Fig. 4.1), while five sets of primers were used to amplify the ICP4 ORF and the non-coding flanking regions (Fig. 4.1). The UL47 and gG genes were amplified with a single set of primers (Fig. 4.1). Primers for amplification were selected in the non-coding flanking regions of the gB, UL32 and gM genes. The UL47 and gG genes were amplified together, while ICP4 was amplified as five separate fragments. Sequencing of the amplified products for all genes was performed with the primers used for amplification plus seven internal primers for gB, five for UL32, two for gM, eight for UL47/gG and ten for ICP4 (Table 4.2) in order to obtained sequences from both strands of the PCR products.

**Polymerase chain reaction (PCR).** All amplifications were performed using high fidelity Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Each amplification reaction was performed in a 50µl volume, containing 200µM of dNTPs, 2mM of $MgSO_4$, 250µM of each primer, 1U Taq polymerase, 5µl of buffer, and 5µl of template DNA. Amplification reactions of gB, UL32, UL47/gG and gM used an initial denaturing step at 94° C for 1 min, followed by 35 amplification cycles of 94° C for 1 min, annealing temperatures ranging from 56° to 60° C, for 45 sec (gB, UL32 and UL47/gG) or 30 sec (gM). Extension was performed at 68° C with extension times that varied accordingly to the size of the target region amplified (gB, UL32 and gM for 3 min; UL47/gG for 4 min), and a final extension at 68° C for 7 min. The ICP4 gene was

amplified in five fragments (ICP4 A, B, C, D, E) (Fig 4.1). All amplification reactions used an initial denaturing step at 94° C for 1 min, followed by 35 amplification cycles of 94° C for 1 min, annealing temperatures of 57° and 58° C, for 30 sec. Extension was performed at 68° C with times that varied accordingly to the size of the target region amplified (ICP4 A, B and D for 2 min; ICP4 C for 3 min; ICP4 E for 3.5 min), and a final extension at 68° C for 5 min (ICP4 A) or 7 min (ICP4 B, C, D and E). To confirm the amplification reaction PCR products were separated by electrophoresis in 1% agarose gels, stained with ethidium bromide, and exposed to ultraviolet light for visualization.

**Nucleotide sequencing and analysis.** The amplified products obtained after PCR were purified with QIAquick PCR purification Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The purified DNA was diluted in 50µl of elution buffer and directly sequenced with PCR primers (Table 4.2) using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Capillary electrophoresis was carried out on an ABI 3730XL DNA Analyzer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced from the nucleotide sequences for all genes. Nucleotide sequence editing, production of nucleotide consensus, and deduced amino acid sequences were obtained using the the SeqMan and EditSeq programs of the LASERGENE software package (DNASTAR Inc., Madison, WI), and the pairwise sequence divergences were calculated using the default setting of the MegAlign program of LASERGENE. The total number of synonymous and non-synonymous nucleotide changes as compared to the USDA reference strain was calculated. To determine the presence of amino acid changes characteristic of different ILTV isolates the deduced amino acid sequences of the five open reading frames for the 19 ILTV isolates were compared to the USDA reference

strain and aligned using the Clustal W software (Thompson et al., 1994). Phylogenetic analyses of the amino acid sequences were conducted using MEGA Version 3.1 (Kumar et al., 2004). All positions with ambiguous codes or alignment gaps were excluded from the phylogenetic analyses. The robustness of the phylogenetic groupings using the neighbor-joining analysis was assessed with 1000 bootstrap resamplings. Nucleotide sequences obtained during this study have been deposited in the GenBank and the accession numbers are: EU104855 to EU104876 (UL47 gene); EU104877 to EU104898 (UL32 gene); EU104899 to EU104920 (ICP4 gene); EU104921 to EU104942 (gM gene); EU104943 to EU104964 (gG gene); EU104965 to EU104986 (gB gene).

**Results**

**Analysis of nucleotide sequences.** No nucleotide changes were observed among isolates that belong to the same PCR-RFLP groups (I, II, III, IV, V, VI). The total number of nucleotide changes, as compared to the USDA reference strain observed per group of isolates is shown in figure 4.2a. A total of 2, 27, 11, 50, 38 and 111 nucleotide changes were observed for the UL32, gB, gM, UL47, gG and the ICP4 gene, respectively. As compared to the USDA reference strain, most of the nucleotide changes were observed for the backyard flock isolates characterized into groups VII, VIII, and IX with a total of 59, 43 and 57 nucleotide changes observed per isolate, respectively (Figure 4.2a).

**Analysis of deduced amino acid sequences.** Of the five genes sequenced, the UL32 gene did not showed any amino acid changes among the 19 isolates analyzed. Only two synonymous

nucleotide substitutions were observed (Figure 4.2a) in the gene UL32for group IX, at position 1677 (C-T), and for group VI at position 1722 (G–A). On the other hand, of 27 nucleotide changes observed among isolates for gB, 23 changes were non-synonymous nucleotide changes that resulted in amino acid substitutions (Figure 4.2a). Figure 4.2b shows sites where amino acid changes in the gB, gM, UL47, gG and ICP4 genes were observed. Each sequence represents the deduced amino acid sequence characteristic of each PCR-RFLP group of isolates as compared to the USDA reference strain. No amino acid substitutions were observed among isolates that belong to the same PCR-RFLP group. A total of 2, 7, 8, 10, and 16 amino acid substitutions were observed for at least one PCR-RFLP group of isolates in the gM, gB, UL47, gG, and ICP4 genes, respectively (Figure 4.2b). Three of the five isolates analyzed from group V (205/J/06/BR; 501/C/06/BR; 19/F/05/BR) showed a deletion of a glutamic acid (E) at position 809 of the gB gene (Figure 4.2b). A deletion of the AAQD motif repeat located at positions 87 to 90 of the ICP4 gene ORF was observed for CEO related groups IV and V isolates (Figure 4.2b). For commercial poultry isolates in group VI, and backyard flock isolates in groups VII, VIII and IX, a six amino acid insertion (QPQEPQ), flanked by EPQ motifs, was observed at position 862 of the ICP4 gene (Figure 4.2b).

**Phylogenetic analysis and classification of ILTV isolates.** A phylogenetic tree was generated using the nucleotide and deduced amino acid sequences of the multiple genes previously targeted by PCR-RFLP analysis (gM, ICP4, UL47 and gG). This analysis separated ILTV isolates and strains in eight clusters (data not shown), with resampling values as high as 63 to 100%. Different from multiple PCR-RFLP, sequencing analysis of the gM, ICP4, UL47 and gG ORF did not separated isolates from PCR-RFLP groups II and III. In a similar fashion sequence

analysis of gB, gM, ICP4, UL47 and gG also separated the ILTV isolates in eight different clusters with resampling values as high as 100% (Figure 4.3). Phylogenetic analysis of the ICP4 sequences separated ILTV viruses in six clusters, the gM gene sequence separated the ILTV viruses in three clusters, and the gG gene, as well as the UL47 gene, separated ILTV isolates in five clusters (data not shown). Sequence analysis of the gB gene allowed separation of commercial poultry isolates (IV, V and VI.). However it did not separate the backyard flock isolates from each other (Figure 4.4).

**Discussion**

The recent PCR-RFLP analysis of US ILTV isolates provided evidence that genetic variation exists among ILTV strains originating from commercial poultry, backyard flocks and vaccine strains. This analysis allowed the differentiation of ILTV viruses from the US (Oldoni and Garcia, 2007). However the specific genome differences and the overall polymorphism of the genome regions targeted by the multiple PCR-RFLP analysis are not known. In this study, sequencing analysis of six genes (gB, UL32, gM, UL47, gG and ICP4) from isolates representative of the different PCR-RFLP groups were compared to the USDA ILTV reference sequences. The USDA strain is derived from a serial of vent brush type laryngotracheitis vaccine manufactured in the early 1960's (NVSL, Ames, IA) and has been routinely used as the challenge strain in protection studies (Davison et al., 2006). Characterization of ILTV isolates by sequencing analysis of the same genes, gM, UL47, gG and ICP4 targeted by PCR-RFLP analysis separated the isolates in eight different clusters as demonstrated by the phylogenetic analysis. The sequencing analysis was able to group all representative viruses similarly to the multiple

PCR-RFLP analyses, with the exception of group II (TCO vaccine), which was not separated from group III (TCO vaccine related). The differentiation of groups II and III by PCR-RFLP analysis relies on one pattern difference of the ICP4 gene. However, the single nucleotide polymorphism (SNP) that allowed the differentiation of these two groups of isolates by PCR-RFLP is located outside to the ICP4 open reading frame, in the gene non-coding region. This sequence was not included in the analysis explaining the lack of discrimination between groups II and III isolates by sequencing analysis. However, sequencing analysis of the six ORF's validated 8 of the 9 genotype groups previously identified by PCR-RFLP analysis; particularly it clearly separated commercial poultry isolates within group VI from vaccine related isolates (Groups III, IV, V).

Analysis of ILTV gene sequences showed that the UL32 and gM genes were highly conserved among commercial poultry and backyard flock isolates. No amino acid changes were observed in UL32, while in gM only two amino acid substitutions were observed characteristics of groups IV (amino acid 42 [A –T]) and VI (amino acid 248 [P –S]). Amino acid substitutions on gG were more frequent for backyard flock isolates, especially isolates from groups VII and IX and for commercial poultry isolates in group VI. As the gG gene, UL47 amino acid sequences were very conserved among commercial poultry isolates and amino acid substitutions were more frequent for the backyard flock isolates. Sequencing characterization of the UL47/gG genes from Canadian isolates showed differences between commercial poultry isolates and vaccine strains (Ojkic et al., 2006). The nucleotide substitutions observed for US isolates and vaccines strains in the UL47 and gG ORFs, as well as in the intragenic region (data not showed), corresponded to those observed for Canadian isolates, indicating that genetically related viruses are circulating in commercial poultry in the US and Canada. The gB gene showed amino acid substitutions among

commercial poultry, as well as backyard flock isolates. The glycoprotein B has been characterized as one of the most immunogenic proteins of ILTV (Kongsuwan et al., 1991) and is one of the protective antigens included in the ITLV pox recombinant vaccine (Davison et al., 2006). The glycoprotein B is located on the viral envelope by a membrane anchor region (amino acids 690-761). Amino acid changes on gB were identified in the protein ectodomain for backyard flock isolates (Groups VII, VII, IX; amino acids 130 and 551), USDA challenge strain (Group I; amino acid 232), TCO vaccine and TCO related isolates (Groups II and III: amino acids 348 and 664), and in the endodomain of backyard flock isolates (groups VII, VIII, IX; amino acid 799), and group VI isolates (amino acid 799 and 805). One amino acid deletion was observed in the endodomain (amino acid 809) of three isolates categorized into group V. This was the only difference observed between isolates that belong to the same group. As gB, the ICP4 gene also showed significant variability. A deletion of four amino acids (AAQD, at position 87-90) was observed for all isolates categorized into groups IV and V, confirming the close relationship of these two groups, which are considered related to the CEO vaccine. Interestingly, an insertion of six amino acids (PQEPQ at position 862) was observed for commercial poultry isolates from group VI, as well as for backyard flock isolates. These results indicated that commercial poultry isolates from group VI are different from those categorized into groups III, IV and V. Other unique sequence changes were observed among the different groups of commercial poultry and backyard flock isolates. The groups II and III, considered TCO vaccine related, have an aspartate (D) at position 280 of UL47 gene, a threonine (T) at position 348 and an isoleucine (I) at position 644 of the gB gene. Two genome changes were associated with virulent ILTV field isolates from Korea in the thymidine kinase gene (Han and Kim, 2001) and in isolates from Northern Ireland in the ICP4 gene (Creelan et al., 2006). US

isolates from commercial poultry, within groups IV, V and VI, have been associated with severe forms of the disease in the field, however, unique sequence changes characteristic of outbreaks related isolates, not shared by vaccine strains, were detected only for group VI commercial poultry isolates. The relation of these particular genome differences with virulence needs to be further investigated.

In summary, sequencing characterization validated the genotype groups previously identified by PCR-RFLP analyses. Also, sequencing characterization showed that backyard flock isolates have more genetic variability than commercial poultry isolates across the different genes, and within commercial poultry isolates, viruses categorized into group VI showed the greatest genetic variability. The UL32 and gM gene sequences were the most conserved and ICP4 and gB gene sequences were the most variable. This study presents the first comparative sequence analysis for a wide variety of ILTV isolates from the US where specific genome differences were identified among commercial poultry and backyard flock isolates. Future studies will be geared towards determining the correlation between the different viral genotypes and pathogenicity.

Table 4.1: Infectious laryngotracheitis virus (ILTV) isolates and strains representative of PCR-RFLP groups used in sequencing analyses.

| Samples IDa/Strain | Age (days) | Vaccination | RFLP Group[c] |
|---|---|---|---|
| USDA[d] | | | I |
| TCO[e] vaccine | | | II |
| 401/A/06/BBR | 280 | Not vaccinated | III |
| 13/E/03/BBR | 441 | TCO | III |
| 14/E/03/BBR | Not available | TCO | III |
| 301/K/06/BR | Not available | Not available | IV |
| 10/C/97/BR | 53 | CEO | IV |
| 21/G/05/BR | 42 | Not vaccinated | IV |
| CEO[f] vaccine | | | IV |
| 19/F/05/BR | 60 | Not vaccinated | V |
| 402/A/06/BR | 54 | Not vaccinated | V |
| 7/B/99/BR | Not available | Not vaccinated | V |
| 102/B/05/BR | 35 | Not vaccinated | V |
| 205/J/06/BR | 52 | Not vaccinated | V |
| 501/C/06/BR | 203 | Pox Vector & TCO vaccines | V |
| 417/A/06/BR | 60 | Not vaccinated | VI |
| 2/A/04/BR | Not available | Not vaccinated | VI |
| 20/F/04/BR | 52 | Not vaccinated | VI |
| 305/K/05/BR | 49 | Not vaccinated | VI |
| 12/D/02/BCY | Not available | Not vaccinated | VII |
| 24/H/91/BCY | 28 to 56 | Not vaccinated | VIII |
| 25/H/88/BCY | 183 to 548 | Not vaccinated | IX |

[a] Samples identified by: sample number, letter, representing different state of origin, year of collection, and type of bird the isolate was collected from (BR: Broiler; BBR: Broiler Breeder; BCY: backyard flock)

[b] Pattern combination that resulted from digestion of four genome regions with ten restriction endonucleases (Oldoni and Garcia, 2007)

[c] RFLP groups categorization according Oldoni and Garcia (2007)

[d] United States Department of Agriculture ILTV reference strain

[e] Tissue culture origin

[f] Chiken embryo origin

Table 4.2: Primers for amplification and sequencing of infectious laryngotracheitis virus genes.

| Primer name | Target genome regions | Sequence ( 5'- 3') |
|---|---|---|
| gBseq 1[a] | gB | AAATTGAAAAGAAAGAAAGAAGCAG |
| gB 2 | gB | AGAGTGTTGCCAAAAAAATCTGC |
| gB 3 | gB | ATACTAGAAGATTTGGACTTTGTGCC |
| gB 4 | gB | CCGAACAAAGATGTCATCAGTG |
| gB 5 | gB | CCTCAGGGTGATAGAATTACTACCC |
| gB 6 | gB | TCCTTTTCTTCCATTGCATCC |
| gB 7 | gB | TACAGGCTAGATCTGTATACCCGTATG |
| gB 8 | gB | TACGGGGCAATGTTTTGC |
| gBseq 9[a] | gB | GAGGAACATCCATTAGTTGCAGT |
| UL32seq 1[a] | UL32 | GCGTATGACAGGAATCGTCA |
| UL32 2 | UL32 | AAATATGTTAGCCGTGGCGA |
| UL32 3 | UL32 | AACCATCACTTGCCATTGG |
| UL32 4 | UL32 | ACATACACTGCGCGGAGC |
| UL32 5 | UL32 | CAAAGCATCCCCCTGTGT |
| UL32seq 6[a] | UL32 | CGGAGGCGATAGGTCATAAA |
| gMseq 1[a] | gM | GCATCCGTACAGTCTTCGTG |
| gM 2 | gM | CACAGCTCAAAAGTGCATGC |
| gM 3 | gM | CGGACAAGGCCATTATAAGC |
| gMseq 4[a] | gM | GCGCTTGTCCCTATTAGACG |
| UL47/gGseq 1[a] | UL47/gG | CTGTTTTCGGCTGAAGAGGA |
| UL47gG 2 | UL47/gG | GGACGTTCTCCAATTAGTGAAGC |
| UL47gG 3 | UL47/gG | CGTAACGCTTTTCTTTGATCG |
| UL47gG 4 | UL47/gG | GACGGTCTTAGCGACCATGT |
| UL47gG 5 | UL47/gG | GCTCCGTTCAGGTACTCGTT |
| UL47gG 6 | UL47/gG | CGTGCCGATTCAATATGAAG |
| UL47gG 7 | UL47/gG | GCTCGTCTATCACCGGCTTA |
| UL47gG 8 | UL47/gG | AACCGCACCACGATTGAG |
| UL47gG 9 | UL47/gG | CACACAGACGCGTTCAGATT |
| UL47/gGseq 10[a] | UL47/gG | AACATTGTCCCCATCTCTCG |
| ICP4seqA 1[a] | ICP4 | AACCTGTAGAGACAGTACCGTGACCC |
| ICP4 2 | ICP4 | GGTGTGGCCAATAACGTACAA |
| ICP4 3 | ICP4 | CAAACCTGAGCCTACTGTAGAACA |
| ICP4seqB 4[a] | ICP4 | GTCAAGAGTATGGGCTGCTGTT |
| ICP4seqA 5[a] | ICP4 | ATGCGCGACTCTCGAAAC |
| ICP4 6 | ICP4 | GTGGACGGGCGAGAAATG |
| ICP4 7 | ICP4 | GAGCGAGTCGATGACCGTAT |
| ICP4 8 | ICP4 | TAGAGCCACTCTGGCGAGTT |
| ICP4 9 | ICP4 | GGATGGGATAATAGCTTCTGGA |
| ICP4 10 | ICP4 | GACCACCCGCAGAAAACTTA |
| ICP4 11 | ICP4 | CAATGCAAGTTCTAGTATGGCG |
| ICP4seqC 12[a] | ICP4 | AGCACGTCACGGACGATT |
| ICP4seqB 13[a] | ICP4 | GCCAGGACTCCCGTAGAGTA |
| ICP4seqD 14[a] | ICP4 | CACCGGAGGGTTTCATCC |
| ICP4 15 | ICP4 | CGGTCGCGGAATAGCTTC |
| ICP4seqC 16[a] | ICP4 | CCATTACTACGTGACCTACATTGAGCC |
| ICP4 17 | ICP4 | TCGCACGCTTACCTTGTAAA |
| ICP4seqD 18[a] | ICP4 | AACGGGGCTAGATCATTTCC |
| ICP4seqE 19[a] | ICP4 | TTAATAGGGCCGCTGAGTTG |
| ICP4seqE 20[a] | ICP4 | TCTTACGCGGATCTGGAAGT |

a: Primers to amplify ILTV genes

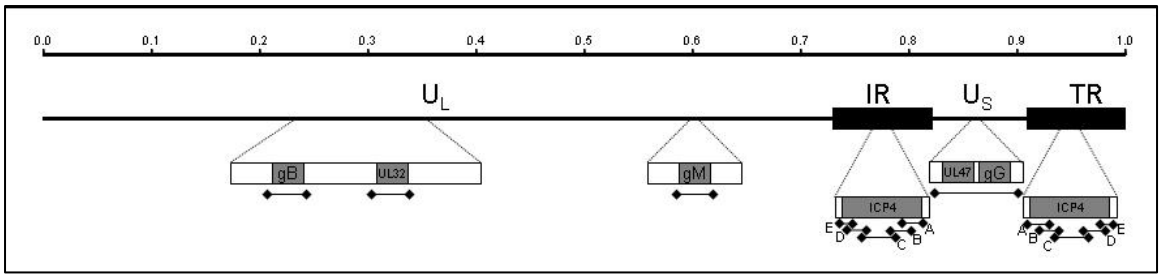


Figure 4.1: Schematic representation of ILTV genome structure. Box in gray represent genes analyzed by sequencing. UL: Unique Long; US: Unique Short; IR: Internal Repeated; TR: Terminal Repeated; = PCR product; ICP4 fragments are represented by letter A, B, C, D, and E.

**a**

| Groups | UL32 | | gB | | gM | | UL47 | | gG | | ICP4 | | Total | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | S | NS | S | NS | S | NS | S | NS | S | NS | S | NS | S | NS |
| II | 0/0 | 0/0 | 0/3 | 3/3 | 1/1 | 0/1 | 0/1 | 1/1 | 0/0 | 0/0 | 3/4 | 1/4 | 4 | 5 |
| III | 0/0 | 0/0 | 0/3 | 3/3 | 1/1 | 0/1 | 0/1 | 1/1 | 0/0 | 0/0 | 3/4 | 1/4 | 4 | 5 |
| IV | 0/0 | 0/0 | 1/2 | 1/2 | 1/2 | 1/2 | 0/0 | 0/0 | 0/0 | 0/0 | 9/15 | 6/15 | 11 | 8 |
| V | 0/0 | 0/0 | 0/1 | 1/1 | 1/1 | 0/1 | 0/0 | 0/0 | 0/0 | 0/0 | 9/15 | 6/15 | 10 | 7 |
| VI | 0/0 | 0/0 | 0/3 | 3/3 | 2/3 | 1/3 | 1/1 | 0/1 | 3/6 | 3/6 | 8/13 | 5/13 | 14 | 12 |
| VII | 1/1 | 0/1 | 0/4 | 4/4 | 1/1 | 0/1 | 11/19 | 8/19 | 5/14 | 9/14 | 8/20 | 12/20 | 26 | 33 |
| VIII | 0/0 | 0/0 | 3/7 | 4/7 | 1/1 | 0/1 | 6/8 | 2/8 | 2/6 | 4/6 | 12/21 | 9/21 | 24 | 19 |
| IX | 1/1 | 0/1 | 0/4 | 4/4 | 1/1 | 0/1 | 13/20 | 7/20 | 3/12 | 9/12 | 9/19 | 10/19 | 27 | 30 |
| **Total** | **2** | **0** | **4** | **23** | **9** | **2** | **31** | **19** | **13** | **25** | **61** | **50** | **120** | **119** |

**b**

| Gens | gG | | | | | | | | | | gM | | UL47 | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid position | | | | | | | | | | | | | | | | | | | | |
| Group | 45 | 51 | 58 | 98 | 115 | 118 | 129 | 220 | 284 | 291 | 42 | 248 | 23 | 35 | 39 | 86 | 87 | 280 | 371 | 532 | 593 |
| I (USDA) | I | R | V | H | G | A | Q | G | F | Q | A | P | H | T | Y | S | - | G | A | M | A |
| II | I | R | V | H | G | A | Q | G | F | Q | A | P | H | T | Y | S | - | D | A | M | A |
| III (TCO) | I | R | V | H | G | A | Q | G | F | Q | A | P | H | T | Y | S | - | D | A | M | A |
| IV (CEO) | I | R | V | H | G | A | Q | G | F | Q | T | P | H | T | Y | S | - | G | A | M | A |
| V | I | R | V | H | G | A | Q | G | F | Q | A | P | H | T | Y | S | - | G | A | M | A |
| VI | I | R | V | N | V | A | Q | L | F | Q | A | S | H | T | Y | S | - | G | A | M | A |
| VII | L | H | G | N | V | V | L | G | L | R | A | P | Q | A | F | P | A | G | V | L | T |
| VIII | I | R | G | N | V | A | Q | G | L | Q | A | P | H | T | Y | S | A | G | A | L | A |
| IX | L | R | G | N | V | V | H | G | L | R | A | P | Q | T | F | P | A | G | V | L | T |

| Gens | gB | | | | | | | | ICP4 | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Amino acid position | | | | | | | | | | | | | | | | | | | | | | | | |
| Group | 130 | 232 | 348 | 551 | 644 | 799 | 805 | 809 | 87-90 | 180 | 200 | 208 | 271 | 747 | 838 | 840 | 842 | 862 | 889 | 1141 | 1302 | 1338 | 1339 | 1433 | 1447 | 1483 |
| I (USDA) | R | K | M | M | T | P | K | E | AAQD | R | V | R | A | N | D | L | S | ------ | I | A | P | G | G | P | T | P |
| II | R | E | T | M | I | P | K | E | AAQD | R | V | R | A | N | D | L | S | ------ | I | A | P | G | D | P | T | P |
| III (TCO) | R | E | T | M | I | P | K | E | AAQD | R | V | R | A | N | D | L | S | ------ | I | A | P | G | D | P | T | P |
| IV (CEO) | R | E | M | M | T | P | K | E | ---- | R | M | R | A | N | D | L | S | ------ | I | V | L | R | D | P | S | P |
| V | R | E | M | M | T | P | K | * | ---- | R | M | R | A | N | D | L | S | ------ | I | V | L | R | D | P | S | P |
| VI | R | E | M | M | T | S | R | E | AAQD | R | M | R | A | N | D | L | S | QPQEPQ | I | A | L | R | D | P | S | P |
| VII | H | E | M | V | T | S | K | E | AAQD | M | V | C | T | D | D | P | P | QPQEPQ | V | A | P | R | D | L | S | T |
| VIII | H | E | M | V | T | S | K | E | AAQD | R | V | R | T | D | Y | P | P | QPQEPQ | V | A | P | R | D | P | S | P |
| IX | H | E | M | V | T | S | K | E | AAQD | R | V | R | T | D | D | P | P | QPQEPQ | V | A | P | R | D | L | S | T |

Figure 4.2: a: Summary of nucleotide changes on ILTV genes as compared to the USDA reference strain; S=synonymous; NS=non-synonymous; b: Summary of amino acid substitutions on ILTV genes as compared to the USDA reference strain. Boxes indicate amino acid changes; * deletion in only isolates (205/J/06/BR, 501/C/06/BR and 19/F/05/BR) from group V.

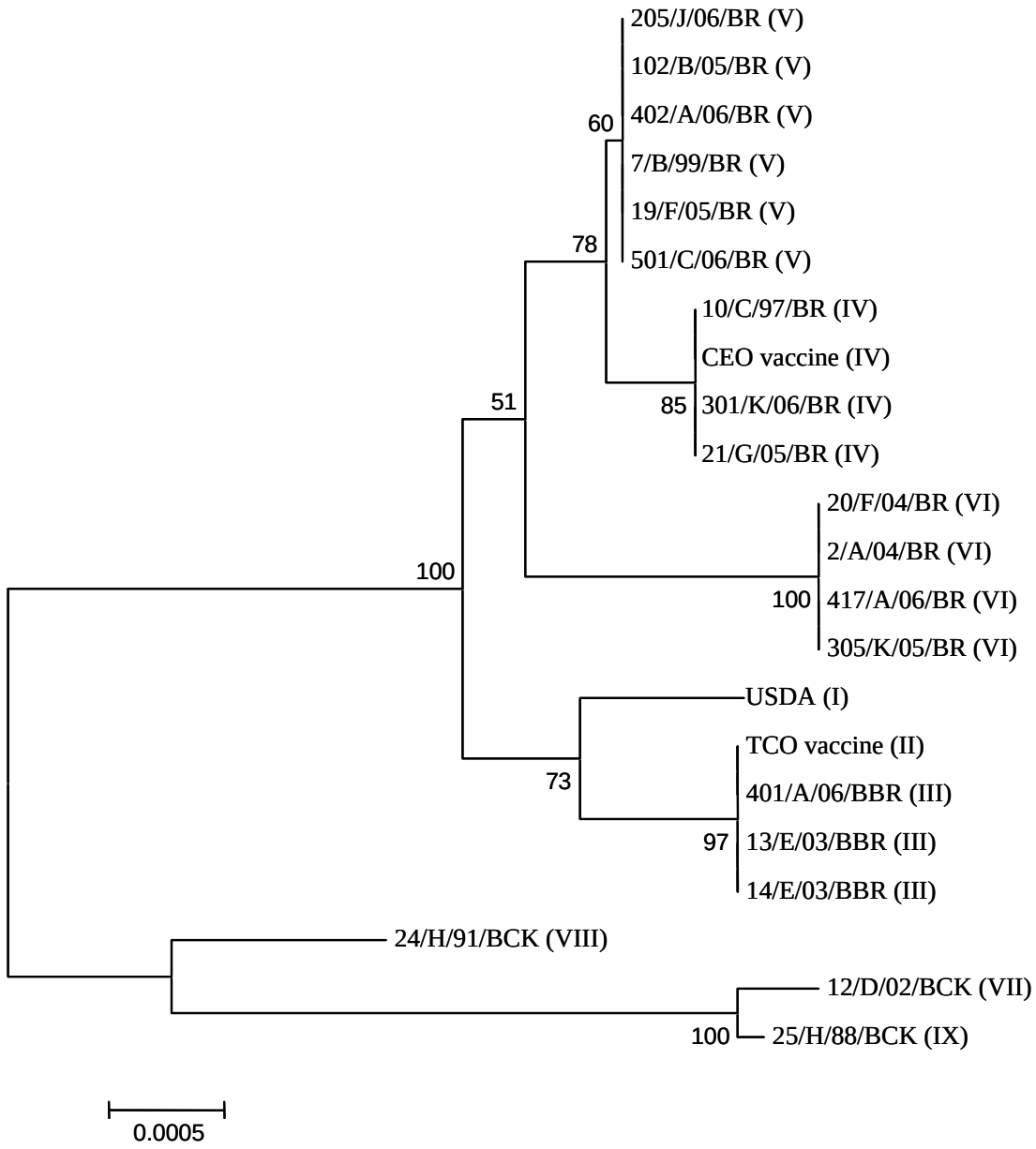


Figure 4.3: Phylogenetic tree generated from the deduced amino acids sequences of the gB, gM, ICP4, UL47 and gG genes of ILTV viruses. The branch lengths represent the genetic distance between the groups, and numbers on the branches are bootstrap values as a percentage at internal nodes (1000 resampling).

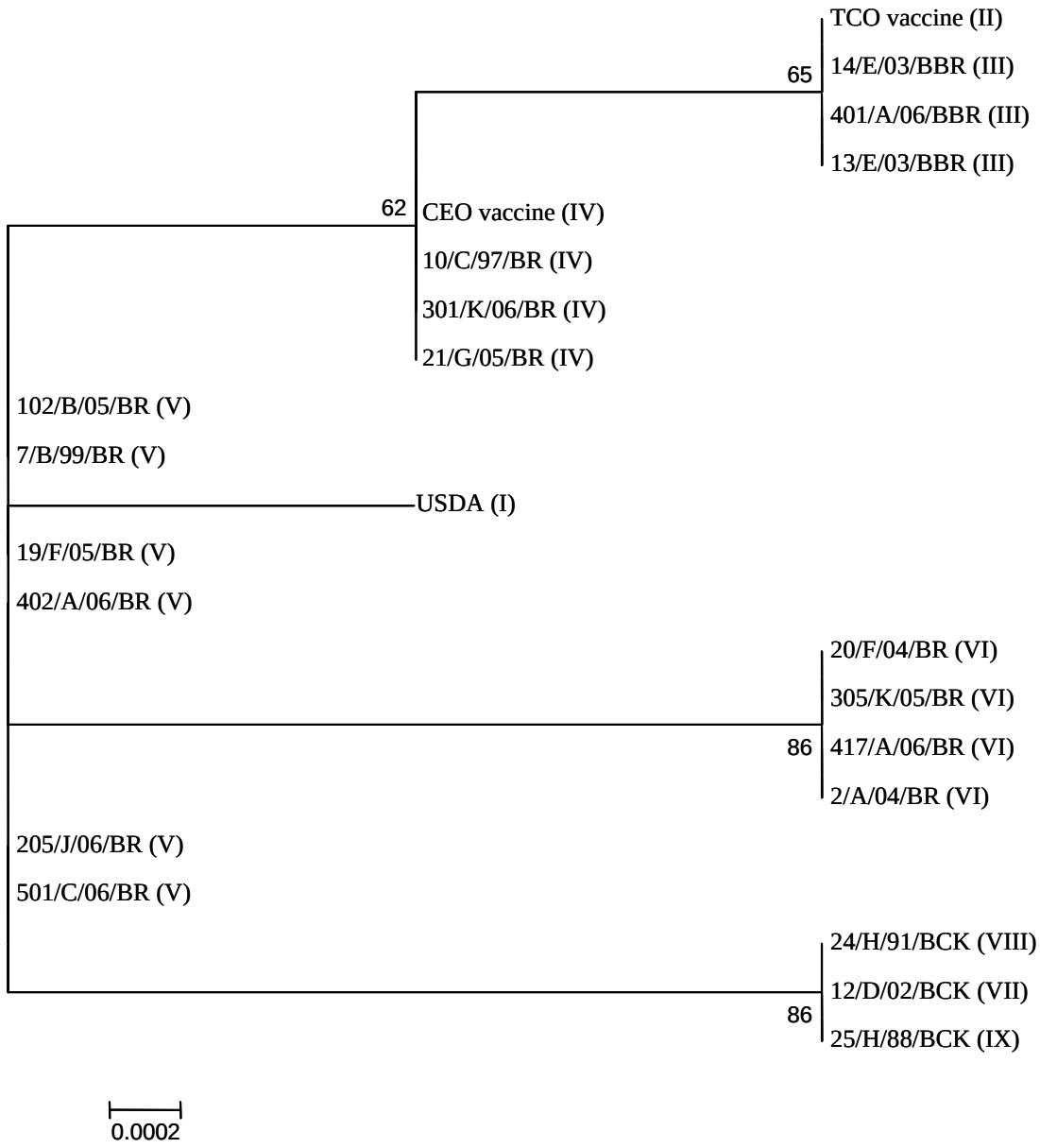


Figure 4.4: Phylogenetic tree generated from the deduced amino acids sequences of the glycoprotein B gene of ILTV viruses. The branch lengths represent the genetic distance between the groups, and numbers on the branches are bootstrap values as a percentage at internal nodes (1000 resampling).

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# CHAPTER 5

# PATHOGENICITY AND GROWTH CHARACTERISTICS OF INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV) ISOLATES FROM UNITED STATES[1]

[1] Oldoni, I., Rodríguez-Avila, A., Riblet, S., Zavala, G., and García, M. To be submitted to Avian Pathology.

## Abstract

Infectious laryngotracheitis (ILT) is an upper respiratory disease of poultry of worldwide distribution. The disease is caused by infectious laryngotracheitis virus (ILTV) a member of family *Herpesviridae*, is characterized by acute respiratory signs, and is common in areas of intense poultry production. The disease is controlled by vaccination with live attenuated vaccines. Once vaccine strains have been introduced in the field the differentiation of ILTV strains is difficult because of the antigenic and genomic homogeneity of the vaccines and field viruses. In a recent study, several US ILTV strains and field isolates were molecularly categorized by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). The aim of this study was to evaluate the pathogenicity in chickens, and viral replication in cell culture for six ILTV isolates, from commercial poultry, representative of PCR-RFLP groups IV, V, VI, as compared to the chicken embryo origin (CEO) vaccine. The evaluation of clinical signs, viral tissue distribution in chickens, viral replication in cell culture, and plaque formation ability showed no biological differences for the two isolates analyzed from group IV, and the two isolates analyzed from group VI. However, growth and pathogenicity differences were found among the different viral groups (IV, V, VI), and between groups V isolates. Group IV isolates and the CEO vaccine were less pathogenic than group V and VI isolates, and showed equal efficiency to replicate in chicken kidney cells. Group VI isolates, characterized as genetically different to the vaccine strains, were equally pathogenic as group V isolates, which are genetically closely related to the CEO vaccines. However, group VI isolates replicate less efficiently in chicken kidney cells, and produce smaller plaques than group V isolates. Therefore,

the genomic differences identified for isolates in groups V and VI may not be not associated with their pathogenicity, but rather associated with their *in vitro* growth characteristics.

**Introduction**

Infectious laryngotracheitis is a highly contagious disease of chickens of worldwide distribution that cause severe production losses due to morbidity, mortality, decreased egg production, weight losses, and/or predisposition to other respiratory avian pathogens (Guy & Bagust, 2003). Infectious laryngotracheitis virus (ILTV), is a member of the genus Iltovirus, belongs to family *Herpesviridae*, subfamily *Alphaherpesvirinae* (Davison *et al.,* 2005). The disease is characterized by acute respiratory signs, and is common in areas of intense poultry production. In the United States (US), two types of live-attenuated vaccines have been traditionally utilized to control ILT; vaccines attenuated by multiple passages in embryonated eggs (chicken embryo origin [CEO]) (Samberg *et al.,* 1971); and the vaccine generated by multiple passages in tissues culture (tissue culture origin [TCO]) (Gelenczei & Marty, 1965). Earlier experimental evidence demonstrated that live attenuated vaccine strains, particularly the CEO vaccines, could easily revert to virulence after bird-to-bird passage (Guy *et al.,* 1991), or after reactivation from latency (Hughes *et al.,* 1991). Molecular epidemiology studies have suggested that most of ILT outbreaks in broilers in the US are caused by viruses closely related to the CEO vaccines, while outbreaks with TCO type isolates are rare (Guy *et al.,* 1989; Keller *et al.,* 1992; Keeler *et al.,* 1993). In a recent study, several US ILTV strains and field isolates were molecularly categorized using polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) (Oldoni & Garcia 2007). As previously found (Guy *et al.,* 1989; Keller *et al.,* 1992; Keeler *et al.,* 1993), multiple PCR-RFLP analysis showed that most of the US isolates from commercial poultry were closely related to the CEO vaccines (Groups IV and V), while a third group of isolates (group VI), also from commercial poultry, showed PCR-RFLP

patterns different from the vaccine strains. Although only four of the 73 ILTV genome predicted open reading frames (Thureen & Keeler, 2006) were analyzed by PCR-RFLP, this analysis allowed the genotypic categorization of US isolates, and provided the framework to compare the pathogenicity and growth characteristics of different viral genotypes.

Different parameters have been utilized to evaluate the biological characteristics of ILTV viruses. Although, ILTV large and small plaques have shown similar virulence in experimental infected chickens, viral plaque size heterogeneity and morphology have been used to phenotypically characterize ILTV field isolates *in vitro* (Russell & Turner, 1983). The pathogenicity of ILTV isolates has been evaluated using different parameters (Guy *et al.,* 1990; Kirkpatrick *et al.,* 2006). For example, viral pathogenicity was measured by the trachea microscopic pathology induced by different ILTV isolates after intra-tracheal inoculation (Guy *et al.,* 1990; Han *et al.,* 2002). Most recently, in addition to the trachea pathology, pathogenicity of vaccines and field strains was evaluated considering clinical signs, body weight gain, and the presence of viral DNA in trachea (Kirkpatrick *et al.,* 2006). It was found that measurement of clinical signs and body weight gain were better indicators of ILTV strain pathogenicity than measurement of the trachea pathology (Kirkpatrick *et al.,* 2006).

The aim of this study was to evaluate the pathogenicity and viral replication of ILTV isolates, from commercial poultry, representative of PCR-RFLP groups IV, V, VI, as compared to the CEO vaccine. The characterization of ILTV isolates was based on clinical signs, examination of viral replication in different tissues during the full course of the disease, and growth characteristics of the different viral isolates in cell culture.

## Materials and Methods

**Viruses and cells.** The commercial ILTV vaccine strain Trachivax® (Schering Plough, Millsboro, DE) and six ILTV isolates were used in this study. Strains and isolates were selected based on their different PCR-RFLP characterization as previously described (Oldoni & Garcia, 2007). Field isolates from PCR-RFLP groups IV, V, and VI were identified as follow: IV (A) for isolate 301/K/06/BR; IV (B) for isolate 10/C/97/BR; V (A) for isolate 19/F/05/BR; V (B) for isolate 402/A/06/BR; VI (A) for isolate 417/A/06/BR; VI (B) for 2/A/04/BR. The six field isolates were recovered from the upper respiratory tract of chickens during severe outbreaks of the disease. All field isolate were pre-incubated with adenovirus and reovirus antisera (NVSL, Ames, IA), and then propagated on the chorioallantoic membrane (CAM) of 10-day-old chicken embryos using standard techniques (Tripathy, 1998). To confirm that viral stocks were free of adenovirus, reovirus and CAV these were tested for adenovirus (Meulemans *et al.,* 2001), chicken anemia virus (Todd *et al.,* 1992), and reovirus (Kapczynski *et al.,* 2002) by PCR. The third CAM passage for the six field isolates was tittered in chicken kidney (CK) cells. Chicken kidney cells were also utilized for growth curve analysis and to expand the USDA reference strain as previously described by Hughes & Jones (1988). Viral plaque assay was performed in the chicken liver tumor cell line, LMH (Kawaguchi *et al.,* 1987), as previously described by Devlin *et al*. (2006).

**Viral titration**. The CEO vaccine, the USDA reference strain, field isolates stocks, and virus working stock dilutions utilized for chicken inoculations were tittered in 96 well plates using a CK cells concentration of $8 \times 10^5$ cells per ml. Viral inoculums were diluted from $10^{-1}$ to

$10^{-9}$ and five replicates of each dilution were inoculated into CK cells. After five days incubation at 37 C, plates were evaluated for the presence of ITLV cytopathic effect. The Reed and Muench method was utilized to estimate the 50% tissue culture infective dose ($TCID_{50}$).

**Experimental design.** One hundred ninety-two white leghorn specific pathogen free (SPF) chickens were divided into eight groups of 24 birds each. Birds were housed in individual stainless steel isolation units with filtered-air and positive-pressure at the Poultry Diagnostic and Research Center (PDRC, Athens, GA), and fed a standard diet and water *ad libitum*. At four weeks of age, six groups of birds were inoculated with field isolates and the CEO vaccine. Each of the six field isolates was applied at a final dose of $10^{2.5}$ $TCID_{50}$ per chicken. The CEO vaccine was applied at a regular dose of $10^{4.3}$ $TCID_{50}$ per chicken. Four aliquots of 50 µl were administered per chicken in each eye and nostril. In the same room a group of 24 birds were inoculated with phosphate buffered saline solution (PBSS) in a similar fashion and kept as the negative control in a separate unit.

**Scoring of clinical signs.** Chickens were recorded, from 1 to 14 days post-inoculation (dpi), with a digital video camera (recording 5 min per group daily). Videos were analyzed and clinical signs were scored on the basis of breathing patterns, conjunctivitis, level of depression, and mortality. Dyspnea was scored on a scale of 0 (normal breathing), 1 (mild dyspnea and open mouth breathing) and 2 (gasping with an extended neck). The condition of the conjunctiva was scored on a scale of 0 (normal), 1 (swollen and/or partial closure of the eyes) and 2 (complete closure of the eyes). The level of depression was scored on a scale of 0 (normal, or not depressed), 1 (depressed) and 2 (severely depressed). For mortality, the score was 0 (no

mortality) and 3 (mortality). A daily total clinical sign score was calculated for each group adding individual chickens clinical sign scores**.** In addition, an overall experiment clinical sign score was calculated per group by adding daily total scores.

**Sample collection and processing.** Samples were collected from two chickens at 2, 4, 5, 7, 9, 11 and 14 dpi from groups inoculated with IV (A), IV (B) V (A), V (B), VI (A), VI (B) viruses, the CEO vaccine, and from one PBSS inoculated chicken. Birds were euthanized by $CO_2$ gas inhalation. Before euthanasia, sinuses, conjunctiva and cloacal swabs were collected and placed in 1 ml of sterile PBSS containing a 2% antibiotic-antimycotic 100X (Gibco, Grand Island, NY) and 2% newborn calf serum (Gibco Grand Island, NY). The trachea was dissected from the larynx to the bronchial bifurcation, and cut longitudinally; half was collected in buffered formalin; the epithelium of the other half was scraped and re-suspended in 1 ml of PBSS. The head was removed and the trigeminal ganglia extracted. After extraction, trigeminal ganglia was collected and minced in 1 ml of PBSS. Both eyelids were excised and collected in buffered formalin. The cecal tonsils were dissected and cut longitudinally, washed, minced, and re-suspended in 1 ml of PBSS. One lung was extracted and a lung section was minced, and re-suspended in 1 ml of PBSS. At 5, 7 and 9 dpi, bursa, thymus and spleen were extracted and sections of these tissues were collected in 1 ml of PBSS as described for other tissues. One section of the thymus was also collected in buffered formalin. All samples collected in PBSS were stored at –80 C until processing for virus isolation and DNA extraction. Before inoculation samples were frozen and thawed three times. Samples were thawed at 37 ºC, vortexed, and frozen for 24 hours at –80 C. After the last thawing samples were vortexed and centrifuged for two minutes at 7500 rpm.

**Histopathology.** Transverse segments of the larynx/trachea, eye-lid and thymus were fixed in buffered formalin for 48h, embedded in paraffin and stained with hematoxylin and eosin for histopathological examinations.

**Virus Isolation**. Virus isolation (VI) was performed in adult chicken kidney (CK) cells as previously described by Rodríguez-Avila *et al.* Bursa, thymus, spleen, lung, trigeminal ganglia, trachea and cecal tonsils tissue suspensions as well as conjunctiva and cloacal swab suspensions were inoculated directly into cells and incubated at 37 ºC, 5% $CO_2$ for five days. Each sample was inoculated in duplicate and all samples were passed three consecutive times in CK cells. Samples were considered positive by virus isolation when the cytopathic effect (CPE) characteristic of ILTV was observed, and were considered negative after three passages without detecting ILTV CPE.

**DNA Extraction.** DNA extraction from tissues and swab suspensions was performed using the MegaZorb DNA Mini-prep 96-weel kit (CORTEX BIOCHEM$_{TM}$, San Leandro, CA) according to the manufacturer's recommendations.

**Real Time PCR Taqman Assay (ReTi-PCR).** ReTi-PCR was executed as previously described (Callison *et al*., 2007). The primers and probe utilized in the assay are located in the viral glycoprotein C gene, were synthesized by IDT (Coralville, IA) and BioSearch Technologies (Novato, CA), respectively. The genome copies number (GCN) $\log_{10}$ per amplification reaction was estimated using the standard curve equation (y = -0.289x + 12.487) generated from the gC

plasmid and expressed as $log_{10}$. The GCN $log_{10}$ value reported was either the average of two samples, when viral DNA was detected in both samples, or the value obtained for one sample.

**One step growth curve**. The six ILTV isolates, the CEO vaccine and the USDA reference strain were inoculated onto CK cells ($8 \times 10^5$ per ml) in 6-well trays, at an m.o.i. of 0.004. After an adsorption period of 1 hour supernatant was removed and replaced with 2.5 ml fresh MEM with 5% of calf serum. Incubation was continued and, at 6, 12, 18, 24, 36, 48, and 72 hours post-incubation (hpi), infected cells and supernatant was harvested and stored at -80 C. All samples were then titrated in triplicate by plaque assay in LMH cells as described bellow. The viral titer for the different time points was expressed as mean plaque forming unit (pfu)/ml and used to plot virus growth curves.

**Plaque formation ability.** A total of 0.5 ml of ILTV isolates, the CEO vaccine and the USDA reference strain stocks resuspended in 0.5ml of minimal essential media with 5% calf serum were inoculated in triplicates onto LMH cell monolayers in 6-well plates. After inoculation the virus was adsorbed for a period of 2 h at room temperature (RT). After adsorption the supernatant was removed and replaced with 2.5 ml of methylcellulose overlay medium containing 5% calf serum and plates were incubated for 96 hpi. After incubation cells were fixed with 2% formaldehyde for 1 h at RT. Plates were washed with running water and stained with 1% crystal violet in 50% ethanol for 15 min at RT. After staining, plates were washed in running water and dried. The diameters (µm) for 100 plaques induced by each virus were measured using the ImageJ software (http://rsb.info.nih.gov/ij). The percentage of plaques within different size ranges were calculated and plotted for each viral isolate, the CEO vaccine

and the USDA strain. The mean plaque diameter for each virus was calculated as well and compared using the statistical analyses of variance (ANOVA-Tukey).

## Results

**Clinical signs.** Cumulative clinical sign scores were recorded daily per group and are summarized in figure 5.1. Chickens from groups IV (A and B) showed mild to moderate dyspnea with open mouth breathing, and moderate depression. Groups V (B), VI (A) and VI (B) showed severe dyspnea between 3 to 7 dpi, with mild to severe conjunctivitis and severe depression, while group V (A) chickens showed the highest clinical sign scores at 9 to 12 dpi. The exception was the CEO vaccine group that showed only mild conjunctivitis and mild depression. For groups V (B), VI (A), and VI (B) the most prevalent clinical sign was depression, followed by conjunctivitis and dyspnea. For group V (A) depression was the most prevalent sign followed by dyspnea and conjunctivitis. Clinical sign for groups IV (A) (Figure 5.1a) and IV (B) (Figure 5.1b) were more marked at 3 to 5 dpi, while clinical signs for groups V (B) (Figure 5.1d), VI (A) (Figure 5.1e), and VI (B) (Figure 5.1f) were more marked at 4 to 8 dpi. Group V (A) (Figure 5.1c) chickens presented more marked clinical signs at 9 to 12 dpi. For groups IV (A) and IV (B) clinical signs cleared by 8 and 9 dpi, for groups V (B), VI (A), VI (B) clinical signs cleared by 11 dpi, while group V (A) chickens showed clinical signs, particularly depression, up to 14 dpi. The peak of clinical sign scores reached by groups V (B), VI (A) and VI (B) ranged from 11 to 13, while groups IV (A) and IV (B) reached highest scores of 8 and 7, respectively (Figure 5.1). The CEO vaccine group (Figure 5.1g) presented the lower clinical sign scores reaching the highest score of 5. The overall cumulative clinical sign scores obtained for groups IV (A), IV (B)

and the CEO vaccine were lower than for groups V and VI (Figure 5.1h). No mortality was observed during the experiment with the exception of one bird from group V (A) at 12 dpi.

**Real Time PCR Taqman Assay (ReTi-PCR).** ILTV DNA was expressed as GCN $\log_{10}$ per sample of the different tissues (Figure 5.2). Results for ReTi-PCR are summarized below for each tissue.

**Eye conjunctiva.** Viral DNA was detected in all inoculated groups (Figure 5.2). For group IV (A), viral DNA was detected between 4 and 14 dpi, with peak of GCN attained at 4 dpi ($10^{4.9}$) (Figure 5.2a). For group IV (B), viral DNA was detected between 2 and 7 dpi, and again at 14 dpi, with peak GCN attained at 5 dpi ($10^{5.4}$) (Figure 5.2b). For the CEO vaccine group, viral DNA was detected between 2 and 7 dpi, with peak of GCN attained at 5 dpi ($10^{4.9}$) (Figure 5.2g). For group V (A), viral DNA was detected between 5 and 9 dpi, and again at 14 dpi, with peak of GCN attained at 9 dpi ($10^{5.1}$) (Figure 5.2c). For group V (B), viral DNA was detected between 2 and 14 dpi, with peak GCN attained at 5 dpi ($10^{5.4}$) (Figure 5.2d). For group VI (A), viral DNA was detected between 4 and 14 dpi, with peak of GCN attained between 4 dpi ($10^{5.4}$) (Figure 5.2e). For group VI (B), viral DNA was detected between 2 and 14 dpi, with peak of GCN attained at 5 dpi ($10^{5.8}$) (Figure 5.2f).

**Trachea.** Viral DNA was detected in the trachea of all ILTV infected groups (Figure 5.2). For group IV (A), viral DNA was detected at 4 ($10^{3.0}$) and 5 ($10^{2.5}$) dpi (Figure 5.2a). For group IV (B), viral DNA was detected only at 5 dpi ($10^{2.5}$) (Figure 5.2b). For the CEO vaccine group, viral DNA was detected at 4 ($10^{2.4}$) and 5 ($10^{2.1}$) dpi (Figure 5.2g). For group V (A), viral

DNA was detected at 5, 7, and 9 dpi, with the highest GCN recorded in the trachea at 9 dpi ($10^6$) (Figure 5.2c). For group V (B), viral DNA was detected at 5 ($10^{3.8}$) and 7 ($10^{2.6}$) dpi (Figure 5.2d). For group VI (A), viral DNA was detected between 4 and 9 dpi, with peak of GCN attained at 4 dpi ($10^{4.6}$) (Figure 5.2e). For group VI (B), viral DNA was detected between 2 and 5 dpi, with peak of GCN attained at 4 dpi ($10^{3.5}$) (Figure 5.2f).

**Sinus cavity.** ILTV DNA was detected on sinuses for all ILTV infected groups (Figure 5.2). For group IV (A), viral DNA was detected between 4 and 11 dpi, with peak of GCN attained at 4 dpi ($10^{6.5}$); IV (B) between 4 and 9 dpi, with peak of GCN attained at 4 dpi ($10^{5.5}$); CEO vaccine group between 2 and 5 dpi, with peak of GCN attained at 4 dpi ($10^{5.7}$); V (A) at 7, 9 and 11 dpi, with peak of GCN attained at 9 dpi ($10^{6.5}$); V (B) between 4 and 11 dpi, with peak of GCN attained at 5 dpi ($10^{5.7}$); VI (A) between 4 and 9 dpi, with peak of GCN attained at 5 dpi ($10^{4.9}$); and VI (B) between 2 and 9 dpi, with peak of GCN attained at 5 dpi ($10^{6.1}$).

**Cecal tonsils and cloaca.** ILTV DNA was detected on the cecal tonsils for chickens in groups IV (B) and the CEO vaccine at 4 dpi; V (A) at 5, 7 and 9 dpi; and on groups V (B), VI (A) and VI (B) at 4 and 5 dpi. No viral DNA was detected on the cecal tonsils for chickens in group IV (A). ILTV DNA was detected in the cloaca of chickens in groups: IV (B) at 5 dpi; CEO vaccine at 4 dpi; V (B) at 5 and 9 dpi; VI (A) at 4dpi; and VI (B) at 5 dpi. No viral DNA was detected in the cloaca of chickens from groups IV (A) and V (A).

**Trigeminal ganglia and lung.** ILTV DNA was detected in the trigeminal ganglia for all groups: For IV (A) between 4 and 7 dpi; IV (B) at 4 and 5 dpi; CEO vaccine between 2 and 5

dpi; V (A) at 5 dpi; V (B) at 4; VI (A) and VI (B) at 4 and 5 dpi. ILTV DNA was detected in the lung for groups IV (B) at 2 dpi, and VI (A) at 4 dpi.

**Thymus, bursa, spleen.** ILTV DNA was detected on the thymus for all ILTV infected groups. For IV (A), IV (B) and CEO vaccine at 5 dpi; V (A) at 9 dpi; V (B) at 5 and 7 dpi; VI (A) at 5 dpi; and VI (B) at 5 and 7 dpi. No viral DNA was detected on bursa and spleen samples.

Parallel samples collected at similar times post-vaccination from PBSS inoculated birds were all negative by Re-Ti PCR.

**Virus Isolation**. Successful virus isolation from the eye-conjunctiva, trachea and sinuses are indicated in figure 5.2. Virus was isolated from the eye conjunctiva and sinus from all ILTV infected groups. Virus was also isolated from the trachea of chickens in groups V (A and B) and VI (A and B), from the thymus of chickens in groups VI (A) and VI (B). No virus was isolated from cecal tonsils, cloacal, trigeminal ganglia, bursa, and spleen. All samples collected from chickens inoculated with PBSS were negative by virus isolation after three passages in CK cells.

**Histopathology examination of larynx/trachea, eyelid and thymus.** Minimal to mild lesions on mucus glands and epithelium, as well as lymphocytic infiltrates, were observed in the trachea for all ILTV infected groups. Minimal to mild lesions in the trachea were observed between 4 and 9 dpi for groups inoculated with group IV (A, and B), V (B), VI (A, B) viruses and the CEO vaccine. Heterophilic infiltrates in the lamina propria with edema, hemorrhage and presence of syncytial cells with intranuclear inclusion bodies (INIB) were observed exclusively, at 9 dpi, in the trachea of chickens inoculated with group V (A) virus. Syncytial cells with INIB

and fibrinoheterophilic infiltrate were also observed in the larynx of chickens inoculated with viruses IV (B), V (A and B) and VI (A and B). In the eyelid, all ILTV infected groups presented syncytial cells with INIB and lymphoheterophilic blepharoconjunctivitis indicating active viral replication in the conjunctiva. Group V (A) also presented catarrhal conjunctivitis. No lesions were observed in the PBSS inoculated birds. Thymus from chickens inoculated with field isolates and vaccine strain did not present any significant lesions.

**Plaque formation ability.** The percentage of plaques within size ranges of < 100, 100 - 400, > 400 μm was calculated for each viral isolate, the CEO vaccine and the USDA strain (Table 5.1). A 70 to 80% of the plaques measured for group IV and VI viruses measure between 100 to 400 μm, while 74 and 97% of the plaques counted for group V viruses measured more than 400 μm. A 100% of the plaques produced by the USDA strain had a diameter of < 100 μM. Mean plaque size for each isolate, the CEO vaccine and the USDA strain are shown in figure 5.3. No significant differences were observed among the mean plaque diameter calculated for isolates categorized into group IV (A: 156±54 μm; B: 172±56 μm; C (CEO): 173±564 μm), as well as for isolates categorized into group VI (A: 163±34 μm; B: 183±40 μm). On the other hand significant differences were observed in the mean plaque diameters (A: 237±49 μm; B: 306±64 μm) obtained for isolates within group V. Overall group V isolates produced larger plaques than any of the viruses analyzed (Figure 5.3). The USDA reference strain, categorized into group I, presented the smallest mean plaque diameter (55±13 μm), of all the viruses analyzed. Isolate VI (A) presented mean plaque diameter similar to isolates from group IV, and isolate VI (B) presented mean plaque diameter similar to isolates IV (B) and the CEO vaccine.

**One step growth curve**. All isolates were capable to replicate in CK cells as shown by growth curves in figure 5.4. The USDA reference strain (group I), started replication earlier than all other isolates and reached the highest titters recorded. Viral replication was detected as early as 18 hours for isolate IV (B) and the CEO vaccine, for the remaining isolates viral replication was detected after 24 hours. With the exception of the CEO vaccine that reached a titer of log 4.0 pfu/ml, isolates form group IV and V reached titers ranging from log 3.1 to 3.4 pfu/ml after 72 hours. As group V isolates, viral replication for group VI isolates was detected after 24 hours, however isolates from group VI reached the lowest titters in CK cells (2.2 to 2.6 log pfu/ml) after 72 hours as compared with group V and VI viruses (Figure 5.4).

## Discussion

This study presents the pathogenicity and cell culture growth characteristics of ILTV isolates from commercial poultry, representative of PCR-RFLP groups IV, V, VI, as compared to the CEO vaccine. The results revealed that although very similar clinical presentations, ILTV isolates do have different biological properties. All field isolates used in this study were able to produce characteristic clinical signs of the disease when inoculated at a dose of $10^{2.5}$ $TCID_{50}$ per chicken. However, viruses from the different groups showed different levels of disease severity and duration. Cumulative scores reflected that the degree of disease produced by isolates from group IV, and the CEO vaccine were lower than isolates categorized in groups V and VI. The duration of clinical signs varied among groups of isolates, with group V (A) presenting the longest duration of clinical signs among all viruses evaluated. Histopathological analysis revealed that all inoculated groups presented syncytia and inclusion bodies characteristic of

ILTV replication in the conjunctiva and the larynx. However, only chickens infected with the V (A) virus presented syncytia and inclusion bodies in the trachea, which may have contributed to the longer duration and slower clearance of clinical signs for chickens inoculated with the V (A) virus. Although duration and severity of clinical signs were different, all isolates analyzed produced similar clinical signs, characterized by moderate to severe depression, moderate to severe conjunctivitis, and dyspnea. Differently from a previous pathogenicity study, where mortality rates reached 100% (Kirkpatrick *et al.,* 2006), in this study mortality was not significant, and although severe clinical signs were observed, no chickens were culled and clinical signs cleared by 10 dpi. The lack of mortality in this study can also be related to the viral dose administered per chicken and the inoculation route utilized.

The virus distribution in the different tissues was assessed by virus isolation and ReTi-PCR. The virus was most frequently isolated from the eye conjunctiva and sinuses, than from the trachea, in all groups of infected chickens; indicating that these were the main sites of viral replication. Although DNA was detected, no virus was recovered from tracheas of chickens inoculated with group IV (A, B) viruses, nor with the CEO vaccine. In this study the decreased trachea replication for viruses from group IV may be related to the use of two combined administration routes (eye-nostril) that may have decreased the overall infectivity of the exposed sites, and consequently decreased the chance of group IV viruses to reach the trachea. In an earlier study it was shown that the predominant sites for virus replication, after eye drop inoculation, were the conjunctiva, the sinuses and to a lesser degree the trachea (Robertson & Egerton 1981). In a more recent study, the CEO vaccine complete dose was administered via a single eye-drop inoculation, and the vaccine was detected in the trachea as early as 2 to 5 days post-vaccination (Rodríguez-Avila *et al.,* 2007). On the other hand, group VI and V (B) viruses,

applied via the eye-nostril combined route as well, were isolated from the trachea during the peak of clinical signs as previously reported for other ILTV strains (Bagust *et al.,* 1986). However, no severe microscopic pathology was observed in the tracheas of chickens inoculated with these viral strains. In agreement with previous reports (Robertson & Egerton 1981; Kirkpatrick *et al.,* 2006), the severity of clinical signs observed for chickens inoculated with V (B), VI (A), VI (B) viruses did not correlated with the trachea pathology.

In addition to the sinuses, eye conjunctiva and trachea, DNA was detected for all ILTV infected groups in the thymus; however, virus was recovered only from the thymus of chickens inoculated with VI (A) and VI (B) viruses. With the exception of chickens infected with IV (A) virus, viral DNA was detected on cecal tonsils of all other viruses infected groups. As previously reported by Rodriguez *et al.* (2007) the detection of viral DNA in the cecal tonsils agreed with the peak of viral DNA in the eye conjunctiva; however no virus was isolated. It has been reported that different strains of ILTV have the ability to infect macrophages (Calnek *et al.,* 1986). In this case, macrophages and/or other cells of the immune system could carry viral particles to the thymus and cecal tonsils but no active viral replication was detected in these sites.

Growth characteristics in cell culture for ILTV isolates and strains also showed differences between the viral groups. The USDA reference strain replicated more efficiently in CK cells than other viruses, reaching the highest titters, and producing the smallest mean plaque diameter. This strain was derived from a serial of vent brush type laryngotracheitis vaccine manufactured in the early sixty's (NVSL, Ames, IA) and since then it has been expanded and adapted to grow in chicken embryonated eggs, what could explain its high replication efficiency. Isolates categorized into group V showed similar efficiency to replicate in CK cells as the CEO vaccine strain; however they produced the largest mean plaque diameter, suggesting that this

group of isolates is more efficient in cell to cell spread than other commercial poultry isolates and the CEO vaccine. Isolates V (A) and V (B) not only were different clinically (duration of clinical signs), but significant differences were observed in plaque size formation. These results suggest that although categorized in the same PCR-RFLP group, these isolates are phenotypically different. Sequence differences between V (A) and V (B) isolates have been detected in the glycoprotein B (data not shown), however the association of gB changes with the different *in vitro* and *in vivo* characteristics of group V isolates remains to be studied.

Similar to the CEO vaccine, group IV viruses showed equal efficiency to replicate in CK cells, and were less pathogenic than groups V and VI isolates. These results suggest that isolates from group IV (A and B) are not only genetically similar, but share *in vivo* and *in vitro* characteristics with the CEO vaccine strain. Different to group IV and V isolates, and the CEO vaccine, group VI isolates replicated less efficiently in CK cells. Furthermore, although genetically different, group V and VI isolates showed similar pathogenic characteristics. Therefore the genomic differences identified for isolates within PCR-RFLP groups V and VI are probably not associated with their pathogenicity, but may be associated with their *in vitro* growth characteristics. Complete genome sequences of isolates from the different PCR-RFLP groups will be necessary to clearly identify molecular determinants of pathogenicity.

Overall, the evaluation of clinical signs, viral tissue distribution, and viral replication in cell culture showed biological differences among US ILTV isolates previously categorized as different genotypes by PCR-RFLP.

Table 5.1: Plaque size distribution for ILTV isolates, CEO vaccine and the USDA strain.

| Diameter Range (μm) | Plaque Percentage | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | IV (A) | IV (B) | V (A) | V (B) | VI (A) | VI (B) | CEO | USDA |
| < 100 | 8 | 3 | 0 | 0 | 0 | 0 | 7 | 100 |
| 100 - 400 | 80 | 72 | 26 | 3 | 88 | 71 | 71 | 0 |
| > 400 | 12 | 25 | 74 | 97 | 12 | 29 | 22 | 0 |

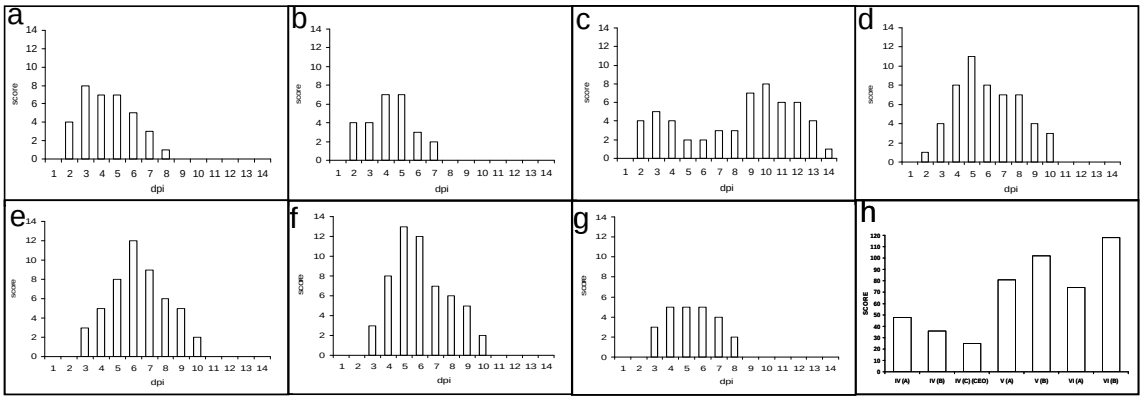


Figure 5.1: Summary of cumulative clinical sign scores recorded daily (a to g) and total scores recorded per group (h); a: IV (A); b: IV (B); c: V (A); d: V (B); e: VI (A); f: VI (B); g: CEO vaccine; h: Cumulative clinical signs by group.

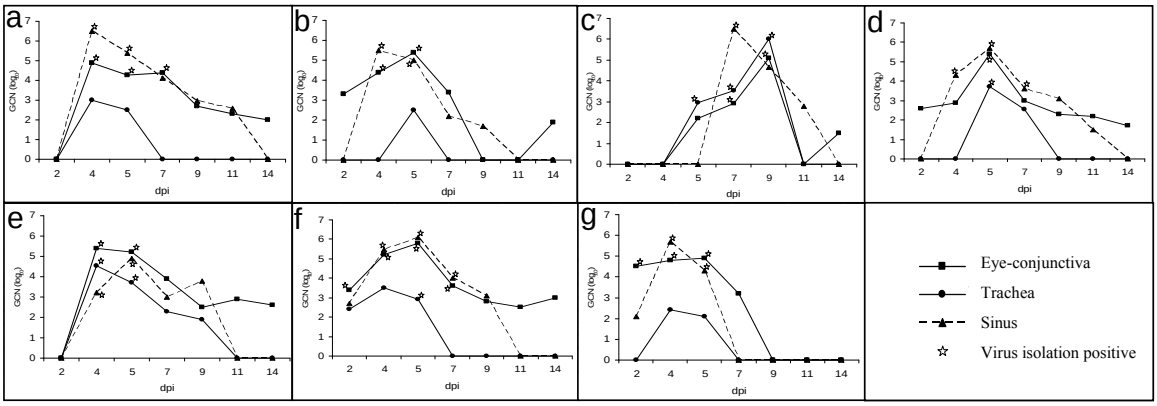


Figure 5.2: Viral genome copy number $\log_{10}$ detected per sample by Real Time PCR Taqman Assay (ReTi-PCR) from trachea, eye-conjunctiva and sinus cavity of chickens inoculated with ILTV isolates; a: IV (A); b: IV (B); c: V (A); d: V (B); e: VI (A); f: VI (B); g: CEO vaccine.

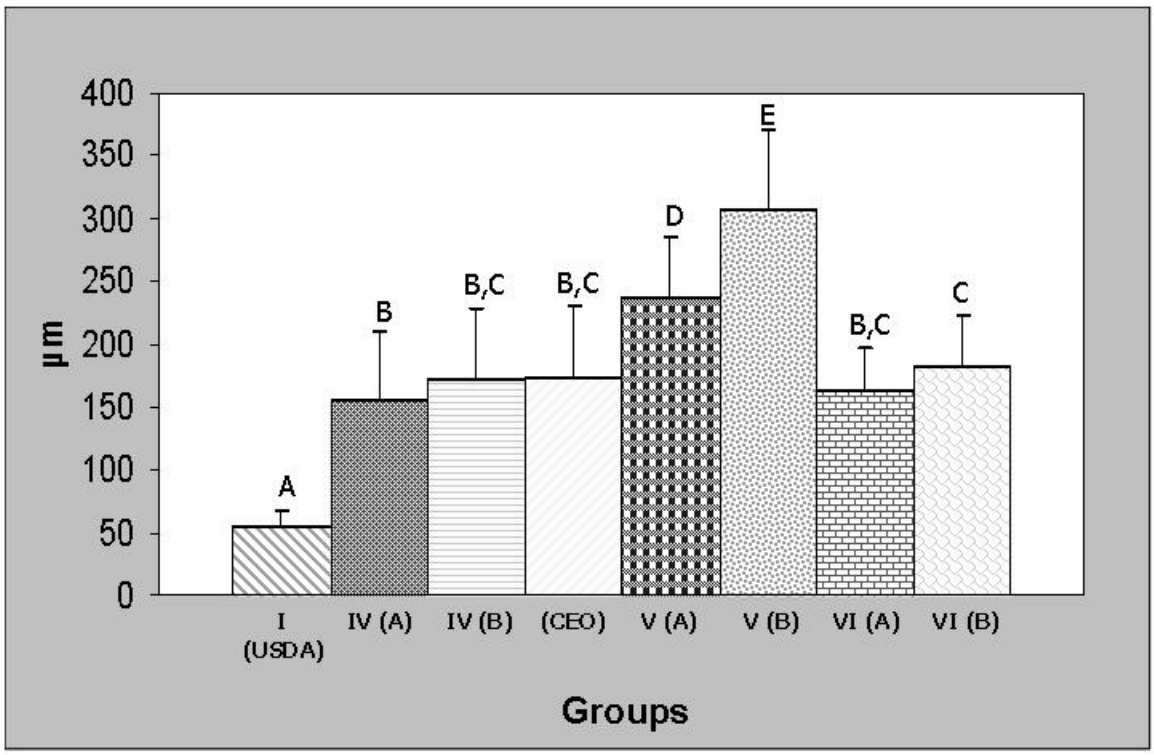


Figure 5.3: ILTV isolates average plaque diameter in LMH cells under semi-solid medium. The average diameters in micrometers (µm) of 100 plaques ± standard deviation was calculated per virus. Different letters represents significant size differences at a p value $\leq 0.05$.

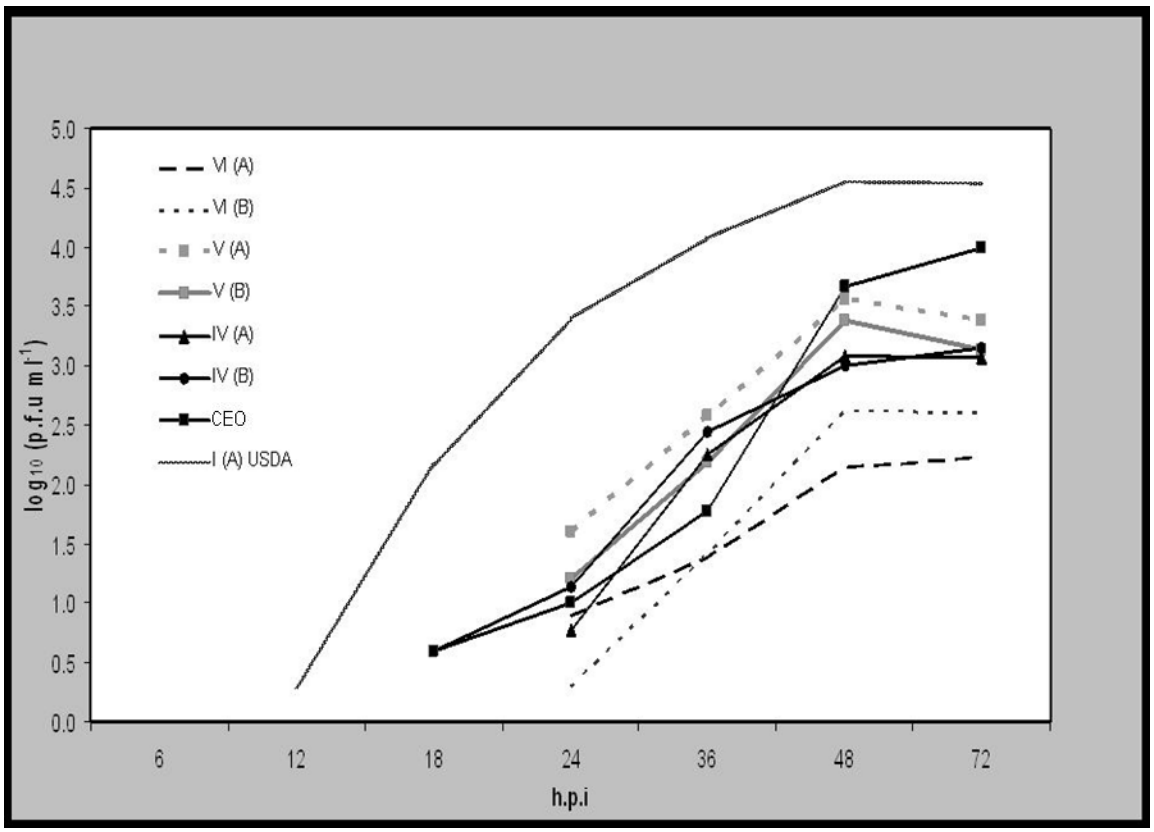


Figure 5.4: One-step growth kinetics for ILTV isolates and strains in chicken kidney cells infected at m.o.i. of 0.004. At the indicated hours post infection (h.p.i) cells were cells and supernatant were collected. Progeny virus titers were determined by plaque assay in LMH cells. The mean $\log_{10}$ pfu/ml of three independent experiments was plotted per time point.

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# CHAPTER 6

# DISCUSSION

Avian infectious laryngotracheitis (ILT) continues to cause cases of respiratory disease in chickens world-wide. While current (modified live) ILT vaccines can offer good protection, the strains of ILTV used in vaccines can also produce latent infections, as well as disease after prolonged bird-to-bird spread in the field. Consequently, the spread of ILTV is attributed to lapses in biosecurity and improper vaccine application.

Our hypothesis is that in the US the intermittent use of CEO vaccines in regions of diverse poultry populations, combined with lax biosecurity, has permitted the emergence of vaccine-related viruses that are well adapted to linger in the field, and incite outbreaks when introduced into naïve flocks. This research concentrated in the study of the molecular and biological characteristics of field isolates involved in ILT outbreaks and their relationship with vaccine strains and the USDA reference strain. Several field isolates and strains were used to identify molecular differences among viruses by PCR-RFLP analysis, which allowed the identification of nine viral genotype groups. Isolates representative of the different PCR-RFLP genotype groups were further selected for sequencing analysis, to study the pathogenicity in specific pathogen free chickens, and to evaluate viral replication and growth characteristics in cell culture.

The first study included the initial genetic characterization of ILTV isolates collected from different regions of dense poultry production in the US.  Single PCR-RFLP analysis was

not enough to separate the field isolates, requiring PCR-RFLP analysis of multiple genome regions to increase the discriminatory power and to precisely differentiate among closely related US ILTV isolates. The development of multiple PCR-RFLP allowed the differentiation of ILTV isolates from commercial poultry in four groups (III, IV, V and VI), and the backyard flock isolates in three separated groups (VII, VIII and IX). Results from this first study also revealed three regions of genetic diversity in the ILTV genome, the ICP4, gM/UL9, and gG/UL47.

Epidemiologically this study confirmed that isolates from PCR-RFLP groups IV and V are closely related to the CEO vaccine strains. These types of isolates were collected from the east, central and western poultry production regions of the US, indicating a widespread prevalence of these viruses in the US. Whether the origin of these isolates is vaccine strains that lost attenuation and consequently persist in the field is still not completely understood. Complete genome sequences of representative isolates and vaccine strains will be necessary to confirm that these isolates are derived from vaccine strains. However, this study provided the initial framework to genetically characterize US ILTV isolates. Not all the commercial poultry isolates analyzed by PCR-RFLP were closely related to the commercial vaccines. A group of isolates (group VI), present in two states, were considerably different from the CEO and TCO vaccines. This result indicated that viruses not related to vaccine were also involved in ILT outbreaks in the US.

In a second study sequencing analysis of ILTV genes that had previously presented genetic variability by multiple PCR-RFLP analysis was performed in order to identify specific genomic differences among US ILTV isolates from commercial poultry, backyard flocks and vaccine strains. In this study the open reading frames (ORF) of the viral glycoprotein genes B (gB), G (gG), M (gM), UL32, the major tegument protein gene UL47, and the immediate early

regulatory gene ICP4 were sequenced for commercial poultry, back yard flock isolates, and vaccine strains. The specific genome differences targeted by the multiple PCR-RFLP analysis and the overall polymorphism of the genome regions were determined. Six ILTV genes (gB, UL32, gM, UL47, gG and ICP4) from representative PCR-RFLP groups isolates were compared to the USDA strain reference sequences. The sequencing analysis was able to group all representative viruses similarly to the multiple PCR-RFLP analyses; particularly it clearly separated commercial poultry isolates within group VI from vaccine related isolates (Groups III, IV, V). This study also revealed specific genomic differences among the analyzed isolates. A deletion of four amino acids (AAQD, at position 87-90) was observed for all isolates categorized into groups IV and V, confirming the close relationship of these two groups, which are considered related to the CEO vaccine by PCR-RFLP analysis. An insertion of six amino acids (PQEPQ at position 862) was observed for commercial poultry isolates from group VI, as well as for backyard flock isolates. These results indicated that commercial poultry isolates from group VI are different from those categorized into groups III, IV and V. Other distinctive sequence changes were observed among isolates within group III, considered closely related to the TCO vaccine.

To evaluate the correlation between the PCR-RFLP viral genotypes and biological differences of these ILTV isolates the pathogenicity and growth characteristics in cell culture of representative isolates from PCR-RFLP groups IV, V, VI, and the CEO vaccine were investigated. The results revealed that ILTV isolates from commercial poultry do have different biological properties, showing different levels of disease severity and duration among the groups. Although duration and severity of clinical signs were different, none of the isolates analyzed

produced unique clinical signs. All isolates were characterized by the production of moderate to severe depression, moderate to severe conjunctivitis, and dyspnea.

Cumulative clinical sign scores reflected that group IV isolates and the CEO vaccine produced milder disease than isolates categorized into groups V and VI. The duration of clinical signs varied among groups of isolates with virus V (A) presenting the longest duration of clinical signs among all viruses evaluated. Histopathological analysis revealed that tracheas collected from chickens infected with the V (A) virus were the only group presenting lesions characteristic of ILTV. Trachea lesions induce by V (A) virus may explain the long duration of clinical signs, and the trachea involvement probably contributed to the slow clearance of clinical signs.

The virus distribution in different tissues was assessed by virus isolation and ReTi-PCR. Virus was most frequently isolated from the eye conjunctiva and sinuses than from the trachea in all groups of infected chickens. This result indicated that these two tissues were the main sites of viral replication. Although DNA was detected, no virus was recovered from tracheas of birds inoculated with both IV (A) and IV (B) viruses, nor with the CEO vaccine. The administration of the dose by two combined routes (eye-nostril) may have decreased the infectivity of group IV and the CEO viruses in the expose sites, and consequently decrease the possibility of these less pathogenic viruses to reach the trachea.

Growth characteristics in cell culture also showed differences between the different viral groups. The USDA reference strain replicated more efficiently in CK cells than other viruses, reaching the highest titters, and producing the smallest mean plaque diameter. Isolates categorized into group V showed similar efficiency to replicate in CK cells as the CEO vaccine strain; however they produced the largest mean plaque diameter, suggesting that this group of isolates has a more efficient cell to cell spread than other commercial poultry isolates and the

CEO vaccine. Isolates V (A) and V (B) not only presented clinical differences in chickens, but also significant differences in plaque size. These results suggest that although categorized in the same PCR-RFLP group these isolates are phenotypically different.

Similar to the CEO vaccine, group IV viruses showed equal efficiency to replicate in CK cells, and were less pathogenic than groups V and VI isolates. These results suggest that isolates from group IV (A and B) are not only genetically, but share *in vivo* and *in vitro* characteristics with the CEO vaccine strain. Different to isolates in groups IV, V, and the CEO vaccine, group VI isolates replicated less efficiently in CK cells. Furthermore, although genetically different, group VI and V isolates showed similar pathogenic characteristics. Therefore the genomic differences identified for isolates within PCR-RFLP groups V and VI are probably not associated with their pathogenicity, but may be associated with their *in vitro* growth characteristics.

In summary, the initial multiple PCR-RFLP study identified genome regions with genetic diversity among US ILTV isolates. Also, the PCR-RFLP study presented the first comprehensive genotyping characterization of a diverse group of isolates from the US and provided a framework for sequencing analysis. Sequencing analysis validated the genotypes identified by PCR-RFLP analysis, identified unique genetic differences for the backyard flock isolates, group VI isolates, and vaccine related isolates (groups III, IV, and V). The evaluation of clinical signs, viral distribution and viral replication in cell culture at different time points presented growth and pathogenicity differences among the different ILTV PCR-RFLP viral groups, and within group V (A and B) isolates. Similar to the CEO vaccine, group IV isolates were less pathogenic than group V and VI isolates. Group VI isolates, characterized as genetically different to the vaccine strains, and was pathogenically similar to group V isolates, which are genetically closely related to the CEO vaccines. Therefore the genomic differences identified for isolates within groups V

and VI are probably not associated with their pathogenicity, and complete genome sequences of isolates from the different PCR-RFLP groups will be necessary to clearly identify molecular determinants of pathogenicity.