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Infectious laryngotracheitis infection in chickens raised in Western Canada: Molecular characterization and vaccine efficacy studies

by

Catalina Barboza Solis

A THESIS

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ABSTRACT

Genomic surveillance of circulating infectious laryngotracheitis virus (ILTV) in specific geographical areas is vital for the control of the disease caused by ILTV, infectious laryngotracheitis (ILT). ILTV is endemic in backyard flocks in some Canadian provinces including Alberta (AB). Sporadic outbreaks of ILTV are reported throughout Canada in both commercial and non-commercial poultry flocks. However, there is a lack of information on the molecular nature of circulating ILTV strains associated with ILT in Canada. Vaccines are used for the control of ILT, and vaccination is employed only in certain provinces due to concerns of limitations of the currently available vaccines. In AB, only breeder flocks are vaccinated routinely in addition to a portion of the backyard flocks. Out of the two commercially available vaccines, the recombinant viral vector vaccines are considered the safest. This is due to their lack of bird-to-bird transmission and reversion to a virulent form.

The first part of the thesis work was focussed on genotyping ILTV isolates linked to ILT clinical cases in AB and British Columbia (BC). Through partial sequencing of open reading frame (ORF) a and b using Sanger sequencing technology, we were able to genotype 27 ILTV isolates from AB and 5 ILTV isolates from BC. We demonstrated that the most common genotype causing ILT outbreaks in AB were chicken embryo origin (CEO) vaccine revertant ILTV strains and then, wild-type ILTV strains. In BC, we identified CEO vaccine and CEO revertant ILTV strains as cause of ILT outbreaks. The second part of this thesis was focussed on determining if recombinant herpesvirus of turkeys- laryngotracheitis (rHVT-LT) commercial vaccine could protect chickens from ILT induced by a wild-type ILTV strain isolated from AB. Our results showed that the rHVT-LT can decrease viral shedding though the oropharyngeal route. However, it did not mitigate clinical signs at the peak of the disease, and it failed to reduce viral replication in the feather tips.

Overall, the work described in the thesis contributed to the knowledge on ILTV molecular epidemiology and vaccine-mediated control of ILT.

Keywords: infectious laryngotracheitis, Sanger sequencing, live attenuated vaccine, rHVT-LT, chicken, back yard poultry

PREFACE

The work described in this thesis was done at the Department of Ecosystems and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada. The work described in this thesis was carried out by me, Catalina Barboza Solis, from July 2019 to May 2020 under the supervision of Dr. M. Faizal Abdul-Careem. Ana Perez Contreras supported me in animal experiments, virus propagation and titration. Shahnas M. Najimuden helped me with animal experiments, tissue processing and lab analysis. Victor A. Palomino-Tapia supported me with vaccination of animals and sequences analysis. Mohamed S. H. Hassan and Sabrina Marsha Buharideen supported me with blood collection for flow cytometry analysis. Ahmed Ali helped me with the examination of the histology sections. This thesis contains materials already published or to be submitted for publication elsewhere, which are listed bellow.

<u>Published Manuscript</u>

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DEDICATION

I would like to dedicate this thesis to my loving parents

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LIST OF ABREVIATIONS

Symbol	Definition
AB	Alberta
AI	Avian influenza
APC	Antigen presenting cell
ARC	Animal Resource Center
ARK	Arkansas
BC	British Columbia
BSA	Bovine serum albuminum
BLAST	Basic Local Alignment Search tool
C	Celsius
CALT	Conjunctival associated lymphoid tissue
CAM	Chorioallantoic membrane
CEF	Chicken embryo fibroblast
CEKC	Chicken embryo kidney cells
CELIC	Chicken embryo liver cells
CEO	Chicken embryo origin
chCXCli2	Chicken chemotactic and angiogenic factor
cm	Centimeter
CO_2	Carbon dioxide
CS	Calf serum
CTL	Cytotoxic T cells
DMEM	Dulbecco's Modified Eagle Medium

dpi	Days post-infection
DSU	Diagnostic Services Unit
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FPV	Fowl pox virus
GaHV-1	Gallid herpesvirus 1
h	Hours
HBSS	Hank’s balanced salt solution
HSACC	Health Science Animal Care Committee
HSV	Herpes simplex virus
HVT	Herpesvirus of turkey
g	Glycoprotein
HG	Harderian gland
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
ICP	Infected cell protein
INF	Interferon
IL	Interleukin
Ig	Immunoglobulin
ILT	Infectious laryngotracheitis
ILTV	Infectious laryngotracheitis virus
IR	Internal Repeat

Kbp	Kilo base pairs
kDA	Kilo Daltons
LMH	Leghorn male hepatocellular carcinoma cell line
μL	Microliter
MASS	Massachusetts
Min	Minutes
mL	Milliliter
MUSCLE	Multiple Sequence Comparison by Log-Expectation
n	Number
NCBI	National Centre for Biotechnology Information
NDV	Newcastle disease virus
nm	Nano meter
NDV	Newcastle disease virus
NK	Natural killer
ON	Ontario
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFU	Plaque forming unit
PHYLM	Phylogenetic inference using Maximum Likelihood
PID	Premises Identification

PK	Proteinase K
PsHV-1	Psittacid herpesvirus type 1
QC	Quebec
RBC	Red blood cells
RFLP	Restriction fragment length polymorphism
rFPV-LT	Recombinant fowl pox virus vector vaccine
rHVT-LT	Recombinant herpes virus of turkeys- laryngotracheitis
RPMI	Roswell Park Memorial Institute medium
SEM	Standard error of means
SNP	Single nucleotide polymorphism
SPF	Specific pathogen free
TCID	Tissue culture infectious dose
TCO	Tissue culture origin
TLRs	Toll-like receptors
TR	Terminal repeat
UL	Unique long
US	Unique short
USA	United States of America
USDA	United States Department of Agriculture

CHAPTER 1: INTRODUCTION

1.1 General introduction

The etiological agent *Gallid herpesvirus 1* (GaHV-1) is also known as infectious laryngotracheitis virus (ILTV), and is responsible of causing infectious laryngotracheitis (ILT), which is an economically important upper respiratory tract disease in chickens (1).

Alberta (AB) has around 10,300 backyard flocks as estimated in 2020 (2). In AB, ILTV is endemic in these non-commercial flocks hence they may act as a constant source of ILTV for potential transmission to naïve chickens and other susceptible species. It has been shown that ILTV could survive in the environment for 3 weeks on carcasses and fomites (3). ILTV spreads through secretions or droplets of infected birds coming into contact with the respiratory tract, oral cavity or eyes of naïve birds (4, 5). There is evidence that shows that ILT outbreaks in unvaccinated broiler flocks could precipitate if ILTV infected backyard flocks are within the radius of a mile (1). Some of the backyard flock keepers move the birds back and forth to trade shows and exhibitions subjecting the birds to stressful events. The stressful events may reactivate latent ILTV leading to viral shedding to the environment (6). ILTV can be shed unto the environment in oropharyngeal contents, feces and as feather dander (7). In AB, only commercial breeders are vaccinated but not the commercial table egg layers and broilers. Consequently, the majority of the commercial flocks are naïve. Given the possibility of ILTV circulation within the backyard flocks, the contamination of environment and lack of immune protection of majority of commercial flocks, the risk of ILT outbreaks in naïve chickens is substantial unless biosecurity measures are optimum. To implement an effective management of the ILT in AB's poultry flocks, characterizing the ILTV linked to the ILT outbreaks is a necessity.

Biosecurity and vaccination are the measures employed by the poultry flock owners to control ILT outbreaks. As the main way of control, there are two types of vaccines commercially available, recombinant viral vector, and live attenuated vaccines. Live attenuated vaccines have two different classification depending on the method of propagation: chicken embryo origin (CEO) (8) and tissue culture origin (TCO) (9). The live attenuated vaccines are effective in managing the disease; however, they possess residual virulence that allows them to replicate, cause disease and/or revert to the original virulent form (10, 11). Also, they establish latency similar to wild-type ILTV and reactivated gaining virulence (10, 11). The alternative option to live attenuated vaccines are the recombinant viral vector vaccines. Two of them are made with a turkey herpesvirus (HVT) and the other one with a fowl pox virus (FPV) as the backbone (12). These vaccines are safer than the live attenuated vaccines but are not as effective as live attenuated vaccines in ILT control (13-16). The Ministry of Alberta Agriculture and Forestry recommends the use of recombinant viral vector vaccines at hatchery and TCO vaccines in the face of outbreaks. TCO vaccines are safer than CEO vaccines as they have a lower transmissibility rate (17-19). There is no experimental data in the effectiveness of recombinant viral vector vaccines against Canadian ILTV strains.

Given the lack of information of molecular nature of ILTV linked to ILT outbreaks in Canada, particularly in AB and efficacy of safer vaccines against these ILTV strains, it is crucial that we characterise ILTV strains linked to ILT outbreaks in AB and evaluate safer vaccines against these ILTV strains. To address these problems, the MSc thesis research was conducted to genetically characterise ILTV strains originating from ILT outbreaks in AB and evaluate recombinant viral vector ILT vaccines against an ILTV strain isolated from a backyard poultry flock.

1.2 Poultry rearing in Alberta

Canada's poultry industry relies on supply management system where the pricing, production and import of poultry products are determined so that the prices are stable for both the consumers and farmers (20). In this way, registered poultry producers may acquire a quota that allows them to produce eggs or poultry meat for a given time. If the farmer decides to work outside the quota system, the number of animals they can raise per year is limited. There are four agencies created since 1972 that establish poultry and egg supply management in Canada, the Chicken Farmers of Canada, the Egg Farmers of Canada, the Turkey Farmers of Canada and the Canadian Hatching Egg Producers (21). These agencies will set the threshold of quota for provinces. Within the province the provincial marketing boards will allocate the quota among their producers, negotiate prices, and set quota transfer rules (22). The poultry marketing boards in AB are the Alberta Chicken producers, the Egg Farmers of Alberta, the Alberta Hatching Egg Producers, and the Alberta Turkey producers. Alberta's poultry industry provides 8031 total jobs and produces $623 million to the gross domestic product. The province's domestic production in 2020 was estimated to be 172.48 million live kilograms and 76.5 million dozen eggs.

1.2.1 Backyard poultry in Alberta

Backyard poultry flocks are also described as small flocks in AB. This term reference to small scale, non-quota/non-commercial poultry production system (23). Non-quota operations are regulated to have less than 300 layers, 2000 or less chickens per year and up to 300 turkeys per year (22). In AB, there are around 10,300 backyard flocks, where the majority are located on acreages (0-40 acres) and run by females between the age of 36-49 years with a background of college/trade education or higher. The majority of these flocks contains less than 50 layer birds

(2). The Animal Health Act states that backyard poultry owners must enrol to obtain a Premises Identification (PID) number. The PID program is part of the Canada's National Livestock Traceability System. Through this system the government of each province effectively locates and notifies owners of emergencies that could have an impact to their poultry flocks such as reportable diseases or natural disasters. The participation in the PID program is mandatory, however, there is no enforcement or policing over by the government. Governmental efforts are targeted to educating the public about their responsibilities. A survey done in 2016 showed that 35.3% of small flocks' owners are not aware of the program (23). A more recent survey in 2020 reported that 27% of backyard flock owners that replied to the survey did not have a PID number (2).

Vaccination practices in these types of flocks vary. Only a low percentage of backyard owners vaccinate their birds. A recent survey of 296 backyard poultry owners reported that ILT had been identified in 3% of the flocks in the past 5 years and 31% were reporting respiratory signs in the past 12 months. This same survey showed that 13% of owners of AB's backyard flocks regularly vaccinate birds against ILT (2). Since 2009, the Alberta Ministry of Agriculture and Forestry recommends backyard flock owners to use TCO vaccines in case of ILT outbreaks.

The most common type of chicken kept by this population are the heritage/fancy breeds. Additionally, the vast majority of owners keep table-egg laying hens out of which 52% are only layers and 48% are a combination of layers and meat birds (2). The rearing of these birds is done using a combination of indoor and outdoor housing. The disposal of birds is usually done in open compost piles (74.4%). Many basic biosecurity measures are usually lacking in these flocks (23).

1.2.2 Commercial Poultry

The majority of commercial poultry flocks rely on indoor production systems and strict biosecurity measures to prevent ILT as opposed to vaccination. Due to thorough biosecurity

practices ILT is uncommon in commercial flocks in AB (2). Nonetheless, the regular appearance of ILT positive cases in backyard flocks are a concern to commercial poultry industry.

AB is one of the major egg producing provinces in Canada and has around 170 layer producers. Layers are housed in different ways in Canada. These housing include the conventional housing, furnished housing, free-run housing and free-range housing. Since December 31, 2014, no new conventional housing systems are allowed to be installed in AB due to animal welfare concerns. Furnished housing is bigger than the conventional facilities and allows the hens to exhibit more of their natural behaviours. Free-run and free-range housing allows free movement within the barn. The latter housing methods also include an outdoor area fenced off and uncovered, with the ground covered with vegetation. Layers are mostly housed in furnished cages, multi-tier floor system or singled-tier floor systems. Farms that have cage systems have around 10,000 – 15,000 hens while non-caged farms have more than 25,000 hens. Birds are mostly kept with LED light with an average of 15.1 ± 0.72 hours of light per day. Around 91% of farmers use controlled ventilation, with temperatures ranging from 19-25°C and relative humidity of 28-70%. Regarding manure management, most farms will do it at the end of the production cycle (24).

AB has 250 regulated broiler producers. These chickens are raised in climate-controlled barns with straw or wood shaving bedding. The most common system to raise broilers is the free-run system, where chickens can move freely in the barn. Broilers can be raised in other types of housings if the animal care requirements are met. Barns will be cleaned out and disinfected after each production cycle. Most broiler produces will sell or use the manure for their own farm for fertilizing crops. Barns are kept at 30-34°C for the first week and it will be lowered 2-3°C per week until they reach 6 weeks of age and temperature is set to 10-27°C. The acceptable humidity

range is 50-70%. Bird density in the barns must be no more than 31 kg/m^2. Broilers take approximately 6-8 weeks to grow to the desired weight (25).

1.3 ILTV classification and morphology

Herpesvirus classification is complex. Viruses within this order have a common morphology and a linear double stranded DNA genome. There are three families in the *Herpesvirales* order: *Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*. Within the *Herpesviridae* family there are three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*. These subfamilies are grouped depending on their common genetic and biological properties (26). The *Alphaherpesvirinae* subfamily have a rapid lytic cycle, lyse infected cells, and establish latent infections in sensory ganglia or peripheral blood mononuclear cells. The *Betaherpesvirinae* subfamily is characterized by a slow replication cycle and delayed cell lysis. The viruses in this subfamily stablish latency in secretory glands, the kidneys and lymphoreticular cells. Lastly, the members of *Gammaherpesvirinae* subfamily are lymphotropic and can establish latency in lymphocytes. Some viruses within this subfamily are linked to oncogenic transformation. The cytolytic infection takes place in epithelial cells and fibroblasts (26, 27).

GaHV-1 is the etiological agent of ILT. It is commonly referred to as ILTV with its old nomenclature. It belongs to the subfamily *Alphaherpesvirinae* and genus *Iltovirus*. Another member of the *Iltovirus* genus is *Psitacid herpesvirus type 1* (PsHV-1), the causative agent of Pacheco's parrot disease. The later disease is a respiratory infection that affects multiple organs in the body including spleen, liver and kidney of psittacine birds (1).

ILTV is a double stranded DNA virus with an icosahedral nucleocapsid and is composed of 162 elongated hollow capsomeres (28). Surrounding the capsid is the tegument enclosed by a

lipoprotein envelope (1). The diameter of the nucleocapsid ranges from 195 to 250 nano meter (nm). Its envelope has a number of glycoprotein (g) spikes on its surface (5).

1.3 Genome

The first large genomic region reported for ILTV was in 1991 (29). With the introduction of new technology, complete genome sequences of attenuated ILTV vaccine and virulent strains have been made available (30). The genome of ILTV ranges from 150 to 155 kilo base pairs (kbp) and it is organized in four distinct regions. These regions are the unique long (UL), unique short (US), internal repeat (IR) and terminal repeat (TR). This last two regions (IR and TR) flank the US region (31) and present in two isomeric orientations (29, 32) (Figure 1.1). The US region has six conserved genes including the gG, gD, gI and gE (33). In the IR an immediate-early gene homologous to the infected cell protein (ICP)4 of other alphaherpesviruses is localized (34). In the right end of the UL region, UL1 to UL5 other homologous genes can be found (35). In the left terminal region of the UL region the UL52 to UL54 are localized (34). The UL region also contains the genes that encode for gB, gC, gD, gE, gH, gI, gJ, gK, gL and gM (36).

Eighty open reading frames (ORFs) can be found in the genome and 79 predicted proteins are encoded (37). Out of these protein coding genes, 65 are located in the UL region, 9 in the US and 6 in the IR region (30). The majority of the ORFs has significant homologies to other mammalian and avian alphaherpesviruses with respect to position and structure (38-40). Among these ORFs, 5 ORFs (a-e) located in the UL region that are unique to the genus *Iltovirus* (41) (Figure 1.1). These specific genes encode protein products that range from 32 to 44 kilo Daltons (kDA) (42). Additionally, genus specific genes of the *Iltovirus* are located between UL-1 and ICP4 (35).

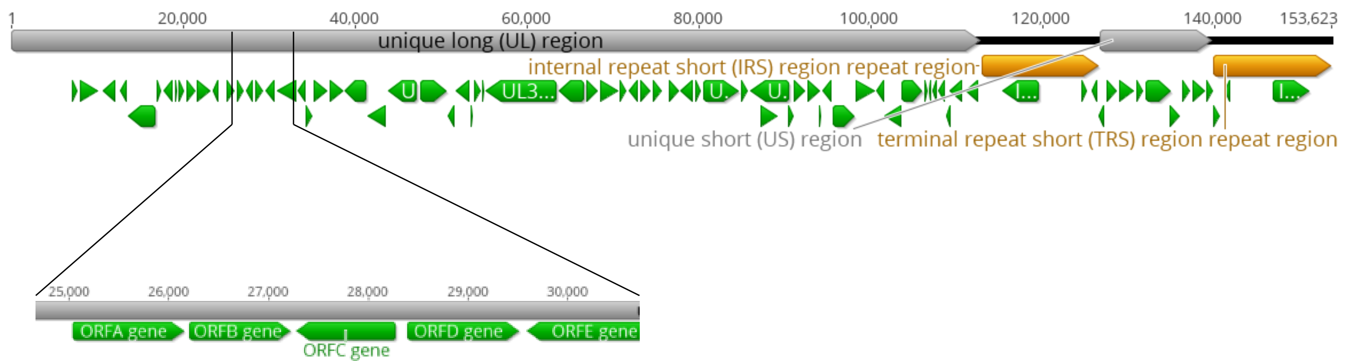

Figure 1.1. Gene map of ILTV strain LT Blen complete genome with closeup to unique region of Iltoviruses, ORF a to e, in the UL genome region. Constructed in Geneious version 10.0 created by Biomatters. Available from http://www.geneious.com using ILTV strain (accession number JQ083493) retrieved from the GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

1.4 Viral replication

ILTV replication is similar to the replication of other alphaherpesviruses (133). A one-step growth analysis of ILTV in chicken embryo kidney (CEK) primary cells, showed that first infectious progeny of viruses are formed between 8 to 12 hours after infection and it reaches a maximum in 24 to 30 hours (43). The replication process is initiated with attachment of several surface glycoproteins followed by fusion of the viral envelope to the host cell membrane (44). Although it is still unknown which specific cell receptor is involved in the attachment of ILTV, there is evidence that show that ILTV gB, gD, gH, gL and mainly gC are involved in viral attachment (45). In herpes simplex (HSV)-1, gC initiates viral attachment to host receptor, but the mechanisms in which it does this are different than the mechanisms of ILTV attachment. This is because ILTV gC lacks the conserved heparin binding domain that HSV-1 uses to attach to the heparan sulfate proteoglycans (46, 47).

Following the fusion of the viral envelope, the nucleocapsid is released into the cytoplasm where it is moved to the nuclear membrane. Then, viral DNA is released and transferred to the nucleus through the nuclear pores (48, 49). Inside the nucleus both transcription and replication take palace in a cascade manner (50). Genes resulted from the transcription are classified according to their order of expression as: immediate early (α), early (β) and late (γ) genes (51). The α genes regulate and play a key role in the expression of the β genes, they also interfere with antigen presentation (52). The β genes are critical for viral replication and also regulate the production of structural proteins encoded by γ genes (50).

Inside the nucleus the capsid proteins are assembled and packed with viral DNA. Then, the newly formed nucleocapsid acquires an envelope by budding out from the inner membrane of the nucleus. Following which, it is taken into the cytoplasm where the nucleocapsid gets a final envelope structure with glycoproteins in a second budding event at the trans-Golgi region. Finally, enveloped virions are released by exocytosis (44).

1.5 Pathogenesis

The ILTV is capable of lysing infected cells or with time, causing degenerative changes in cells due to membrane damages associated with egress (26). Initial replication takes place in the epithelial cells of the conjunctiva, larynx, sinuses and upper respiratory tract following entry of the virus *via* respiratory and ocular routes (1, 53). After natural infection, the incubation period ranges from 6-12 days. In the case of experimental intratracheal infection, the incubation period is much shorter (2-4 days). The peak of viral replication in primary sites is between 4 to 6 days post-infection (dpi). The highest viral loads are observed in trachea and the virus will remains in

secretions between 6 to 10 dpi (54). The lytic cycle results in damaged tracheal and conjunctival epithelia that leads to hemorrhages (55, 56).

Although viremia has not been described yet, there is *in vitro* evidence showing that ILTV can infect macrophages thus providing a potential way of spreading to non-respiratory sites *via* lymphatics (57). ILTV has been isolated in liver, cecal tonsils and cloaca (54, 58). Being a herpesvirus, ILTV causes lifelong latent infection after an initial phase of lytic replication, in the trigeminal ganglion (54) and to a lesser degree in trachea (54). Reactivation may go unnoticed or can lead to ILTV replication in the respiratory tract leading to viral shedding, which provides a source of virus for susceptible birds (6).

1.6 Hosts

Like other herpesviruses, ILTV has a narrow host range. ILTV mainly targets chickens, but it can also infect pheasants and peafowls (57). Additionally, it has been shown that turkeys are susceptible to experimental infection (59). For *in vitro* studies, the chorioallantoic membrane (CAM) of embryonated eggs and primary kidney and liver cells of chickens are used (60, 61). There is only one immortal cell line derived from chicken liver tumors (Leghorn male hepatoma, LMH) that allows ILTV replication (62, 63).

1.7 Transmission

The ILT outbreaks are most observed in high density poultry production systems. The virus is transmitted horizontally (64) and there is no evidence of vertical transmission (65). Horizontal transmission results through direct bird-to-bird contact and/or through indirect transmission through contaminated equipment, cloths and exposure to improperly disposed litter, manure and

carcases (3). ILTV can remain detectable after 3 to 14 days, in swabs left at a range of temperatures (room temperature, 0°C and 5°C). It can also be detected in contaminated litter for 3 to 20 days (3).

It has been observed that farms that have ILT outbreaks are more likely to be within the wind direction of infected flocks (66). Further, it has been observed that infected farms can transmit the virus through the wind up to 1 mile away (1). A recent study using air sampling for detection of ILTV was able to isolate the virus as high as 120 centimeters (67). These data suggests that ILTV is an air-borne pathogen.

ILTV is capable of affecting chickens of any age, reports in United States of America (USA) have documented age ranges as early as 3 weeks of age up to 76 weeks (3). Backyard flocks are an important source of viral transmission to commercial flocks due to poor biosecurity standards and housing multi-age birds (66). These settings are ideal for the maintenance of ILTV in the environment.

1.8 Clinical signs

The severity of the disease caused by ILTV differs depending on the infecting strain. Recent studies have demonstrated that ILTV strains related to CEO vaccines are more virulent than the wild-type ILTV strains (7, 10, 11). The typical clinical signs of ILT include conjunctivitis, nasal discharge, drop in egg production, sinusitis, delay in body weight gain, predisposition to other respiratory pathogens and low mortality (1). The severe infections are characterised by necrosis, hemorrhage and ulceration of larynx and tracheal mucosa leading to the formation of diphtheritic membranes that may obstruct the airway and increased mortality (26). Depend on the severity of the lesions, the course of the disease can be extended. Under ordinary conditions, the

recovery from ILT occur between 10-14 dpi (1). The morbidity and mortality rates also vary, for example, enzootic form of ILT results in high morbidity rates (90-100%), but a variable mortality rate (5-70%). On the other hand, mild enzootic strains have a morbidity rate as low as 5% and a mortality of 0.1%-2% (1).

1.9 Immune response

Virus neutralizing antibodies can be detected between 5 to 7 dpi with a peak at 21 dpi (68). Local antibodies in trachea can be detected 7 dpi and plateau at 10-28 dpi (54, 69). The antibody titers in serum and mucosal surfaces can be correlated to the stage of infection. The number of cells producing immunoglobulin (Ig)A and IgG in trachea increase between 3 to 7 dpi under experimental conditions (69). Nonetheless. there is a poor correlation between the antibody-mediated immune response and protection against ILTV infection (70-73). Additionally, there is evidence that shows that maternal antibodies against ILTV do not protect the offspring against infection or affects vaccination (70). It has been demonstrated that ILTV gG enhances the humoral immune response resulting in an increase of circulating antibodies against ILTV (74) as an immune evasion strategy. These results in increased level of histopathological lesions in the trachea due to poor induction of cellular immune response (57). Other studies have also indicated that this immunomodulating effect of ILTV results in viral persistence in the target tissues (75).

On the other hand, it is well documented that the cell-mediated immune response is effective against ILTV infection (70, 76-78). Additionally, studies suggest that specific T cell subsets have an important role in the resistance against ILT and vaccine mediated protection (70-72). However, the exact mechanisms of the cell-mediated immunity and the interactions it has with the innate immune system are not fully described (71-73, 78). Epithelial cells of the mucosa and

other cells that are part of the innate immune system, recognize ILTV through Toll-like receptors (TLRs). There is documentation that supports that TLR2 can recognize herpesvirus and other DNA viruses. Together with TLR1 or TLR6 it has been shown that TLR2 recognizes gB and gH proteins of ILTV (79, 80). After recognition, a signaling cascade leading to the production of cytokines and chemokines is triggered (81). The cytokines such as chicken chemotactic and angiogenic factor (chCXCli2) and interleukin (IL)-1β are involved in the recruitment of inflammatory cells to the affected tissues. The IL-10 down regulates the inflammatory response thus aiding in the repair of damaged tissue caused inflammatory response. These cytokines and interferon (INF) –α have been detected in *in vivo* studies at the peak of viral replication (3 dpi) (82, 83).

Studies have highlighted that local inflammatory processes have an important role in the pathology of the disease and modulate the response of the adaptive immune response (57). Specifically, it has been demonstrated that local innate immune activation is important in modulating the speed and efficacy of the adaptive immune response (75). *In vivo* studies have demonstrated that acute ILTV infection shift subsets of antigen presenting cells (APC) and lymphocytes in the conjunctival associated lymphoid tissue (CALT) and harderian gland (HG) in early infection (84). A study showed that different tissues had different mechanisms to eliminate ILTV. In conjunctiva, CD8+ cytotoxic T cells (CTLs) and natural killer (NK) cells are the primary cells responsible for ILTV clearance. In the HG, viral clearance is achieved by the generation of IgA-secreting cells, expansion of CD4+ cells and production of Granzyme A by CD8+ CTLs (75).

1.10 Vaccine mediated ILT control

Control efforts against ILT depend on rapid diagnosis, increased biosecurity, usage of geographic data, cleaning and disinfection procedures, vaccination and cooperation and dialogue

between farms (85). Brush vent application was the first method attempted for vaccination; however, it did not prevent viral shedding (86). Currently, the vaccine mediated control of ILT depends on two types of vaccines, recombinant viral vector, and live attenuated vaccines (Figure 1.2).

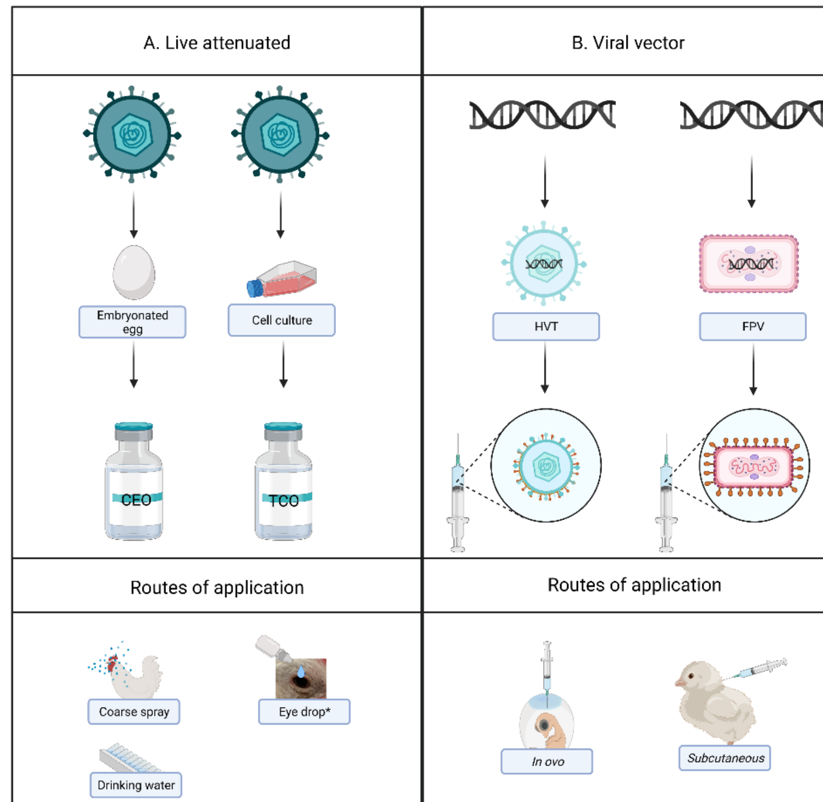

Figure 1.2. The current control of ILT is attempted using two types of vaccines. **A.** Live attenuated vaccines made following propagation in CEO or TCO. **B.** Recombinant viral vectored vaccines are developed using HVT or FPV as vectors. * = TCO vaccine is only licensed to be used via eye drop. Created with BioRender.com.

The live attenuated vaccines are comprised of a less virulent version of the ILTV. The main purpose of attenuation of ILTV is to mimic the type of protective immunity induced after surviving infection, but without leading to serious disease outcomes. With this type of vaccines both

antibody- and cell-mediated immune responses are stimulated (87). Two culture systems are used to make live attenuated vaccines against ILTV. The first one depends on serial passages of ILTV in specific pathogen free (SPF) embryonated eggs and these vaccines are known as CEO vaccines (11). The CEO vaccines are the "gold standard" for protection against a virulent ILTV challenge (1). This vaccine is approved to be used *via* drinking water or coarse spray. The second method of attenuation depends on propagation of ILTV in chicken embryo liver cells (CELIC) and CEK from chicken and turkey origin and these vaccines are known as tissue culture origin or TCO vaccines (Figure 1A). The later vaccine is licensed to be used *via* eye drop (9).

ILT vaccination using live attenuated vaccines is the most effective means of ILT control (1). However, because of their residual virulence, they can replicate, cause mild disease, establish latency and revert to the original virulent form (10, 88). Out of live attenuated vaccines, the TCO vaccines have shown less risk of reversion to virulence (11, 19). However, studies have showed that TCO vaccine strains have acted as parental strains leading to recombinant ILTV strains with higher virulence (89, 90). A common concern of ILT vaccination programs is interference of vaccine induced immune response due to vaccines directed against other poultry diseases. For example, it is possible that multivalent Newcastle disease virus (NDV) and/or the Arkansas (Ark) and Massachusetts (Mass) serotypes of infectious bronchitis virus (IBV) vaccines may interfere with the protection induced by TCO vaccine and CEO vaccines. Additionally, ILT live attenuated vaccines could reduce antibody response elicited by NDV and IBV vaccines (91).

Outbreaks of ILT primarily related to CEO vaccines have been observed in USA (88, 92), Eastern Canada (93, 94) and South America (95). In recent years, reports of recombinant ILTV strains with CEO vaccine have been observed in Egypt, Korea and Australia (37, 89, 96, 97).

Recently, evidence of ILTV recombination in Canada involving TCO and CEO vaccines has surfaced (90).

When compared to live attenuated vaccines, recombinant viral vector vaccines are safer but they are expensive and do not decrease viral shedding (92). FPV and HVT are the two viral vectors utilized in these recombinant vaccines. These viral vector vaccines express ILTV proteins that induce immune response against a ILTV infection. The rFPV-LT (licensed in Canada and manufacture by CEVA Biomune, Lenexa, KS, USA) has the gB and UL32 genes from ILTV (12). The rHVT-LT (licensed in Canada and manufactured by Merck Animal Health, Madison, NJ, USA) carries the gI and gD genes and a more recent one has only the gB gene (licensed in Canada and manufactured by CEVA Biomune, Lenexa, KS, USA) (98). Recombinant vaccines with HVT backbone induce immune response against ILT and Marek's disease whereas recombinant vaccines with FPV backbone induce immune response against ILT and fowl pox (99, 100). The rFPV-LT vaccine was initially recommended for wing web application at 6 weeks of age but was later approved for *in ovo* (at embryo day 18) application. The rHVT-LT vaccine has been licensed to be applied *in ovo* at embryo day 18 and subcutaneously at 1 day of age. Some of the advantages of these recombinant vaccines are the lack of transmission of the vaccine virus between chickens (101), they do not revert to virulence and do not generate ILTV latent infection (102). Viral vector vaccines have shown evidence of decreasing clinical signs; however, they are not as successful as live attenuated vaccines in reducing viral shedding (13).

When comparing the performance of both recombinant vaccines, it has been shown that rHVT-LT is superior to rFPV-LT vaccines (14, 15). Additionally, studies have shown that FPV vectors can be compromised by existing immune response in chickens against the virus (13). Recombinant HVT vaccines can replicate in the chicken's spleen after *in ovo* and subcutaneous

administrations (101). Nevertheless, expression of ILTV antigens in the lungs, following vaccination with rHVT-LT vaccines, is lower thus inducing a lower mucosal immune response in the respiratory tract (99).

The efficacy of rHVT-LT vaccine has been assessed under different experimental conditions. Studies suggest that vaccine efficacy is better against ILT when challenge is done at 7 to 35 weeks of age following vaccination (15, 16, 101, 103). These studies have been shown that the capability of rHVT-LT to decrease viral shedding through the trachea but this decrease did not result in the reduction of transmission (16).

1.11 Surveillance of ILTV infection

To carry out an effective management of ILT outbreaks, the surveillance of circulating ILTV is needed, and this information is required to understand how ILTV evolved over time in a geographical area changing its virulence. In order to accomplish reliable surveillance, rapid sequencing of the ILTV genome is needed. Different molecular assays are utilized to distinguish wild-type strains from vaccine related ILTV, which is essential for ILTV control (104). The polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) targeting multiple ILTV gene regions, is one of the most common assays used in the past (93). However, this is a time-consuming assay, and requires a large amount of DNA (105). There is also a problem with the lack of standardization in cataloging ILTV strains due to the fact that each country using solely the strains circulating in their territory (106). Wild-type and vaccine ILTV strains have shown to have antigenic and genetic homogeneity (only 0.1–0.8% nucleotide sequence difference exist between wild-type and vaccine ILTV strains), which further complicate the discrimination between the two ILTV strains (107).

Sequencing of specific ILTV gene fragments has proven to be useful in the differentiation of wild-type and vaccine related ILTV strains (10, 89, 105, 108). Spatz and colleagues (2019) shown that Sanger sequencing of partial ORF a and b genes of ILTV may be useful in differentiating wild-type ILTV from ILT vaccine related strains (106). Sanger sequencing technology has been demonstrated to be rapid and cost effective than the next generation sequencing when targeting fewer genomic regions (109). This method groups together TCO vaccine related clusters (I, II, III) through their common ancestry (USA Department of Agriculture or USDA ILTV strain). Clusters VII, VIII and IX are also combined through their relationship to Australian ILTV strains.

STATEMENT OF RATIONALE

ILTV infection is endemic in backyard poultry flocks in certain provinces of Canada including AB and Ontario (ON) (94). There are sporadic reports of ILT outbreaks in commercial and backyard flocks in Canada (110-114). However, studies addressing the molecular nature of the ILTV strains associated with clinical disease in Canada are scarce. A few studies focussed on ILT outbreaks occurred in commercial and backyard flocks in ON and characterised the ILTV strains based on partial sequences of UL47 and gG genes of ILTV (93, 94). In Western Canada, particularly in AB and British Columbia (BC), ILT outbreaks involving backyard flocks are occurring annually and the samples are archived at Agri Food Laboratories, AB Agriculture and Forestry and BC Animal Health Center. Since, the Ministry of Alberta Agriculture and Forestry recommends using TCO vaccines for about a decade on the face of ILT outbreaks, it is possible multiple ILTV strains are involved in these outbreaks. Therefore, investigations characterizing ILTV strains linked to ILT outbreaks in AB and BC is needed for deeper understanding of the predominant ILTV strains and to use this information to formulate effective control measures.

In addition to biosecurity, ILT control in Canada relies on vaccination. In Canada, both live attenuated and recombinant viral vector vaccines are commercially available. However, the poultry industry prefers recombinant viral vaccines due to minimal adverse reactions when compared to the live attenuated vaccines (92, 115). In AB, recombinant viral vector vaccines are recommended to be administered at hatchery *in ovo* or on the day of hatch, but their efficacy has not been evaluated against ILTV strains linked to ILT outbreaks in Canada. There is experimental evidence demonstrating that recombinant vaccines used *in ovo* or on the day of hatch, although useful in diminishing clinical signs, are not as efficient as ILT live attenuated vaccines in decreasing viral shedding through respiratory routes in chickens following ILTV infection done at

1 month of age (13, 16, 103, 116). Recently, it has been shown that Canadian ILTV strains are replicating in feather tips and are also, present in cloacal swabs. There is no experimental data that demonstrated the efficacy of ILT recombinant viral vector vaccines against ILTV shedding in feathers and cloacal routes.

HYPOTHESES

The hypotheses of the studies presented in this thesis are:

1. Most of the Canadian ILTV isolates linked to ILT outbreaks in AB and BC are CEO vaccine related.
2. The use of rHVT-LT vaccine against wild-type ILTV linked to ILT outbreaks in AB, will mitigate clinical signs but fail to prevent ILTV shedding.

OBJECTIVES AND EXPERIMENTAL APPROACH

Objective 1

To characterize ILTV isolates associated with several ILT cases in Western Canada recorded during 2009–2018, to discriminate between wild type and vaccine strains.

Key steps involved:

- Obtained archived samples related to ILT outbreaks in AB and BC.
- Extracted nucleic acid from clinical samples.
- Performed PCR targeting ORF a and b on isolates obtained from AB and BC for Sanger sequencing.

- Propagated ILTV positive isolates with low viral loads in SPF embryonated eggs and CELIC before submitting for Sanger sequencing.
- Assembled sequences and done multiple sequence alignments using Geneious software.
- Performed phylogenetic analysis using Geneious software.

Objective 2

Evaluate the efficacy of a commercial rHVT-LT vaccine against ILT induced by a wild-type Canadian ILTV isolate.

Key steps involved:

- Forty-four one-day old SPF chickens were separated into 4 different groups. Two of these groups were vaccinated with rHVT-LT vaccine as prescribed by the manufacturer and the other 2 groups were mock vaccinated with the vaccine diluent.
- At 3 weeks of age, one of the vaccinated and one of the mock vaccinated groups were infected with Canadian wild-type strain, AB-S63 *via* ocular and intra-tracheal routes with an infectious dose of $10^{3.5}$ median tissue culture infective dose ($TCID_{50}$).
- The experimental chickens were monitored for clinical signs for 14 days following ILTV infection.
- At 3, 7, 10 and 14 dpi, oropharyngeal swabs, cloacal swabs and feather tips were collected for ILTV genome quantification, and bodyweights were also recorded.
- At 5 and 12 dpi whole blood was collected to quantify CD4+ and CD8+ T cells in peripheral blood mononuclear cells (PBMCs).
- At the end point (14 dpi), animals were euthanized, and tissue samples collected to quantify ILTV genome loads and for histological observations.

CHAPTER 2: GENOTYPING OF ILTV ISOLATES FROM WESTERN CANADIAN PROVINCES OF ALBERTA AND BRITISH COLUMBIA BASED ON PARTIAL ORF A AND B

2.1 Abstract

ILTV causes an acute upper respiratory disease in chickens called ILT. Live attenuated vaccines are effective in disease control; however, they have residual virulence, which makes them able to replicate, cause disease and revert to the original virulent form. Information is scarce on the molecular nature of ILTV that is linked to ILT in Canada. This study aims to determine whether isolates originating from ILT cases in Western Canada are a wild type or vaccine origin. Samples submitted for the diagnosis of ILT between 2009–2018 were obtained from AB (n = 46) and BC (n = 9). For genotyping, a Sanger sequencing of ORF a and b was used. A total of 27 from AB, and 5 from BC samples yielded a fragment of 1751 bp. Three of the BC samples classified as group IV (CEO vaccine strains) and 2 as group V (CEO revertant). Of the AB samples, 22 samples clustered with group V, 3 with group VI (wild-type), and 2 with group VII, VIII, and IX (wild-type). Overall, 17 non-synonymous SNPs were detected. Further studies are underway to ascertain the virulence and transmission potential of these isolates.

2.2 Introduction

The etiological agent responsible for ILT, an acute upper respiratory tract disease in chickens, is GaHV-1 (ILTV). ILT is most commonly seen in large, high-density production areas where it is horizontally transmitted (64). Enzootic forms of ILT show high morbidity (90–100%), and depending on the infecting strain, more variable mortality (5–70%) can be observed (5). Even

though viremia has not yet been described, in vitro experiments indicated that ILTV can infect macrophages, potentially leading to infection of non-respiratory sites (57). The incubation period following natural infection ranges from 6 to 12 days (117, 118), while experimental intratracheal challenge usually results within 2 to 4 days in clinical signs (119). Lifelong latent infections will be established following the acute phase. This was first described in the trachea (54) and further demonstrated in the recent year (120). However, the predominant site of latency is the trigeminal ganglia (95, 121-123).

Pathogenicity may differ between isolates, but typical acute clinical signs are conjunctivitis, nasal discharge, drop in egg production, sinusitis resulting in decreased body weight gain, and predisposition to other respiratory pathogens (93). In the case of severe infection, inflammation, necrosis, hemorrhage, and ulceration of upper respiratory tract are found on pathology; furthermore, the formation of diphtheritic membranes may obstruct the airways resulting in death from asphyxia (26). ILT can affect chickens at any age, although most often at four weeks of age, or even younger (3).

ILT is endemic in backyard flocks of Canada (124), and infrequent ILT outbreaks are recorded in commercial poultry operations in many parts of the country (93, 113, 114, 125), most recently, (2017–2019) an ILT outbreak in the provinces of ON and Québec (QC) involved commercial chickens (126, 127).

There are two types of ILTV vaccines commercially available: A live attenuated and a recombinant viral vector. Live attenuated virus vaccines can be either CEO or TCO and are satisfactory choices in ILT control (1). On the downside, their ability to replicate and residual virulence can still cause disease (10, 88). Outbreaks of ILT in the USA were traced back to CEO vaccines (88). In Canada, the TCO vaccine and CEO vaccines are licensed and manufactured and

distributed by Merck Animal Health (Madison, NJ, USA) and Merial Select Inc. (Gainesville, GA, USA) respectively. On the other hand, recombinant viral vector vaccines are safer. Although they are more expensive and when compared to live attenuated vaccines, studies have shown that they are not as effective as the CEO vaccine in reducing viral shedding (13, 14, 92). FPV and HVT are the two viral vectors utilized in these recombinant vaccines. The FPV has the gB and UL 32 gene from ILTV (licensed in Canada and manufacture by CEVA Biomune, Lenexa, KS, USA) (12). The HVT carries the gI and D (licensed in Canada and manufactured by Merck Animal Health, Madison, NJ, USA), and a more recent one has the glycoprotein B (licensed in Canada and manufactured by CEVA Biomune, Lenexa, KS, USA) (98). Although all these vaccines are licensed in Canada, usage varies in the different provinces. For example, in AB, recombinant viral vector vaccines and TCO vaccines are recommended by the Ministry of Alberta Agriculture and Forestry since 2009.

There is an array of molecular assays available for the purpose of differentiating wild-type strains from vaccine viruses. One of the most common assays used is the PCR-RFLP that targets multiple ILTV genome regions (17, 128-130). However, this assay is time-consuming and expensive. Recently partial sequencing (using Sanger sequencing technology), of the gene ORF a and b (106), which are unique to the *Iltovirus* genus (31), was developed as a faster alternative. This method enables the differentiation of vaccine and wild-type ILTV strains. In this way, strains are classified as either TCO vaccine-related, CEO vaccine, CEO revertant, or wild-type through six SNPs. The ability to differentiate circulating wild-type and modified live vaccine viruses, is essential for ILT control (104).

Genomic surveillance of the circulating ILTV strains in a geographical area is essential for the development of control measures (109). The most recent genetic data of ILTV strains in Canada

dates from 2006 (93), and this previous study examined samples from a 2004–2005 outbreak of ILT in the Niagara Peninsula in Southern Ontario. This later study demonstrated that both wild-type and CEO vaccine-derived ILTV strains were circulating.

The objective of this study was to characterize ILTV isolates associated with several ILT cases in Western Canada recorded during 2009–2018, to discriminate between wild-type and vaccine strains.

2.3 Materials and Methods

2.3.1 ILTV Isolates

Between 2009 and 2018, qPCR positive clinical ILT samples were collected along with their background information from Agri Food Laboratories, Alberta Agriculture and Forestry, AB (n = 46) (Table 2.1) and from Animal Health Center, BC (n = 9) (Table 2.2). The samples obtained from AB were comprised of tracheas and tracheal swabs in viral transport medium. Samples obtained from BC had been propagated in CEKC from tracheal swabs. Tracheal swabs samples and cell culture samples were aliquoted and stored at −80 °C along with the tissues until further processing.

Table 2.1 Background information of samples from Alberta provided by Agri Food Laboratories, Alberta Agriculture and Forestry.

Samples are arranged by year of submission and the names are color-coded as the phylogenetic tree.

Sample	*Age*	*Sample Submission Year and Month*	*Number of birds in the Flock*	*Number of Birds Affected*	*Mortality*	*Clinical Signs*	*Gross Lesions*
AB-S3-ILTV	6–24 months	oct-13	100	90	15	Sneezing; frothy eyes; runny nose; rattle breathing; swollen heads; crusting eyes.	Mild catarrhal tracheitis
AB-S7-ILTV	Unknown	feb-14	28	6	6	Lethargic, puffy eyes, gurgling breathing and found dead	Fibrinonecrotizing laryngotracheitis, catarrhal sinusitis, conjunctivitis
AB-S11-ILTV	12–16 months	feb-14	50	9	9	Sudden deaths	Fibrinonecrotizing laryngotracheitis
AB-S13-ILTV	7 months	jun-14	60	2	2	Sneezing, watery eyes	Catarrhal sinusitis and tracheitis
AB-S15-ILTV	1.5 months	jun-14	250	10	10	Lethargy, gasping, gurgling, and neck stretching	Hemorrhagic tracheitis
AB-S20-ILTV	10 months	mar-15	150	4	4	Puffed-up eyes, difficult breathing, sudden death	Catarrhal laryngotracheitis
AB-S23-ILTV	7–18 months	apr-15	40	9	9	Sneezing, watery eyes	Tracheitis

AB-S31-ILTV	8 months	apr-15	10	10	1	Sneezing, cough, raspy breathing	Catarrhal and hemorrhagic tracheitis
AB-S35-ILTV	5 months	sep-15	120	Unknown	1	Coughing, bubbly eyes, snotty beak	Catarrhal and hemorrhagic tracheitis
AB-S38-ILTV	4 months	sep-15	20	Unknown	1	Lethargy	Mild catarrhal tracheitis
AB-S41-ILTV	18 months	apr-16	56	22	4	Ruffled feathers, crusty eyes	Hemorrhagic and necrotizing laryngotracheitis
AB-S42-ILTV	15 months	may-16	56	22	4	Gasping, gurgling and difficult breathing	Fibrino-hemorrhagic and necrotizing laryngotracheitis
AB-S44-ILTV	12 months	sep-16	20	Unknown	1	Wheezing respiratory sounds	Catarrhal sinusitis and tracheitis
AB-S45-ILTV	6 months	sep-16	50	4	4	Puffed-up eyes with ocular secretions, conjunctivitis	Fibrinonecrotizing laryngotracheitis
AB-S50-ILTV	2.5 months	oct-16	475	400	40	Difficult breathing, neck stretching and gurgling sounds	Fibrinohemorrhagic and necrotizing laryngotracheitis
AB-S53-ILTV	Unknown	dec-16	30	20	2	Breathing difficulties, sneezing	Catarrhal and hemorrhagic tracheitis; catarrhal sinusitis
AB-S54-ILTV	Unknown	dec-16	30	Unknown	2	Lethargy	Necrotizing laryngotracheitis

AB-S55-ILTV	6 months	dec-16	12	Unknown	1	Swollen eyes with discharge and sneezing	catarrhal sinusitis and necrotizing laryngotracheitis
AB-S56-ILTV	Unknown	jan-17	Unknown	Unknown	8	Sneezing, respiratory distress, conjunctivitis	Laryngotracheitis, caseous conjunctivitis and sinusitis
AB-S62-ILTV	24–36 months	mar-17	100	100	100	Breathing difficulties before death	Fibrinonecrotizing tracheitis
AB-S61-ILTV	24 months	mar-17	50	15	15	Gurgling sounds when breathing	Fibrinonecrotizing laryngotracheitis
AB-S63-ILTV	1.5 months	apr-17	150	5	5	Nasal and ocular secretions, facial swelling and depression	Fibrinohemorrhagic laryngotracheitis
AB-S72-ILTV	3 months	jun-17	70	5	5	Breathing difficulties	Mucohemorrhagic laryngotracehitis
AB-S77-ILTV	20 months	sep-17	150	7	4	Puffy swollen eyes, gasping and neck stretching	Catarrhal and necrohemorrhagic laryngotracheitis
AB-S80-ILTV	7 months	dec-17	100	21	21	Gasping, runny eyes and sneezing	Fibrinonecrotizing laryngotracheitis
AB-S84-ILTV	5.5 months	dec-17	50	5	0	Swollen eyes and sneezing	Fibrinonecrotizing laryngotracheitis
AB-T85-ILTV	10 months	jan-18	120	80	80	Lethargy, swollen eyes and gasping	Fibrinonecrotizing laryngotracheitis
AB-S87-ILTV	24 months	feb-18	8	2	2	Breathing difficulties	Catarrhal sinusitis and tracheitis

Table 2.2 Background information of samples from British Columbia provided by Animal Health Center. Samples are arranged by year of submission and the names are color-coded as the phylogenetic tree.

Sample	***Age***	***Sample Submission Year***	***Type of Flock***	***Type of Operation***	***Flock Size***	***Clinical Signs and History***
CAN/BC-9-3204	38 days	2009	Commercial	Broiler chicken	7000	Submitted fresh broiler tissue for ILT by PCR.
CAN/BC-9-2276	12 weeks	2009	Commercial	Broiler chicken	12,000	Mortality of 50 birds. Noticed gasping and mucus discharge on birds.
CAN/BC-10-1122	11 weeks	2010	Commercial	Layer chicken	45,000	Increased in mortality. Condition suspected: Respiratory.
CAN/BC-12-1949	36 days	2012	Commercial	Broiler chicken	13,000	Submitted fresh broiler trachea and lungs for ILT by virology/PCR and histopathology. Post-mortem findings: fibrinohemorrhagic exudate in trachea and red congested lungs.
CAN/BC-14-6034	6 to10 months	2014	Backyard	Layer chicken	Not available	Submitted two laying hens for post-mortem. All seemed to be healthy. Started losing two birds per week. Sound phlegmy (mucus sounds from throat). Eating and drinking normally. Lost 29 birds in the last 7-10 days

2.3.2 ILTV Propagation in Chicken Embryo Liver Cell

The CELIC were prepared using liver tissues harvested from 14-day-old chicken embryos (131). The maintenance media comprised of Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), and 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Carlsbad, CA, USA). The cells were incubated at 37 °C with 5% CO2, and at >80% confluence, they were infected with 100 uL of the ILTV isolates using DMEM containing 2% calf serum (CS) and 100 U/mL penicillin and 100 µg/mL streptomycin. The inoculum consisted of trachea homogenized in 1 mL of phosphate-buffered saline (PBS, Lonza, Walkersville, MD, USA) and 15 uL of antibiotic (100 U/mL penicillin and 100 µg/mL streptomycin). At five days following infection or when the cytopathic effect was extensive, the cells were harvested by freezing and thawing for 30 min three times. The propagated samples were aliquoted and kept at −80 °C.

PCR targets the ORF a and b partial sequence (USDA reference genome coordinates 21,703–23,895) was used to verify successful virus propagation. Samples that remained negative by PCR after three cell passages, were subsequently propagated in embryonated eggs from the original sample (Table 2.3).

Table 2.3 Samples propagated in cell culture and embryonated eggs and number of passages.

Sample	*Method of Propagation*	*Number of Propagations*
AB-S20-ILTV	CELIC [1]	2
AB-S23-ILTV	CAM [2]	2
AB-S45-ILTV	CELIC	1
AB-S61-ILTV	CAM	1
AB-S63-ILTV	CELIC	2
AB-S84-ILTV	CAM	1
AB-S87-ILTV	CAM	1

[1] CELIC: chicken embryo liver cells. [2] CAM: chorioallantoic membrane.

2.3.3 CAM Inoculation

For the propagation of ILTV in embryonated chicken eggs, 10-day-old SPF eggs were used. Briefly, a small hole was drilled in the air cell, and the egg was placed horizontally, then the air was drawn out with a rubber bulb to create a new, artificial, air cell in which the inoculum was placed using a 25-gauge needle (131). After five days of inoculation, the eggs were placed at 4 °C for 24 h. The infected CAMs were extracted and thoroughly minced, then homogenized with a mini homogenizer, and aliquots were stored at −80 °C. After the CAMs extraction, they were observed for the presence of pock lesions, however none were observed.

2.3.4 DNA Extraction

DNA extraction from tracheal tissues (n = 10), tracheal swabs (n = 15), and cell culture supernatants (n = 7) were performed using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) based on manufacturer's instructions. Briefly, tracheas were homogenized with a mini homogenizer and aliquoted. The volume utilized for the DNA extraction was 200 uL. Later, the extracted DNA was quantified with the Nanodrop 1000 spectrophotometer (ThermoScientific, Wilmington DE, USA) with absorbance at 260 nm.

2.3.5 PCR and Amplicon Purification

Two separate PCR reactions were performed targeting two different regions of ORF a and b. The first reaction, made with a total volume of 50 uL, was based on already published protocol with modifications (106). The reaction included 200 nM of primers ILTVF1_F2 (5′ TTTTGTGCTCATCGCTGTTC3′) and ILTV 1R_R1 (5′CAGCGTTGTGAATT GCTTGT3′) (USDA strain, accession number JN542534) in a reaction containing 2.5 U Taq DNA polymerase

(non-high-fidelity enzyme) per reaction, 0.2 mM of 10 mM dNTP mix, 1X of 10X PCR Buffer, -Mg, 1.5 mM of 50 mM MgCl2 and 100 ng of DNA template (Invitrogen, Burlington, ON, Canada) and resulted in a 1751 bp amplicon. The thermocycler conditions were: Initial denaturation at 94 °C for 2 min, followed by 40 cycles of a three-step amplification protocol: Denaturation at 94 °C for 30 s, annealing at 58 °C and elongation at 68 °C for 60 s each.

However, after sending the samples for sequencing, the laboratory reported that some samples presented secondary structures or loss of signal, making the sequencing incomplete. Thus, a second reaction targeting a 1000 bp amplicon within the first target was performed to fill the gaps in the first obtained sequence. This reaction was similar but with a different set of primers; ILTVF (5′ CGAATGCATCCTTAGACGGG 3′) and ILTVR (5′ AGCTCGAGAAATTGCAGCG 3′) (USDA reference genome coordinates 22,364-23,364). The primers were designed using the Primer3 web version 4.0.0 with the default settings (https://primer3plus.com/primer3web/primer3web_input.htm). For this second reaction, thermocycler conditions were: Initial denaturation at 94 °C for 2 min, followed by 40 cycles of a three-step amplification protocol: Denaturation at 94 °C for 30 s, annealing at 60 °C and elongation at 68 °C for 60 s each. All PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany).

After purification, samples were sent for Sanger sequencing at the University of Calgary Core DNA Services (Calgary AB, Canada). This facility used an Applied Biosystems 3730xl (96 capillary) genetic analyzer.

2.3.6 Phylogenetic Analysis

Sequences were subjected to the Basic Local Alignment Search tool (BLAST) analysis in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm nucleotide identity. A sequence was generated by analyzing the forward and reverse sequences of the first reaction obtained with each sample using the Geneious version 10.0.9 (Biomatters Ltd., Auckland, New Zealand). The sequences obtained from the second PCR reaction were used when a big gap between the reverse and forward sequence of the first reaction was found. The nucleotide sequence of the 32 partial ORF a and b gene segments obtained through this study were aligned with 34 reference ILTV strains downloaded from the GenBank (https://www.ncbi.nlm.nih.gov/genbank/)

The alignment was performed using MUltiple Sequence Comparison by Log-Expectation (MUSCLE) on Geneious version 10.0.9 (Biomatters Ltd., Auckland, New Zealand). To do the analysis, some sequences had to be reduced in size (1599 bp), so that a larger number of samples could be added to the analysis. The reduction was made by ensuring that the critically informative SNPs necessary for genotyping, mention in the previous study [34], would be included.

A phylogenetic tree was generated with Phylogenetic inferences using Maximum Likelihood (PHYML) (132) in Geneious set to 1000 bootstrap replicates using the concatenated sequences.

To identify single nucleotide polymorphisms that led to non-synonymous substitutions, all ILTV strains were translated into amino acids and realigned using multiple sequence alignment MUSCLE on the Geneious version 10.0.9 (Biomatters Ltd., Auckland, New Zealand).

The nucleotide sequences were uploaded to the GenBank (accession numbers are in Table 2.5).

2.4 Results

The background information relevant to the analyzed ILT samples is depicted in Table 2.1 and Table 2.2. BC samples originated from commercial broiler and layer flocks. Only the sample CAN/BC-10-1122, was accompanied by a vaccination history. All the AB samples originated from chickens from non-commercial farms (in AB, less than 2000 broilers and 300 layers per household/per year are allowed without quota, and defined as non-commercial poultry flocks), only sample AB-S15-ILTV came from a vaccinated flock. Samples obtained from AB came from birds with different ages (1.5 to 24 months), as well as samples obtained from BC (36 days to 12 weeks).

Conventional PCR assays conducted targeting two areas of ORF a and b, and the resulting products that were sequenced are shown in Figure 2.1 and Figure 2.2.

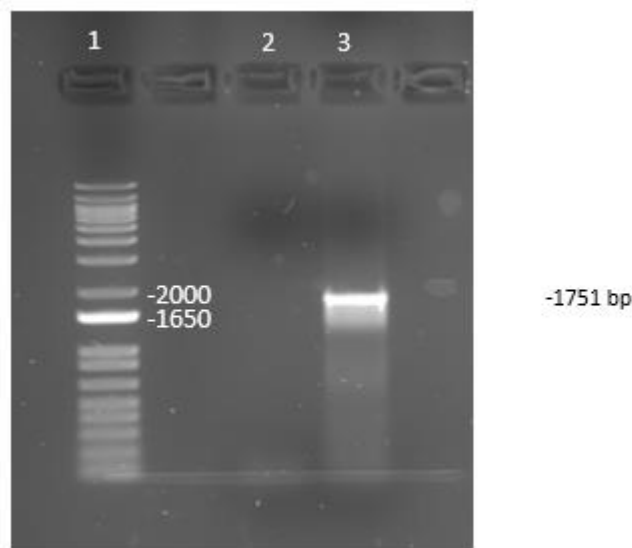

Figure 2.1 Visualization of PCR product run in a 1% agarose gel of PCR targeting ORF a and b. The amplicon size is 1751 bp (USDA reference genome coordinates 21,928–23,679. The DNA ladder used was 1 kbp+. Lane 1 is the DNA ladder. Lane labeled 2 is the negative control. Lane labeled 3 is a known positive sample to ILTV.

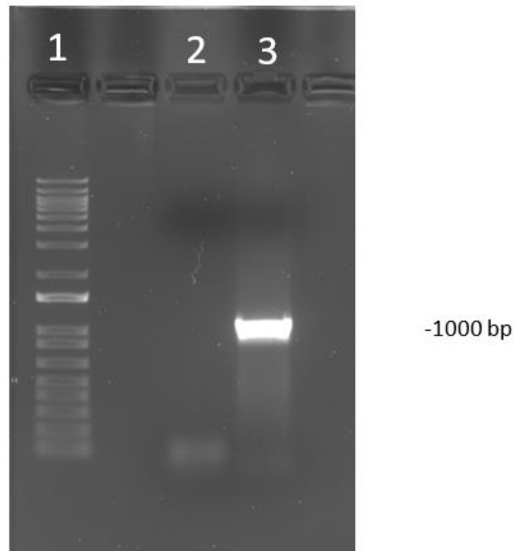

Figure 2.2 Visualization of PCR product run on a 1% agarose gel of PCR targeting ORF a and b (USDA reference genome coordinates 22,364–23,364). The amplicon size is 1000 bp. The DNA ladder used was 1 kbp+. Lane 1 is the DNA ladder. The lane labeled 2 is a negative control. Lane labeled as 3 is a known positive sample to an ILTV.

From the initial 55 samples, only 32 were successfully sequenced. The other 23 were either negative in the PCR for sequencing, or the results after the sequencing were of poor quality. Of the 32 PCR positive samples, 27 were from AB, and 5 from BC (Table 2.4).

Table 2.4 Classification of ILTV isolates from AB and BC is arranged by the year. The table includes the type of flock, province of origin, and if the flock was vaccinated. Sample's name and genotype have been color-coded as the phylogenetic tree.

Isolates	*Origin*	*Year*	*ORF a and b Gene Genotyping*	*Province*	*GenBank Accession Number*	*Vaccinated*
CAN/BC-9-2276	Broiler	2009	IV	British Columbia	MT639625	No
CAN/BC-9-3204	Broiler	2009	V	British Columbia	MT639621	Unknown
CAN/BC-10-1122	Layer	2010	IV	British Columbia	MT639626	Yes
CAN/BC-12-1949	Broiler	2012	IV	British Columbia	MT639627	No
AB-S3-ILTV	Backyard chicken	2013	V	Alberta	MT639620	No
CAN/BC-14-6034	Layer	2014	V	British Columbia	MT639619	Unknown
AB-S7-ILTV	Backyard chicken	2014	V	Alberta	MT639603	No
AB-S11-ILTV	Backyard chicken	2014	V	Alberta	MT639601	No
AB-S13-ILTV	Backyard chicken	2014	V	Alberta	MT639605	No
AB-S15-ILTV	Backyard chicken	2014	V	Alberta	MT639602	Yes
AB-S20-ILTV	Backyard chicken	2015	VII, VIII, IX	Alberta	MT639631	No
AB-S23-ILTV	Backyard chicken	2015	V	Alberta	MT639606	No
AB-S31-ILTV	Backyard chicken	2015	V	Alberta	MT639607	No
AB-S35-ILTV	Backyard chicken	2015	VI	Alberta	MT639628	No
AB-S38-ILTV	Backyard chicken	2015	V	Alberta	MT639608	No
AB-S41-ILTV	Backyard chicken	2016	V	Alberta	MT639609	No
AB-S42-ILTV	Backyard chicken	2016	V	Alberta	MT639610	No
AB-S45-ILTV	Backyard chicken	2016	V	Alberta	MT639611	No
AB-S50-ILTV	Backyard chicken	2016	V	Alberta	MT639613	No
AB-S53-ILTV	Backyard chicken	2016	VII, VIII, IX	Alberta	MT639632	No
AB-S54-ILTV	Backyard chicken	2016	V	Alberta	MT639622	No
AB-S55-ILTV	Backyard chicken	2016	VI	Alberta	MT639629	No
AB-S56-ILTV	Backyard chicken	2017	V	Alberta	MT639604	No
AB-S61-ILTV	Backyard chicken	2017	V	Alberta	MT639623	No
AB-S62-ILTV	Backyard chicken	2017	V	Alberta	MT639624	No
AB-S63-ILTV	Backyard chicken	2017	VI	Alberta	MT639630	No
AB-S72-ILTV	Backyard chicken	2017	V	Alberta	MT639615	No

AB-S77-ILTV	Backyard chicken	2017	V	Alberta	MT639616	No
AB-S80-ILTV	Backyard chicken	2017	V	Alberta	MT639617	No
AB-S84-ILTV	Backyard chicken	2017	V	Alberta	MT639618	No
AB-S85-ILTV	Backyard chicken	2018	V	Alberta	MT639612	No
AB-S87-ILTV	Backyard chicken	2018	V	Alberta	MT639614	No

The resulted nucleotide sequences of the 32 ORF a and b gene segments were aligned with 34 reference ILTV strains (Table 2.5) illustrated in Figure 2.3.

Table 2.5 Reference strains used in phylogenetic study arranged by country of origin.

Isolates	***Origin***	***Year***	***ORF a and b Gene Genotyping***	***Country***	***GenBank Accession Number***
3.26.90	Backyard flock	1990	IV	USA [1]	MF417809
6.48.88	Backyard flock	1988	VII, VIII, IX	USA	MF417810
14.939	Broiler	2014	V	USA	MF417811
1874C5	Broiler	2004	VI	USA	JN542533
J2	Game chickens	2008	VI	USA	MF417808
63140	Broiler	2006	V	USA	JN542536
USDA	Challenge strain	1960	I, II, III	USA	JN542534
81658	Broiler breeder	2010	I, II, III	USA	JN542535
S2.816	Pea fowl	2002	VII, VIII, IX	USA	MF417807
CEO_HPc	CEO [2] high passage	1990	IV	USA	JN580316
CEO_LPc	CEO low passage	1990	IV	USA	JN580317
CEO_TRVX	Vaccine	1983	IV	USA	JN580313
Nobilis Laringovac(R)	CEO vaccine	1975	IV	USA	KP677881
Laryngo-Vac	CEO vaccine	1975	IV	USA	JQ083494
LT-Blen	CEO vaccine	1975	IV	USA	JQ083493
TCO_HP	TCO [3] high passage	1991	I, II, III	USA	JN580314
TCO_LP	TCO low passage	1991	I, II, III	USA	JN580315
TCO-IVAX	Vaccine IVAX	1983	I, II, III	USA	JN580312
VFAR-043	Field Isolate	2014	VI	Peru	MG775218
A20	Vaccine	1966	VII, VIII, IX	Australia	JN596963
ACC78	Broilers/layers	2008	IV	Australia	JN804826
CSW-1	Layer	1970	VI	Australia	JX646899
CL9	Broilers	2008	IV	Australia	JN804827
SA2	Vaccine	1983	VII, VIII, IX	Australia	JN596962
SERVA	European CEO vaccine	2011	IV	Australia	HQ630064
V1-99	Layer	1999	VI	Australia	JX646898
LJS09	Layer	2009	IV	China	JX458822
K317	Layer		IV	China	JX458824
WG	Vaccine	1950	IV	China	JX458823

30678/14/Ko	Commercial flock		IV	Korea	MH937565
0206/14/Ko	Commercial flock		IV	Korea	MH937564
Rus/Ck/Penza/2013/2701		2013	V	Russia	MF405080
757/11	Field Isolate	2011	V	Italy	KP677884
4787/80	Field Isolate	1980	V	Italy	KP677885

[1] USA: United States of America. [2] CEO: Chicken embryo origin. [3] TCO: Tissue culture origin.

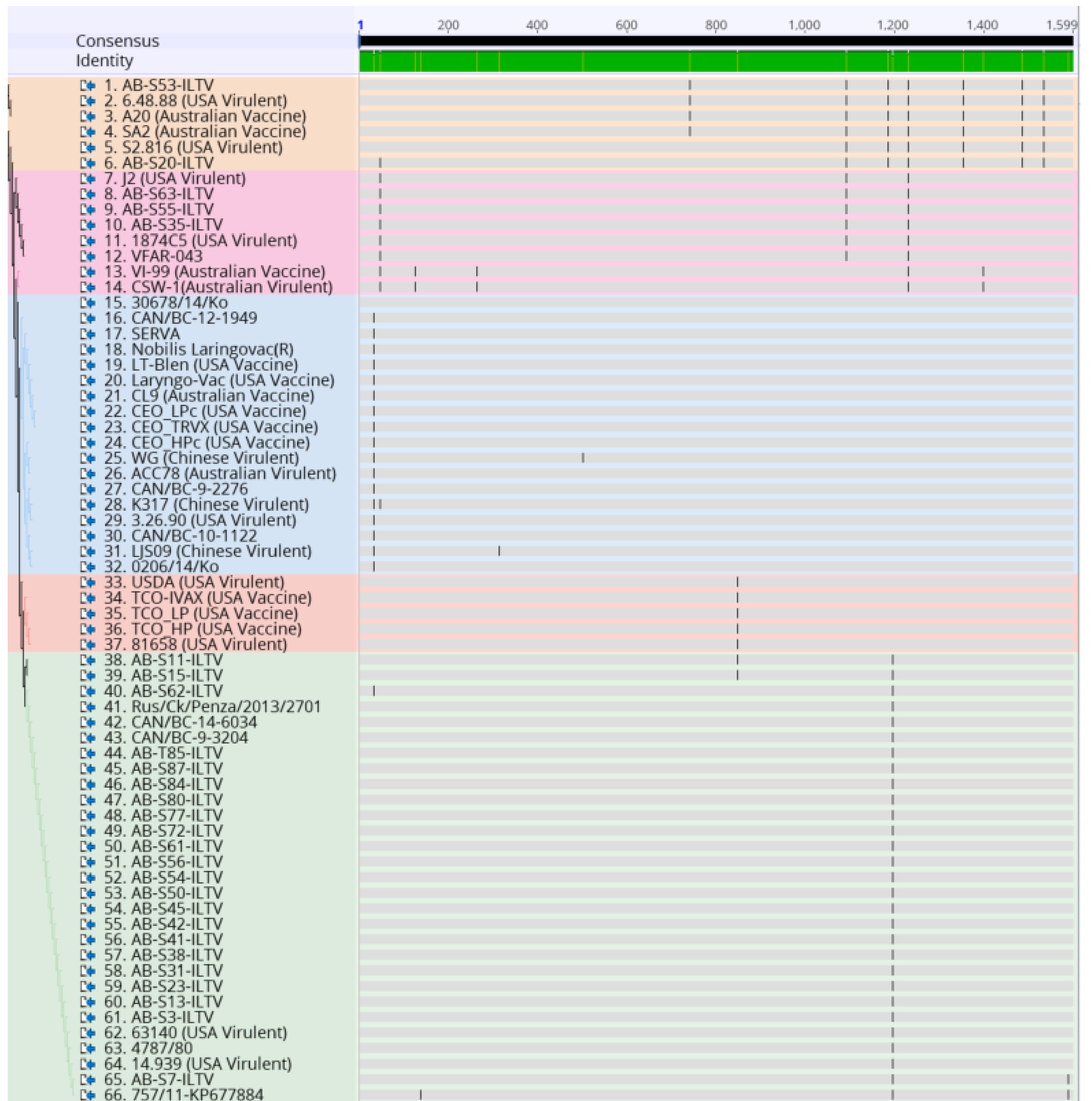

Figure 2.3 Alignment of the nucleotide sequences of 32 Canadian ILTV strains and 34 ILTV reference strains using MUSCLE and Geneious software package. Vertical lines indicate SNP positions. Samples are color-coded as the phylogenetic tree.

Further analysis of amino acid sequences of current ORF a and b segments and reference amino acid sequences are shown in Figure 2.4. Overall, 17 SNPs that lead to non-synonymous substitutions were found in the processed samples and references (Table 2.6).

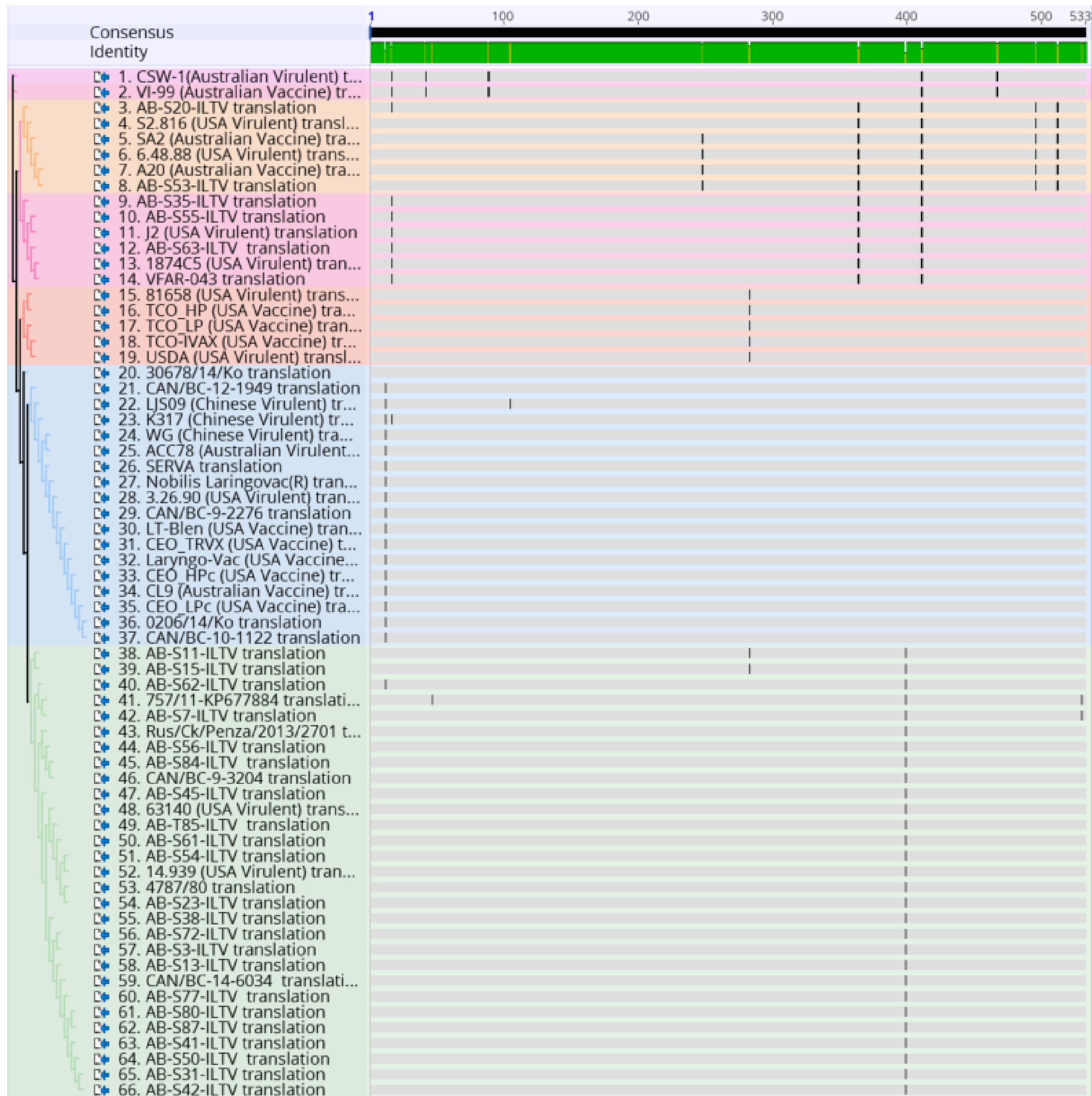

Figure 2.4 Alignment of the amino acid sequence of 32 Canadian ILTV strains and 36 ILTV reference strains using MUSCLE and Geneious software package. Vertical lines indicate nucleotide change positions. Samples are color-coded as the phylogenetic tree.

Table 2.6 Single nucleotide polymorphisms and amino acid change in reference strains and processed samples compared to LT-Blen (CEO genotype) as reference. Changes are recorded as shared with all samples that are classified into a specific genotype or as being present in specific strains.

SNP Position	***Reference Nucleotide***	***Nucleotide Change***	***Type of Nucleotide Change***	***AA Change***	***Reference AA***	***AA Change***	***Genotype and/or Isolates with Same Change***
34	A	C	Tn **	12	Lys	Gln	All except IV
49	G	A	Tr ***	17	Ala	Thr	K317
50	**C***	**T**	Tr	17	Ala	Val	VI
126	T	A	Tn	42	Cys	*	VI-99 and CSW-1
139	C	T	Tr	47	Pro	Ser	757/11
265	G	A	Tr	89	Val	Met	VI-99 and CSW-1
314	T	C	Tr	105	Leu	Ser	LJS09
742	A	G	Tr	248	Thr	Ala	VII, VIII, IX except AB-S20-ILTV and S2.816
848	**T**	**C**	Tr	283	Val	Ala	I, II, III, and AB-S11-ILTV and AB-S15-ILTV
1091	T	C	Tr	364	Val	Ala	VI, VII, VIII, IX
1196	**A**	**C**	Tn	399	Gln	Pro	V
1231	**G**	**T**	Tn	411	Gly	Cys	VI, VII, VIII, IX
1399	G	A	Tr	467	Glu	Lys	VI-99 and CSW-1
1487	A	G	Tr	496	Lys	Arg	VII, VIII, IX
1533	**AG**	**GA**	Tr	512	Ala	Thr	VII, VIII, IX
1588	T	C	Tr	530	Trp	Arg	AB-S7-ILTV and 757/11

* Nucleotide changes in bold—same as already published findings (106). ** Tn—transversion. *** Tr—transition.

These ILTV strains could be grouped into four previously published clusters (106), as shown in Figure 2.5. Of the BC ILTV strains, three were classified in group IV (CEO vaccine), and the remaining two in group V (CEO revertant). Of the AB isolates, 22 clustered in group V, 3 in cluster VI (wild-type), and 2 in cluster VII, VIII, and IX (wild-type) (Figure 2.5).

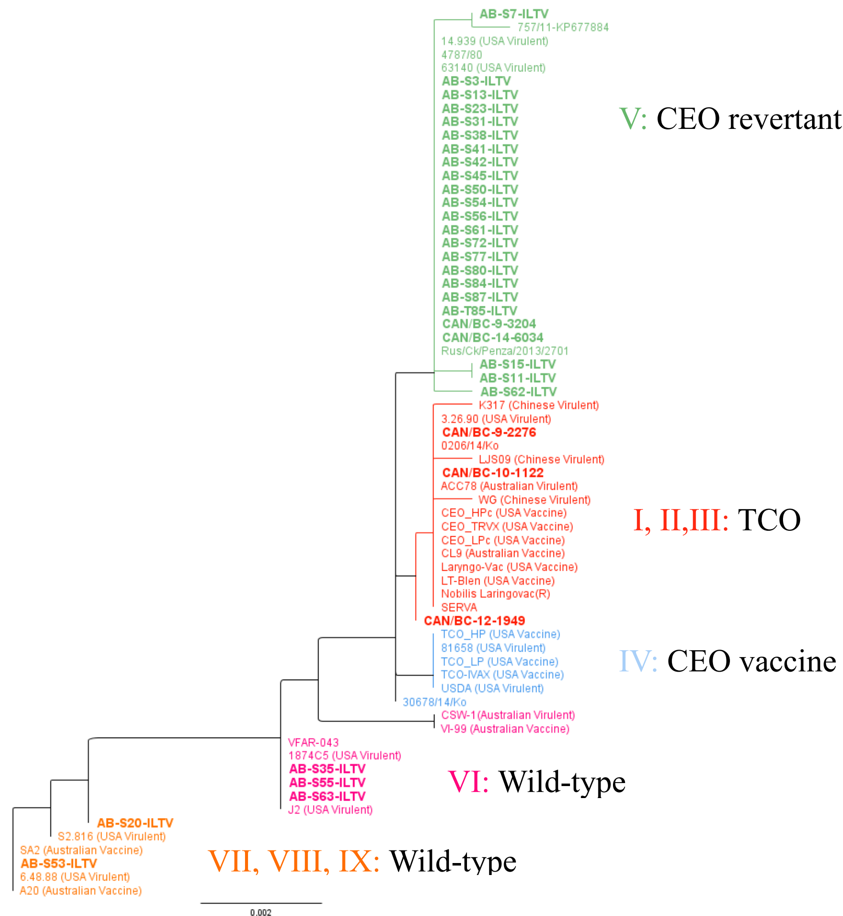

Figure 2.5 PHYML phylogenetic tree with nucleotide sequences of 66 partial ORF a and b of ILTV. The cladogram includes 34 ILTV reference strains downloaded from the GenBank and 32 ILTV Canadian strains characterized in the current study in bold (AB = 27, BC = 5). The genotype nomenclature used is based on (106). Geneious version 10.0 was created by Biomatters. Available from http://www.geneious.com.

Of the totality of the processed samples, none clustered in group I, II, and III (TCO related). Of the samples from AB, none clustered in group IV, whereas three of the BC samples did. The nucleotide identity of ILTV isolates classified in group IV varied from 99.9% to 100%. The BC samples showed the lowest nucleotide identity (99.9%) to the rest of the strains in the group.

The ILTV clustering in group V were 99.8–100% similar. The Italian strain 757/11 was the most distant of the ILTV in group V; however, AB-S7-ILTV showed a 99.9% identity to the Italian strain 757/11. Samples AB-S11-ILTV and AB-S15-ILTV were genetically identical and were 99.9% similar to the rest of the strains in group V. It should be noted that both samples were from the same year and could be the same ILTV strain, however, analysis of more variable genome regions are necessary for confirmation. In group VI, all the sequences obtained were identical except for the Australian strains (99.8%). In the final clusters (VII, VIII, and IX), AB-S53-ILTV was identical to Australian vaccine strains and US virulent strain 6.48.88. In the case of sample AB-S20-ILTV, it was more similar to the US virulent strain S2.816 (99.9%).

2.5 Discussion

The aim of the current study was to characterize the ILTV isolates that originated from various ILT outbreaks in chickens raised in Western Canada. The molecular epidemiology of recent outbreaks of ILTV in chickens from Western Canada is poorly defined. The current study genetically characterized parts of the ORF a and b genes of 32 ILTV isolates collected during ILT outbreaks in Western Canada. It evaluated mostly non-vaccinated flocks, on which the predominant cause for outbreaks were strains related to CEO vaccine strains. These results contribute to the ongoing discussion related to the safety of using the CEO vaccine and the use of safer options like the recombinant viral vector vaccines. Additionally, these findings support the

need to implement better biosecurity measures and effective vaccination strategies in backyard flocks.

ILT outbreaks, related to TCO vaccine strains, are globally reported, but not as frequent as CEO related ones (18, 88, 89, 108). In our study, none of the examined AB and BC ILTV isolates were identify as TCO vaccine strains. This is an interesting finding since TCO vaccines are recommended by the Ministry of Alberta Agriculture and Forestry and used by flock owners since 2009. Our findings are in agreement with previous studies that recorded lower transmissibility of TCO vaccine strains compared to CEO vaccines (17-19). It has also been shown that TCO vaccines do not revert to virulence like CEO vaccines following the passage for 20 times in vivo (11). However, this could be due to low pathogenicity or low transmission of TCO vaccine strains into non-commercial poultry, there is evidence that shows that TCO vaccine strains acted as parental strains contributing to the emergence of recombinant strains with higher virulence (89). To be able to determine if the currently studied ILTV strains are the product of recombination, full genome sequence or sequencing of a bigger genomic region is needed.

On the other hand, CEO related strains are identified in recent outbreaks of ILT in the USA (92), and in ON, Canada (93). In the current study, CEO vaccine ILTV strains were identified in samples from BC but not from AB, possibly because the Ministry of Alberta Agriculture and Forestry recommends the use of TCO and not CEO for backyard poultry producers in the last decade. In addition, Egypt, Korea, and Australia have also reported that some ILTV strains are the result of recombination, with at least one of the parental strains being a CEO vaccine virus (89, 96, 97, 133). To ascertain whether ILTV strains circulating in BC are a product of recombination events, full genome sequence analysis is necessary. Although CEO vaccine viruses are attenuated, bird-to-bird passages can enable the reversion to a virulent state, which can cause disease (11).

Most of the AB samples classified as CEO revertant (Group V) even though CEO vaccines were discouraged from being used for decades. Similarly, a recent study in Argentina found circulating strains were CEO revertants, even though the use of CEO vaccines has been prohibited for the last 10 years (108). The hypothesis was that the CEO vaccine strains may have still been circulating, due to various bird-to-bird passage gaining virulence back (11), leading to ILT outbreaks (108). In AB, ILTV is endemic in non-commercial flocks, and it is possible that CEO vaccines used a decade ago are still circulating because ILTV infection is a lifelong infection (with an established carrier state), and multi-age birds are common in backyard flocks, providing a perfect scenario for the ongoing spread of the CEO related ILTV. These poultry flocks could potentially be constant sources of this virus for naïve chickens, since the movement of birds through long distances, particularly the rare breeds for various shows and competitions, is common. An alternative explanation may be the transmission of CEO vaccines or revertant strains from neighboring provinces with heavy use of CEO vaccines, which was demonstrated in Brazil (95). Studies have shown that the combined usage of live attenuated and recombinant vaccines may produce long term protection against CEO revertant strain 63140 (15). However, to the best of the knowledge of the authors, short term protection of combination or single vaccines against CEO revertant strains have not been studied.

In AB, five of the ILTV isolates were wild-type and not related to ILT live attenuated vaccines. It is well established that live attenuated ILT vaccines prevent clinical signs, but do not completely protect birds from wild-type ILTV replication (92, 95). The ILT outbreaks caused by wild-type ILTV strains are reported in different countries, although to a lesser degree than CEO vaccine-related ILT outbreaks (88, 93, 105). The findings of this study are in agreement with the previous study done in ON (93). Wild-type ILTV caused outbreaks are not uncommon in Canada, but in a

lesser degree than vaccine-related ILT. It would be interesting to compare our data to data of more recent outbreaks in a commercial flock in QC.

Through our analysis, we were able to detect 17 SNPs that were non-synonymous. Previously published data reported six SNPs in positions 50, 848, 1196, 1231, 1533, and 1534 (106) that were critically informative to phylogenetically separate the ILTV groups are also identified in our samples. Additionally, 11 different SNPs were found from which four were shared by specific groups. One was shared by all viruses of group IV in position 34. Two that were found only in group VII, VIII, and IX, and one that was shared by all wild type viruses in position 742. The other seven SNPs were unique to different strains not shared within their group (Table 2.6). At comparing CEO vaccine strains and CEO revertant, they differ in two different mutations. CEO vaccine has a transversion mutation in position 34 (Lys12Gln) that is unique to the genotype; on the other hand, CEO revertant has a transversion mutation in position 1196 (Gln399Pro) that is unique to the cluster. Wild-type clusters share two common SNPs in position 1091 (Val364Ala) and 1231 (Gly411Cys). However, cluster VI has a unique SNP in position 50 (Ala12Val), and cluster VII, VIII, IX has three unique SNPs in position 742 (Leu105Ser), 1487 (Lys496Arg), and 1533 (Ala512Thr).

A previous study has found that mutations on 12 different genes (ORF c, ORF e, ORF f, UL23, UL39, UL36, UL26, UL28, UL17, UL27, UL10, and UL8) that are exclusive to a CEO revertant strain (63140) in comparison to CEO vaccines from the US; however, ORF a and b were not included in this study (64). Additionally, it describes seven non-synonymous substitutions in five genes (ICP4, UL54, UL5, UL38, and UL8) involved in virulence/attenuation phenotypes for the CEO group and three mutations in the UL5 gene for the TCO group.

The ORF a and b are two of the five unique genes in the UL region that characterize the *Iltovirus* genus from other alpha herpesviruses (31). These specific genes encode a protein product of 40 kDa in the case of ORF a and 34 kDa in the case of ORF b (42). According to database searches, they seem to have no significant homologies to other viral proteins and appear to not have conserved motifs that may point to function or location within virions (134). Nonetheless, RNA analysis indicates that they might express during virus infection. It was later found that these genes are dispensable for ILTV replication in tissue culture but might aid in immune evasion or species specificity (41). Another study suggested that ORF a and b function for virus replication might be important but redundant (31). It then would be difficult to ascertain what these specific amino acid changes might do in terms of virulence or immune evasion, as it is still unclear the specific role these genes have. However, if or when, further studies are done to ascertain the functions of these specific genes, these mutations might provide useful information to determine differences between genotypes.

CHAPTER 3: EVALUATION OF A rHVT-LT VACCINE AGAINST GENOTYPE VI CANADIAN WILD-TYPE ILTV INFECTION

3.1 Abstract

In Canada, ILTV infection is endemic in backyard poultry flocks; however, outbreaks are only sporadically observed in commercial flocks. In addition to ILTV vaccine revertant strains, wild-type strains are among the most common causes of ILT. Given the surge in live attenuated vaccine-related outbreaks, the goal of this study was to assess the efficacy of a rHVT-LT vaccine against a genotype VI Canadian wild-type ILTV infection. One-day-old SPF White Leghorn chickens were vaccinated with the rHVT-LT vaccine or mock vaccinated. At three weeks of age, half of the vaccinated and of the mock-vaccinated animals were challenged. Throughout the experiment, weights were recorded, and feather tips, cloacal and oropharyngeal swabs were collected for ILTV genome quantification. Blood was collected to isolate PBMC and quantify CD4+ and CD8+ cells. At 14 dpi, the chickens were euthanized, and respiratory tissues were collected to quantify genome loads and histological examination. Results showed that the vaccine failed to decrease the clinical signs at 6 dpi. However, it was able to significantly reduce ILTV shedding through the oropharyngeal route. Overall, rHVT-LT produced a partial protection against genotype VI ILTV infection.

3.2 Introduction

GaHV-1 or ILTV (by its historic nomenclature) is an alphaherpesvirus belonging to the genus *Iltovirus* and family *Herpesviridae,* which causes ILT in chickens (37). There is also published evidence that shows that ILTV is capable of infecting pheasants, peafowls and turkeys (28, 59, 86, 135). ILT is an acute upper respiratory disease that impacts mainly chickens reared in high-density populations (1). The transmission of ILTV is horizontal; no vertical transmission could be demonstrated (65). The pathogenicity of the virus is dependent on the isolate, age of the bird and the infective dose (1). Chickens of all ages are susceptible to ILTV infection, with reports as early as 3 weeks of age (70). Some of the clinical signs observed are conjunctivitis, nasal discharge, drop in egg production and reduced weight gain (1). As a herpesviruses, ILTV can establish latency mainly in the trigeminal ganglion (123) and to a lesser degree in trachea (54).

In 1925, the first case of ILT was reported in Canada (86); since then, outbreaks in backyard and commercial poultry flocks in the country have been reported (90, 93, 111, 126, 127, 136, 137). In ON and AB, ILT is endemic in backyard poultry flocks that are known to house multi-age chickens and generally have low biosecurity standards. These practices promote ILTV establishment and maintenance, causing a continuous risk for commercial poultry operations (105). To tackle ILT, a combination of biosecurity measures and vaccination strategies were implemented.

The live attenuated vaccine was the first of its type to be commercially available against ILT (8). Based on the method of attenuation, there are two types of live attenuated vaccines: the CEO, derived from consecutive passages in embryonated chicken eggs (8), and the TCO, produced by consecutive passages of the ILTV ASL L-6 strain in primary avian-origin cells (9). Some limitations of the live attenuated ILT vaccines are their ability to regain virulence, following bird-

to-bird passage (10), and establish latency (11). CEO vaccine-related ILTV strains cause frequent ILT outbreaks in North America (92, 93, 136). Additionally, there has been a surge in ILTV recombination involving CEO or TCO vaccinal ILTV strains (89, 90, 96, 136, 138). This has resulted in ILT vaccine failures, leading to ILT outbreaks in vaccinated flocks (139).

There have been several outbreaks of ILT in recent years, predominantly involving CEO revertant and wild-type strains in Canada (90, 136, 140). In AB, outbreaks are more commonly seen in backyard flocks where only 13% are vaccinated against ILTV (2). In addition to the live attenuated vaccines, in AB, recombinant vaccines are recommended to be administered at hatchery in ovo or post-hatch. These viral vector vaccines replicate to a similar degree in the secondary lymphoid organs of chickens, such as the spleen, regardless of the application route (99). There are three recombinant viral vector vaccines licensed in Canada. One uses FPV as a vector, and the other two use a HVT as a backbone. The rFPV-LT vaccine uses gB and UL-32 genes of ILTV as inserts (Vectormune® FP LT+AE, CEVA Biomune, Lenexa, KS, USA) (12). On the other hand, both rHVT-LTs use ILTV gI and gD (Innovax® ILT, Merck Animal Health, Summit, NJ, USA) (99) or the gB and UL-32 genes (Vectormune® HVT LT, CEVA Biomune, Lenexa, KS, USA) (101) as inserts. Some of the advantages of these vaccines are the prevention of bird-to-bird transmission (101) and reversion to virulence (102).

The efficacy of rHVT-LT and rFPV-LT vaccines is variable (13-16, 101, 103). It has been suggested that rHVT-LT has a slower onset of immunity (14, 101) and is most efficacious after 7 weeks of age (101). This prompted us to investigate if an immune response is induced when the vaccinated chickens are infected at 3 weeks post-vaccination, since ILTV can occur earlier. Previous studies have only investigated the humoral immune response, where results showed no correlation between antibodies and protection elicited by vaccination (13, 14, 103). It has been

noted that performing vaccine evaluation studies using field-derived challenge strains closely mimics field conditions (139). At this stage, there are no experimental data on the protective efficacy of the rHVT-LT vaccine against Canadian ILTV field strains. In this study, the main objective was to evaluate the efficacy of a commercial rHVT-LT vaccine against ILT induced by a wild-type Canadian ILTV isolate.

3.3 Materials and Methods

3.3.1 Animals and ethics statement

The HSACC of the University of Calgary, AB, Canada (Protocol number: AC19-0013), approved the use of SPF eggs, embryos, and live chickens. The SPF eggs and SPF chickens were obtained from the Canadian Food Inspection Agency, Ottawa, Canada. The ILTV-infected chickens were maintained in high containment poultry isolators (Plas Labs™, Fisher scientific, Ottawa, ON, Canada) at the Prion/Virology Facility at the Foothills Campus of the University of Calgary. Mock-infected chickens were kept in the ARC at the Foothills Campus of the University of Calgary. The experimental animals were provided with ad libitum access to food and water, and veterinary care was provided by resident veterinarians.

3.3.2 ILTV virus

ILTV AB-S63, which belongs to genotype VI (136), was obtained from the Agri Food Laboratories (Alberta Agriculture and Forestry, Edmonton, AB, Canada). This ILTV strain was isolated from an ILT outbreak in backyard chicken flock in AB, Canada (90). The virus was propagated in the CAM of 10-day-old SPF chicken embryos as previously described (136). The virus stock was titrated in CELIC prepared from 14-day-old chicken embryos as previously

described (7). The $TCID_{50}$ was calculated following the Reed and Muench method (141). Previously, it has been shown that ILTV AB-S63 was virulent and induced ILT in SPF chickens (7).

3.3.3 Vaccine and vaccine titration

The recombinant vaccine, rHVT-LT (Innovax® ILT, Merck Animal Health, Summit, NJ, USA), was used to vaccinate the chickens. The vaccine was titrated in a secondary chicken embryo fibroblast (CEF) monolayer in 60 mm plates. Ten-fold dilutions of the reconstituted vaccine were produced in DMEM, and 200 µL of each dilution was added to three plate replicates. The inoculated cells were incubated at 37°C and 5% CO_2 for 5 days. At day 5 following inoculation, plaques were microscopically counted. Virus titer was calculated and recorded as 6360 plaque forming units (PFU)/per dose.

3.3.4 Experimental design

The SPF eggs were incubated in digital egg incubators (Kingsuromax 20 and Rcom MARU Deluxe max, Autoelex Co., Ltd., GimHae, GyeongNam, Korea) according to the manufacturer's instructions. Upon hatching, 44 1-day-old chickens were randomly assigned to four different groups (n=11 per group). Two groups of chickens were vaccinated with a full dose of rHVT-LT subcutaneously, as prescribed by the manufacturer. The other two groups were mock vaccinated with the vaccine diluent provided by the manufacturer (Merck Animal Health, Summit, NJ, USA). At three weeks of age, one group of the vaccinated and one group of the mock-vaccinated chickens were infected with the ILTV AB-S63 strain. A dose of $10^{3.5}$ $TCID_{50}$ in a total volume of 200 µL per chicken (100 µL was delivered via intratracheal administration and 50 µL per eye mucosal

surface) (7). The other two groups were mock infected with phosphate buffered saline (PBS) (Table 3.1).

Table 3.1 Experimental design.

Group	**n**	***Vaccination (1 day* of age*)***	***ILTV* infection *(3 weeks* of age*)***
MV-MI[1]	11	0.2 ml of vaccine diluent	0.2 ml of PBS
V-MI[2]	11	0.2 ml of Innovax® ILT vaccine	0.2 ml of PBS
V-I[3]	11	0.2 ml of Innovax® ILT vaccine	0.2 ml of AB-S63 ILTV
MV-I [4]	11	0.2 ml of vaccine diluent	0.2 ml of AB-S63 ILTV

[1]Mock vaccinated and mock infected; [2]vaccinated and mock infected; [3]vaccinated and infected; [4]mock-vaccinated and infected.

Following ILTV infection, the chickens were observed twice a day for 14 days to record clinical signs. The clinical signs were scored with a value from 1 to 4 depending on their severity, as described previously (7). Briefly, the clinical signs of ruffled feathers, droopy wings, depression and bodyweight loss received a score of 1. The clinical signs of increased respiratory rate with an open beak and conjunctivitis received a score of 2. A severe increase in respiratory rate, marked by gasping and bloody mucus expectoration, received a score of 3 or 4, respectively. The obtained clinical signs scores were added together to obtain a cumulative clinical score per day. A cumulative clinical score of 4 was considered the humane end point. Oropharyngeal and cloacal swabs were collected at 3, 7, 10 and 14 days dpi. All the collected swabs were placed in 1 ml of viral transport medium DMEM supplemented with 3% FBS, 0.02M HEPES and 0.25 mg/ml of penicillin and streptomycin (Gibco, Carlsbad, 144 California, USA). At the same time points, body weights were recorded, and feather tips were collected for DNA extraction (RNA Save, BI,

Cromwell, CT, USA). The samples were stored at -80ºC until processed. At 5 and 12 dpi, 1.5 ml of blood was collected from the jugular vein with syringes previously filled with sodium citrate (142). The samples were stored on ice until processed for PBMC isolation. At 14 dpi, all the chickens were euthanized, and post-mortem examination was performed to record gross lesions in the respiratory tissues. Trachea and lung samples were collected in 10% buffered formalin (VWR International, Radnor, PA, USA) for histological examination. Additionally, the trachea, lung and spleen were collected in RNA Save (Biological Industries, FroggaBio, Toronto ON, Canada) for ILTV genome load quantification and HVT genome load quantification. At the end of the experiment, serum samples were also collected for the quantification of anti-ILTV antibodies.

3.3.5 Serology

The blood samples were kept overnight at room temperature, and serum was collected following centrifugation at 2500 RPM for 15 minutes (4°C). The serum samples were analyzed for the quantification of anti-ILTV antibodies using a commercial enzyme-linked immunosorbent assay (ELISA) kit (ProFLOCK LT ELISA Kit; Synbiotics Corp., San Diego, California, USA). The sample to positive value (s/p) and titer calculations were performed according to the manufacturer's instructions. This kit does not detect antibodies produced by the rHVT-LT vaccine (99).

3.3.6 DNA extraction and quantitative polymerase chain reaction (qPCR) assay

DNA extraction from swabs, feather tips and tissues (trachea, lung and spleen) were performed using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) based on the manufacturer's instructions. The extracted DNA was quantified with the Nanodrop 1000

spectrophotometer (Thermo Scientific, Wilmington DE, USA). The qPCR assay was carried out using a CFX96-c1000 Thermocycler (Bio Rad laboratories, Mississauga, ON, Canada), as previously described (143). For the ILTV genome load quantification, the primers targeted the proteinase K (PK) gene. For the genome load quantification of the vaccine, the primers targeted the gB gene of HVT (F: 5'- GCC AGT TGG ATA TCT GCC G-3' and R: 5'- CGG CCA ATC ATC GTA GGT AC-3'). Additionally, the β-actin gene (F: 5'–CAA CAC AGT GCT GTC TGG TGG TA–3' and R: 5'–ATC GTA CTC CTG CTT GCT GAT CC–3') was used in each sample to normalize variation in the template amount. The total volume per reaction was 20 µL; this included DNA as a template (20 ng for swabs, 200 ng for tissues and 100 ng for feather tips), 10 pmol/µL of forward and reverse primer, 10 µL of SYBR Green Master Mix (Invitrogen, Burlington, ON, Canada) and DNAse/RNAse-free water (Thermo Scientific, Wilmington, DE, USA). Thermocycler conditions for PK and β-actin genes were as follows: 95 °C for 20 seconds (s) for initial denaturation, then 40 cycles of denaturation at 95 °C for 3 s, annealing at 57 °C for 30 s and elongation at 95 °C for 10 s. Thermocycler conditions for the gB gene were as follows: 95 °C for 20 s for initial denaturation, then 40 cycles of denaturation at 95 °C for 3 s, annealing at 60 °C for 30 s and elongation at 95 °C for 10 s

3.3.7 PBMC isolation

The collected whole blood (1.5 mL) samples were diluted in 2.5 mL of Hank's balanced salt solution (HBSS) and carefully layered into 15 mL SepMate™ tubes (StemCell Technologies, Vancouver, BC, Canada) containing 3.5 mL Ficoll Plaque Premium (GE Healthcare, Chicago, IL, USA). The tubes were centrifuged for 15 minutes (min) at 1200 xg, 20°C. The upper layer consisting of PBMCs was decanted into a 15 ml falcon tube containing 7 mL of HBSS. Following

mixing, the tubes were centrifuged at 400 xg (18°C) for 15 minutes. Following centrifugation, the supernatant was discarded, and the pellet was resuspended in 7 mL of HBSS. The resuspended cells were centrifuged again under the same conditions. Then, the supernatant was discarded, and the pellet was resuspended in Roswell Park Memorial Institute medium (RPMI; Gibco, Carlsbad, CA, USA) containing 1% l-glutamine, 1 % of antibiotic (100 U/mL penicillin and 100 µg/mL streptomycin) and 10% FBS (Gibco, Carlsbad, CA, USA). The PBMCs in each sample were counted using LUNA™ Cell Counting Slides (Logos Biosystems, Annandale, VA, USA) with the Luna™ Automated Cell Counter (Logos Biosystems, Annandale, VA, USA).

3.3.8 Staining for flow cytometry analysis

The obtained PBMCs were strained with monoclonal antibodies as previously described (144). Briefly, cells were washed with 1% bovine serum albumin (BSA) fraction V (OmniPur, EMD, Darmstadt, Germany) made in PBS and centrifuged at 211 xg for 10 min at 4 °C. They were then suspended in 100 µL of 1:100 chicken serum (diluted in 1% BSA) for Fc blocking. After 10 min, the plate was centrifuged at 211 xg for 10 min at 4°C, and the cells were resuspended and incubated in the dark with phycoerythrin (PE)-conjugated mouse anti-chicken CD4 (Southern Biotech, Birmingham, AL, USA) and fluorescein isothiocyanate (FITC)-conjugated mouse anti-chicken CD8 (Southern Biotech, Birmingham, AL, USA). Their respective isotypes were used as controls, and 1% BSA was used for the unstained control. Samples were incubated in the dark for 30 min and spun at 211 xg for 10 min (4°C). The supernatant was discarded, and the pellet was resuspended in 1% paraformaldehyde (145) and submitted to the University of Calgary's Flow Cytometry Core Facility (Calgary AB, Canada). Flow cytometry was performed with a D LSR 11 (BD Bioscience, San Jose CA, USA) with 4 lasers: violet, blue, yellow-green and red laser. Data

analysis was performed with BD FACSDiva software version 6.1.3 (BD Bioscience, San Jose CA, USA).

3.3.9 Histology

Tissue samples of the trachea preserved in 10% formalin were submitted to the Diagnostic Services Unit (DSU) of the University of Calgary Faculty of Veterinary Medicine (UCVM) to produce hematoxylin and eosin (H&E) stained sections.

3.3.10 Data and statistical analyses

The ILTV and HVT viral load quantification was based on standard curves of the PK and gB gene plasmids; in both cases, the β-actin gene was used as a housekeeping gene. For absolute quantification of the genome loads, threshold cycle (Ct) values were plotted against the standard curve data. To determine the starting quantities, the following formula was used:

$$\text{Log starting quantity} = \frac{[Ct - intercept]}{m}$$

$$\text{Starting quantity} = 10^{\text{logstarting quantity}}$$

m=Y-C of the standard curve

The ELISA data processing, sample to positive value (s/p value) and titer calculations were performed according to the manufacturer's instructions. Serum samples with a s/p ratio value of ≤ 0.150 received a "0" titer value and were considered negative for ILT antibody.

The tracheal histopathology was scored by a scale (0-5) developed by Guy and colleagues (146). In this scale, a score of 0 represents normal tissue (thin pseudostratified columnar epithelium and normal mucous glands), a score of 1 represents minimal changes (normal epithelium with mild to moderate infiltration of lymphocytes, rare heterophils, normal mucous glands, no syncytia or

cells with intranuclear inclusion bodies), a score of 2 represents mild changes (mucosa thickened due to mild to moderate cell infiltration and/or an essentially normal epithelium, except for foci of syncytia with intranuclear inclusion bodies; hyperemia, occasionally with cell cuffs), a score of 3 represents moderate changes (mucosa thickened due to moderate to marked cell infiltration; numerous syncytia with intranuclear inclusion bodies; patches of affected epithelium often separating from or, less commonly, sloughed from the lamina propria; mucosal surface well covered by normal or affected epithelium; mucous glands reduced; marked hyperemia; cuffs of mononuclear cells around vessels outside the mucosa), a score of 4 represents severe changes (mucosa thickened due to edema, proteinaceous fluid, cellular exudate or adherent fibrinohemorrhagic to cellular pseudo membrane on the surface; normal epithelium absent; mucosal surface covered by a thin layer of basal cells; syncytia with inclusion bodies sometimes present) and a score of 5 represents very severe changes (same as a score of 4, except that the mucosa has no residual epithelium, and syncytia with inclusion bodies are rarely found).

For the statistical analysis of the data, GraphPad Prism 9.0.0 (GraphPad Software, San Diego, CA, USA) was used. Kruskal–Wallis test and Dunn's multiple comparison test were used to analyze bodyweight data, clinical scores, viral genome load, anti-ILTV antibody titer, CD4+ and CD8+ cell fractions. The percentage of remaining animals following the euthanasia of chickens that reach humane end points were analyzed using the log rank (Mantel–Cox) test and Gehan–Breslow–Wilcoxon test. The group differences were considered significant at $p \leq 0.05$.

3.4 Results

3.4.1 HVT genome loads

The HVT genome loads in spleen samples obtained at 14 dpi are illustrated in Figure 3.1 as an indication of correct rHVT-LT vaccine application. The HVT genome could be quantified in all spleen samples from the vaccinated groups (V-MI and V-I). No HVT genome was detectable in the spleens of the mock-vaccinated groups (MV-MI and MV-I). There was no statistically significant difference between the vaccinated groups (P>0.05).

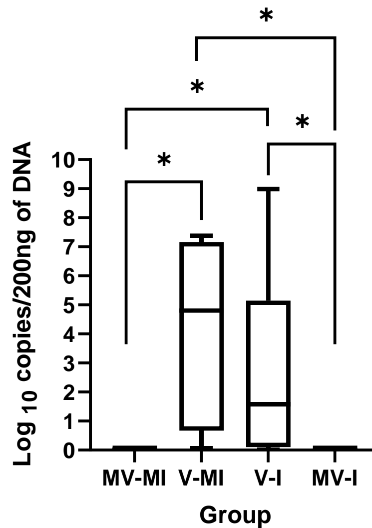

Figure 3.1 rHVT-LT genome load in spleen at 5 weeks post-vaccination (14 dpi) as an indicator of rHVT-LT vaccine successful application. The rHVT-LT genome loads targeting the gB gene of the HVT were quantified using the qPCR technique. The Kruskal–Wallis test followed by Dunn's multiple comparison test was used to compare group differences. *=P<0.05.

3.4.2 Percentages of remaining animals following euthanasia of chickens reaching humane endpoint

The clinical signs were scored. The chicken accumulating a critical clinical score were humanely euthanized and the remaining animals are illustrated as percentages of survival in Figure

3.2a. No difference was found between the groups' percentage of survival. Two chickens were euthanized, one at 5 and the other at 6 dpi. In the post-mortem examination of the one chicken that reached endpoint at 6 dpi, we observed a fibrinous exudate buildup in the lumen of the trachea (Figure 3.2b).

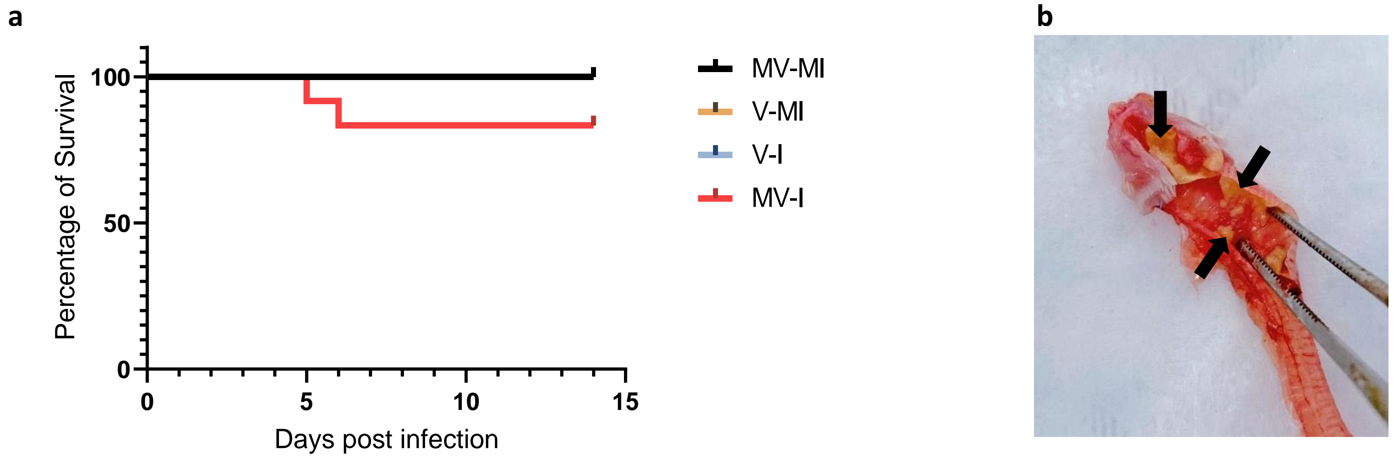

Figure 3.2. Percentage of Survival (a) Percentages of remaining chicken following euthanasia of chickens reaching humane end point. Log rank (Mantel–Cox) test and Gehan–Breslow–Wilcoxon test were performed to identify group differences. (b) Post-mortem examination of trachea of a chicken euthanized at 6 dpi with fibrinous exudate in trachea (black arrows).

3.4.4 Clinical signs

None of the mock-infected animals (vaccinated and unvaccinated) showed clinical signs throughout the experimental period. At 4 dpi, the mean clinical scores of the mock-vaccinated and ILTV-infected (MV-I) group were significantly higher than in the mock-vaccinated and mock-infected (MV-MI) ($P<0.05$), vaccinated and mock-infected (V-MI) ($P<0.05$) and vaccinated and ILTV-infected (V-I) ($P<0.05$) groups. The peak of clinical signs was observed at 6 dpi in both

ILTV-infected groups. At 6 dpi, the vaccinated and ILTV-infected (V-I) groups had higher clinical scores than the mock-vaccinated and mock-infected (MV-MI) ($P<0.05$) and vaccinated and mock-infected (V-MI) ($P<0.05$) groups. The mock-vaccinated and ILTV-infected (MV-I) group also had higher clinical scores than the mock-vaccinated and mock-infected (MV-MI) ($P<0.05$) and vaccinated and mock-infected (V-MI) ($P<0.05$) groups. However, at the peak of the disease (6 dpi) there was no difference between the infected groups (Figure 3.3, $P>0.05$).

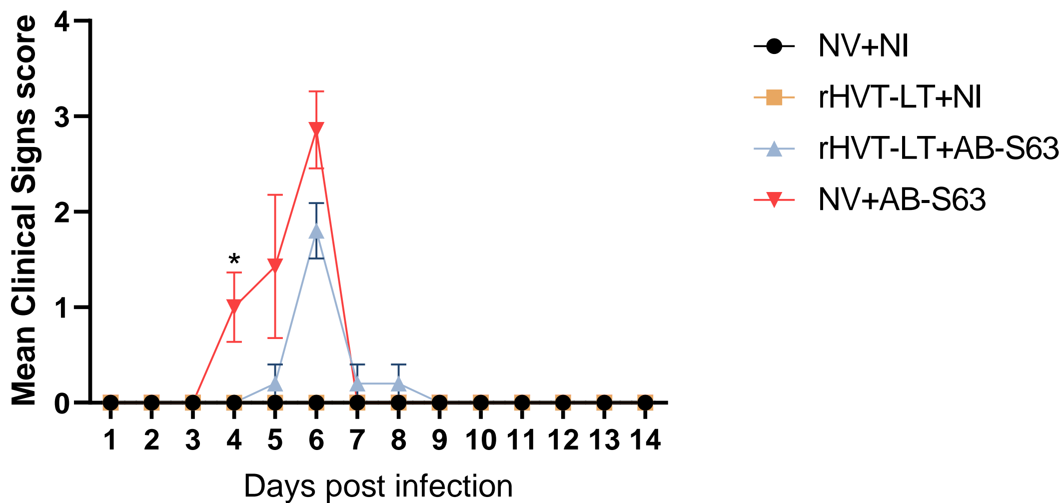

Figure 3.3 The observed mean clinical scores during experimental period. The mean clinical scores are given, and the error bars represent the standard error of means (SEM). Kruskal–Wallis test followed by Dunn's multiple comparison test was performed to compare mean clinical scores of groups. *=$P<0.05$.

The clinical signs observed in the vaccinated and ILTV-infected (V-I) group were open beak respiration, depression, and conjunctivitis. On the other hand, the mock-vaccinated and ILTV-infected (MV-I) group exhibited ruffled feathers, depression, constant open beak respiration, severe dyspnea marked by gasping, bodyweight loss and conjunctivitis.

3.4.5 Bodyweight

Mean bodyweights at 0, 3, 7, 10 and 14 dpi are shown in Figure 3.4. At 14 dpi, the mock-vaccinated and ILTV-infected (MV-I) group had significantly lower mean bodyweights than the mock-vaccinated and mock-infected (MV-MI) group (P<0.05). However, the difference between the vaccinated and infected (V-I) and mock-vaccinated and infected (MV-I) groups was not significant (P>0.05).

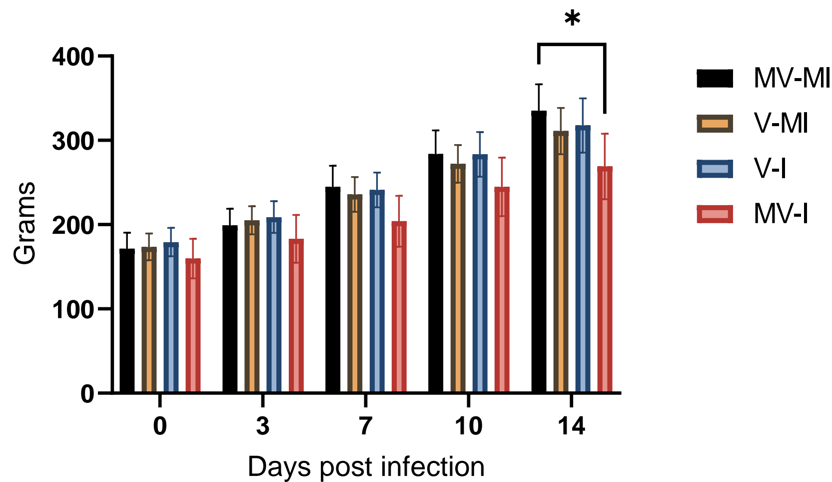

Figure 3.4 Bodyweight gains of experimental chickens at 0, 3, 7, 10 and 14 days post-infection (dpi). Data are presented as mean, and the error bars represent the standard error of means (SEM). Kruskal–Wallis test followed by Dunn's multiple comparison test was used to identify group differences. *=P<0.05.

3.4.6 ILTV genome loads

3.4.6.1 ILTV genome loads in oropharyngeal swabs

The ILTV genome loads in oropharyngeal swabs are illustrated in Figure 3.5a. As expected, the mock-infected groups were negative for the ILTV genome at the observed time

points. At 3, 7 and 10 dpi, the ILTV genome loads in the mock-vaccinated and ILTV-infected (MV-I) group were significantly higher than those observed in the vaccinated and ILTV-infected (V-I) ($P<0.05$), vaccinated and mock-infected (V-MI) ($P<0.05$) and mock-vaccinated and mock-infected (MV-MI) groups ($P<0.05$). At 14 dpi, none of the ILTV-infected groups were positive for the ILTV genome.

3.4.6.2 ILTV genome loads in cloacal swabs

The ILTV genome loads in cloacal swabs are illustrated in Figure 3.5b. Only one bird in the mock-vaccinated and infected (MV-I) group was positive at 3 and 10 dpi. There were no statistically significant differences between the groups at any time point ($P>0.05$).

3.4.6.3 ILTV genome loads in feather tips

The ILTV genome loads in feather tips are illustrated in Figure 3.5c. None of the groups were positive for the ILTV genome at 3 and 14 dpi. There was no difference between the vaccinated and unvaccinated ILTV-infected groups at 7 and 10 dpi ($P>0.05$).

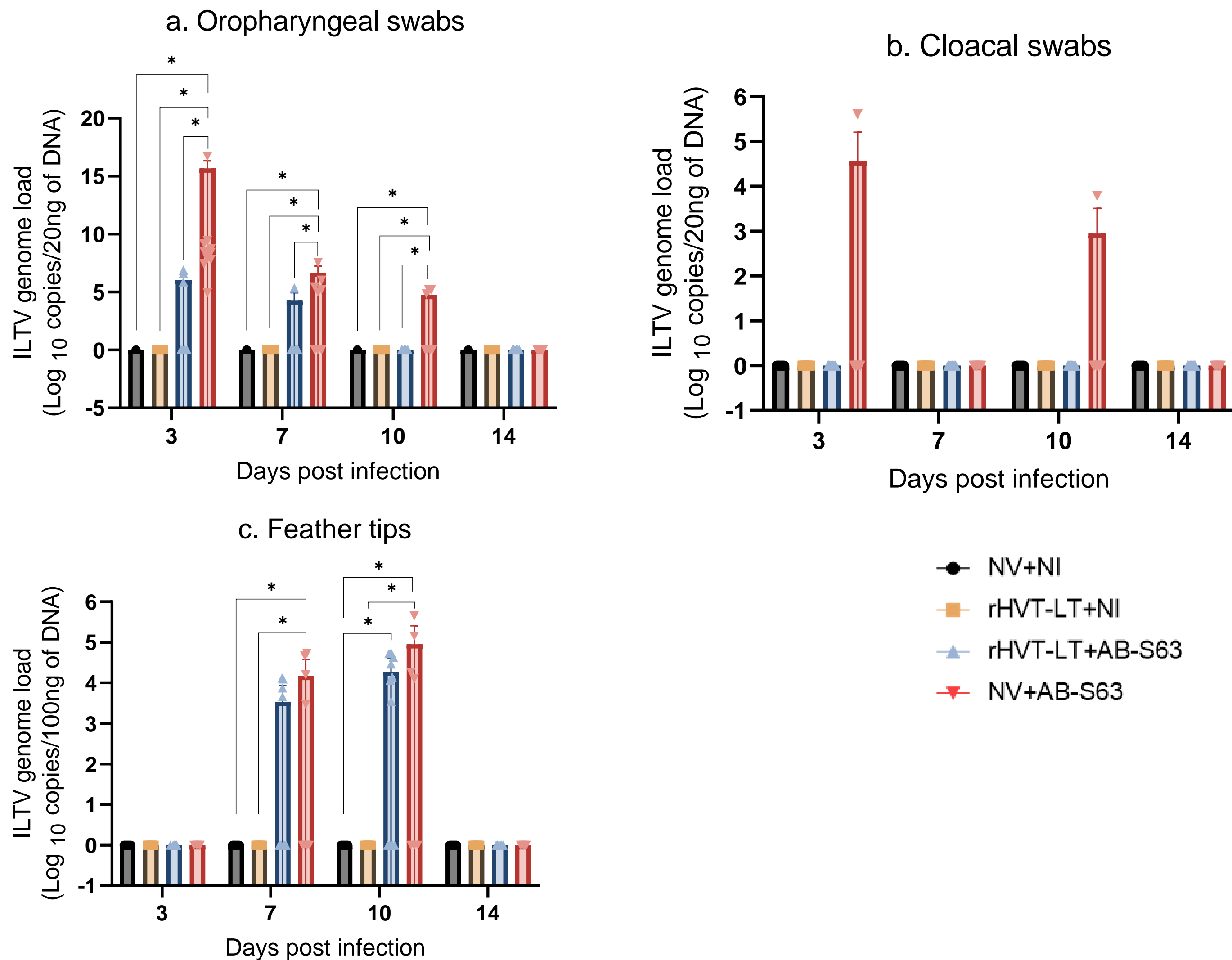

Figure 3.5 ILTV genome loads quantified targeting the PK gene at 3, 7, 10 and 14 days post-infection (dpi). The bars represent the mean, and error bars indicate the standard error of mean (SEM). Kruskal–Wallis test followed by Dunn's multiple comparison test was used to identify group differences. (a) ILTV genome loads in oropharyngeal swabs; (b) ILTV genome loads in cloacal swabs; (c) ILTV genome loads in feather tips. *=P<0.05.

3.4.6.4 ILTV genome loads in trachea and lungs

The ILTV genome loads in trachea and lungs quantified at 14 dpi are illustrated in Figure 3.6a and b, respectively. The mock-vaccinated and ILTV-infected group (MV-I) had higher ILTV genome loads in the trachea when compared to the mock-vaccinated and mock-infected (MV-MI) ($P<0.05$) and vaccinated and mock-infected (V-MI) ($P<0.05$) groups. Similarly, the vaccinated and ILTV-infected (V-I) group had significantly higher ILTV genome loads in the trachea when compared to the mock-vaccinated and mock-infected (MV-MI) ($P<0.05$) and vaccinated and mock-infected (V-MI) ($P<0.05$) groups. However, ILTV genome loads in the trachea between the mock-vaccinated and ILTV-infected (MV-I) group and the vaccinated and ILTV infected (V-I) group showed no difference ($P>0.05$). Although we observed a similar pattern of ILTV genome loads in the trachea and lungs (Figure 3.6a and b), there were no significant differences in the ILTV genome loads in the lungs between the groups ($P>0.05$).

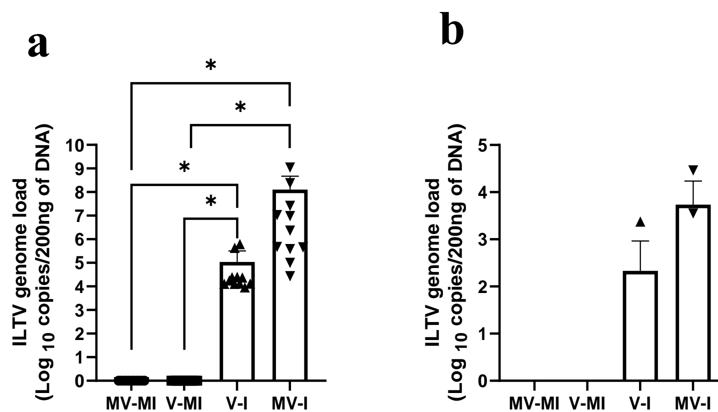

Figure 3.6 ILTV genome loads in trachea (a) and lungs (b) at 14 dpi. The quantification of ILTV genome loads was performed targeting the ILTV PK gene using qPCR assay. Mean genome load is plotted in $\log_{10}$ scale and represented in bars with standard error of means (SEM). Kruskal–Wallis test followed by Dunn's multiple comparison test was performed to identify group differences. *=$P<0.05$.

3.4.7 Peripheral blood CD4+ and CD8+ T cells

The results of the flow cytometry analysis of peripheral blood CD4+ and CD8+ T cells are illustrated in Figure 3.7a and b, respectively. The percentage of CD8+ T cells in the mock-vaccinated and ILTV-infected (MV-I) group at 5 dpi was significantly lower when compared with the mock-vaccinated and mock-infected (MV-MI) (P<0.05) and vaccinated and ILTV-infected (V-I) (P<0.05) groups. There was no statistical difference in CD8+ cell percentage at 12 dpi between the groups. Additionally, we did not observe group differences in CD4+ T-cell percentages in any of the observed time points (P>0.05).

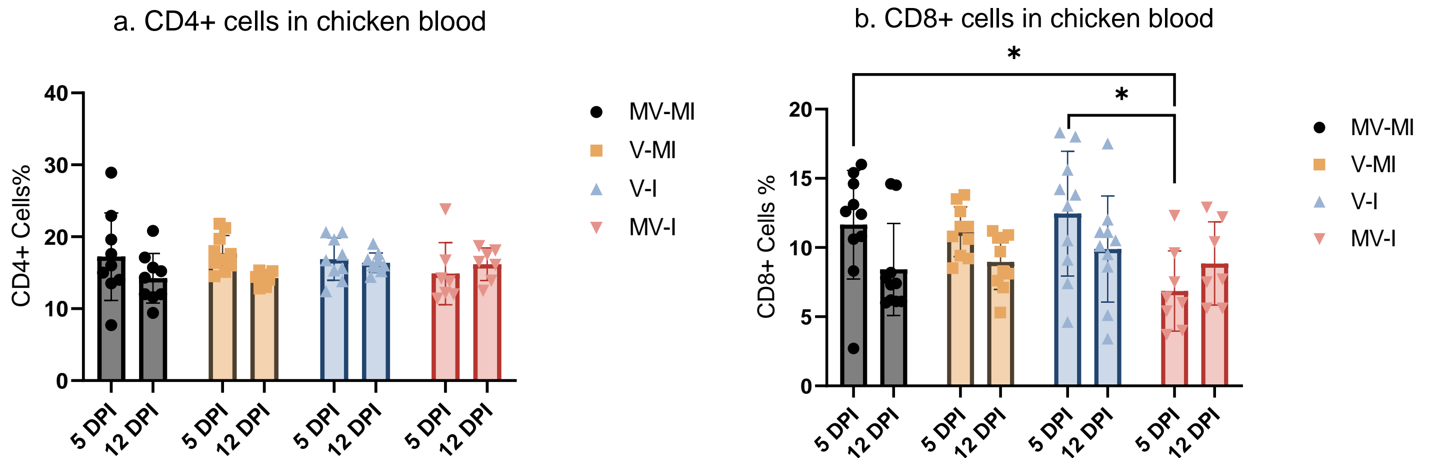

Figure 3.7 Percentage of a) CD4+ and b) CD8+ cells in peripheral blood mononuclear cells (PBMCs) at 5 and 12 dpi. Mean percentage of CD4+ and CD8+ cells is given, and error bars represent the standard error of means (SEM). Kruskal–Wallis test was performed followed by Dunn's multiple comparison test to identify the group differences. *= P < 0.05.

3.4.8 Anti-ILTV antibody response

The s/p values obtained by ELISA at 14 dpi are illustrated in Figure 3.8. The mock-vaccinated and ILTV-infected (MV-I) group had a significantly higher s/p value than the mock-

vaccinated and mock-infected (MV-MI) group (P<0.05) and the vaccinated and mock-infected (V-MI) group (P<0.05). The vaccinated and ILTV-infected (V-I) group had a statistically higher s/p value than the mock-vaccinated and mock-infected group (MV-MI) (P<0.05). Neither the vaccinated and mock-infected group (V-MI) nor the mock-vaccinated and mock-infected group (MV-MI) had values above the cut-off point (0.15). There was no statistically significant difference between the infected groups.

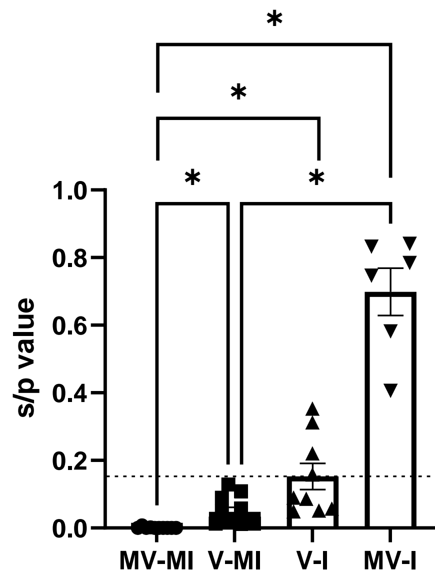

Figure 3.8 Serum sample to positive value (s/p) at 14 dpi. The bars represent mean; the geometric figures, the individual values; and the error bars, the standard error of mean (SEM). The horizontal discontinuous line represents the cut-off value of negative to positive serological diagnostic results, corresponding to 0.15. Kruskal–Wallis test followed by Dunn's multiple comparison test was used to compare the group differences. *= P <0.05.

3.4.9 Histology

In general, the most severe microscopic lesions were detected in the trachea after the exposure of chickens to ILTV isolate in comparison to the vaccinated and infected group and the

controls (Table 3.2). It was clear that there were variations in tracheal lesion scores among birds of the same group (Figure 3.9). The mock-vaccinated mock-infected group (MV-MI) had very minimal pathological lesions (Figure 3.9h) with a mean tracheal lesion score of 0.

Table 3.2 The mean microscopic lesions detected in 4 groups

Microscopic lesions	***MV-I[1]***	***V-I[2]***	***V-MI[3]***	***MV-MI[4]***
Lamina epithelialis and intratracheal lumen:				
Necrotic desquamated epithelial cells and deciliation	8/11	3/11	1/11	0/11
Cellular exudate (heterophils, necrotic epithelium, erythrocytes)	3/11	0/11	0/11	0/11
Fibrinohemorrhagic exudate	2/11	0/11	0/11	0/11
Eosinophilic intra nuclear viral inclusions	2/11	0/11	0/11	0/11
Lamina propria:				
Mononuclear cell infiltration	8/11	2/11	3/11	0/11
Hyperemia	3/11	1/11	2/11	1/11

[1]Mock vaccinated and infected; [2]vaccinated and infected; [3]vaccinated and mock infected; [4]mock vaccinated and mock infected.

Initially, the mock-vaccinated and ILTV-infected group (MV-I) was associated with wide patches of necrosis, desquamation of the epithelial lining with deciliation, intraluminal fibrinohemorrhagic and cellular exudates (heterophils and necrotic epithelial cells); the lamina propria was infiltrated with mononuclear cells; and the underlying connective tissue was projected into the tracheal lumen (tracheal lesion score = 5) (Figure 3.9a, b). In some cases, there were infiltrated heterophils together with mononuclear cells in the lamina propria, and the tracheal lumen was filled with abundant necrotic epithelial cells; fibrin, heterophils and intranuclear viral inclusion could be observed in some epithelial cells (tracheal lesion score=4) (Figure 9c). Additionally, the epithelial lining revealed squamous metaplasia and vacuolar degeneration with

the formation of vacuolar spaces containing cellular debris, the sub epithelial tissue was infiltrated with mononuclear cell infiltration (tracheal lesion score = 3) (Figure 3.9d).

In the ILT-vaccinated and infected group (V-I), the trachea showed necrosis with desquamation of the epithelial lining and ciliary loss and intraluminal ciliated cuffs of necrotic cells admixed with mucous, and hyperemia was observed in the lamina propria (tracheal lesion score = 3) (Figure 3.9e). Focal mononuclear cell infiltrations, hyperemia and edema were also observed (tracheal lesion score = 2) (Figure 3.9f).

The ILTV-vaccinated group (V-MI) showed necrosis and sloughed epithelium with ciliary loss in some areas and cuffs of desquamated ciliated epithelial cells in the tracheal lumen (tracheal lesion score = 3) (Figure 3.9g).

The quantitative tracheal histological scores are given in Figure 3.9i.

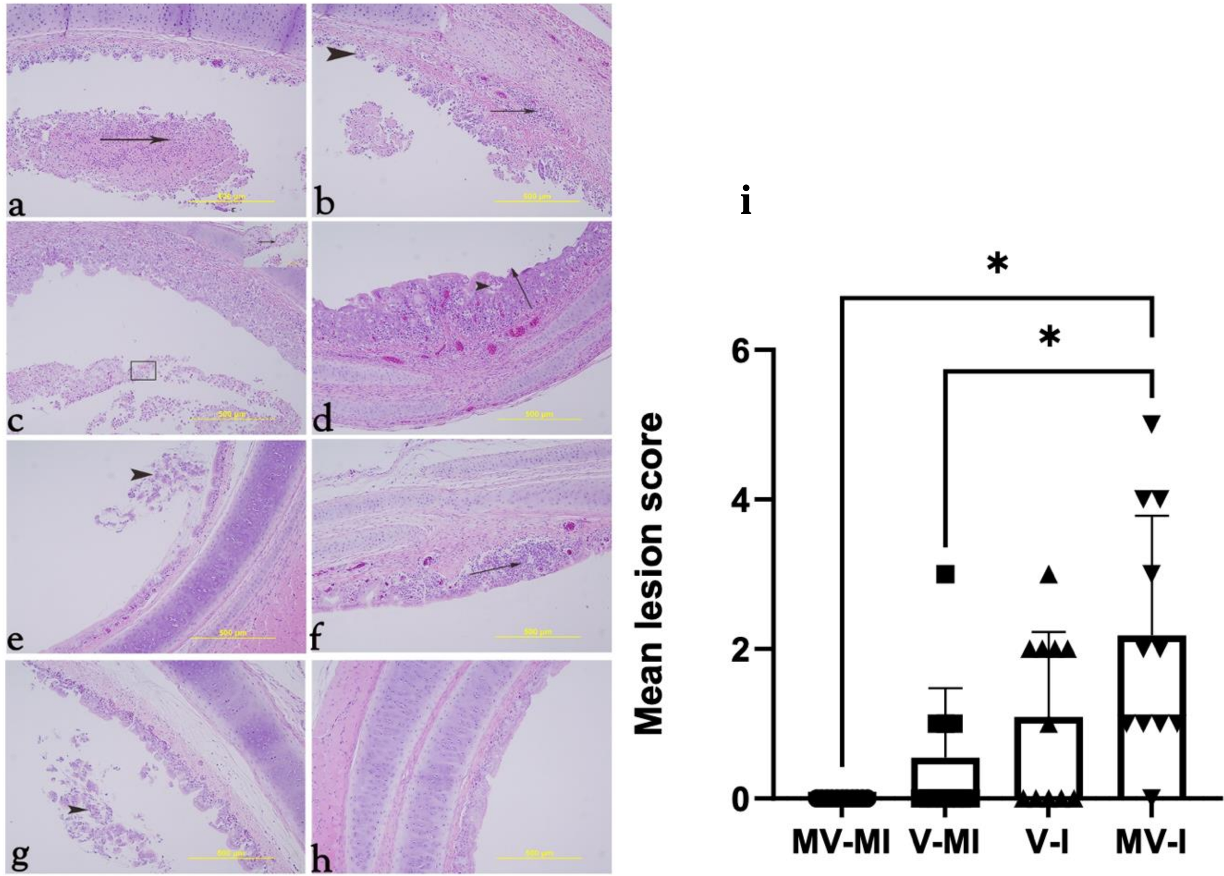

Figure 3.9 Representative histology images of trachea at 14 dpi and quantitative tracheal histological score. a: Black arrow represents fibrinohemorrhagic membrane containing cellular exudates. b: Black arrow represents mononuclear cell infiltration and hyperemia; head arrow indicates projection of the underlying connective tissue into the lumen. c: Eosinophilic intranuclear viral inclusion inside sloughed epithelial cells around heterophilic infiltrates (inset). d: Vacuolar spaces containing cellular debris and mononuclear cell infiltration with hyperemia in l. propria (head arrow); the epithelium lining exhibited squamous metaplasia (black arrow). e: Black head arrow represents cuffs of sloughed epithelial cells in the tracheal lumen. f: Black arrow represents mononuclear cell infiltration, hyperemia and edema. g: Black head arrow represents intraluminal sloughed epithelial cells. h: Normal trachea without pathological lesion. i: The mean lesion scores are given, and the error bars represent the standard error of means (SEM). Kruskal–Wallis test

followed by Dunn's multiple comparison test was performed to compare mean clinical scores of groups. *=P<0.05.

3.5 Discussion

Several of the expected outcomes of vaccination in poultry are as follows: protection against clinical disease, and a reduction in susceptibility to infection and reduction in viral shedding (147). The vaccine protection of a rHVT-LT-based vaccine was assessed after challenge with a field strain of ILTV AB-S63 by comparing weight gain, histopathological changes and clinical sign scores. The rHVT-LT vaccine induced a level of protection in our study. This was demonstrated by a slower onset of clinical signs in the infected and vaccinated group (V-I). At the peak of the clinical signs (6 dpi), there was no difference between the infected groups. Previous studies have shown that the recombinant vaccine administered to chicken that were challenged between 28 days to 35 weeks of age appears to reduce clinical signs (14-16, 101, 103). Nonetheless, protection elicited by the rHVT-LT vaccine administered in chickens challenged at 74 weeks of age was minimal (15). In the case of chickens given rHVT-LT that contained gB only and challenged at a younger age (21 days of age), the protection against clinical disease was partial, with 67% of vaccinated birds protected (101).

We evaluated ILTV genome loads as an indicator of ILTV shedding in feather tips, oropharyngeal and cloacal swabs. In agreement with our observation of decreased viral shedding via the oropharyngeal route, previous studies have demonstrated that the rHVT-LT vaccine can significantly reduce viral shedding (3 dpi) as indicated by viral loads determined using tracheal swabs (14, 16, 103). However, it is worth noting that some of these studies did not record significant reductions in viral loads in tracheal swabs at 7 dpi (14, 103) or beyond this time point

(103), contradicting our findings. Although it is difficult to explain the difference in respiratory tract viral shedding between the current study and previous studies, it is possible that it may be related to the difference in time between vaccination and ILTV infection (21 days vs. 55-57 days). In support of this hypothesis, it has been shown that the vaccine induced immune response wanes 57 days after vaccination (14). Another study showed no significant viral reduction using tracheal swabs at 5 and 8 dpi in the rHVT-LT vaccinated group (13). This study attributed the failure in protection efficacy due to incorrect in ovo delivery of the vaccine. It is also important to note that some of these studies used live attenuated vaccine-related ILTV strains as the challenge strain (13-15, 101). In our study, we used a Canadian origin genotype VI wild-type ILTV. One of the chickens out of the mock-vaccinated and challenged group (MV-I) tested positive for ILTV genome at 3 and 10 dpi. Previously, it has been shown that the ILTV is quantifiable in feather tips (7, 148, 149). However, we did not observe that the rHVT-LT vaccine given at 1 day of age is effective in reducing ILTV genome loads in feather tips.

However, we observed that ILTV genome loads in oropharyngeal swabs were reduced in the vaccinated group; we did not observe a difference in ILTV genome loads in the lungs and trachea (14 dpi) between these two groups. Our observation agrees with the previous literature, which indicates that ILTV can persist at 14 dpi due to the reactivation of latent ILTV infection or viral residue from a previous lytic infection (120, 150). A similar trend was observed in the histopathological lesion scores with no difference in lesion scores between vaccinated and infected and mock-vaccinated and infected groups.

It is known that cellular immune response plays an essential role in vaccine-induced antiviral immunity and viral infections (151, 152). In the case of ILTV infections, it is well documented that cell-mediated immunity is the primary immune response (70, 71, 78). While there

are no prior studies that evaluate the dynamics of T cells in PBMCs after ILTV infection or rHVT-LT vaccination in chickens, there are studies that have investigated T-cell response in the context of other respiratory viruses, such as NDV and avian influenza virus (AIV) (144, 153-155). Our experimental findings suggested that the rHVT-LT vaccine promotes the presence of CD8+ in early infection (5 dpi). This could explain the lower clinical sign score in the vaccinated and challenged group (V-I) at 4 dpi. Similarly, one study showed a significant increase in CD8+ cells at 2, 3 and 7 dpi when compared to the non-vaccinated and NDV-infected group (154). In a different context, a significant increase in CD8+ cells in vaccinated chickens has also been shown (144). On the other hand, researchers observing the cellular and humoral immune response after challenge with a low pathogenic AIV found a significant increase in CD4+ cells in the challenged group at 14 dpi (153). However, in our study, we did not observe a difference in CD4+ at any of the sampling time points among the groups.

In the current study, we observed that the s/p value of mock-vaccinated and ILTV-infected (MV-I) chickens did not exhibit a statistically significant difference when compared to vaccinated and ILTV-infected (V-I) chickens. Additionally, we observed that none of the birds in the vaccinated and mock-infected group had s/p values that reached the cut-off value. The role of antibody-mediated immune response against ILTV infection is unclear (70-73). It is also important to note that previous studies have demonstrated that the gG of ILTV can inhibit T-cell responses and favor the humoral immune response as an immune evasion strategy (74, 156).

CHAPTER 4: GENERAL DISCUSSION

5.1. Implications

ILTV causes multimillion-dollar losses to USA's poultry industry due to mortality and decreased egg production (26). Economic impact of ILT is not only limited to North America, but these losses are recorded worldwide. The Canadian poultry industry contributes around $6.8 billion to the gross domestic products creating 87,200 employment opportunities (157). ILTV is endemic in backyard flocks in some Canadian provinces including AB and ON, where they may potentially act as a transmission source for naïve poultry flocks. Therefore, characterization of circulating ILTV strains within a region is of extreme importance as has been the investigations focusing on the efficacy of vaccines against common ILTV genotypes.

In the first part of the thesis work, we identified that CEO revertant strains are the main cause of ILTV outbreaks in AB backyard flocks followed by wild-type strains. This trend is similar to what has been observed in the USA (92) and some provinces of Canada such as ON (93). It is puzzling, how CEO vaccine related ILTV become dominant in AB backyard flocks since the provincial government recommends using TCO vaccines rather than CEO vaccines for ILT control in backyard flocks in AB for more than a decade. One of the possibilities is that the CEO vaccines used previous to this period may still have been circulating in backyard flocks since ILTV causes a life-long infection (11, 54) and the ILTV may have been carried to present period due to bird-to-bird passage (10). The second possibility is that ILTV may have reached these backyard flocks from neighboring provinces where CEO vaccines have been used in the recent past. This is feasible since ILTV can be carried long distances *via* contaminated inert objects, soil, and manure (1, 3, 66).

Evidence of CEO revertant and wild-type ILTV linked to ILT breaks in AB is significant in many ways. First, it has been shown that CEO revertant ILTV strains are more virulent (7, 10, 11, 146) and have higher transmission potential than the wild-type ILTV strains (7, 138, 158). A recent survey estimated that AB has about 10,300 backyard flocks distributed throughout the province (2). This same study also indicated that 3% of the flocks surveyed have experienced ILT outbreaks. If the survey results are extrapolated considering total AB backyard poultry flocks, potentially 309 flocks have been infected with ILTV. We know that the birds in backyard flocks are taken to trade shows and exhibitions and this stress factor may potentially reactivate the latent ILTV (6) and virus excretion and contamination of locations far away from these flocks is possible. For two reasons this can be a threat to commercial poultry operations in AB. First, commercial poultry flocks in AB are not vaccinated against ILT and consequently, the chickens are naïve and highly vulnerable. Second, during last several years, multiple ILT outbreaks have been observed in commercial poultry flocks in QC with similar biosecurity standard to AB commercial poultry flocks (90, 127). In addition, the work of the first objective indicated that heavy use of TCO vaccines for ILT control in AB backyard flocks for more than a decade, had minimum impact on the epidemiology of ILTV infection and this is a better vaccine choice than the CEO live attenuated vaccines for ILT control. It has been shown that TCO vaccines are as efficacious as CEO vaccines against ILT (14, 15, 19, 121, 159). However, live attenuated vaccines are effective in controlling ILT but not ILTV infection (92, 95) and, consequently, the source of ILTV will not be reduced over time. The best approach to reduce this source of ILTV is to test and cull the ILTV positive backyard flocks (3, 85, 160, 161). However, this might prove difficult as apparently healthy flocks might be carrying the latent ILTV (162) and detecting latent ILTV infection is challenging (150).

Although there have been experimental tests used to detect latent ILTV, there is still not a standardized diagnostic test to detect birds with latent ILTV infection (54, 123, 150).

Although the use of live attenuated ILT vaccines is effective in controlling ILT (1), they possess several limitations. The limitations of live attenuated vaccines include establishment of latency (11, 122), residual virulence, reversion to virulence after *in vivo* passages (88, 146), bird-to-bird transmission (163), *in vivo* recombination (89, 90, 96, 164) and interaction with immunity induced by other vaccines such as NDV and IBV vaccines (91). Given this situation, in our second study, we investigated the efficacy of a recombinant viral vector ILT vaccine against one of the wild-type ILTV strains linked ILT outbreaks in AB backyard poultry flocks. We demonstrated that vaccination done at day 1 with a rHVT-LT vaccine leads only to partial protection against ILTV wild-type genotype VI strain infection done at 3 weeks of age. This means that the vaccine was able to decrease viral shedding through OP route but failed to mitigate clinical signs at the peak of the disease and reduce viral replication in feather tips.

The use of recombinant ILT vaccines are not suitable for backyard flock settings since these vaccines are given at the hatchery via *in ovo* or subcutaneous routes on the day of hatch (92). However, commercial poultry producers have more suitable settings making this type of vaccine is commonly used in some Canadian provinces. A recent ILT outbreak in QC was observed in chickens vaccinated with recombinant viral vector vaccine. The isolate was sequenced and identified as CEO revertant (90). Although studies have found that CEO related strains are more pathogenic than wild-type strains (7, 10, 11), our findings suggest that recombinant viral vector vaccines will not fully protect chickens against wild-type ILTV outbreaks either.

Although various options are available for vaccine mediated ILT control, both backyard and commercial poultry sectors are in a dilemma as there are still outbreaks occurring. Given this

situation, strengthening the biosecurity measure could help significantly decrease the risk of ILTV transmission among commercial and backyard poultry flocks. Lastly, this work adds to the constant conversation of the need to develop better vaccines that are as efficacious as the live attenuated vaccine but without the risk of reactivation or establishment of latency inherent to live attenuated vaccines (101, 102). These new vaccines also need to be accessible to backyard flock owners.

5.2 Limitations

Although both objectives of the thesis yielded novel data relevant to ILTV epidemiology and control, the experimental design and the techniques used would have been improved. To achieve our first objective, we used several approaches to propagate the ILTV isolates. The propagation of ILTV is a challenging procedure. The available options for ILTV propagation are CELIC (165), CAM (60), CEKC (166) and LHM cells (61, 63). However, among these techniques, the use of primary cells is the preferred method of ILTV propagation. A study comparing the sensitivity of CELIC, CEKC and CAM for primary isolation demonstrated that CELIC is the most sensitive and rapid method for ILTV isolation form trachea (61). Nonetheless, due to the short life span of primary cells and cost and time required to produce primary cell cultures, the propagation of ILTV has become a difficult task (167). On the other hand, despite LMH cells being permissive to ILTV replication, the virus needs to be adapted to be propagated in this system (63). Furthermore, another challenge in the propagation of ILTV is that some strains are difficult to propagate in primary cell lines (63). We used CELIC for up to 3 passages to minimize changes in the original ILTV genome as previous studies have demonstrated genetic changes after 7-10 passages (9, 63). When the samples were still not yielding the desired results following

propagation in CELIC, we switched to CAM inoculation for further propagation of the virus. However, we were unable to successfully propagate 19 of the AB samples and 4 of the BC samples. This might have been due to a low ILTV genome content (Ct value above 30) or lack of viable virus in the original sample.

The work of objective 1 was done sequencing ORF a and b of ILTV and that approach allowed to characterise ILTV strains linked to ILT outbreaks in AB and BC. The limitation of this approach is that the method was unable to distinguish recombinant ILTV strains. One of the samples from BC (CAN/BC-10-1122) was classified in our study as CEO vaccine. However, in a later study using whole genome sequencing, it was determined that it has undergone recombination events with TCO vaccine and CEO vaccines (90). In recent years, there has been a surge of recombinant strains (37, 89, 96, 97). In Victoria, Australia, these ILTV strains have even become the dominant circulating strain in poultry production areas (96).

The second part of the thesis contains data of a vaccine efficacy study, and we were able to get significant information about the efficacy of rHVT-LT vaccine against a genotype VI Canadian ILTV strain. However, it would have also been of value to conduct animal experiments with other Canadian ILTV strains, as their pathogenicity and transmission potential are different (7). Second, although our analysis yielded significant findings relevant to T cell immune response in the peripheral blood compartment, we did not examine the other tissues such as respiratory tract (84) and head-associate lymphoid tissue as has been observed in other studies (75, 84). Including the analysis of these tissues could further expand our knowledge on how the rHVT-LT vaccine enhances the immune response against ILT. Third, we were able to demonstrate reduction of ILTV genome loads in OP swabs. However, to prove if this reduction in ILTV genome loads in OP swabs leads to reduced transmission, we did not add contact birds in the experiment due to logistical

reasons. Previously, it has been shown that contact chickens can be used to determine the transmission potential of ILTV (7, 138, 158).

For quantification of ILTV in our swabs and tissue samples, we used SYBR green based qPCR assay. This is a highly sensitive molecular assay, and ILTV genome load data have a good correlation with active ILTV infection (123, 168). The disadvantage of this technique is that it is not possible to distinguish replicating virus from non-replicating virus (169). To be able to determine the quantity of replicating virus, viral plaque assay in primary cell culture is needed. The limitation of this assay is that the requirement of a large quantity of embryonated eggs and it is also a less sensitive compared to the qPCR technique (123, 170, 171).

For immune cell quantification, we used flow cytometry technique and we do not know if the measured immune cells were antigen specific. To observe if these T cell subsets were antigen specific, we would have measured the production of intracellular interferon (IFN)-γ in CD4+ and CD8+ T cells using flowcytometry technique following restimulation with ILTV antigens (172-174). The production of IFN-γ in response to ILTV infection has been shown previously (175, 176).

In our study we used SPF chicken to conduct our *in vivo* experiments since we wanted to minimise compounding factors that complicate data interpretation. Although the information that resulted from this experiment was of great value to the poultry industry, there are significant differences once the vaccine is used in field conditions or in commercial chickens (15). For example, there is a study that shows that SPF chickens are more susceptible than commercial broilers to infectious bursal disease virus (IBDV), showing a difference in their immune response (177). Maternal antibodies do not protect chickens against ILTV infection or affect vaccination with live attenuated vaccines (70, 178-180). Studies looking into maternal antibody interference

against ND and IBD recombinant viral vector vaccines using HVT and FPV as vectors, showed that systemic antibody response was limited to the viruses but the protection against the disease was not compromised (181-183). Furthermore, since rHVT vaccines are cell associated, they are considered to be less susceptible to maternal antibodies than the rFPV (92). However, there are no studies that assess if maternal antibodies do affect the efficacy of rFPV-LT and rHVT-LT vaccines against ILT.

5.3 Future directions

Taking the limitations into account, further studies should be designed to determine if the rHVT-LT vaccine is able to halt or reduce the transmission of ILTV. For this we would introduce contact birds to the isolator for 3 days as previously described (7). During this period naïve chickens would be exposed to the virus and acquire the disease, depending on the efficacy of the vaccine to reduce viral shedding in the challenged chickens. Additionally, there is no experimental data on the local immune response elicited by the rHVT-LT vaccine. Further studies on this could help come up with strategies to enhance the immune response in the upper respiratory tract to make the vaccine more effective.

It is known that virulence and transmission potential between ILTV strains are different (7, 138, 158). To fully assess the vaccine efficacy and its shortcomings, future studies should be conducted with ILTV strains belonging to different genotypes. Furthermore, studies that reproduce the field conditions and practices are also needed. Additionally, studies investigating the efficacy of TCO and rFPV-LT vaccines against these Canadian ILTV strains are necessary to compare the efficacy of all current vaccines being utilized in AB. Lastly, there are studies that have demonstrated that the combination of vaccines or using a second dose of the same vaccine to

booster the immune response, provides better protection against ILTV infection (15, 16, 159). Studies that focused on different vaccination strategies against the most virulent and transmissible ILTV strains would be helpful to formulate better vaccination programs.

5.4 Conclusions

In conclusion, the majority (>80%) of the ILT outbreaks in backyard poultry flocks from AB were found to be CEO revertant strains (22 of 27). Although CEO vaccines have not been used for decades, it is possible that underground transmission of CEO revertant strains in unvaccinated chickens had been taking place for decades resulting in these recent ILT outbreaks. On the other hand, we also determined that wild-type outbreaks are not uncommon and can be observed in unvaccinated flocks. ILT outbreaks in backyard flocks could be a source of transmission to commercial operations; however, additional studies and information is necessary to trace transmission between commercial and non-commercial operations.

Regarding the vaccine efficacy study, we concluded that the rHVT-LT vaccine diminishes viral shedding and augments the peripheral blood CD8+ T-cell response. Nonetheless, it does not reduce the clinical signs at the peak of the disease or prevent ILTV genome loads or lesions in respiratory tissues. Additional studies with contact birds are needed to assess if a reduction in viral shedding might reduce the transmission of ILTV.

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APPENDICES

As the co-authors that contributed to the paper "**Barboza-Solis C**, Perez-Contreras A, Palomino-Tapia V, Joseph T, King R, Ravi M, Peters D, Fonseca K, Gagnon CA, van der Meer F, and Abdul-Careem MF. Genotyping of infectious laryngotracheitis virus (ILTV) isolates from Western Canadian provinces of Alberta and British Columbia based on partial open reading frame (ORF) a and b" We permit using this paper as Chapter 2 of Catalina Barboza Solis's thesis entitled "Infectious laryngotracheitis infection in chickens raised in Western Canada: Molecular characterization and vaccine efficacy studies" that will be submitted to the Faculty of Graduate Studies at the University of Calgary in July 20201.

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