Tissue tropism and latency of infectious laryngotracheitis virus: A study in the natural host

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ABSTRACT

Herpesviruses are evolutionarily successful pathogens that infect a large number of animal species. This success is partly attributed to their capacity to establish latency in the host. The avian alphaherpesvirus infectious laryngotracheitis virus (ILTV) causes an acute upper respiratory tract infection in chickens that has considerable impacts on world poultry economy and welfare. The disease caused by ILTV, infectious laryngotracheitis (ILT), is currently controlled by vaccination principally with live attenuated vaccines. Limitations associated with live attenuated vaccines, including the ability to establish latency, may provide avenues for the emergence of novel, more virulent, recombinant strains of ILTV, further complicating the epidemiology of ILTV.

In this project, a sensitive nested polymerase chain reaction (NPCR) protocol was developed and the sensitivity of this assay was compared with that of other commonly used PCRs in ILTV research. A trigeminal ganglia (TG) co-culture system was established and optimised and a tracheal co-culture system was reproduced to study *in vitro* reactivation of latent ILTV. A genotyping system based on allelic variations in multiple genomic regions of ILTV to discriminate ILTV strains prevalent in Victoria, Australia, is also described.

These methodologies revealed that a large proportion of the ILTV-vaccinated birds in a commercial layer flock, close to the end of their productive laying period, were shedding multiple vaccine strains of ILTV in the upper respiratory tract, presumably due to reactivation of latent infection. Further, co-culture systems showed *in vitro* reactivation of latent ILTV in TG and trachea of these birds.

The capacity of four vaccine strains of ILTV (SA2, A20, Serva and a glycoprotein G deleted mutant vaccine candidate; ΔgG) to establish latency in specific pathogen free

chicken (SPF) following eye-drop vaccination was investigated *in vivo*. This study revealed ILTV vaccines differed in their capacities to establish latency in TG, and also showed that nearly half of the population had detectable ILTV in their upper respiratory tract (URT), 21 days post vaccination, possibly due to reactivation of infection.

A second *in vivo* experiment was performed to study latency characteristics and late systemic lymphocyte responses in SPF chickens following intratracheal inoculation with a vaccine ILTV (SA2) or a virulent field ILTV (class 9; CL9) strain. Results from this study indicated that latency characteristics did not significantly differ between these strains at 21 days post inoculation (dpi) or at 35 dpi, and suggested that the trachea may be a more significant site of latency and reactivation than the TG. Moreover, regardless of the ILTV strain inoculated, SPF birds showed lymphocytosis during the latent stage of infection.

Additionally, tissue tropism of two newly emerged recombinant strains of ILTV (CL9 and class 10; CL10) was investigated using commercial broiler and SPF chickens. The possibility of using feathers as a diagnostic sample was explored. This study revealed that both CL9 and CL10 ILTV strains caused severe disease in both types of birds, distributed to visceral organs and persisted for up to 14 dpi in URT. The NPCR developed in this project detected ILTV DNA in feathers of infected broiler and SPF birds at 14 dpi.

Taken together, these studies have shown that tissue tropism and latency is a complex area of research and to a large extent these properties are strain dependent. The studies reported in this thesis have enriched the literature on tissue tropism and latency of ILTV. The results will be valuable for future latency studies and for selection of vaccines to control ILTV.

DECLARATION

I hereby certify that in this thesis

- I have presented my original work performed to fulfill the degree of doctorate of philosophy.
- Due acknowledgement has been made in the preface and within the text for the external support received and for all the material used during this work.
- Word count is less than 100,000 excluding the tables and references.

Dulari S Thilakarathne

PREFACE

The work presented in this thesis, including data curation, formal analysis and writing was performed by Dulari S Thilakarathne to fulfill the requirements for the degree of doctorate of philosophy. Housing and care of the chickens used in the *in vivo* experiments were carried out by Dr Pollob Shil, Ms June Daly and Ms Jenece Wheeler. Samples for Sanger sequencing were sent to either the Centre for Translational Pathology, University of Melbourne or the Australian Genome Research Facility (AGRF), Melbourne.

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Dulari Thilakarathne, Joanne Devlin, Andres Diaz, Carol Hartley, Jose Quinteros & Mauricio Coppo. Systemic lymphocyte response in specific pathogen-free chickens long after an intratracheal challenge with vaccine or field strain of infectious laryngotracheitis virus. The Victorian Infection and Immunity Network Symposium, Melbourne, Australia (18th October 2018)

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I wish to express my sincere gratitude to all the wonderful members in the Microbiology group at the Faculty of Veterinary and Agricultural Sciences, the University of Melbourne for helping me in various ways to complete this work.

I am especially grateful to my husband, Suraj Niroshan and to my dearest daughter, Tharushi Amaya for all the sacrifices they made for me to reach my goals. Also for the love and support they extended in all the moments of my life.

Last but not least, I wish to state that I am eternally grateful to my parents and my siblings for helping me throughout my life and for their unconditional love.

DEDICATION

This thesis is dedicated to the memory of my beloved mother Magret Samarasinghe

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ABBREVIATIONS

AGRF	Australian Genome Research Facility
ANOVA	One-way analysis of variance
APCAH	Asia-Pacific Centre for Animal Health
bp	Base pair
CAM	Chorioallantoic membrane
CBT	Control brain tissue
CEK	Chicken embryo kidney
CEL	Chicken embryo liver
CEO	Chicken embryo origin (vaccines)
CK	Chicken kidney
CL	Class
CoREST	Co repressor element-1silencing transcriptional factor
CPE	Cytopathic effects
Ct	Cycle threshold
DIVA	Differentiation between infected and vaccinated animals
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dpe	Days post explant
dpi	Days post inoculation
dpv	Days post vaccination
dsDNA	Double stranded DNA
E	Early (phase)
EID50	Median embryo infective dose
EL	Early late (phase)
ELISA	Enzyme-linked immunosorbent assay
FAM	Six-carboxyfluorescein
FBS	Foetal bovine serum
gB	Glycoprotein B
gC	Glycoprotein C
gD	Glycoprotein D
gG	Glycoprotein G
gH	Glycoprotein H
gJ	Glycoprotein J
gL	Glycoprotein L
GCN	Genome copy number
GHV-1	Gallid herpesvirus type 1
GM	Growth media
H&E	Haematoxylin and eosin
HCF-1	Host cell factor 1
HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen

HSPG	Host cell heparan sulfate proteoglycan
HSV-1	Herpes simplex virus-1
IE	Immediate early (phase)
IFN	Interferon
ILT	Infectious laryngotracheitis
ILTV	Infectious laryngotracheitis virus
IP	Incubation period
IR	Internal repeat
L	Late (phase)
LAT	Latency associated transcripts
LMH	Leghorn male hepatoma (cell line)
LSD1	Lysine specific demethylase 1
MM	Maintenance medium
miRNA	MicroRNA
NGF	Nerve growth factor
NPCR	Nested polymerase chain reaction
Oct-1	Octamer binding protein 1
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PCR-HRM	Polymerase chain reaction-high resolution melting curve analysis
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism
PFU	Plaque forming units
PILR	Paired immunoglobulin-like type 2 receptor
qPCR	Quantitative polymerase chain reaction
REST	RE 1- silencing transcriptional factor
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
SH-SY5Y	Human origin neuroblastoma
SNPs	Single nucleotide polymorphisms
SPF	Specific pathogen free
sRNA	Small RNA
Ta	Annealing temperature
TCO	Tissue culture origin (vaccines)
TG	Trigeminal ganglia
TGVTMs	Viral transport media used to transport the trigeminal ganglia that were used in fresh culture
TOC	Tracheal organ co-cultures
TR	Terminal repeat
UHV	Universal herpes virus
UHVNPCR	Universal herpesvirus nested PCR
UL	Unique long (genomic region)
UL15NPCR	UL15 gene targeted nested PCR
UL15qPCR	UL15 gene targeted quantitative PCR

URT	Upper respiratory tract
US	Unique short (genomic region)
VP-16	Virion protein 16
VRE	VP16 response element
VTM	Viral transport media
α	Alpha
β	Beta
γ	Gamma
ΔgG	Glycoprotein G deletion mutant vaccine candidate

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Chapter 1-Literature review

1. Infectious laryngotracheitis virus

1.1. Overview and nomenclature of the virus

Infectious laryngotracheitis virus (ILTV) is the causative agent of infectious laryngotracheitis (ILT), an upper respiratory disease of chickens which is reported worldwide (1-13). This virus was first reported in 1920s from Canada and United States (15) but within the next two decades the virus had been reported from at least 40 countries (16). It has been given several names over time, such as Canadian flu, avian diphtheria, infectious bronchitis, infectious tracheitis and tracheolaryngitis (15). Then in 1931 the American Medical Association named this virus as infectious laryngotracheitis virus (15) and now it is taxonomically identified as *Gallid alphaherpesvirus type 1* (GHV-1) and categorized under the genus *Iltovirus*, family *Herpesviridae*, subfamily *alphaherpesvirinae*, within the order of *Herpesvirales* (17).

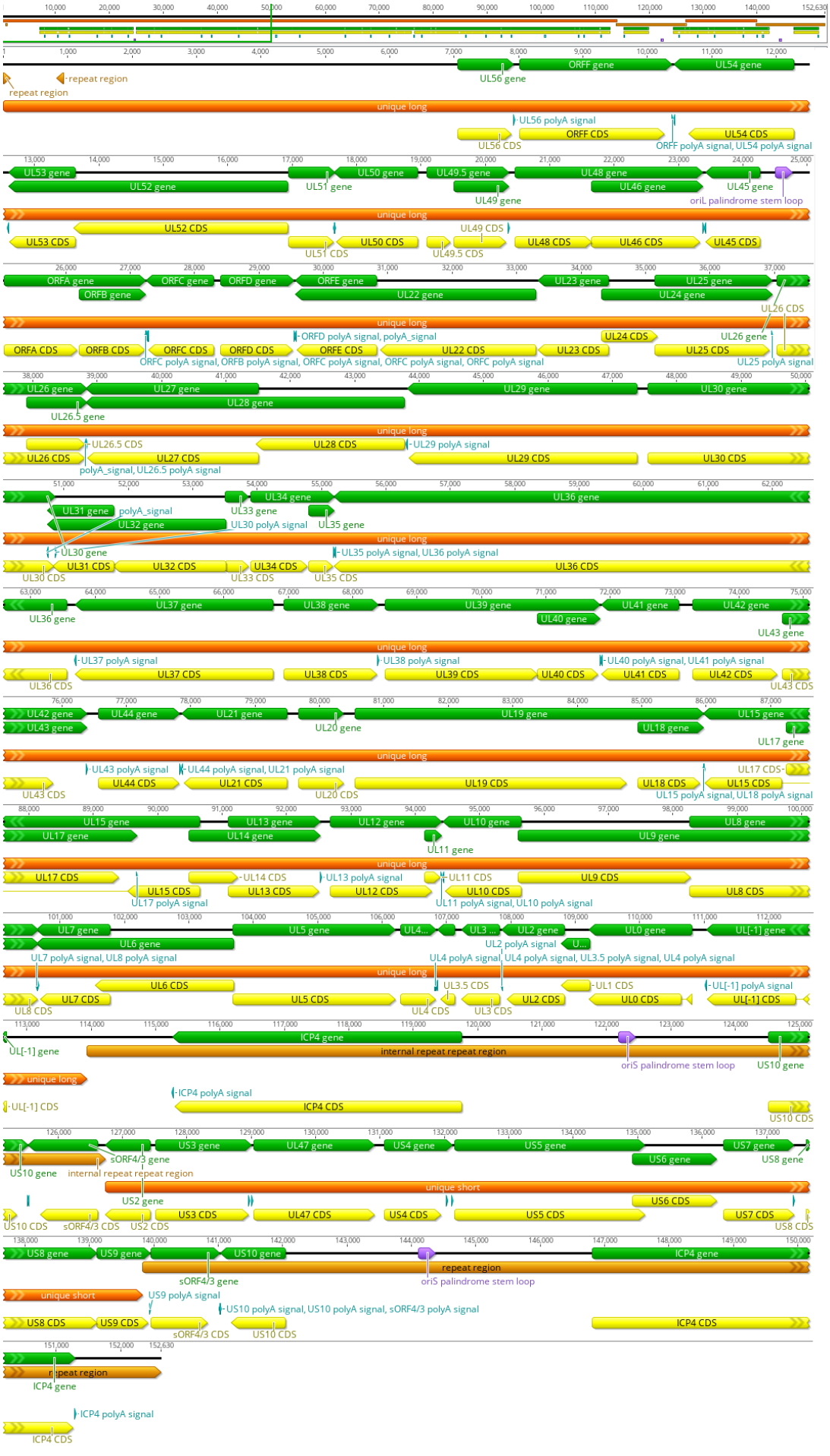
1.2. Morphology

As with other herpesviruses, ILTV consists of an envelope, tegument and a capsid enclosing a double stranded deoxyribonucleic acid (dsDNA) genome. The outermost layer is the irregular envelope which results in a viral diameter of 195 – 250 nm (18). The envelope is a bilayer lipid membrane that is acquired during two different stages of ILTV replication; budding through the nuclear membrane (primary envelopment) and maturation in the trans Golgi region (secondary envelopment) (19). Glycoprotein projections are found embedded in the envelope and hitherto, 12 surface glycoproteins have been recognized in ILTV (20). Most

of these glycoproteins are reported to have key roles in viral replication, pathogenesis and immunomodulation in the natural host (21). Between the envelope and the nucleocapsid is the tegument, a layer of protein which is incorporated into the virus during viral maturation at the trans-Golgi region (19). The nucleocapsid lies at the core of the virus and is an 80 – 100 nm diameter hexagonal structure with icosohedral symmetry (18).

1.3. Genome

The viral genome is enclosed by a nucleocapsid. According to complete genome sequence of Serva vaccine strain (Figure 1), the genome is made up of 148,687 base pairs of dsDNA, and with 48.2% guanosine + cytosine content (22). The arrangement of genome is type D; it consists of a unique long (U_L) and a unique short region (U_S) where each side of U_S consists of two repeating codes; internal repeat (IR) and terminal repeat (TR) (23). Lee et al. (22) have predicted a total of 80 open reading frames (ORFs) in the Serva genome, of which 65 are within the U_L, nine within the U_S and six within the repeat regions. Similar to other herpesviruses, ILTV has three DNA replication origins, of which two are located in the repeat regions (OriS) and the other within U_L (OriL). Compared to other alphaherpesviruses with type D genomes, the unique feature observed in the ILTV genome is that the U_L region is inverted. Furthermore the U_L segment contains a unique set of conserved genes; UL 22 to UL 44 and these are located after a block of five ORFs; ORF A to ORF E. Additionally, even though the ILTV genome is generally homologous to the herpes simplex virus-1 (HSV-1) genome, the homolog to the HSV-1 UL47 gene is located in U_S region of ILTV (24).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UL7 CDS
UL5 CDS
UL2 CDS
UL0 CDS
UL[-1] CDS
UL8 CDS
UL4 CDS
UL3 CDS
ICP4 gene
UL[-1] gene
internal repeat repeat region
oriS palindrome stem loop
US10 gene
unique long
ICP4 polyA signal
UL[-1] CDS
ICP4 CDS
US10 CDS
US3 gene
UL47 gene
US4 gene
US5 gene
US7 gene
sORF4/3 gene
US6 gene
US8 gene
US10 gene
internal repeat repeat region
unique short
US2 gene
US6 CDS
US3 CDS
UL47 CDS
US4 CDS
US5 CDS
US7 CDS
US10 CDS
sORF4/3 CDS
US2 CDS
US8 CDS
US8 gene
US9 gene
US10 gene
ICP4 gene
repeat region
sORF4/3 gene
oriS palindrome stem loop
unique short
US9 polyA signal
US10 polyA signal, US10 polyA signal, sORF4/3 polyA signal
US8 CDS
US9 CDS
ICP4 CDS
sORF4/3 CDS
US10 CDS
repeat region
ICP4 gene
ICP4 polyA signal
ICP4 CDS

Figure 1: A schematic of the Serva ILTV genome (Gene bank accession number HQ630064)

The Serva ILTV genome has 148,687 base pairs with a guanosine + cytosine content of 48.2%. The genome consists of a unique long (U_L) and a unique short region (U_S). Two repeating regions, termed the internal repeat (IR) and terminal repeat (TR) regions are located on either side of the U_S region. A total of 80 open reading frames (ORFs) has been predicted in the Serva genome. The ILTV genome has three DNA origins of replications, of which two are located in the repeat regions (OriS) and the other within U_L (OriL).

1.4. Strains of ILTV and classification

Viral genome based assays have often been used to classify ILTV strains because early studies have shown that ILTV strains are antigenically homogenous (25). Classification by restriction endonuclease analysis has been attempted (26, 27) but this technique became redundant as the technique could not accurately distinguish modified live vaccines from field strains and strains with similar restriction patterns varied in their pathogenicity or virulence (5, 28, 29). Polymerase chain reaction (PCR) combined with restriction fragment length polymorphism (RFLP) has been a powerful method in discriminating strains (9, 30-36). A single gene (8, 35, 36) or multiple genes (9, 31-34) have been used as the targets in PCR-RFLP strain discrimination and strain prediction.

With the advancement of sequencing technologies (Sanger sequencing and next generation sequencing) ILTV genome sequencing became a common way to classify strains. As genome sequencing provides more precise genomic information, many researchers around the world have performed genome sequencing of vaccine or field ILTV isolates (2, 6, 14, 22, 37-43). Knowledge of whole genome sequences of ILTV strains has identified specific single nucleotide polymorphisms (SNPs) (44-46) that can be identified by quantitative PCR-high resolution melting analysis to categorize ILTV strains (47).

1.5. Natural hosts

Infectious laryngotracheitis virus infects chickens of all ages, however clinical signs and lesions are prominent when birds are infected with ILTV around three

weeks of age (48, 49). Other than chickens, there have also been reports on ILTV induced respiratory signs in pheasants and peafowls (50). Moreover, ILTV is capable of causing natural infection in turkeys as well (51).

1.6. Transmission

Infectious laryngotracheitis virus gains entry to natural hosts via inhalation, ocular deposition or less commonly by ingestion (49). ILTV does not spread vertically via eggs (48). Direct transmission from acutely infected birds is common as they excrete virus in nasal, oropharyngeal, tracheal and ocular discharges (52). Moreover, Roy et al. (53) found that viral DNA could be detected in feces of infected birds at 7 days post inoculation (dpi) and up to 28 dpi. Recovered or vaccinated birds carry latent infection (54) therefore they can shed the virus if undergoing stressful events which cause reactivation of latent infection (11, 55). Backyard poultry have been identified to be a critical source of infection (56). As with many other viral infections, contaminated fomites and mechanical carriers can indirectly contribute to transmission of ILTV (48, 54, 57). Darkling beetles (*Alphitobius diaperinus*; Panzer) have been suggested as a potential mechanical vector for ILTV (58). Crows, cats and dogs have been recognized as indirect sources of ILTV transmission (59). Wind appears to be important in transmitting ILTV (60), as transmission mainly occurs by means of aerosols or expectoration (56). Virions of ILTV have survived on wooden surfaces, away from light, for up to 3 months and it has been shown that ILTV can survive in deep litter for about 20 days and in the carcasses of birds that have died due to ILTV infection for up to 3 weeks after death (56).

1.7. Viral attachment and entry

Upon deposition on the upper respiratory tract or ocular membrane, ILTV attaches and invades epithelial cells where primary replication occurs (11, 61). The precise molecular mechanism of ILTV attachment or invasion has not been directly identified but is likely to be similar to other alphaherpesviruses, such as HSV-1 (49). HSV-1 binds to the host cell surface using viral glycoprotein B (gB) and glycoprotein C (gC) (62). These glycoproteins mainly interact with host cell heparan sulfate proteoglycan (HSPG) (63) however, Satoh et al. (64) suggest that gB may also bind to the paired immunoglobulin-like type 2 receptor (PILR) on the cell surface. Studies conducted using ILTV have shown the amino acid content and protein charge of gB and gC are different to that of HSV-1 and that ILTV does not interact with cell surface heparan sulfate, indicating that ILTV has a different method of attachment and cell fusion (65). According to the model proposed for HSV-1, attachment of the virus to the host cell mainly occurs at filopodia (F-actin rich membrane protrusions in host cells), sites rich in HSPG (66). HSV-1 penetration into the host cell may occur mainly by fusion of the envelope with the plasma membrane. Fusion in non-immune cells is brought about by interaction of viral glycoprotein D (gD) to one of its cognate receptors; nectin-1 or nectin-2 (67, 68). This causes a conformational change in gD which activates the glycoprotein H (gH) and glycoprotein L (gL) complex; gH/gL in the virus envelope (62, 69). The activated gH/gL complex will promote the action of gB as a viral fusion protein (62). This results in the release of tegument protein and nucleocapsid into the cytoplasm (63, 70).

In the cytoplasm nucleocapsids become dissociated from the tegument and engage in microtubules and dynein-mediated transport to localize in the host nuclear membrane (71). Tegument proteins play a key role in transportation of nucleocapsids along microtubules to the perinuclear region. For example viral tegument protein VP26 is required for the interaction between the capsid and dynein (72) and VP1/2 is essential for directing the nucleocapsid to the nuclear pore complex and docking at the site (73). The host cell factor, heat shock protein 90 has also found to participate in capsid transport (74). When nucleocapsids reach the host nuclear membrane, VP1/2 and nucleoporins (Nup358 and Nup214) facilitate the binding of the capsid to the nucleus (75). Integrin-β and viral UL25 interact with nucleoporins (CAN/Nup214 and hCG1) assisting the docking of the capsid, while interactions between UL25, UL6 and VP1/2 triggers injection of viral DNA to nucleus (76, 77).

Viral tegument proteins, particularly VP16, are fundamental in initiation and modulation of herpesvirus gene expression and also termination of host protein synthesis (63). These are discussed in sub section 3.1.1.

1.8. Transcription and replication of ILTV

Once the viral genome is injected to the nucleus a series of molecular events occur in the nucleus and cytoplasm facilitating the transcription and replication of the viral genome. Transcription takes place in the nucleus whereas translation of protein occurs in the cytoplasm (78). A typical alphaherpesvirus such as HSV-1 that undergoes a lytic cycle of replication is reported to have three phases of viral genome transcription; immediate early (IE) or alpha (α), early (E) or beta (β), and

late (L) or gamma (γ). These phases have been based on the temporal pattern and interdependent nature of gene expression (79). Essential transcription factors are encoded in IE genes and they reach peak rate of synthesis between 2 - 4 h post infection. Products required for viral DNA metabolism and replication are coded in E genes and their synthesis reaches a peak 6 -12 h after infection. Viral genes in which the expression is completely or partially dependent on IE and E are categorized as L and these genes encode structural and/or other proteins necessary for viral assembly. It takes 10 -16 h for L genes to reach the peak of synthesis (78, 80).

The classification of gene expression may be more complex in ILTV. Mahmoudian et al., (81) have revealed an additional phase, early late (EL), in ILTV gene expression. According to the study most of the ILTV genes are expressed within an hour after infection. This has been explained as due to either the "leaky" nature of transcription of the genome or the presence of a more complex regulatory system in gene transcription than in other alphaherpesvirus (81).

Replication mechanisms are largely conserved in herpes viruses (82). As there is no sufficient information on the complete process whereby ILTV DNA is replicated, cleaved and packed into capsids, mechanisms of ILTV are understood using studies conducted for mostly studied herpes virus, HSV-1. A detailed description on HSV-1 DNA replication, cleavage and packing can be found in section 3.1.1.

The primary sites of ILTV replication include the epithelial lining of upper trachea and conjunctiva (61). Kirkpatrick et al. (83) have shown different strains of ILTV have varying affinity towards replication in trachea or conjunctiva. Peak ILTV replication occurs around 4 - 6 dpi (10). Viruses enter the basement membranes of epithelial cells slower than occurs during HSV-1 infection (61, 84). The capacity to cross basement membrane is proposed to be a strain dependent property in ILTV (61), allowing ILTV to spread in to non-respiratory tissues and sites of latency.

1.9. ILTV recombinant strains

When two strains of viruses replicate within the same host cell at the same time it is possible that progeny viruses contain genetic material from both parental strains. This molecular process is termed recombination and it has been observed with many alphaherpesviruses (85) including ILTV (44, 86). Many alphaherpesviruses undergo high rates of homologous recombination (87, 88) which occurs between strains of the same virus, or closely related viruses. Late stages of viral DNA replication involves the formation of concatermeric DNA (89) (described in detail in section 3.1.1.) that accumulates within the nucleus (90) before proceeding to cleavage and packing. This presumably favours recombination of the genomes of two strains replicating within the same cell at the same time. Researchers have observed extensively branched networks of DNA as a product of late DNA replication (91) and it has been assumed that recombination of concatemeric DNA is responsible for generation of highly branched replication intermediates during the viral life cycle (92). It has been shown that recombination in herpesviruses is coupled with DNA replication (93)

and thus replication is said to be recombination dependent (94, 95). Host factors (genetics or immune status) and viral factors (virulence, invasiveness, latency, tissue distribution and genetic homology) will influence the emergence of recombinants (85). Inter-species recombination (96) as well as intra-species recombination (97) has been observed in alphaherpesviruses however, the latter is more efficient and more common compared to the former.

Numerous studies have reported natural recombination between ILTV vaccine and field strains (2, 3, 14, 98). Recombination can favor survival and evolution of the virus and can result in more virulent strains that replace previously dominant strains. The most recent example in Australia is the emergence of virulent recombinant ILTV strains (classes 8, 9 and 10) after the introduction of the European ILTV vaccine strain; Serva (2, 3, 99). Lee et al. (14) have shown that a large part of the sequence of the genome of these virulent strains is almost identical to the genome of Serva. The genome structures of these three recombinant strains are shown in Figure 2. Further, Lee et al. (99) has shown class 9 has replaced the previously dominant class 2 strain in the field, possibly due to the improvement in fitness it acquired after recombination.

More studies on ILTV recombination have been done recently and they have shown a high proportion of recombinants when SPF chickens are coinoculated with two different ILTV strains (44, 86), where a higher infectious doses may increase the diversity of the recombinants. A 20,000 to 50,000 bp segment of the U_L region of the ILTV genome has been identified as a recombination “hot spot” as this region is prone to be involved in recombination more frequently than other

regions of the genome (86). Moreover, Loncoman et al. (100) found vaccination (in particular with Serva ILTV strain) can limit the number of recombinant progeny viruses as well as their diversity.

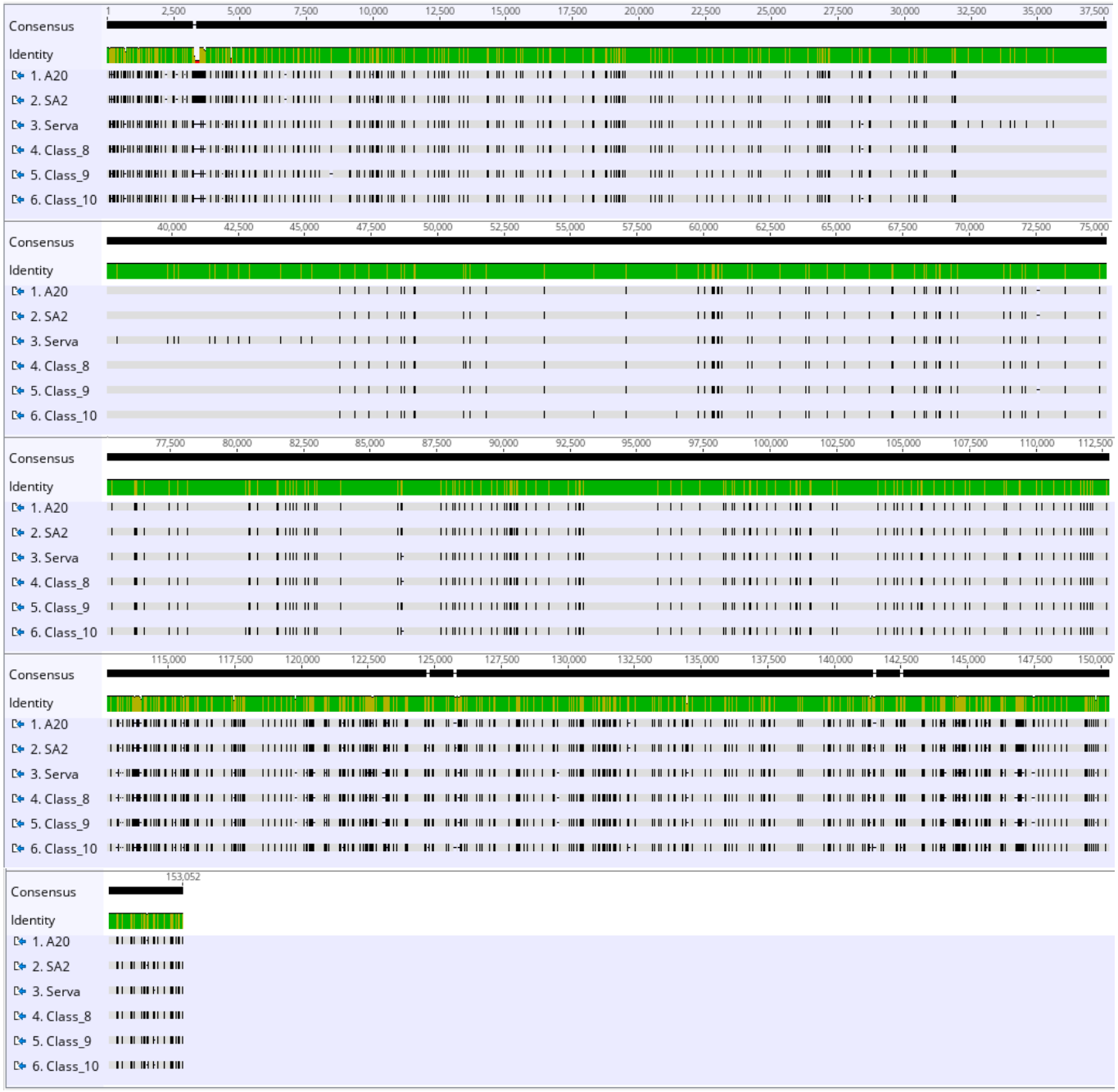

Figure 2: Whole-genome alignments of the field strains of class 8 (CL8), class 9 (CL9), class 10 (CL10) ILTV, along with the vaccine strains of SA2, A20 and Serva ILTV.

Vertical black lines indicate single nucleotide differences. Dashes indicate gaps. Adapted from Lee et al. (14) and Agnew-Crumpton et al. (2).

1.10. Tissue tropism of ILTV

Even though the respiratory tract and eye are the primary replication sites for ILTV, virus has been detected in many visceral organs (10, 53, 101-103) and in feather pulp (104-107). The first report on studying tissue tropism of ILTV is by Bagust et al. (108) where ILTV was isolated within 4 to 7 days post inoculation (dpi) in non-respiratory/ocular sites such as lung, liver, brain and trigeminal ganglia (TG) but not from peripheral blood leukocytes or lymphoid organs (spleen, bursa, thymus). Two decades later, ILTV DNA has been detected by quantitative polymerase chain reaction (qPCR) in thymus and cecal tonsils, as well as respiratory/ocular sites and TG (10, 109). During the same period, Davidson et al. (104, 105) were able to demonstrate ILTV could spread into feather shafts during the acute phase of ILTV infection. This finding provided an avenue for convenient detection and even monitoring of ILTV vaccination programs (106, 107). Zhao et al. (103) showed qPCR detectable ILTV in almost all visceral organs (lung, heart, kidney, spleen, thymus, bursa, cecal tonsils, tongue, glandular stomach, duodenum, pancreas, liver, small intestine, large intestine, cecum and brain) as well as in primary replication sites (laryngotrachea) even up to 28 dpi. Wang et al. (102) detected ILTV DNA by qPCR in throat, trachea, lung, cecum, kidney, pancreas, thymus and esophagus from 3 to 8 dpi . Wang et al. (102) also detected histopathological changes such as characteristic necrosis and inflammatory cell infiltration in all qPCR positive organs starting from 5 dpi suggesting these organs may play a role in acute ILTV infection. However, the study done by Silvereen et al. (101) on naturally infected chickens revealed qPCR detection of ILTV only in oculo-respiratory sites. The latest ILTV

tissue tropism study was by Roy et al. (53) where they report sustained ILTV DNA detection by qPCR in the trachea, harderian gland, lung and kidney up to 28 dpi. The mechanisms by which ILTV spreads in to other visceral organs are not completely understood.

2. Infectious Laryngotracheitis - Clinical disease

2.1. Incubation and clinical signs

Lytic cycles of ILTV replication leads to clinical disease. (55). The incubation period (IP) varies depending on the route of infection (110). Usually IP ranges between 3 - 14 days following natural exposure (111, 112). However, IP can be as low as 2 - 4 days after intratracheal inoculation of virus (113). Clinical signs depend on the pathogenicity of the strain causing the infection and the immune status of the host (6, 52). Less virulent strains cause mild forms of disease in which clinical signs include sneezing, mild coughing, watery eyes mild/moderate hemorrhagic conjunctivitis, swelling of the infraorbital sinuses, persistent nasal discharges, mild mucoid tracheitis and respiratory rales. Depression, general unthriftiness and reduced egg production can also be seen with ILT infection (13, 54, 113, 114). Virulent strains cause severe infections exhibiting signs such as gasping, loud coughing, marked dyspnoea, expectoration of blood stained mucus and moderate to severe conjunctivitis (13, 114, 115). Peracute infections may lead to death of birds within 2 - 3 days after onset of clinical signs (114). After 10 - 14 days of natural infection birds will recover but they can become persistent carriers stage of disease and the virus becomes latent (110).

2.2. Pathology

Lesions are correlated with the virulence of the strain involved (83) and are mainly found in the upper respiratory tract and conjunctiva (116). In mild cases, typical lesions of mucoid tracheitis are seen with varying levels of hemorrhage. In severe ILT infections, lesions indicative of local inflammation can be seen at an early stage (13, 114). Diffuse hemorrhage, degeneration, necrosis and diphtheritic lesions (with blood/mucoid casts) are visible in the trachea and larynx in severe cases (114, 117). Hemorrhagic tracheitis and obstruction of the trachea with blood casts or blood stained mucus can be found in birds that have died with peracute infections. When infected with highly virulent strains lesions may even be found in the bronchi, lungs and air sacs (117). Varying degree of conjunctivitis, nasal exudates, sinusitis and oropharyngeal lesions may also be present and the extent of these lesions is related to the pathogenicity of the strain (13, 116).

Microscopic lesions are linked to the stage of disease. At early stages of infection loss of goblet cells and infiltration of inflammatory cells in the tracheal mucosa can be apparent. Tracheal epithelial cells become enlarged and edematous eventually leading to giant multinuclear cell (syncytia) formation. Eosinophilic intranuclear inclusion bodies can found in both tracheal and conjunctival epithelial cells which is a pathognomonic feature of ILT infection. With further progression of infection desquamation of tracheal epithelium occurs, exposing blood vessels in the edematous lamina propria to the tracheal lumen (13, 116, 117).

2.3. Morbidity mortality & economic significance

High morbidity (90 - 100%) has been observed in infections involving virulent strains but mortality more typically varies from 5 - 70% (12). Less virulent strains produce lower levels of morbidity and these also cause a lower varying degree of mortality (0.1-2%) (13, 49). Economic losses due to mortality, unthriftiness and reduced egg production have been estimated to be substantial (115).

2.4. Diagnosis of ILT

The use of laboratory diagnostics, in addition to observing clinical signs of disease, is recommended to aid to diagnosis of ILTV infection as many other respiratory pathogens can induce similar signs (49). The diagnostic methods available are histopathological examination, isolation of virus, detection of antibody using serology, and detection of viral antigens and/or viral DNA (118).

2.4.1. Histopathology

Intranuclear inclusion bodies that develop in respiratory and conjunctival epithelia are pathognomonic in ILTV and detection of these is considered to be a rapid (detection within 24 hours) and sensitive method of diagnosing ILT (13, 119).

2.4.2. Isolation and Identification of ILTV

Samples collected in the early course of infection are better to isolate ILTV (10, 118). Swabs, tissue homogenates and exudates from the upper respiratory tract or conjunctiva can be used as samples (118). Embryonated chicken eggs or susceptible cell cultures are the laboratory host systems in use to propagate the virus (49). When inoculating embryonated eggs, 9 - 12 day old eggs are chosen and the chorioallantoic membrane (CAM) is the recommended route for

inoculation (114). At about 2 dpi ILTV produces viral plaques on the CAM ; the appearance and size of plaques vary with the viral strain (54). ILTV can grow in a variety of cell culture systems; chicken embryo liver (CEL) , chicken embryo lung, chicken embryo kidney (CEK) and chicken kidney (CK) cells are the primary cell lines used, however some ILTV strains also grow well in the Leghorn male hepatoma (LMH) continuous cell line (49, 120). Cytopathic effects (CPE) are visible within 4 - 6 hours of inoculation and those effects include ballooning of cells, displacement of chromatin with rounding of nucleoli and formation of syncytial cells. Twelve hours post inoculation pathognomonic intranuclear inclusion bodies can be detected and these progresses with the time course of cellular infection (49).

2.4.3. Detection using serology

Serological assays such as agar gel immunodiffusion assay, virus neutralization assay, indirect fluorescent antibody assay and enzyme-linked immunosorbent assay (ELISA) have been used in the past to identify antibody against ILT (118). However the results of serological tests should be interpreted carefully, as detection of antibody does not always correlate to an active infection (120). Serodiagnosis may be used to observe seroconversion after vaccination (49) however these tests are not suitable for monitoring the protection status of flocks as antibodies are not protective against challenge (121).

2.4.4. Detection of viral antigens and viral DNA

Viral antigens have been detected in epithelial tissues of larynx and trachea using tests such as direct fluorescent antibody assay, immunohistochemical staining and

indirect florescent antibody assay (118). Further ELISA techniques have been developed to detect viral antigens *in situ* (118). However, since the advancement of PCR based methods, these have become popular in diagnosing ILTV because they provide rapid and sensitive diagnosis (49). Conventional PCR has been used to detect ILTV from trachea, conjunctiva (122), trigeminal ganglion (TG) (11) and various other internal organs (11, 101, 103). Further, to enhance the sensitivity of detection of ILTV nested PCR have been developed (123), whilst to test multiple respiratory pathogens at a single run multiplex PCR systems have been developed and validated (124, 125). To achieve quantitative diagnosis real-time polymerase assays (RT-PCR/qPCR) are used. Two such qPCR based quantification techniques that have been recently validated; SYBR Green qPCR assay of ILTV (126) and TaqMan assay for rapid detection and quantification of ILTV (103, 127).

2.5. Immunity to ILTV

Herpesviruses, including ILTV, are successful pathogens that are capable of causing not only acute infection but also persistent lifelong infection termed latent infection (61). The exact mechanism by which ILTV overcomes innate and adaptive immune mechanisms to establish these two forms of infections are not fully understood (55). However, inflammatory cell recruitment during the innate response is a significant driver of the outcome of the infection (128). Cell mediated immunity, but not humoral immunity, confers protection against ILTV (55, 129). An interesting feature observed with some alphaherpesviruses is that they use their glycoprotein G (gG) as a broad spectrum viral chemokine binding molecule for immune evasion (130). In particular, the gG of ILTV inhibits

chemokine function to result in a reduction of heterophil migration to the primary viral replication sites. This molecule may skew the host immunity towards the humoral pathway which is non-protective (128). Thus to provide protective immunity against ILT enhancing cell mediated immunity in birds has been the prime target in current vaccine development efforts (121). Some reports suggest that the capacity of ILTV to infect leukocytes, in particular macrophages (131) may explain differences in ILTV resistance or susceptibility in different bird lines (132, 133).

2.6. Vaccination

Vaccines, together with strict bio-security measures, have been use to control ILT in intensively reared poultry (134). Three types of ILTV vaccines have been in commercial or research use; attenuated vaccines, new generation virally vectored recombinant vaccines, and recombinant deletion mutant vaccines (121). However none of these vaccines has been able to provide complete protection against ILT and all of them have advantages and disadvantages (55).

Traditionally attenuated vaccines are produced by serial passaging of ILTV either in embryonated chicken eggs (chicken embryo origin vaccines, CEO) or tissue cultures (tissue culture origin vaccines, TCO) (135). These vaccines have been in use for a long time and they provide variable level of protection depending on the route of administration (109). However, these vaccines have several drawbacks including residual virulence, having the potential to revert into virulent status during *in vivo* passaging, ability to transmit from vaccinated birds to naive birds,

establishment of latent infection resulting in lifelong viral carriers and contributing to the generation of more virulent recombinants (109, 121, 134, 135).

Virally vectored recombinant vaccines have been designed to overcome the above limitations (134). These vaccines do not establish latency, they lack the capacity to revert into virulent forms and allow differentiation between infected and vaccinated animals (DIVA); a useful strategy for successful control of disease using vaccination (99, 135). However they only provide partial protection against ILT (99) and exhibit delayed onset of immunity (55, 135). Currently Fowl pox vectored vaccine (expressing ILTV gB and U_L-32 gene) and herpesvirus of turkey vectored vaccines (encoding ILTV glycoprotein I; gI and gD) are commercially available in some countries (134).

Developing deletion mutant vaccines is another approach to ILTV vaccine development. These vaccines contain an ILTV strain that has had the genome engineered to be deficient in a complete or partial gene encoding a virulence gene or a gene required for efficient replication. Hitherto several deletion mutants of ILTV (gG (136), gC (112), glycoprotein J; gJ (137), thymidine kinase (138), U_L0 (139), U_L47 (111), U_L50 (140) genes and ORF A-E (141)) have been tested. Generally mutant vaccines are highly attenuated and provide a high level of protection (55). Moreover, gG, gC and gJ mutants have been identified as suitable for DIVA control strategies (112, 137, 142). Nonetheless each of these has their own limitations for example gJ mutant show reduced growth *in vitro* whereas gG mutant result in a low titred antibody response which is a restriction in serological

testing (137). Thus to date no ideal vaccine candidate exists to completely control ILT (121).

Other approaches to ILTV vaccines have also been attempted and have included immunization using subunit vaccines (143) or improved DNA vaccines (144, 145) however these approaches have not progressed to the development of commercial vaccines. More recently vaccination using virus-like particles displaying ILTV gB or gG on their surface has been studied however this approach induced a humoral immune response with only a minimal cell mediated immune response (129). Inactivated, subunit or DNA vaccines do not elicit strong cell mediated immune response compared to live attenuated vaccines (146) and their use is not favored when compared to the (55, 129)strong cell mediated immune responses that are generated by replication competent live attenuated vaccines which are associated with protection against ILTV (55, 129).

The route of administration has a substantial impact on the efficacy of immunity induced by vaccination (109). For instance, eye drop administration of the gG deletion mutant ILTV vaccine is recommended rather than administration via drinking water or aerosol spray. That is because vaccine administration via drinking water has been associated with suboptimal efficacy and vaccination via fine spray has produced suboptimal results in terms of vaccine safety (147). *In ovo* administration is the proposed ideal route of vaccination for poultry as it allows 100% coverage and does not cause stress to birds. Further this route allows fast delivery of vaccines and hence the associated cost for vaccination is low (84).

Virally vectored ILTV vaccines (134) are used commercially and deletion mutants of gG (148) and gJ (84) have been tested for *in ovo* delivery.

Despite advances in vaccine development and the implementation of strict biosecurity measures, ILT continues to challenge poultry industries worldwide (48, 54). The complexity of the disease and lack of an in depth understanding about key aspects of ILTV biology have limited the ability to completely control the disease (55). Latency and reactivation are important characteristics of ILTV that contribute to the complexity of the disease and have not been studied in detail (55).

3. Latency of neurotropic alphaherpesviruses

The ability to establish latency makes herpesviruses highly successful viruses (149). During latent infection viral gene expression gets restricted (150), no progeny viruses or infectious particles are produced, disease is absent in the host and transmission does not occur (151). Latent viruses are maintained in an immunoprivileged site within the host (152), without being detected by the immune system and they evade the host immune system by producing non-immunogenic molecules (153). Further, latent viruses promote host cell survival (154), they also monitor and manipulate the microenvironment (151, 155). At times a microenvironment suitable for a lytic cycle of virus replication leads to virus reactivation resulting in clinical disease (151).

The mechanism of ILTV latency has not been investigated in detail (55). It is assumed that the mechanism is similar to other alphaherpesviruses (109). Herpes simplex virus-1 (HSV) is one of mostly studied alphaherpesvirus but even in the

case of HSV-1 several limitations hinder complete understanding latency; *in vitro* models of HSV-1 are artificial, and *in vivo* models in heterologous species can behave differently to the natural host (156). Conflicting observations have been made by different groups of researchers and despite extensive research on herpesvirus latency, further studies are required to ascertain the mechanism of latency (151). In the absence of a sufficient body of work describing ILTV latency, the following sub-sections describe some key features of HSV-1 latency and lytic infection that could serve as a starting point to understanding latency in ILTV.

3.1. Molecular mechanisms of HSV-1 latency

During lytic cycles in primary replication sites, virus infects neuronal dendrites of secondary ganglia that innervate these sites. Then viruses undergo retrograde transport along microtubules to reach the neuronal cell bodies of these neurons ultimately entering sensory ganglia (157). Studies with experimental mouse models have shown HSV-1 can have lytic cycles of replication in some neurons of sensory ganglia, while in some other neurons viruses undergo a latent stage (158, 159). Establishment of HSV-1 latency in sensory ganglia happens during the acute phase of infection (160) which typically occurs within 21 dpi.

Understanding the molecular basis of lytic replication is pivotal to observing the differences between lytic and latent stages. Hence the molecular mechanisms of the HSV-1 lytic cycle have been reviewed briefly below.

3.1.1. The process of productive/ lytic HSV-1 replication

As mentioned in section 1.7, following fusion of the viral envelope with the host cellular membrane, the capsid together with tegument proteins gets released into the cytoplasm (61). The capsid is then transported along the microtubule network (71) and docks to the nuclear pore where rearrangement of capsid proteins leads to release of viral DNA into the nucleus (161). Viral DNA and tegument proteins will concurrently undergo series of events (Figure 3).

Immediately after entry, viral DNA gets circularized, coated by histones and cellular repressors of HCLR complex. This repressor complex derives its names from its component parts of *h*istone deacetylase (HDAC), *c*o repressor element-1 silencing transcriptional factor (CoREST), *l*ysine specific demethylase 1(LSD1) and *R*E 1- silencing transcriptional factor (REST). This viral DNA and protein complex is a heterochromatin structure that functions to repress the neuronal genes in non-neuronal cells. An additional protein, nuclear domain 10(ND_{10}) assemble at this heterochromatin structure silencing the viral DNA (80).

Virion protein 16 (VP-16) is a tegument protein introduced into the cytoplasm during viral entry. It acts as the main transcriptional activator of IE genes. As the initial step VP16 binds with host cell factor 1 (HCF-1) in the cytoplasm. HCF-1 is a protein that has a nuclear localization sequence and therefore once VP16 is associated with HCF-1 it gets transported into the nucleus (162).

All IE genes (genes corresponding to ICP0, ICP4, ICP22, ICP27, ICP47 and $U_S1.5$) have VP16 response element (VRE) within their cognate promoters. Once VP16 and HCF-1 reach the nucleus these two proteins interact with Octamer

binding protein 1 (Oct-1) to form a trimeric complex. Interaction takes place specifically on the consensus sequence VRE present in each IE promoter (151, 162). In addition to Oct-1, VP16 recruits LSD-1 within the nucleus. This leads to depression of transcriptional factors and disassembly of ND_{10} bodies. As a consequence IE gene transcription ensues producing ICP proteins (163).

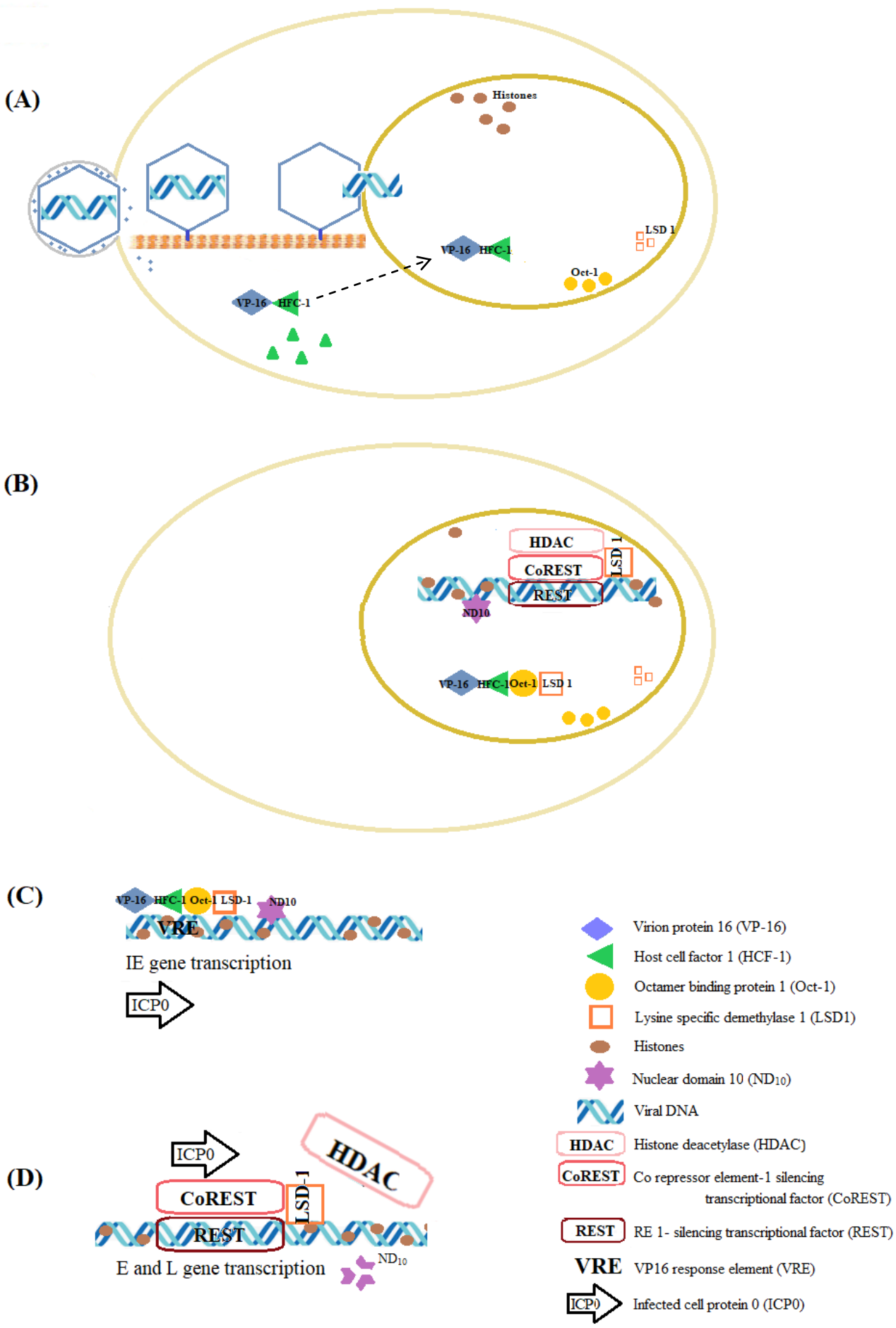
(A)
Histones
LSD 1
VP-16 HFC-1
Oct-1
VP-16 HFC-1
(B)
HDAC
CoREST
REST
LSD 1
ND10
VP-16 HFC-1 Oct-1 LSD 1
(C)
VP-16 HFC-1 Oct-1 LSD-1 ND10
VRE
IE gene transcription
ICP0
(D)
ICP0
HDAC
CoREST
REST
LSD-1
E and L gene transcription
ND10
Virion protein 16 (VP-16)
Host cell factor 1 (HCF-1)
Octamer binding protein 1 (Oct-1)
Lysine specific demethylase 1 (LSD1)
Histones
Nuclear domain 10 (ND10)
Viral DNA
HDAC Histone deacetylase (HDAC)
CoREST Co repressor element-1 silencing transcriptional factor (CoREST)
REST RE 1- silencing transcriptional factor (REST)
VRE VP16 response element (VRE)
ICP0 Infected cell protein 0 (ICP0)

Figure 3: Molecular mechanisms of HSV-1 immediate early (IE), early (E) and late (L) gene transcription

(A) Fusion of the viral envelope with the host cellular membrane and release of the nucleocapsid and tegument proteins into the cytoplasm. Nucleocapsids are transported along the microtubule network, docked at the nuclear pore and viral DNA is released into the host cell nucleus. Virion protein 16 (VP16) binds with host cell factor 1 (HCF-1) in the cytoplasm and is translocated to the nucleus **(B)** In the nucleus viral DNA becomes coated with histones forming heterochromatin. The cellular repressors of HCLR complex and nuclear domain 10 (ND_{10}) are assembled at the heterochromatin structure. VP16 and HCF-1 interact with Octamer binding protein 1 (Oct-1) to form a trimeric complex and recruits LSD -1 within the nucleus. **(C)** Interaction of trimeric complex with the VP16 response element (VRE) leading to depression of transcriptional factors, disassembly of ND_{10} bodies and transcription of immediate early (IE) genes including infected cell protein 0 (ICP0). **(D)** ICP0 binds with the co repressor element-1 silencing transcriptional factor (CoREST) component, dislodges the histone deacetylase (HDAC) and degrades ND_{10} domains to remove the repressor effect and enable early (E) and late (L) gene transcription.

The multifunctional protein ICP0 has a significant influence on transcription of E and L genes. Once produced, ICP0 binds with the CoREST component of HCLR complex and dislodges the HDAC element. Further, ICP0 can degrade ND_{10} domains thus removing the repressor effect of ND_{10}. This initiates transcription of E and L genes (163). The E and L gene transcription produces enzymes required for viral DNA replication and structural components for viral assembly (151). Although VP16 and ICP0 seem critical in viral gene transcription, the deletion mutant of VP16 and one or more IE genes are still capable of undergoing productive replication but with reduced efficiency (151).

Replication of HSV-1 DNA is another cascade of molecular events. As previously mentioned DNA replication machinery is largely conserved among herpes viruses (164) and in all herpes viruses DNA replication occurs with the contribution of 2 components; viral DNA sequences responsible for origin of replication and transacting proteins which facilitate the replication (82). The HSV-1 genome contains three origins (Ori) of replication: OriL in the UL region flanked by UL29 and UL30 genes, and two OriS sequences in IR and TR regions found either sided of the US region of the genome (165). Seven essential transacting proteins have been described for HSV-1: a single-strand DNA binding protein (known as ICP8 or UL29), a two-subunit DNA polymerase (Pol/UL42), a three subunit helicase/primase complex (H/P:UL5,UL8, and UL52) and a origin binding protein (UL9). Another 6 nonessential proteins which play auxiliary role have also been described in HSV-1 viral DNA synthesis and they are the proteins coded by UL23, UL39, UL40, UL2, UL50 and UL12 genes (82).

Early DNA sedimentation and electron microscopy based studies suggest 2 consecutive modes of HSV-1 DNA replication: an early bidirectional theta mode where circular DNA is synthesized and a late sigma mode which result concatemeric form of viral DNA (166-168). Initiation of DNA synthesis is marked by the distortion and destabilization of viral DNA at one of the three Ori regions (169). UL9 and UL29 attach to either side of the functional Ori and undergo a series of conformational changes leading to distortion of DNA via an ATP independent process (170). Next they enter an ATP dependent process whereby they induce formation of a hairpin at the functional Ori (171, 172). Then the H/P complex is recruited and is involved in unwinding the duplex DNA and synthesizing short RNA primers (173). Direct interaction of the active H/P complex with Pol/UL42 and/or synthesized RNA primers is important to attract the Pol/UL42 to the replication fork (174). Recursion of Pol/UL42 promotes leading and lagging strand synthesis (82).

The exact mechanism by which the theta replication mode switches to the sigma replication mode is not fully understood (175). In the sigma mode it has been proposed that rolling cycles of DNA synthesis occur generating long head-to-tail concatemers which form the substrate for cleavage and packing (176). However, other evidence suggests HSV replication may be more complex and may be recombination dependent, as occurs in dsDNA bacteriophages (e.g. λ) (177). Products of late DNA replication have been seen as extensively branched networks (91) and it has been proposed that they have arisen due to inter and/or intramolecular recombination of concatemeric DNA (94).

Concatemeric forms of DNA are cleaved at regular intervals (178). In the TR regions of the genome *cis*-acting sequences act as cleavage sites (179). Unit-length genomes are then packed into preassembled capsids (180). These capsids are synthesized as a result of translation of L genes in the cytoplasm and are translocated to the nucleus with the aid of nuclear localization sequences (73, 181). Three forms of capsids (type A, B and C) have been recognized and this classification has been based on the viral material present within the cavity. Type C capsids contain the viral DNA genome whereas type B capsids contain the scaffolding protein. Type A capsids contain neither the DNA nor the scaffolding protein (182). Excision into unit-length genomes and encapsulation is a coupled process and in HSV-1 it requires at least 7 gene products: (UL6, UL15, UL17, UL25, UL28, UL32 and UL33 (175). Further it has been found that packing in HSV-1 is a polar process occuring from L terminus to S terminus (175).

Once encapsulation is completed the viral capsids undergo maturation within the nucleus. At this stage viral tegument proteins VP1/2 and UL37 will be added to the capsid (183). Virus particles leave through the inner nuclear membrane into the perinuclear lumen which leads to acquisition of the primary envelop of the virion (184). Protein UL34 and UL31 constitutes the nuclear egress complex anchored into the primary envelope (185). Enveloped viral particles in the perinuclear space will then fuse with the outer nuclear membrane releasing the capsid and tegument proteins to the cytoplasm for further maturation (19).

In the cytoplasm additional tegument proteins (UL7, UL11, UL16 and UL51) are incorperated into the capsid (186). HSV-1 glycoproteins are translated in the

endoplasmic reticulum and through the *trans* Golgi network these will be directed to multi-vesicular bodies (187). Multi vesicular bodies carry these glycoproteins to the plasma membrane where endocytosis will occur to generate early endosomes (188). Virus particles that mature in the cytoplasm will fuse with these HSV-1 glycoprotein containing endosomes to form infectious virions that ultimately get secreted in to extracellular medium (189).

3.1.2. Establishment of latency by HSV-1

According prevailing hypotheses, HSV-1 undergo latency in sensory ganglia because the virus encounters a restricted availability of key components required for IE transcription (80). For instance, VP16 is thought to be inefficiently transported in the cell body of sensory neurons to exert its function (190). Further Kolb and Kristei (2008) have shown that HCF-1 is only available in the cytoplasm of sensory neuronal cells and hence unlikely to associate with VP16 at nerve terminals (191). Furthermore, Oct-1 is down regulated in neuronal cells (192). As a consequence viral DNA entering the neuronal nucleus will be silenced by repressive histone modification and HCLR complex (151). However, since REST is not a normal constituent of HCLR complex of neurons (163) it has been suggested that in the establishment of HSV-1 latency, REST is induced or recruited from adjacent satellite cells (163).

Since VP16, HCF-1 and Oct-1 trimetric complex is not available to induce IE transcription, viral DNA remains as heterochromatin (162). Unlike the linear dsDNA found in the virus particle, HSV-1 DNA is maintained as a circular episome during latency with histone proteins attached to latent genomes forming

nucleosomes (193) . The only transcripts that are expressed during latency is a prominent non coding intron called ‘latency associated transcripts (LAT)(194, 195). LAT comprise a set of co-linear RNA (195) in particular, a set of small ribonucleic acid (sRNA) and micro ribonucleic acid (miRNA) (80). Surveying of the micro environment of the neuron during latency is done by these RNAs (155).

3.1.3. Maintenance of latency

Once latency has been established, the maintenance phase is characterized by persistence of the silent viral genome for the life of the host, evading immune detection and promoting host cell survival (162). It has been suggested that LAT encoded siRNA or miRNA play a key role in maintenance of latency (151, 155). However, expression of LATs is not only restricted to the maintenance phase, but some LATs are also expressed during acute HSV-1 infection (158). HSV-1 can establish, maintain and reactivate from latency even without LATs in their genome (196, 197). This suggests non-LAT measures are also involved in maintenance of latency (151).

3.1.3.1. LATs

As mentioned earlier LATs are the only transcript that can be detected throughout the period of latency (80, 151, 162). LATs are encoded within the repeat regions of HSV-1 genome (repeat regions flanking U_L region of the genome). Even though ORFs are found in LAT coding regions it has never been possible to detect proteins produced from these ORFs (162). Initial transcription of these codes produces a primary transcript known as “minor LAT” which is 8.3 kbp in size. This primary transcript is then spliced producing 2.0 kbp and 1.5 kbp introns

respectively. These two RNA transcripts are together termed “major LATs” because they are comparatively abundant. Major LATs have a stable structure compared to minor LATs (198).

Once transcribed, LATs can down regulate lytic genes which eventually facilitates maintenance of latency (199, 200). Moreover, LAT encoded mRNA has the potential to down regulate translation of viral genes, restricting expression of viral protein. This favors maintenance of latency as it aids immune evasion (155). While HSV-1 genomes encode several genes that interfere with apoptosis during productive cycles of lytic infection, these genes are not expressed during latency and an alternative mechanism is required to promote host cell survival. During latency, this achieved by the anti-apoptotic function of LATs (201). Interestingly, this anti-apoptotic activity is brought about a larger RNA which cannot be categorized as miRNA (151).

LATs are an attractive means of regulating key events of latency because of following features. Firstly, they have the capacity to regulate multiple transcripts and can directly act on both viral and cellular targets. Secondly, they can exert their effects without producing proteins. Also, because these small RNAs are intrinsically non-immunogenic, viruses do not need extra measures to evade immune mechanisms during latency. Therefore, it is apparent that the evolutionary success of herpesviruses has been aided by the use of LATs to regulate latency in an efficient manner (151).

3.1.3.2. Non-LAT measures involved in maintenance of HSV-1 latency

CD8+ T cell mechanism of maintaining HSV-1 latency

CD8+ T cells that are juxtaposed to HSV-1 latently infected neurons can inhibit neuronal HSV-1 replication in a non-cytolytic fashion. CD8+ T cells recognize the immunodominant gB epitope in infected cells which leads to production of IFNɤ and CD8+ T cell derived granzyme B. This enzyme degrades ICP4 protein which is an IE protein required for further viral gene expression and eventually viral genome expression is arrested. (202, 203).

Epigenetic mechanisms of regulating viral lytic gene expression

In beta- and gammaherpesviruses, methylation of DNA is important in the regulation of lytic gene expression. However, HSV-1 lytic gene expression is not regulated by DNA methylation and instead appears to utilize epigenetic modification of histones. As mentioned above, when naked viral DNA enters the nucleus it is condensed to form chromatin and becomes associated with octamers of histone protein to form a nucleosome. The N- termini of histones then undergoes post transitional modifications forming euchromatin or heterochromatin. Both forms of chromatin have been identified in latent HSV genomes, which indicates lytic gene expression of HSV is subjected to epigenetic modifications (151, 204, 205).

3.1.3.3. Non-uniformity of HSV latency

Another striking feature of HSV latency is that the non-uniformity of the latent stage, with latently infected sensory neurons exhibiting differences between each

other. For instance, Herpes simplex viruses (HSV) show a preference towards certain types of sensory neurons in which to establish latent infection. Within sensory ganglia different populations of sensory neurons have been recognized and they have been classified according to their cellular morphology, physiological response properties, and patterns of gene expression. A5 and KH10 are two such functionally distinct nociceptive neuronal populations. A5 neurons express Galβ1-4GlcNAc-R epitopes and TrkA, the high-affinity NGF receptor. In contrast KH10 neurons express Galα1-3Galβ1-4NAc-R epitopes and little or no TrkA (206, 207). Further, A5 neurones project their A and C fibers to laminae I and II (outer) of the dorsal horn while KH10 neurons project C fibers to lamina II (inner) of the dorsal horn (208). Margolis et al. (209) have shown HSV-1 and HSV-2 preferentially establish latent infection in A5 and KH10 positive cells, respectively. Further, by conducting studies using a chimeric HSV-2 mutant expressing HSV-1 LAT coding regions, Margolis et al. (209) showed chimeric HSV-2 to exhibit an HSV-1 phenotype that was capable of establishing latent infection in A5 positive neurons. Thus it was been proposed that the difference in the preference is brought about by the LATs coding region. Shortly afterwards Imai et al., (206) used HSV-1 LAT deletion mutants to demonstrate that most of the LAT 5' exon is not required for HSV-1 to preferentially establish latent infection in A5-positive neurons. More recent findings with the use of adult murine TG neurons *in vitro* suggest HSV-1 enters the majority of sensory neurons however productive infections and latent infections occur preferentially in KH10 and A5 neurons, respectively, while reactivation can be induced only from A5 neurons (210).

Viral DNA is not evenly distributed among latently infected groups of sensory neurons. The majority of latently infected neurons will contain 10 - 100 genome copy numbers (GCN) whilst some may contain more than 1000 GCN (211). The reason for this uneven distribution of GCN is not clear but several possibilities have been put forward to explain this phenomenon. One interpretation is related to preference of HSV-1 towards different sensory neurons. The cellular marker A5 positive neurons do not support the lytic HSV-1 infection (210). These neurons may act as a dead end for productive replication and therefore viral DNA that sequentially enter these cells during productive replication accumulates leading to a larger copy number per cell. According to another explanation these cells are permissive but productive infection has been silenced at a later stage, perhaps by CD8+ T cell inhibition of neuronal HSV replication. Therefore, replicated genomes can accumulate in these cells (151). These high GCN bearing cells represent a reservoir of viral genomes that have the potential to be reactivated (212).

Non uniformity is also found with respect to the number of latently infected neurons that express LATs in a latently infected ganglion. Of the viral DNA harboring latently infected neurons, only 5-30% of the neurons transcribe LATs (213-216). According to Proenca et al. (217) level of LATs expression is dynamic during the period of latent infection.

Additionally, HSV-1 latent genomes are enriched with two distict forms of heterochromatin; facultative and constitutive (218). Heterochromatin can be facultative, constitutive or any intermediate form between the two extremes.

Facultative heterochromatin is reversible and can get converted to euchromatin which actively engage in replication. Constitutive heterochromatin is more stable and is found associated with structures such as centromeres and telomers of mammalian DNA (151). Cliffe et al. (219) and Kwaiatkowski et al. (218) have observed that lytic gene promoters of HSV-1 latent are enriched with both types of heterochromatin.

3.1.4. Reactivation from latency

Reactivation is the production of infectious viral particles from the latently infected tissues/cells (162). Since no lytic gene products are detected during latency it is believed that reactivation initiates without preformed viral proteins and perhaps exclusively using cellular mechanisms (151).

A variety of stimuli have been reported to trigger reactivation from latency. Natural exposures to UV light (220), emotional stress (221, 222), fever, tissue damage (223, 224) and immune suppression (225) have caused HSV-1 reactivation in humans. Stimuli such as transient induction of hyperthermia (226), UV irradiation (227), CD8+ cell depletion (228) and corneal scarification (227) have been used to induce reactivation of latent HSV-1 in mouse models *in vivo*. Provision of compounds such as HSV protein (VP16 and ICP4) (229), the histone deacetylase inhibitors (230), forskolin (231), inducible cyclic AMP early repressor (232), capsaicin (233) and caspase-3-activator C2 ceramide has reactivated HSV-1 latency in neuronal cell cultures. Addition of dexamethasone (234, 235), withdrawal of nerve growth factor (NGF) (236) and induction of

hyperthermia (234) have also been used to reactivate latent HSV-1 infection in neuronal cultures.

How all these stimuli provoke the signaling cascade that reactivate latent infection is still unclear, however it has been revealed that only a small fraction of latently infected cells are capable of reactivating following stimuli (237). Moreover, many factors can influence the efficiency of reactivation. Some of such factors include latent DNA copy number, level of LAT expression, neuronal type involved, degree of genome expression and level of CD8+ T cell immune surveillance (151).

Molecular mechanisms of reactivation are highly regulated. Which viral promoters respond to the triggers of reactivation and which viral genes are responsible for initiation of lytic cycle breaking the phase of latency is still unclear (151). Lytic cycles of reactivation appear to be different to that of primary lytic cycles (162) since the latent genome does not have preformed tegument proteins (especially VP16) or its co-factors to initiate the process (151, 162). Initial studies have identified ICP0 as a significant driver of reactivation. This view was supported by observations such as the inability of an ICP0 deficient mutant of HSV-1 to reactivate from latency in an i*n vivo* model (238), ICP0 being the only HSV-1 protein that reversed the quiescent state of HSV-1 in non-neuronal tissue *in vitro* (239), the capacity of ICP0 to degrade ND_{10} domains (240) and because ICP0 is a non-selective activator of viral gene expression (240). However, there have been contradictory observations as well. Thompson and Sawtell (241) have shown similar number of latently infected neurons exit

from latency in both groups of mice infected with ICP0 null mutants of HSV-1 and its parent strain of HSV-1. In the same year Miller et al., (242) have demonstrated it is not ICP0 but the acidic activation domain of VP16 is required for efficient reactivation HSV-1 using quiescently infected neuronal cells.

Attention has been drawn to VP16 as an important inducer of reactivation in recent years. Studies conducted on VP16 have shown that it has a profound regulatory effect on neurons (243). VP16 contains chromatin remodeling features (244) and is a potent transcriptional activator (Section 3.1.1) (245). Further, VP16 is essential for viral lytic cycles in both primary infection of neurons and in reactivation from latency. In the latter circumstance it is assumed that *de novo* synthesized VP16 is involved (162). In a study that tested reactivation competency of different HSV-1 mutants; deleted ICP0, deleted ICP4 or, a virus with mutated VP16 region, only the VP16 mutant failed to reactivate efficiently from latency in neuronally differentiated PC12 cells following a stress stimulus (242).

Given that VP16 functions with HCF-1, Oct-1 and LSD-1 to induce transcription during lytic infection (section 3.1.1), it is logical that VP16 require co-factors Oct-1 and HCF-1 to exert its effect in reactivation from latency. Supporting this logic, ganglionic explant experiments have shown that Oct-1 expression is induced in sensory neurons (246) while HCF-1 re-localizes from the Golgi to the nucleus (191). Moreover, HCF-1 recruits LSD-1 to virus IE promotors to derepress the latent genome and facilitate the lytic phase of replication within sensory neuron (247).

3.1.5. Fate of reactivated viruses and viral shedding

Completion of reactivation, of which production of progeny viruses becomes the end point, is essential for recurrence of HSV-1 infection (162). Upon completion, infectious particles are produced within the sensory neurons that are then transported anterograde to specific sites. This facilitates recurrence of infection in peripheral epithelia and transmission to naïve hosts (151).

Thus, the mechanisms of latency are complex and still incompletely understood. The lack of an ideal model to be used in latency experiments contributed to our incomplete understanding of mechanisms of HSV-1 latency.

3.2. Models for study HSV-1 latency and reactivation

Ethical and practical considerations have prevented the use of natural host species in HSV-1 experiments. Many aspects of latency and reactivation have been studied using *in vivo* (experimental animals) and *in vitro* (cell cultures) models. Even though plenty of useful and important information had been revealed using these models, none of the models have been able to fully replicate the events that occur in the natural host (156).

3.2.1. *In vivo* models

In vivo models of HSV-1 include experimental infection in mice (217, 219, 241, 243, 248-253), rabbits (254-257) and guinea pigs (258, 259). Of these, the mouse is the predominantly used animal model as it can be supported with preferential gene manipulations such as gene knock-out, and transgenesis (260). Upon

peripheral inoculation (scarification of cornea (206, 219, 241, 243, 250-252, 261, 262), ear pinna (217), foot pad inoculation (218), or other (263)) of HSV-1, mice develop acute disease leading to latency. However, mouse models exhibit limited spontaneous clinical reactivations or recurrent lesions similar to that of natural host (260). Hence many latency trials that have been based on mouse model are followed by induction of *in vivo* reactivation using a stimulus, for example hyperthermia treatment (226, 241, 243) and followed by *ex vivo* reactivation. In contrast, rabbit model has shown to support HSV-1 spontaneous reactivation. In rabbit eye model of HSV-1 spontaneous reactivation leads to shed the virus in tears (257). The rabbit model is considered to be the gold standard for study recurrent HSV-1 infections (156). Recently a human leukocyte antigen (HLA) transgenic rabbit model was developed that mounts "human-like" CD8+ T-cell immune responses against HSV-1 (264).

3.2.2. *Ex vivo* models

Ganglia explant (230, 241, 243, 250, 251, 265), ganglia co-culture (238, 248, 249, 252), dissociated ganglia cell explant (266) and dissociated ganglia cell co-culture (267) have been performed as *ex-vivo* models of studying HSV-1 reactivation. *Ex vivo* reactivation always follows *in vivo* infection and around 30 dpi, latently infected sensory ganglia (TG or dorsal root ganglia) are dissected and placed in culture. Ganglia are often explanted as is (241, 243, 250, 251), cut in to several pieces before culture (230, 238, 252, 268, 269) or dissociated before culture using enzymes such as collagenase to make a single suspension (249, 266). Ganglia tissues or cell suspensions are cultured in a favorable media with (co-culture) or without (explant) a permissive monolayer.

During incubation, latent HSV-1 genomes within the neuronal cells of these sensory ganglia are reactivated and progeny viruses are released into culture media, where heat stress (43 °C for 3 hours) can accelerate HSV-1 *ex vivo* reactivation (238, 252). HSV-1 reactivation in explants has been detected by transferring culture supernatant into a secondary monolayer and observing CPE (230, 238, 250, 251, 266, 269). Other methods of detecting *ex vivo* reactivation include plaque assay of the homogenized explanted ganglia (241, 243), DNA extraction and qPCR (217) of explanted tissue or *in situ* hybridization (268). The type of the *ex vivo* model used, as well as the method used to detect reactivation, have an impact on the final observation (249, 250). Chen et al., (249) have observed significant improvements in HSV-1 reactivation rates in co-cultured dissociated ganglia compared to co-cultured minced ganglia. Doll and Sawtell (250) have shown plaque assay of the homogenized explanted ganglia is a better method of detecting the initial time point of *ex vivo* HSV-1 reactivation compared to testing the culture supernatant in a secondary monolayer. *Ex vivo* HSV-1 reactivation is seen as early as 12 hours post explant when explanted TG are homogenized and used to perform a plaque assay (268). However, the first time point of detection can take as long as 3-4 days post explant in TG co-cultures if a supporting monolayer is used to detect CPE (249, 252). Reactivation rates are relatively low in ganglion explant models, with only 5 - 10% of latently infected neurons in a ganglia reactivated upon explant (156). Thus the models used to study HSV-1 latency and reactivation vary widely and the choice of the model is based on the objective of the research.

3.2.3. *In vitro* models

In vitro systems are used frequently in HSV-1 research and can overcome some of the limitations associated with *in vivo* models. When compared with *in vivo* models, *in vitro* models are more easily controlled and convenient (156, 270). *In vitro* models generally include cell culture models (of non-neuronal or neuronal origin), ganglion explant models and ganglion co-culture models.

Non-neuronal cell lines include human fibroblasts (271, 272), human keratinocyte cell lines (273, 274) and rat pheochromocytoma (PC12)(275, 276). Rat prenatal sympathetic neurons (277, 278), rat neonatal/adult dorsal root ganglion (279) or superior cervical ganglion neuron (280) cells are the commonly used mouse-origin neuronal cell lines. Human-origin neuroblastoma (SH-SY5Y) cell lines (281, 282), stem cells(283, 284) and immortalized ganglion cell lines(285) have been used in HSV-1 experiments. Each of these cell lines has their own advantages and disadvantages. Latency is usually achieved in these models by treating with antiviral drugs such as acyclovir (277;Camarena, 2010 #262, 280) to block the lytic replication cycles, or by the use of elevated temperatures (271) or replication defective mutants(279). Various stimuli such as withdrawal of NGF (279) and treatment with chemicals (eg. trichostatin A (277), PI3-kinase inhibitor (286, 287)) can be used to reactivate latency in these models. Therefore, HSV-1 latency studies in cell culture models have been often criticized for use of highly artificial conditions and HSV-1 does not establish true latency in non-neuronal cell culture models For example when non-neuronal cell cultures such as human fibroblasts are infected with HSV-1 the viral genomes undergo a tightly repressed stage called ‘quiescence’ which is different to true latency. These quiescent

genomes are non-responsive to many reactivation stimuli that can reactivate latency in neuronal cell line (151, 270).

4. What is known so far about ILTV latency?

Similar to HSV, latency has largely contributed to the success and persistence of ILTV in host populations (56). During initial replication in respiratory and ocular tissue, ILTV reaches the TG which has been identified as the main site for latency (288). The mechanism by which ILTV enters into TG has not been described but it has been assumed that the mechanisms are similar to those used by other alphaherpesviruses, such as HSV (109). There have been reports of recovering latent viruses from trachea via tracheal organ cultures indicating that trachea may also be a site for latency (289). By performing virus isolation Bagust et al. (108) have detected ILTV in TG 4-7 days after intra-tracheal challenge with the CSW-1 strain whereas Han et al. (290) have demonstrated both vaccine and field virus in TG 7 dpi. The exact location of the latent ILTV in trachea has not been determined and therefore the cell types where latent ILTV genome may be harboured in the trachea is currently unknown. It is not clear if the recovered virus in the trachea represents a true latent infection or a persistent infection.

Johnson et al. (291) have identified potential LATs in the ICP-4 region of ILTV. Viral miRNA appeared to play a key role in regulation of latency (292-294) and two such important miRNA have been identified from putative LATs in the ICP-4 region of ILTV (295). It has been predicted that these miRNA are involved in switching between the lytic and latent infections (295, 296).

Reactivation of ILTV has been coupled with stressors such as onset of laying or re-housing of birds and it can occur from time-to-time throughout the life of the host. With reactivation virus resumes its lytic cycle and hence birds start shedding the virus thus infecting in contact susceptible birds (56).

Available information regarding latency of ILTV is limited and there is much that remains to be discovered about the complex molecular and immunological events occurring in establishment, viral persistent and reactivation of ILTV in its natural host (121). In an era where ILT is controlled using vaccines (84), the latency characteristics of the commonly used vaccines will be an important factor to consider in the control of the disease. Further, with the recent evidence of emergence of multiple virulent recombinant strains of ILTV, it will be useful to assess the capacity of recombination of ILTV after latency and reactivation.

5. Research Aims

The purpose of this study was to investigate tissue tropism and latency characteristics of ILTV in the natural host (chickens) and thereby advance our understanding of ILTV pathogenesis.

The specific aims of this investigation were to:

1. Optimise molecular techniques to detect ILTV genome using PCR and to develop a TG culture techniques to *in vitro* reactivate latent ILTV genome
2. Test the capacity of ILTV vaccines (SA2, A20, Serva and glycoprotein G deleted vaccine candidate) to establish latency
3. Compare latency and late systemic lymphocyte responses induced by a vaccine (SA2) and a virulent field strain of ILTV (CL9)
4. Investigate tissue tropism of class 9 and class 10 ILTV in Australian SPF and meat chickens

Chapter 2

Development and application of a combined molecular and tissue culture based approach to detect latent infectious laryngotracheitis virus (ILTV) in chickens

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Development and application of a combined molecular and tissue culture-based approach to detect latent infectious laryngotracheitis virus (ILTV) in chickens

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ABSTRACT

Infectious laryngotracheitis virus (ILTV) causes severe respiratory disease in chickens. ILTV can establish latency and reactivate later in life, but there have been few investigations of ILTV latency. This study aimed to contribute to the methodologies available to detect latent ILTV. A nested PCR was developed which was more sensitive than three other molecular methods investigated in this study. This nested PCR was then used in conjunction with *in vitro* reactivation culture methods that were optimized and applied to trigeminal ganglia (TG) and tracheal samples from ILTV-vaccinated commercial layer birds (n = 30). ILTV DNA was detected by nested PCR in the upper respiratory tract (URT) or eye of 22 birds. Of the remaining 8 birds, ILTV could be detected by co-culture in TG of 5 birds, with reactivated virus mostly detected 6 days post-explant (dpe). ILTV was also detected in tracheal cultures by 6 dpe. In the ILTV-positive URT samples, the virus could be characterised as vaccine strains SA2 (n = 9) or A20 (n = 5). This study provides evidence for reactivation and shedding of vaccine ILTV in commercial layer birds. Moreover, this study produced a molecular and *in-vitro* culture method to detect latent viral infection.

1. Introduction

Infectious laryngotracheitis virus (ILTV, *Gallid alphaherpesvirus 1*) (Davison et al., 2009), causes a highly contagious, acute respiratory tract disease in chickens (Kirkpatrick et al., 2006a; Lee et al., 2015; Vagnozzi et al., 2015), with significant economic losses especially to intensive poultry production systems (Chin et al., 2009; Guy et al., 1991). The severity of the disease, infectious laryngotracheitis (ILT), can vary from mild to severe depending on the strain involved (Chacón et al., 2015; Graham et al., 2000; Moreno et al., 2010; Neff et al., 2008; Ojkic et al., 2006; Oldoni et al., 2008). Attenuated strains of ILTV are widely used as vaccines to control the disease (García et al., 2013). However, there are known limitations associated with some of these attenuated vaccines which include reverting back to a virulent form (Guy et al., 1990), spreading to naïve populations (Chacón et al., 2015; Oldoni et al., 2008) and facilitating the generation of recombinant viruses that are more virulent (Lee et al., 2013, 2012) contributing to the fitness of strains that might overtake previously predominant field strains (Agnew-Crumpton et al., 2016; Blacker et al., 2011; Lee et al., 2015).

Both, field and vaccine strains of ILTV have been reported to establish latent infection in the trachea (Bagust, 1986) and trigeminal ganglia (TG) (Chacón et al., 2015; Han and Kim, 2003; Hughes et al., 1991; Parra et al., 2015; Williams et al., 1992). Of these two sites, the latter has been recognized as the predominant site of latency, consistent with most other alphaherpesviruses (Williams et al., 1992). Latency is considered to be a major reason why complete control of ILTV cannot be achieved (Bagust and Johnson, 1995). To date, few studies have been published on ILTV latency and these have largely relied on PCR to detect viral genomes in sites of latency, without examination of reactivation potential of the latent viruses *in-vivo* or *in-vitro* (Chacón et al., 2015; Han and Kim, 2003; Parra et al., 2015; Rodríguez-Avila et al., 2007; Williams et al., 1992).

Latency in human herpes simplex virus (HSV) -1 has been classically defined as the capacity of a viral genome to be maintained in a tissue without generating infectious particles, lytic viral transcripts or viral proteins (Stevens, 1989). This state results in concealing the virus from the host immune system while maintaining the capacity to reactivate when the micro-environment is suitable for lytic replication (Stevens,

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1989). Detection of viral genomes in the sites of latency alone is insufficient evidence to conclude that viruses are latent, as the capacity for virus to be reactivated is unknown. Mouse models of HSV-1 infection have shown that latent genomes are not usually present in high copy numbers at the sites of latency unless the initial viral load is high or it is in the stage of reactivation (Sawtell, 1997) and the latent genome copy numbers can differ depending on a range of variables including titre of the inoculum and viral genetic factors (Sawtell, 1997; Sawtell et al., 1998). Latency associated transcripts (LATs) have been identified and studied in other herpesviruses (Sawtell, 1997; Stevens et al., 1987; Tenser et al., 1989), but there is no information regarding LATs for ILTV.

Despite being a relatively under-studied area of ILTV biology, ILTV latency remains a concern and is repeatedly highlighted in discussions and reviews regarding the epidemiology of ILTV (Coppo et al., 2013a, b; Fuchs et al., 2007). Therefore, identifying a sensitive approach for detecting latently infected chickens will be valuable in future ILTV studies. The current study aimed to combine a sensitive molecular assay with optimised culture-based methods of virus reactivation to detect latent virus in ILTV-infected commercial chickens.

2. Methodology

2.1. ILTV strains

Five field ILTV strains; V1-99, CSW-1, Class 8 (CL8), Class 9 (CL9) and Class 10 (CL10) ILTV and four ILTV vaccine strains; glycoprotein G deleted mutant (ΔgG), Serva (MSD Nobilis® ILT, MSD), A20 (Poulvac® Laryngo A20, Zoetis), and SA2 (Poulvac® Laryngo SA2, Zoetis) were used in this study. All field ILTV strains and the ΔgG vaccine candidate were propagated and titrated in Leghorn Male Hepatoma (LMH) cell line as previously described (Devlin et al., 2007). Serva, A20 and SA2 vaccine strains were propagated and titrated on chicken embryo kidney cell (CEK) monolayers as previously described (Lukert, 1965).

2.2. Comparing limits of detection of PCR assays

The sensitivity of four different molecular (PCR-based) assays to detect ILTV DNA were compared with the aim of identifying a suitable assay to use in conjunction with culture-based approaches for the detection of latent virus in ILTV-infected birds.

2.2.1. Dilutions and DNA extraction

Ten-fold serial dilutions were prepared using viral stocks of six different ILTV strains; V1-99, CSW-1, ΔgG, Serva, A20, and SA2. Automated DNA extraction was performed on 200 µl of each dilution using the PureLink® Pro 96 viral RNA/DNA purification kit (Invitrogen®, Carlsbad, California, United States) and the Corbett Xtractor Gene Robot (Corbett Life Science Pty Ltd, Sydney, Australia). DNA extracted from these dilutions was eluted in 200 µl of elution buffer and used as template in 4 separate PCR assays (described in 3.1–3.4 below) to directly compare the limits of detection of each assay.

2.2.2. Specific TaqMan PCRs to detect different strains of ILTV

To explore the use of TaqMan PCRs for the detection of specific ILTV strains we first developed specific TaqMan PCRs for 5 different ILTV strains and assessed the level of sensitivity of each assay. As these TaqMan PCRs were not as sensitive as other methods of ILTV detection, the development of TaqMan PCRs for the other strains was not pursued. The full genome sequences of 5 ILTV strains; CSW-1 (used for the design of ΔgG probe), V1-99, Serva, SA2, and A20 (Genbank accession JX646899, JX646898, HQ630064, JN596962 and JN596963, respectively) were aligned using the MUSCLE alignment function in Geneious 9.1 software (Kearse et al., 2012). The alignment was scanned to identify strain-specific single nucleotide polymorphisms (SNPs). All the SNPs identified by genome alignment of published sequences were confirmed by Sanger-sequencing of the target region following amplification with the oligonucleotide primers listed in Table A1. Two hundred base pair (bp) fragments of sequence around the SNPs of interest were extracted and uploaded to RealTime Design Software, Biosearch Technologies (https://www.biosearchtech.com/realtimedesign) to design TaqMan probes, targeting the SNPs, and their respective primers. Six-carboxy-fluorescein (FAM) was used as the dye. Generated TaqMan probe sequences were analysed using the Oligoevaluator option (https://www.biosearchtech.com/ProbeITy/design/OligoEvaluator.aspx). The primers and probes that were evaluated as 'excellent' by the software were chosen for further testing. The probes and primers finally used in these qPCRs are shown in Table A2.

The master mix and templates were dispensed using a QIAgility Robot (QIAGEN). The 12.5 µl TaqMan PCR reaction included 6.25 µl of the TaqManGTXpress Master Mix (Applied Biosystems), 0.25 µM of the probe, 0.5 µM of the relevant upstream and downstream primers, 2.5 µl of DNA template and nuclease-free water to reach the final volume.

Thermal cycling was performed in an MX3000 real-time thermocycler (Stratagene). Cycling conditions included an initial step at 95 °C for 2 min, 40 cycles of 95 °C for 30 s and at annealing temperature (Ta) of the probe (Table A2) for 1 min. At the end of each cycle fluorescence acquisition was performed and any sample with cycle threshold (Ct) value ≤ 40 was considered positive. Appropriate positive (ILTV strain specific for the probe) and negative (ILTV strain non-specific for each probe and template negative) control samples were included in each run to validate the TaqMan assay.

2.2.3. UL15 gene targeted quantitative PCR (UL15qPCR)

Oligonucleotide primers for this qPCR have been previously described by Mahmoudian et al. (2011). The modified protocol included the use of GoTaq® system (Promega, Madison, Wisconsin, USA) with 0.1 mM of each deoxyribonucleotide (dNTP), 2 mM magnesium chloride, 4 µM Syto9 (Invitrogen, Eugene, Oregon, USA), 0.8 µM of each oligonucleotide primer, 0.05 U of Go*Taq*®DNA Polymerase (Promega), 2 µl of template DNA or plasmid dilution and nuclease-free water to reach a final reaction volume of 25 µl. For each run, a standard curve was generated using triplicates of ten-fold dilutions of a solution containing pGEM-T (Promega) bearing the 113 bp fragment of UL15 gene amplified from SA2 ILTV as the template (from 10^8 to 10^1 genome copy number/µl). A Qubit fluorometer (Life Technologies) was used to determine the concentration of pGEM-T in stock solutions prior to ten-fold dilutions following the manufacturer's instructions. The QIAgility System (QIAGEN) for automated PCR setup was used to prepare the standard serial dilution of the plasmid and dispensing the PCR master mix and template into Rotor-Discs. Triplicates of each viral dilution were subjected to PCR. The Rotor-Gene Q thermocycler (QIAGEN) was used for thermocycling and data acquisition. The PCR reactions had two initial holds at 50 °C for 2 min and then at 95 °C for 5 min. Then the reactions were subjected for 40 cycles of denaturation (95 °C for 5 s), annealing (60 °C for 30 s) and extension (72 °C for 30 s) with fluorescence acquisition at the end of each extension step. Following another hold at 72 °C for 3 min and 95 °C for 1 min, high resolution melting analysis was done with temperature increase steps of 0.3 °C from 50 °C to 95 °C. Under these conditions, the linear range of detection was 2×10^8 to 2×10^2 genome copies per reaction, hence 200 copies per reaction was considered as the cut-off value for this assay. Any amplicon with a deviated melting pattern to that of the standards was excluded and the viral copy numbers in each sample were calculated based on the standard curve generated by the Rotor-Gene Q series software (version 2.1.0.9 - Windows platforms, QIAGEN).

2.2.4. Universal herpesvirus nested PCR (UHVNPCR)

Degenerate oligonucleotide primers for this nested PCR, targeting a conserved region of herpesvirus DNA polymerase gene, have been previously described by VanDevanter et al. (1996). In the current study 25 µl reactions were performed in both rounds using the Go*Taq*® system

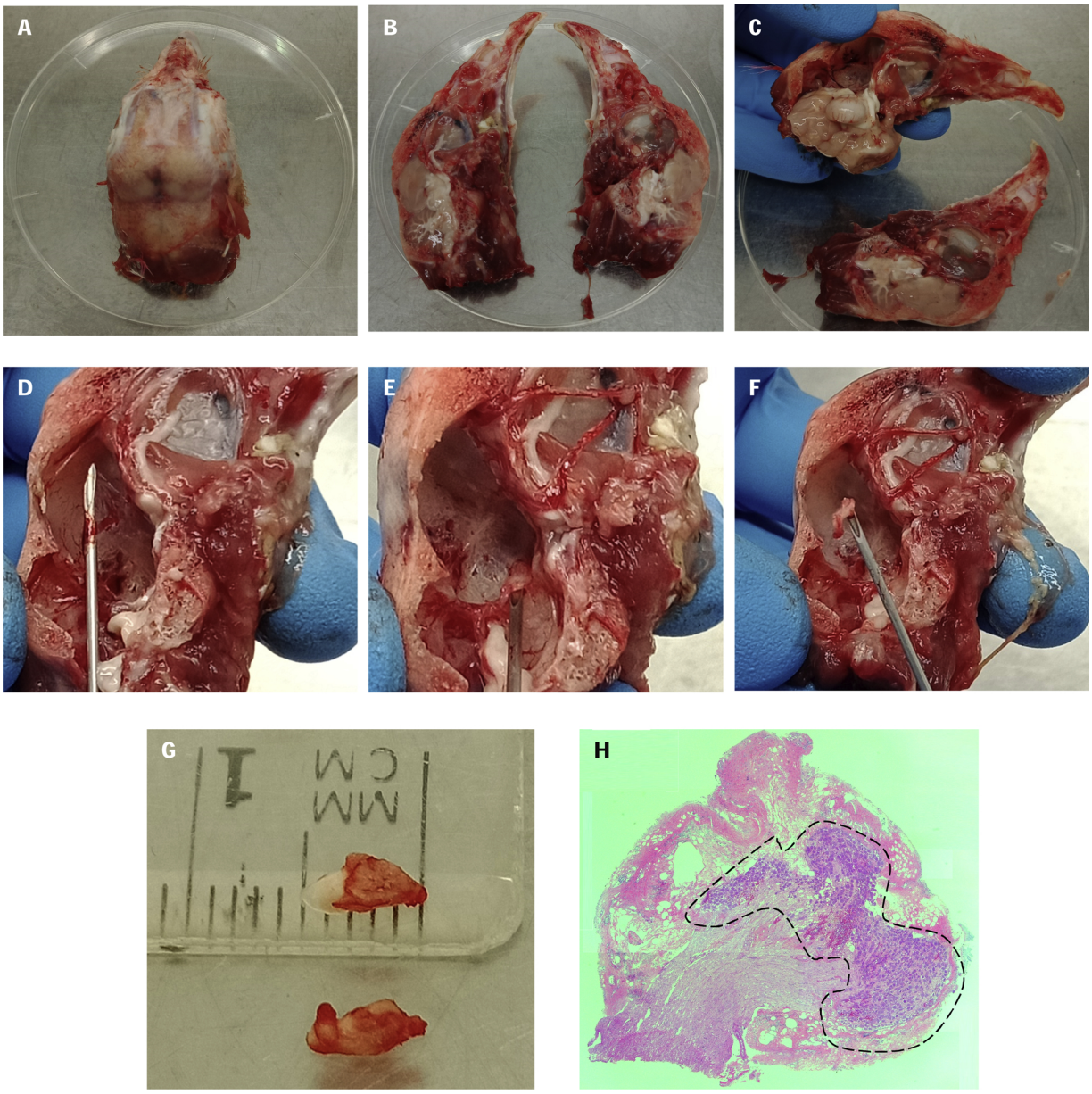

Fig. 1. Steps of neuro-dissection to collect TG and H&E stained section of a trigeminal ganglion.
During neuro-dissection, skin is removed over the cranium after decapitation (A), an incision is made along the sagittal plane (B), the cerebrum and the cerebellum are exteriorized by detaching from the brain stem (C), the meninges are removed (D), the trigeminal ganglion is located on the floor of the cranial cavity (E), the trigeminal ganglion is dissected out using 18G needle (F), yielding a trigeminal ganglion of approximately 5 mm in length (G). Dashed line indicates the aggregation of neuronal cell bodies within an H&E stained section of the excised trigeminal ganglion (H).

(Promega, Madison, Wisconsin, USA) according to manufacturer's instructions. Reactions contained 0.2 mM of each dNTP, 2 mM magnesium chloride, 0.04 U of Go*Taq*®DNA Polymerase (Promega) and 5 µl of template DNA. In the first round, 0.4 µM of each of the 3 oligonucleotide primers (DFA, ILK and KG1) was used. Following a hot start at 95 °C, reactions were thermocycled (BioRad®) 45 rounds of denaturation (95 °C for 30 s), annealing (46 °C for 60 s) and extension (72 °C for 90 s). The final extension step was at 72 °C for 5 min. In the second round, 5 µl of the first round PCR product was used as the template and 2 µM of each primer (TGV and IYG) was used in each reaction. The thermal profile of the second PCR reaction (nested) was similar to that of the first round except that annealing and extension times were 30 s and 60 s, respectively. After the final extension, the PCR reactions were held at 4 °C until gel electrophoresis in 2 % agarose (brand) prepared in SYBR safe (Invitrogen) and image capture with a ChemiDoc XRS + imaging system (BioRad®).

2.2.5. *UL15 gene targeted nested PCR (UL15NPCR)*

This nested PCR was developed to address the limitations associated with the low specificity of the UHVNPCR, which was designed to amplify DNA from any member of the *Herpesviridae* family (VanDevanter et al., 1996). An ILTV-specific nested PCR would overcome this limitation while maintaining high levels of analytical sensitivity. For this, a new pair of oligonucleotide primers was designed flanking the binding sites of the oligonucleotide primers described by Mahmoudian et al. that amplify a 113 bp fragment of the UL15 gene coding sequence. This was done using the Primer3 application imbedded within the Geneious software version 9.1.3 (Kearse et al., 2012). The new set of oligonucleotide primers, UL15Ex-F (5′-TCAGACTTGACAAACCCCCG-3′) and UL15Ex -R (5′- AAGCGTGCATTGCCCCTATA -3′), amplify a 315 bp

segment of the UL15 gene and were used in the first round of PCR amplification (Fig. A1). The second round of this nested PCR used the oligonucleotide primers described by Mahmoudian et al., (Mahmoudian et al., 2011). In both PCR rounds, Go*Taq*® system (Promega, Madison, Wisconsin, USA) was used according to manufacturer's instructions. The UL15NPCR reaction mix contained 2 mM magnesium chloride, 0.1 mM of each dNTP, 0.5 µM of each forward and reverse oligonucleotide primers, 0.05 U of Go*Taq* DNA polymerase and 5 µl of template DNA in a final reaction volume of 25 µL. The first round of PCR had a hot start at 95 °C, immediately followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Amplicons were further extended at 72 °C for 5 min. At the end of the first round, 5 ul of the PCR reaction was used as template for the second PCR reaction. The second round of amplification was performed using the same thermal profile as above, except for the annealing time which was reduced to 15 s in each cycle. All PCRs were performed in a BioRad T100™ thermocycler. At the end of the second round, PCR amplicons were resolved in a 2 % agarose gel prepared in SYBR-safe and DNA fragments visualised in a ChemiDoc™ XRS + imaging system (BioRad®).

2.3. Optimisation of a co-culture system for in vitro reactivation of latent ILTV in trigeminal ganglia and trachea and development of a genotyping system to differentiate ILTV strains detected in trachea

TG explants (Doll and Sawtell, 2017; Matundan et al., 2016), TG co-cultures (Mostafa et al., 2011) or co-culture of dissociated cells from TG (Yu et al., 2018) have often been used to induce *in vitro* reactivation of herpesvirus infection in latently infected tissues. The experiments described in the following section were aimed at optimising the culture conditions to *in vitro* reactivate latent ILTV from trigeminal ganglia and tracheal samples.

2.3.1. Chickens and sample collection

Samples were collected from two groups of 15 commercial layer birds with a history of vaccination against ILT which were at the end of their laying period (approximately 70–75 weeks of age). During post-mortem examination, swabs were collected from the conjunctiva, palatine cleft, and trachea from all birds and placed in 1 ml of viral transport media (VTM, DMEM supplemented with 3 % v/v FBS, 10 mM HEPES and 0.25 mg/ml of each gentamicin and ampicillin). These samples were stored at −80 °C until DNA extraction and viral determination using the UL15NPCR as described above. In order to confirm the accuracy of TG neuro-dissection, TG from five additional chickens were collected, fixed in a 10 % formalin solution, processed for histological examination and stained with haematoxylin and eosin before microscopic examination.

2.3.2. Neuro-dissection of the TG

The process of neuro-dissection of the TG is shown in Fig. 1. Aseptic dissection procedures were used, these included dipping the birds in water-chlorohexidine solution, changing gloves, scalpel blades and dissection needles between animals, dipping dissection instruments in ethanol (70 %v/v) and flaming between individual cuts, and thorough cleaning and disinfection of dissection tools between animals. During post-mortem examination, the head of each chicken was excised at the atlanto-occipital joint and placed on a sterile Petri dish. An incision was made using a sterile scalpel blade along the median plane to divide the head into left and right halves. The left or right hemispheres of cerebellum together with cerebrum were removed. The tip of a sterile 18 G needle was then used to transect the attachment of TG to the brainstem (pons), thus leaving the TG covered in dura mater attached to the skull. Using the same needle, the meningeal covering attached to inner surface of the skull was removed and the TG was dissected out, dissociating it from the mandibular, maxillary and opthalmic nerves. Both left and right TG from each bird were exteriorised and placed on a sterile Petri dish before being divided into two fractions. One fraction from each TG (left or right) was submerged in 0.5 ml of VTM and stored on ice until further processing for fresh co-culture. The remaining fraction was snap-frozen in dry ice and stored at −80 °C until later use in frozen-thawed culture.

The freezing-thawing procedure is a standard method for lysis of mammalian cells and release of intra-cellular infectious virions (Shehadul Islam et al., 2017). Assuming that latently infected cells would contain only viral DNA and not infectious virus particles, it was hypothesised that latently infected cells would show cytopathic effects due to viral infection only after fresh co-culture but not after co-culture of frozen-thawed samples (Fig. A2). Based on this hypothesis, following neuro-dissection, TG tissues were divided in two fractions where one was immediately frozen on dry ice, while the second fraction was chilled and prepared for immediate co-culture.

2.3.3. Culturing of fresh TG

A selected number of TG (n = 5) were explanted directly onto cell culture plates without a cell monolayer and the others (n = 25) were co-cultured with sub confluent LMH monolayers grown on 12-well-plates. Although LMH cells are not equally permissive to all vaccine strains of ILTV these cells were chosen for the current study due to the practical difficulties encountered with the use of CEK cells in our previous study (Thilakarathne et al., 2019). LMH growth media on the pre-established LMH-cell monolayers was removed and replaced with 1 ml of maintenance medium (MM-TG, DMEM supplemented with 5 % v/v FBS, 2 µM L-glutamine, 5 µg/ml amphotericin B and 200 µg/ml of each ampicillin and gentamicin). TG tissue from each bird was processed separately within a class II biological safety cabinet. TG fractions were transferred aseptically into a sterile Petri dish and were further minced using a fresh sterile scalpel blade (one per TG) before being transferred using a 200 µl pipette tip into a single tissue culture well containing an LMH-cell monolayer. Co-cultures were incubated at 37 °C in a humidified atmosphere of 5 % v/v CO_2 in air for up to 12 days.

2.3.4. Frozen-thawed TG culture

TG fractions stored in −80 °C were thawed to room temperature. Within a class II biological safety cabinet, TG tissues were transferred into a 1.5 ml microcentrifuge tube containing 0.5 ml of MM-TG. Each TG tissue was disrupted using a sterile plastic pellet pestle (Sigma-Aldrich). Tubes with TG tissue suspensions were frozen-thawed twice at −80 °C to rupture the cells. After removal of growth media from sub confluent LMH monolayers in 12 well plates, the frozen-thawed TG tissue suspensions were added on top of the monolayer and 1 -h incubation time was provided at 37 °C in a humidified atmosphere of 5 % v/v CO_2 in air with the rocking of plates at 15 min intervals. At the end of incubation, 1 ml of MM-TG was added to each well and cultures were incubated at the same incubation conditions for up to 6 days.

2.3.5. Tracheal organ co-cultures (TOC)

Twenty birds were randomly selected for TOC. At post-mortem, the intact trachea together with larynx was excised from each bird at the level of the thoracic inlet and placed in a 50 ml centrifuge tube containing 20 ml of VTM. These samples were kept on ice until further processing. In a class II biological safety cabinet, each trachea was transferred to a sterile Petri dish. Adipose and connective tissue surrounding the trachea were removed. The trachea was then divided into three equal size segments: upper, middle and lower trachea. Each segment was sliced transversely to yield 4 tracheal rings approximately 3 mm in thickness. Four tracheal rings from just below the larynx from the upper tracheal segment, centre of the middle tracheal segment and at the proximal end of the lower tracheal segment were used for TOC. Aseptic techniques and sterile instruments were used throughout this process.

Tracheal rings were cultured in 12 well tissue culture trays. Tracheal rings from the upper, middle and lower segments of the tracheas obtained from five birds were cultured without an LMH-cell

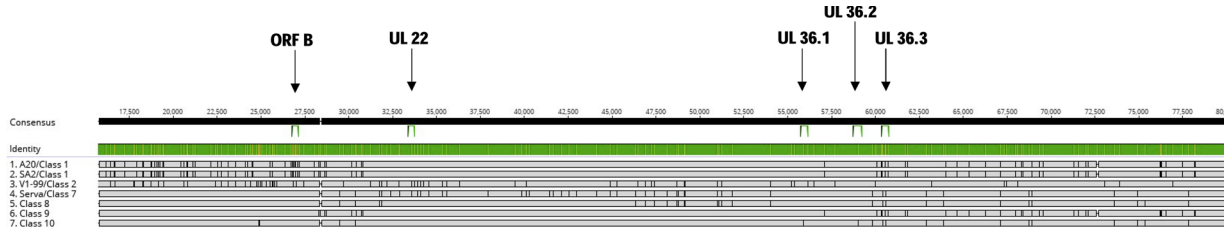

Fig. 2. Nucleotide sequence alignment of the seven ILTV strains and the locations of the selected variable regions.
Alignment of the complete genomes was performed by MAFFT software. Vertical lines indicate single nucleotide polymorphisms and horizontal lines indicate gaps in the sequence. Arrows point out the primer binding sites.

monolayer. The tracheal rings from the remaining 15 birds were cultured on sub-confluent LMH monolayers. Growth media on the LMH monolayers in each well was removed and replaced with 1 ml of MM-TG. Four tracheal rings from each segment were transferred using sterile forceps to a single well of these 12 well trays. Aseptic techniques and sterile instruments were used when transecting and transferring the tracheal rings to the tissue culture trays. Culture trays were then incubated at 37 °C in a humidified atmosphere of 5 % v/v CO_2 in air for up to 12 days.

2.3.6. Maintenance and sample collection from cultures

Samples of co-culture supernatant (500 ul in volume) were collected at 3-day intervals from all cultures, including those of fresh TG, frozen-thawed TG and TOC. The volume of culture supernatant removed was replaced by 500 u l of fresh MM-TG medium. In consideration of the integrity and viability of the LMH-cell monolayers, TG tissues were transferred to a new sub confluent monolayer every 6 days. When replacing the monolayer all media in each well was also collected. Culture supernatants collected during co-culture were stored at −80 °C until further use in nucleic acid extractions and viral DNA detection by PCR, or detection of infectious virus by inoculating a secondary monolayer of LMH cells using this material.

2.3.7. DNA extractions and screening for viral DNA

DNA was extracted from the swab material collected during post-mortem examination and the co-culture supernatants collected at 3-day intervals during culture. In addition, DNA extractions were also performed on samples of the VTM used to transport the TGs that were used in fresh culture (TGVTMs). All collected specimens of each sample type were subjected to automated DNA extraction using 200 µl of sample loaded onto PureLink® Pro 96 viral RNA/DNA purification kit (Invitrogen®, Carlsbad, California, United States) and using the Corbett X-tractor Gene Robot. Extracted DNA was eluted in 75 µl and screened for the presence of ILTV DNA using the UL15NPCR as described above. Swabs that were positive with the UL15NPCR assay were subsequently subjected to the UL15qPCR for viral copy number quantification.

2.3.8. Testing culture supernatants for infectious ILTV

Pre-established LMH monolayers, approximately 70 % confluent, were used. Growth medium was removed and the monolayers were washed twice with PBS. 200 µl of each UL15NPCR positive culture supernatant was added to a single well on 12-well tissue culture plates. The culture plate was gently swirled to cover the monolayer with the culture supernatant. The plates were incubated for 1 h in a humidified atmosphere of 5 % v/v CO_2 in air at 37 °C to allow viral adsorption and this was further facilitated by gentle swirling of the plates/flasks in 15 min intervals. After 1 h incubation, 1 ml LMH growth medium was added and incubated up to 72 h under the same conditions.

2.3.9. Histopathology of TG

Formalin-fixed TG sections were sent to the Veterinary Histology Laboratory at University of Melbourne Histopathology Laboratory for paraffin-embedding, sectioning, slide preparation and haematoxylin and eosin (H&E) staining. The stained TG sections were examined using a light microscope.

2.3.10. Genotyping field strains detected in the samples from the layer birds

As the TaqMan probes described in Section 2.3.2 are not capable of differentiating all the ILTV strains that may be present in Victoria, and the products of the UL15NPCR are not sufficiently variable for genotyping, a panel of 5 additional PCR assays was developed and used to identify the genotype of the viruses detected in the samples collected from the layer birds used in this study. The full genome sequences of ILTV strains (Table A3) known to be present in Victoria (Blacker et al., 2011) were aligned using the MAFFT alignment function in Geneious 9.1 software (Fig. 2). The alignments were screened to identify variable genomic areas between the strains. Classification of the different strains, according to the SNPs identified in the 5 variable genomic regions, is shown in Table A3. Oligonucleotide primers were designed to amplify the five variable genomic target regions (Table A4). To test the genotyping system, DNA was extracted from 200 µl of either pure cell culture grown ILTV strains (V1-99, CL8, CL9 & CL10) or reconstituted commercial vaccine stock (Serva, SA2 & A20) using the same extraction kit and equipment as described above. These positive controls and UL15NPCR positive URT swab samples (one sample for each bird) were subjected to PCR using the oligonucleotide primers described above (Table A4) and PCR amplicons sequenced using BigDye terminator v3.1 (Applied Biosystems™). Capillary electrophoresis was performed at the Australian Genome Research Facility (AGRF), Melbourne.

3. Results

3.1. Analytical sensitivities of PCR assays

The comparative analytical sensitivity of each PCR was determined by testing DNA extracted from a serial dilution of six different ILTV strains (Table 1). Since the initial titres of the ILTV stocks were not identical, the comparison of limits of detection of each PCR relied on the genome copy number calculated for each of these virus dilutions as determined by the UL15qPCR (Table 1). The lower limit of detection for the UL15qPCR was 200 copies ($10^{2.30}$) and so this PCR was used to determine the genome copy number in all samples that contained 200 or more copies. The genome copy number in samples diluted beyond this were calculated using the genome copy number in samples above the lower limit of the assay along with the dilution factor of the sample.

Both of the qualitative nested PCR assays (UHVNPCR and U15NPCR) were able to detect dilutions of virus containing 200 genome copies per reaction or less. The UL15NPCR was the most sensitive assay compared to the other qualitative PCR, detecting between 1 and 3 additional 10-fold dilutions of all ILTV strains except CSW-1 where the two nested PCRs performed similarly. The UHVNPCR generally showed equivalent analytical sensitivity to the UL15qPCR in detecting 200 copies per reaction of all ILTV strains with the exceptions of CSW-1 and V1-99 strains, which were detected in one further viral dilution.

The sensitivity of the TaqMan assays varied across the different ILTV strains they were designed to detect. These assays detected one

Table 1
Sensitivity of different PCR techniques to detect ILTV DNA in serial dilutions of ILTV stocks.

ILTV strain	V1-99				CSW				ΔgG				A20				SA2				Serva			
Titre log_{10}PFU¤/ml	3.35‡				6.21‡				5.99‡				6.70§				5.08§				6.36§			
Dilution	UL15qPCR «	TaqMan PCR with V-99_271 probe	UHVNPCR	UL15NPCR	UL15qPCR	TaqMan PCR with CSW_99117 probe	UHVNPCR	UL15NPCR	UL15qPCR	TaqMan PCR with CSW_99117 probe	UHVNPCR	UL15NPCR	UL15qPCR	TaqMan PCR with A20.SA2_88253_99117 probe	UHVNPCR	UL15NPCR	UL15qPCR	TaqMan PCR with A20.SA2_88253_99117 probe	UHVNPCR	UL15NPCR	UL15qPCR	TaqMan PCR with Serva_22805 probe	UHVNPCR	UL15NPCR
Neat	**6.10**	+++Θ	+	+	**8.02**	+++	+	+	**7.77**	+++	+	+	**8.17**	++N	+	+	**7.52**	NNN	+	+	**8.18**	+++	+	+
-1	**5.19**	+++	+	+	**7.13**	+++	+	+	**7.18**	+++	+	+	**7.43**	+NN	+	+	**7.55**	NNN	+	+	**7.55**	+++	+	+
-2	**3.99**	+++	+	+	**6.13**	+++	+	+	**6.41**	+++	+	+	**6.48**	+++	+	+	**6.84**	+++	+	+	**6.30**	+++	+	+
-3	**2.88**	+++	+	+	**5.00**	+++	+	+	**4.77**	+++	+	+	**5.27**	+++	+	+	**5.98**	+++	+	+	**5.12**	++N	+	+
-4	1.78Φ	+NN	+	+	**3.48**	+++	+	+	**3.79**	+++	+	+	**4.16**	+++	+	+	**4.88**	+++	+	+	**4.28**	+++	+	+
-5	1.53	NNN	N#	+	**2.30**	+++	+	+	1.00	+++	+	+	**3.03**	+++	+	+	**3.34**	+++	+	+	**2.74**	+++	+	+
-6	1.79	NNN	N	N	1.29	+++	+	+	1.59	+NN	N	+	1.91	+++	N	+	**2.42**	+++	+	+	1.57	+++	N	+
-7	No Ct	NNN	N	N	1.12	NNN	N	N	0.92	NNN	N	+	1.45	NNN	N	+	1.74	+++	N	+	1.47	+NN	N	+
-8	1.36	NNN	N	N	1.06	NNN	N	N	0.82	NNN	N	N	1.49	NNN	N	+	D	+++	N	+	1.15	NNN	N	+
-9	1.08	NNN	N	N	1.42	NNN	N	N	1.65	NNN	N	N	1.33	NNN	N	N	D	NNN	N	N	1.34	NNN	N	N
-10	D	NNN	N	N	1.19	NNN	N	N	1.00	NNN	N	N	1.43	NNN	N	N	1.55	NNN	N	N	1.28	NNN	N	N

¤PFU: Plaque forming unit.
‡ILTV strain propagated and titrated on LMH monolayers.
§ILTV strain propagated and titrated on CEK monolayers.
« UL15qPCR reported as log_{10} copies/reaction.
#N: Negative.
+Positive.
ΦqPCR copy numbers indicated in grey are outside the linear range of detection (Linear range of this assay (log_{10}) = 8.3–2.3).
Ddeselected because the melting curve of the sample does not follow the melting pattern of the standards.
ΘTaqman PCR were performed in triplicates.

further dilution compared to the UL15qPCR, except for the SA2 and Serva ILTV vaccine strains, where these PCRs detected ILTV DNA in 2 additional 10-fold dilutions.

3.2. In vitro reactivation of latent ILTV

This study aimed to identify birds in which ILTV could only be detected and reactivated from the TG without detectable virus in any of the URT or conjunctival swabs. This approach helps to prevent any potential for cross-contamination between sites of active replication in the URT and conjunctiva, and the TG. Commercial layer birds (n = 30) were screened for ILTV in their conjunctiva and URT (palatine cleft and tracheal swabs) at post mortem with the UL15NPCR (Table 2). Results showed that 22/30 of the layers birds were positive for ILTV DNA in at least one of the conjunctival or URT sites examined, where 17/30 layers were positive for viral DNA in the palatine cleft and/or tracheal swabs and only one bird was positive for ILTV DNA in the conjunctival swab. Only 8 of the 35 swabs from the URT or conjunctiva that were positive in the UL15NPCR URT had quantifiable ILTV DNA, which ranged between log_{10} 2.00–3.00 genome copies per reaction (Table 2). ILTV was not detected in any conjunctival or respiratory sites in 8/30 birds, and as such these birds were selected for investigating latent ILTV infection (Table 3).

A reliable TG neuro-dissection technique was optimised (Fig. 1) with minimal contamination from the URT. To identify and exclude any possibility of viral cross-contamination during neuro-dissection, the VTM used to transport the TGs were also tested by UL15NPCR. ILTV DNA was detected in (3/30) of these VTM samples (Table 3). These samples were excluded from further investigation.

Following TG co-culture, evidence of virus reactivation was sought in those birds (n = 8) that had no detectable ILTV in URT and conjunctival swabs. *In vitro* reactivation was indicated when ILTV DNA was detected by UL15NPCR in supernatants collected from the co-culture of fresh TG at any time within 12 days post explant (dpe) but not in the frozen-thawed TG culture up to 6 dpe. ILTV DNA was detected in 5/8 of freshly co-cultured TG. This ILTV DNA was detected in co-culture supernatants at 6 dpe or later (Table 3). *In vitro* reactivation was also detected when fresh TG were not supported by an LMH monolayer

Table 2
Detection and quantification of ILTV DNA in swabs collected from the upper respiratory tract or eye of layer hens previously vaccinated against ILTV^.

Bird Number	UL15NPCR			UL15qPCR (Log_{10} Genome copy numbers per reaction)		
	Eye	Palatine cleft	Trachea	Eye	Palatine cleft	Trachea
1	–	+	+	NT#	N*	N
2	+	+	+	N	N	N
6	–	+	–	NT	2.47	NT
7	–	+	–	NT	N	NT
9	–	+	+	NT	2.51	N
10	–	+	+	NT	N	2.56
12	–	–	+	NT	NT	N
13	–	+	+	NT	N	2.74
14	–	+	+	NT	N	N
15	–	+	+	NT	N	N
16	–	+	+	NT	N	2.53
17	–	+	+	NT	N	N
18	–	–	+	NT	NT	2.77
19	–	–	+	NT	NT	N
20	–	–	+	NT	NT	N
22	–	+	+	NT	3.06	3.07
23	–	+	+	NT	N	2.23
24	–	+	–	NT	N	NT
25	–	–	+	NT	NT	N
28	–	+	+	NT	N	N
29	–	+	–	NT	N	NT
30	–	+	–	NT	N	NT

^ Results are only shown for those birds that returned positive samples. Quantification of ILTV using the UL15qPCR was only attempted in samples that were positive using the UL15NPCR.
# NT: not tested.
* N: negative.

(Table 3). ILTV DNA was detected in the supernatants of only 1/8 of TGs in both fresh and frozenthawed TG co-culture, demonstrating the presence of lytic ILTV genomes in this sample. In 2/8 co-cultured TGs there was no detection of ILTV DNA in either fresh or frozen-thawed culture (Table 3).

Five potentially latently infected birds were found among the birds

Table 3
UL15 nested PCR to detect ILTV DNA in swab suspensions, viral transport media in which TG were transported (TGVTM) and culture supernatant collected during fresh and frozen-thawed trigeminal ganglia co-culture with LMH-cell monolayers.

Bird Number	Swabs			Fresh TG culture supernatants (Days post explant)					Frozen and thawed TG culture supernatants (Days post explant)		Culture detection summary¤
	Eye	Palatine cleft	Trachea	TGVTM « D0	D3	D6	D9	D12	D3	D6	
1	-	+	+	-	+	+	-	-	-	-	**Fresh only**
2	+	+	+	-	+	-	+	-	+	-	Fresh and F/T
3	-	-	-	-	-	+	-	-	-	-	**Fresh only**
4	-	-	-	-	-	+	+	-	-	-	**Fresh only**
5	-	-	-	-	-	-	-	-	-	-	Not detected
6 Φ	-	+	-	-	+	-	-	-	+	-	Fresh and F/T
7	-	+	-	-	-	-	-	-	-	-	Not detected
8	-	-	-	-	-	+	-	-	-	-	**Fresh only**
9	-	+	+	-	-	-	-	-	-	-	Not detected
10	-	+	+	+	-	-	+	+	-	+	Fresh and F/T
11	-	-	-	-	+	-	+	+	+	-	Fresh & F/T
12	-	-	+	-	-	-	-	+	-	-	**Fresh only**
13	-	+	+	-	-	+	-	-	-	-	**Fresh only**
14	-	+	+	-	-	+	+	-	-	-	**Fresh only**
15	-	+	+	-	-	-	-	-	-	-	Not detected
16	-	+	+	-	-	+	-	+	+	-	Fresh & F/T
17	-	+	+	-	+	-	+	-	-	-	**Fresh only**
18	-	-	+	-	+	-	-	-	-	-	**Fresh only**
19	-	-	+	-	+	-	+	-	-	-	**Fresh only**
20	-	-	+	-	-	-	+	-	+	-	Fresh & F/T
21	-	-	-	-	-	-	-	-	-	-	Not detected
22	-	+	+	-	-	-	-	-	-	-	Not detected
23	-	+	+	-	-	-	-	+	-	+	Fresh and F/T
24	-	+	-	+	+	+	-	-	-	-	**Fresh only**
25	-	-	+	-	+	-	-	-	+	+	Fresh and F/T
26	-	-	-	-	-	-	-	+	-	-	**Fresh only**
27	-	-	-	-	-	+	-	-	-	-	**Fresh only**
28	-	+	+	-	+	-	-	-	+	-	Fresh and F/T
29	-	+	-	+	-	-	-	-	-	-	Not detected
30	-	+	-	-	-	-	+	+	-	+	Fresh and F/T

^Highlighted cells are from those birds that had URT and eye samples that were negative by UL15NPCR.
«TGVTM- VTM of which the TG for immediate (fresh) culture were stored.
ΦFresh TG cultures from bird 6–10 was not performed on LMH cell monolayers.
¤Culture detection summary; F/T indicates detection in frozen/thawed samples, fresh indicates detection in fresh samples. Detection of virus in fresh samples only (bold text) suggests reactivation of latent virus. Detection of virus in fresh and thawed samples indicates the presence of lytic virus, which may be present due to contamination from another site, or the presence of lytic virus at the TG site.

Table 4
UL15 nested PCR of tracheal organ culture supernatant collected at 3 days intervals post explant.

	Tracheal organ culture supernatants (days post explant)											
	Day 3			Day 6			Day 9			Day 12		
Bird Number	Upper trachea	Middle trachea	Lower trachea	Upper trachea	Middle trachea	Lower trachea	Upper trachea	Middle trachea	Lower trachea	Upper trachea	Middle trachea	Lower trachea
1	-	-	-	+	+	-	-	-	+	+	-	-
2	-	-	-	-	+	-	+	-	-	-	-	-
3	-	-	-	-	+	-	-	-	-	-	-	-
4	-	-	-	+	-	-	+	-	-	-	+	-
5	-	-	-	+	-	+	-	-	-	-	-	+
6 Φ	-	+	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	+	-	-	-	-	-	+	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	+	-	-	+	-	-	+	+	-	+	-	-
11	-	-	-	-	-	-	-	-	-	+	-	-
12	+	+	+	-	-	-	-	+	-	+	-	-
13	+	-	+	+	+	-	+	-	-	+	-	-
14	-	-	-	-	+	-	-	-	-	-	-	-
15	+	+	-	+	-	-	+	-	-	+	-	-
16	+	+	+	+	+	-	+	-	-	+	+	-
17	-	-	+	-	-	-	-	-	-	-	-	-
18	+	+	+	+	+	+	+	-	-	+	-	-
19	-	-	-	+	-	+	+	-	-	+	-	-
20	+	-	-	+	-	-	-	-	-	+	-	-

^Highlighted cells are from those birds that had URT and eye samples that were negative by UL15NPCR.
ΦFresh TG cultures from bird 6–10 was not performed on LMH cell monolayers.

recruited for TOC. Of these, one was among the group of tracheas cultured without an LMH-cell monolayer. ILTV DNA was detected in the culture supernatant of TOC from these five birds by day 6 post explantation or later (Table 4). In most of these samples (3/5), ILTV DNA was first detected in cultures containing upper trachea whereas the others (2/5) were detected in TOC from the middle trachea.

Table 5
Genotyping outcome for the layers that were positive for ILTV DNA in upper respiratory tract at the time of sample collection.

Bird number	Swab from which the ILTV DNA extracted from	ILTV strain detected by the genotyping system
1	Palatine cleft	A20
2	Palatine cleft	SA2
6	Palatine cleft	SA2
7	Palatine cleft	unconfirmed
9	Palatine cleft	SA2
10	Palatine cleft	unconfirmed
12	Trachea	A20
13	Palatine cleft	unconfirmed
14	Palatine cleft	unconfirmed
15	Palatine cleft	SA2
16	Palatine cleft	unconfirmed
17	Palatine cleft	SA2
18	Trachea	SA2
19	Trachea	A20
20	Trachea	A20
22	Palatine cleft	SA2
23	Palatine cleft	SA2
24	Palatine cleft	unconfirmed
25	Trachea	unconfirmed
28	Palatine cleft	unconfirmed
29	Palatine cleft	A20
30	Palatine cleft	SA2

Neither the UL15NPCR-positive TG nor the TOC co-culture supernatants showed cytopathic effects on the secondary LMH monolayers.

3.3. Genotypes of the ILTV present in URT of the layers

Selected variable genomic regions contained at least two SNPs unique to each of the seven ILTV strains known to be present in Victoria. Sequencing results of the PCR amplified genomic regions effectively differentiated the seven strains of ILTV and their alignments were in accordance with *in-silico* alignments (Fig. 2). Using this method, ILTV strain identification could be achieved in 14 of the 22 layers that had ILTV DNA in their respiratory tract. According to the genotyping system established in this study, 9/14 layers were positive for ILTV vaccine strain SA2. The remaining 5 birds were positive for ILTV vaccine strain A20 (Table 5).

4. Discussion

This study optimised and compared a range of PCR assays for the detection of ILTV DNA. The most sensitive assay was then used in conjunction with *in vitro* culture systems to facilitate *in vitro* reactivation and detection of latent ILTV from recognised sites of latency. These systems were applied to samples collected from commercial birds to optimise the detection of latently infected individuals.

The analytical sensitivity of the different PCRs used in the current study (i.e.: UHVNPCR, UL15qPCR, UL15NPCR and TaqMan PCRs) was performed using serial 10-fold dilutions of ILTV strains. This approach was preferred to using a cloned template DNA for each of the oligonucleotide pairs used in these PCR protocols, as PCR outcomes using cloned template DNA may differ to those obtained using viral DNA extracted from test samples. Therefore, to achieve an unbiased comparison the same set of viral serial dilutions were tested with all the mentioned PCR techniques. The quantitative UL15 PCR provided reproducible results but had a relatively high limit of detection (200 copies per reaction) which limits the usefulness of the assay when applied to samples where virus is not abundant. The TaqMan PCRs offer some unique advantages, such as discrimination and quantification of alleles in a sample containing a mixture of viral strains (Loncoman et al., 2017), however the results of this study showed that the sensitivity between probes varied significantly. In addition, issues with reproducibility were observed with some of the probes designed in this study. Thus, results would need to be carefully interpreted if the probes were used to detect and quantify ILTV in samples where a mixed viral population may exist. Of the two nested PCR protocols used in the current study, the UHVPCR is widely used for herpesvirus screening worldwide (Amery-Gale et al., 2018; Dalton et al., 2017; Geldenhuys et al., 2018; Grattarola et al., 2018; Marenzoni et al., 2018; Wisely et al., 2018). This PCR detected 200 or less copy numbers of ILTV genomes per reaction, but this PCR is designed to detect all herpesviruses. Therefore, sequencing of the amplicon is required to confirm the viral species detected, as the assay would also detect Marek's disease virus (*Gallid alphaherpesvirus 2*) and Turkey herpesvirus (*Meleagrid herpesvirus 1*) if either of these viruses were present in clinical samples.

The UL15NPCR developed in the current study was specifically designed to provide high levels of sensitivity and specificity. This UL15NPCR was consistently more sensitive than any of the other PCRs tested and was selected for further use to detect latently infected birds in conjunction with TOC and TG culture approaches. Although not performed in this current study, the UL15NPCR assay may also be suitable for the sensitive detection of ILTV directly in TGs although the results would need to be carefully interpreted as detection of ILTV DNA at this site does not necessarily indicate latent infection with the capacity for reactivation (Thilakarathne et al., 2019).

Screening the URT or eye swabs from layers with the UL15NPCR revealed a high percentage (22/30) of ILTV positive birds. When quantified, eight of these positive birds had ILTV DNA ranging from 2.00 to 3.00 $\log_{10}$ viral genomes per reaction in either the palatine cleft and/or tracheal swabs without any obvious clinical signs or gross pathological findings consistent with ILT. This finding suggests that, at any one time, a relatively high proportion of birds in commercial settings may be shedding virus into the environment at a low level. The sampled population also showed an equal proportion of birds that were UL15NPCR ILTV positive in the palatine cleft and tracheal swabs (17/30). However, most of the samples with quantifiable amounts of DNA were found in tracheal swabs. Nevertheless, these results suggest that palatine cleft swabs may be a convenient site of sampling for detection of ILTV in live chickens. This site has been widely used for detection of the respiratory pathogen *Mycoplasma gallisepticum* (Sprygin et al., 2011).

Identification of latently infected birds was informed by the assumption that latent genomes would require intact host cells for reactivation (Sawtell and Thompson, 1992), with cell lysis through repeated cycles of freeze-thawing allowing release of intra-cellular infectious virions (Shehadul Islam et al., 2017). Therefore, it was expected the latent genomes would only generate viral progeny in the fresh TG co-cultures, while the lytic genomes would produce viral progeny in both the fresh and the frozen-thawed TG co-cultures. Amongst the eight birds that had no detectable ILTV in the URT or conjunctiva, latent infection of the TG was detected in only 5/8 layers, while lytic and latent infections were detected in the TG of 1/8 birds. Given the negative detection of ILTV DNA in the URT or conjunctival swabs collected from this bird (using a highly sensitive nested PCR) it is unlikely that lytic ILTV detected in the frozen-thawed TG represents cross-contamination from respiratory sites. According to a more recent understanding of alphaherpesvirus latency, at any given time point the sites of latency are in a dynamic state (Bloom, 2016). Which means only a certain fraction of the latently infected cells will remain latent, but some other latently infected cells may express lytic transcripts. In mouse models of HSV-1 small amounts of infectious viruses have been detected in latent TG as a result of spontaneous reactivations in a limited number of neurones (Margolis et al., 2007). Since we have excluded the cross-contamination from sites of the URT or conjunctiva in the eight birds that were negative at the URT and conjunctival sites, it is possible that ILTV detection in both fresh and frozen thawed culture in one of these birds reflects the dynamic nature of ILTV latency in TG.

This study revealed that a number of birds (n = 8) had detectable levels of ILTV DNA in conjunctival and or URT site but produced TG culture results that were positive in the fresh TGs only (and negative in the frozen-thawed TG samples). These results are likely to indicate the birds are latently infected with ILTV in their TG. It is possible that reactivation of latent genomes from an additional latency site, such as trachea (Bagust, 1986), is contributing to the lytic viruses seen in eye or URT of these birds.

It is interesting to note that when lytic virions were detected in the TG (in fresh and frozen-thawed co-culture), UL15NPCR positive supernatants at the first sampling point (3 dpe) remained positive for most of the subsequent sampling time points (6, 9 and 12 dpe). In contrast, when the TG contained latent genomes only, culture supernatants became UL15NPCR positive at 6 dpe or later, with most co-culture supernatant samples negative at subsequent time points. Additionally, cytopathic effects were not observed in the primary monolayers. This could be due to either a low number of progeny viruses being released, or the progeny viruses having lost their infection capacity within the *in vitro* system. Alternatively, LMH cells at full confluency may have become non-permissive. Additionally, adding fresh media every 3 days to replace supernatant volumes sampled at each time point may have further diluted the number of released virions and had an impact on the viral adsorption to the monolayer. An additional technique such as *in-situ* hybridization (ISH) or UL15NPCR on the TG tissue would have been useful to investigate latent infection in TG. However, the small size of the TGs limits the amount of tissue available for testing. Due to this limitation, further dividing the TG for testing in additional assays had the potential to negatively affect the outcomes of the *in vitro* co-culture system and reducing the sensitivity of the chosen ILTV detection protocols. No studies have previously reported successful *in vitro* reactivation of latent ILTV from TG (Bagust, 1986). However, when findings from the current study are compared to those of HSV-1 TG *in vitro* reactivation systems, initial ILTV detection was detected approximately 3 days later than in HSV-1 infection models where initial detection of reactivation has been observed from 3 to 4 dpe and the peak reactivation between 5–10 dpe (Balliet et al., 2007; Balliet and Schaffer, 2006; Chen et al., 2006; Mostafa et al., 2011).

All five birds in which latent virus was detected in the TG only, were among the birds recruited for TOC, and these had UL15NPCR positive TOC supernatants by 6 dpe or later, mostly from the upper or middle tracheal segments. Whether these viruses were residing within tracheal epithelial cells from a previous lytic infection or as latent viral genomes in the peripheral ganglia (or other tissues) associated with the trachea is currently unknown. These data and those from Bagust (1986) indicate that the trachea may play an important role in vaccine ILTV latency and reactivation. Since the establishment of latent infection in neurones requires that ILTV reaches sensory nerve terminals, it is possible that the ability of some viral strains to replicate more effectively in the trachea may enhance the establishment of latent infections. Our previous study (Thilakarathne et al., 2019) showed that different attenuated ILTV vaccine strains replicated at different magnitude in trachea and also differed in their capacity to establish latency in TG following eye-drop vaccination. However, that study was not able to conclude that latent infections established in TG by ILTV vaccine strains is replication-dependent, and TOCs were not performed. The only previous study attempting to recover ILTV from trachea long after infection by Bagust (1986) reported that 70 % of the tracheal tissue was explanted in the form of tracheal rings, without the support of a monolayer, and reactivated viable viruses in TOC supernatants was demonstrated by sub-culture into secondary chicken kidney cell monolayers (Bagust, 1986). This 1986 study showed *in vitro* reactivation in 38 % of tracheas from ILTV field strain (CSW ILTV strain) infected birds 3–15 months post infection and in 44 % of tracheas from vaccine strain (SA2 ILTV) infected birds after 2–10 months post infection. The higher reactivation rate (100 %) detected in TOCs in the current study may be attributed to ILTV detection by PCR rather than detection of viable viruses producing CPE in that previous study (Bagust, 1986).

The genotyping system developed during this study could differentiate all the anticipated ILTV strains without any sequence variation to those of published sequences available in Genbank. Well established ILTV PCR-RFLP genotyping systems are available in Australia (Kirkpatrick et al., 2006b) and elsewhere. However, the application of PCR-RFLP can be expensive and often it is difficult to extract sufficient amounts of good quality DNA from field samples, especially long after infection or vaccination. Since none of the TG culture supernatants that were positive in the UL15NPCR resulted in observable cytopathic effect on secondary monolayers for further analysis, it was not possible to genotype the ILTV that reactivated from the TG. Moreover, if a product was amplified with the genotyping PCRs (Table A4), not enough product could be purified for sequencing. As a result, genotyping of the ILTV isolates recovered from palatine cleft or trachea was performed. The use of the PCR-Sanger sequencing genotyping system on respiratory isolates revealed that the layer hens used in the current study were harbouring and/or shedding SA2 or A20 ILTV vaccine strains. This suggests that birds in the same flock had been vaccinated with both vaccine strains. Traditionally in Victoria, long-lived birds such as commercial layers would typically be vaccinated at a young age (< 6 week-old) with the highly attenuated A20 vaccine, and then receive a booster with the SA2 vaccine at an older age (16 week-old) (Kirkpatrick et al., 2006a). Findings from the current study are consistent with this vaccination regime, where samples from these commercial layer birds were positive for both vaccine strains. The vaccine strains detected in this study belong to the same genotype, class 1. Previous studies have reported the recombination between Australian vaccine strains in genotype class 1 and a European vaccine strain (genotype class 7). This resulted in the emergence of new more virulent recombinant viruses with genotypes classified in classes 8, 9 and 10 ILTV (Agnew-Crumpton et al., 2016; Blacker et al., 2011; Lee et al., 2013, 2012). It has been hypothesised that vaccination of flocks with different vaccine strains (belonging to genotype classes 1 and 7) may have played a role in the emergence of these recombinants (Lee et al., 2012). Results from the current study support the notion that multiple vaccine viruses are frequently shed by latently vaccinated birds and circulating among vaccinated flocks, a finding with important implications for the epidemiology of ILT.

Thus, by conducting these studies, tools that could be efficiently used to study ILTV latency in chickens were developed. These methods will contribute to future research of ILTV latency and understanding of the epidemiology of ILT.

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CRediT authorship contribution statement

Dulari S. Thilakarathne: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Carol A. Hartley:** Conceptualization, Methodology, Validation, Investigation, Resources, Writing - review & editing, Supervision. **Andrés Diaz-Méndez:** Validation, Writing - review & editing, Supervision. **Mauricio J.C. Coppo:** Conceptualization, Validation, Investigation, Writing - review & editing, Resources, Supervision. **Joanne M. Devlin:** Conceptualization, Methodology, Validation, Writing - review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

No competing interests.

Appendix A. Supplementary data

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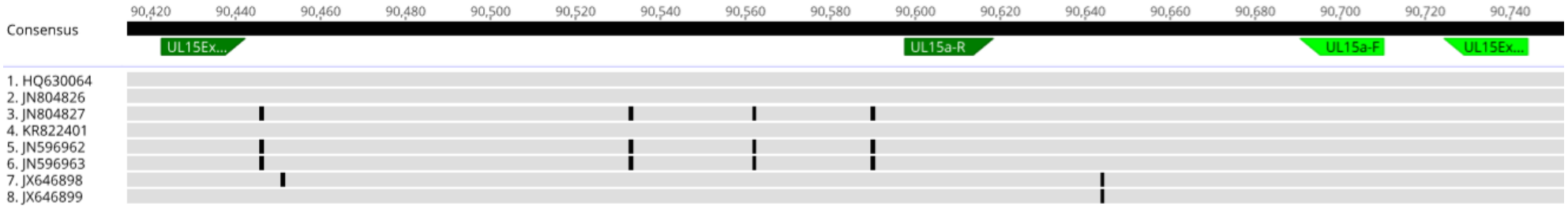
Supplementary figure 1



Supplementary figure 2

Immediate culture	+	+	–
Frozen thawed culture	+	–	–
	Contamination /Infectious viruses	Latent genome	Negative/fail to reactivate

Supplementary table 1: Oligonucleotide primers for sequence confirmation of strain specific SNPs targeted for strain identification

Primer Name	Sequence (5' - 3')	Direction	Sequence Length	Annealing Temperature (°C)	Product size (bp*)
CSW SNP 99117	GATTGATCACTAAGGTCTGC	Forward	20	54	250
	TTTCCTCTTCCATCAATCCA	Reverse	20		
V1-99 SNP 271	CAATCGCTCCCCTACTTT	Forward	18	54	250
	GAAAGGGTCAATGGGAAG	Reverse	18		
Serva SNP 22805	AGCTACCATCCTCCCTGCTA	Forward	20	54	750
	AGGTCAATCTCCCCAGAGCT	Reverse	20		
A20.SA2 SNP 88253	CCGGCAATATGTAATAAGCGCA	Forward	22	57	750
	CTCGGGTCCCTAGCGTTTTT	Reverse	20		

* Base pair

Supplementary Table 2: Properties of the oligonucleotide probes and primers generated using Real Time Design Software to target the identified single nucleotide polymorphisms within the CSW-1, V1-99, Serva, A20 and SA2 ILTV genomes

Probe name	Target gene	SNP position in alignment	Nucleotide Identity					Fluorogenic probe sequence (5'-BHQ-1 plus 3')	Primer sequence (5'-3')	Annealing temperature (°C)
			V1-99	CSW	A20	SA-2	Serva			
V1-99_271	Intergenic	271	C	T	T	T	T	AGCTCCCCGGCATC	F: TTAGGCGCGGTGTTGCTA R: TGGGTGCTTGCCTGCATA	64
CSW_99117	UL 8	99117	A	G	A	A	A	CGTTCAATATAACTTGCTT	F: GTTCACAACAATGTCCGGCTTG R: CACGTATGGCAACTGCATCTTAC	60
A20.SA2_88253	UL15 & UL17	88253	A	A	G	G	A	TCAGCCAAGCAGGCT	F: ATGCGCCACCGGACAC R: AAGCGCCTGCCACCTG	64
Serva_22805	UL 46	22805	G	G	G	G	A	TTATGGATACACACGAAACG	F: GGCTCAAGCTCTGACTGGAAT R: TCATGGCCTCCGAAGAATACC	64

F: forward

R: reverse

Supplementary Table 3: Classification of predicted ILTV strains in Victoria based on the variations in the target genomic regions

Strain	GenBank accession number	Target genomic region					Pattern
		ORF B	UL22	UL36.1	UL36.2	UL36.3	
V1-99	JX646898	A	A	A	A	A	AAAAA
Serva	HQ630064	B	B	B	A	B	BBBAB
CL8	JN804826	B	C	B	A	B	BCBAB
CL9	JN804827	B	C	B	A	C	BCBAC
CL10	KR822401	B	C	C	B	B	BCCBB
SA2	JN596962	C	C	B	A	C	CCBAC
A20	JN596963	D	C	B	A	C	DCBAC

Supplementary Table 4: Sequence of the oligonucleotide primers used to amplify the selected variable genomic regions within the ILTV genomes used to differentiate between prevalent ILTV strains in Victoria

Name	Sequence (5' to 3')	Expected PCR Product size (bp*)
ORF B - F	CTAAGAAGGGCGGGGTGTAC	357
ORF B - R	TGCCGACGGTCATGAAATGT	
UL22 - F	CCTTTGCCTTGTCATCCCCT	376
UL22 - R	AGAGGGGCTTCGCAAATACC	
UL36.1 - F	TGATCGTTCCATCCCGCAAT	398
UL36.1 - R	CACAGACCCGGAACCAGAAG	
UL36.2 - F	AGTTCAAGTCTTAGCAGT	497
UL36.2 - R	AAAAAGCGATTGGATGAA	
UL36.3 - F	GCCGAAAACTTAGCACTGCC	407
UL36.3 - R	AGAGGGGCTTCGCAAATACC	

*base pair

Chapter 3

Attenuated infectious laryngotracheitis virus vaccines differ in their capacity to establish latency in the trigeminal ganglia of specific pathogen free chickens following eye drop inoculation

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PLOS ONE

RESEARCH ARTICLE

Attenuated infectious laryngotracheitis virus vaccines differ in their capacity to establish latency in the trigeminal ganglia of specific pathogen free chickens following eye drop inoculation

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Abstract

Infectious laryngotracheitis (ILT) is a respiratory disease that affects chickens. It is caused by the alphaherpesvirus, infectious laryngotracheitis virus (ILTV). This virus undergoes lytic replication in the epithelial cells of the trachea and upper respiratory tract (URT) and establishes latent infection in the trigeminal ganglia (TG) and trachea. Live attenuated vaccines are widely used to control ILT. At least one of these vaccines can establish latent infections in chickens, but this has not been demonstrated for all vaccines. The aim of the current study was to determine the capacity of three commercially available vaccines (SA2, A20 and Serva) and a glycoprotein G deletion mutant vaccine candidate (ΔgG ILTV) to establish latent infection in the TG of specific pathogen free (SPF) chickens. Five groups of 7-day-old SPF chickens were eye-drop vaccinated with either one of the vaccine strains or mock-vaccinated with sterile media and followed until 20 or 21 days post-vaccination (dpv). ILTV DNA was detected at 20–21 dpv in the TG of 23/40 (57.5%) vaccinated SPF chickens (SA2 = 10/10; A20 = 6/10; Serva = 3/10; ΔgG = 4/10) by PCR, but virus could not be reactivated from TG co-cultivated with primary chicken embryo kidney cells. In the birds from which ILTV DNA was detected in the TG, ILTV DNA could not be detected in the URT or trachea of 3 birds in each of the SA2, A20 and Serva vaccinated groups, and in 4 birds in the ΔgG vaccinated group, indicating that these birds were latently infected in the absence of active lytic replication and virus shedding. Results from this study demonstrate the capacity of commercial ILTV vaccines to establish latent infections and underline their importance in the epidemiology of this disease.

analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Introduction

Infectious laryngotracheitis virus (ILTV) is the causative organism of infectious laryngotracheitis (ILT). ILTV is an alphaherpesvirus that infects the trachea and upper respiratory tract of chickens, causing disease with high morbidity and variable mortality determined by the virulence of the strain involved [1–3]. In the event of an outbreak, ILT causes substantial economic losses associated with drop in egg production [4], weight loss and mortality [1]. Considering the potential risk, especially in areas where the disease is enzootic, intensive poultry producers vaccinate their flocks against ILT [5]. Hitherto, four types of ILTV vaccines have been developed: live vaccines attenuated by sequential passage in embryonated eggs [6] or in tissue culture [7,8], virally vectored recombinant vaccines [9,10] and new generation recombinant deletion mutant vaccines [11–16]. Live attenuated vaccines are the most widely used vaccines worldwide [5]. New generation recombinant deletion mutant vaccines are not yet commercially available. Despite advances in vaccinology, ILTV still represents a significant burden to the poultry industry [17] causing disease in both unvaccinated and vaccinated flocks [18].

Live attenuated ILTV vaccines have several limitations that contribute to the persistence of live viruses in poultry production systems. These viruses multiply within the respiratory tract in birds on administration [19–21] and they are capable of spreading from bird-to-bird within flocks [20–22]. They can revert to virulence after multiple consecutive *in vivo* passages [18], and can recombine and result in the emergence of virulent ILTV strains [23,24]. Importantly, attenuated strains of ILTV also have the potential to establish latent infections in vaccinated birds [25,26].

Latency is a phenomenon observed in all herpesviruses, where much of the knowledge regarding the establishment of latency has been derived from the study of herpes simplex virus 1(HSV-1) [27,28]. During the acute phase of infection, alphaherpesviruses infect the sensory nerve terminals that innervate the primary replication sites and are retrogradely transported to the sensory ganglion where neurone bodies are located. Upon reaching the neuronal nucleus, viral DNA enters the nucleus leaving the capsid behind. The viral DNA genome then becomes associated with histones, is circularised and either undergoes lytic gene expression or suppression, which is thought to be determined by the type of the sensory neurone invaded. Viral genomes that have silenced their lytic gene expression persist within the nucleus as an episome for the life of the host and resume the lytic cycles (reactivation) given the appropriate stimulus [27].

Similar to other alphaherpesviruses, the trigeminal ganglia (TG) has been recognized as the main site of ILTV latency for both vaccine and field strain of virus [21,29–31]. Additionally, Bagust (1986) identified the trachea as a site of ILTV latency by detection of reactivated vaccine viruses in tracheal organ cultures 2, 5, or 10 months post vaccination [32]. Stressors such as changes in housing and onset of sexual maturity act as stimuli for reactivation [26], and these reactivation events either lead to sub-clinical, self-limiting infections or clinical infection that may result in an outbreak of disease [30]. Reactivation of ILTV in vaccinated chickens has been postulated to be an important source of ILTV transmission, especially in long-lived birds such as layers or breeders [33], and may provide a potential source of virus for recombination with other circulating ILTV in a flock [34].

Despite a number of studies showing that attenuated ILTV vaccine strains can be detected in the TG [21,25,29,32], to date no comprehensive comparison has been performed to compare the latency characteristics of different commercially available ILTV vaccines. The aim of this study was to evaluate three commercially available ILTV vaccine strains and a glycoprotein

G (gG) deletion mutant ILTV vaccine candidate (ΔgG ILTV) [11] for their ability to establish latent infections in TG of specific pathogen free (SPF) birds following eye-drop inoculation.

Materials and methods

Vaccines viruses and cell culture

Three commercial attenuated vaccines MSD Nobilis ILT (MSD, Serva strain), Poulvac Laryngo A20 (Zoetis) and Poulvac Laryngo SA2 (Zoetis) were obtained from the manufacturers for use in this trial. These vaccines were administered at the dose recommended by the manufacturers (≥ 2.5 $\log_{10}$ median embryo infective dose (EID_{50}), $\geq 10^{3.5}$ plaque forming units (PFU) and $\geq 10^{4.1}$ PFU, respectively), as determined by the label information. The ΔgG ILTV vaccine candidate developed by Devlin *et al*. [11] was sourced from our laboratory. This vaccine candidate was propagated and titrated in chicken leghorn male hepatoma (LMH) cells as previously described [11]. This vaccine was administered to birds at a dose of $10^{4.0}$ PFU per bird, as determined by PFU assay on LMH cells. After administration to the birds, all vaccine strains were re-titrated in a PFU assay using chicken embryo kidney (CEK) cell monolayers, as all the vaccine viruses can be successfully propagated using CEKs. To prepare CEK monolayers, kidneys harvested from the embryos of embryonated SPF eggs at 18 days of incubation were digested with 0.125% w/v trypsin overnight to recover CEK cells. Each well of a twelve-well plate was seeded with approximately $10^{5.0}$ cells to produce a CEK monolayer as previously described [35].

In vivo study design

This study was conducted with the approval of the Animal Ethics Committee at the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne (Animal Ethics ID: 1513713.1). One hundred SPF hybrid White Leghorn chickens (Australian SPF Services Pty Ltd, Woodend, Australia) were divided into five groups of 20 birds when they were one day old. Each group of birds was housed in separate Horsfall-Bauer type isolators which were equipped with negative pressure HEPA filtered air. Birds received irradiated feed and water *ad libitum*. Wing tags were used for individual bird identification. At seven days of age, all birds in each group were eye-drop vaccinated with one dose of one of the four ILTV vaccines; Serva, A20, SA2, or ΔgG ILTV, or were mock inoculated with sterile cell culture medium (Dulbecco's Modified Eagle's Medium [DMEM] supplemented with 10% v/v foetal bovine serum [FBS]). In all groups, the volume of the inoculum was 30 μl, which was administered to the left eye of each bird. Birds were monitored for up to 21 days post vaccination (dpv) for clinical signs of ILT.

Sample collection

Tracheal swabs were collected from all birds 4 and 14 dpv. On 20 and 21 dpv, 10 birds in each group were euthanised by halothane exposure and a detailed post-mortem examination was conducted on each bird. Swabs were collected from the conjunctival mucosa, palatine cleft, infraorbital sinus and trachea of each bird, to test for the presence of vaccine virus in primary replication sites. All samples were collected into 1 ml of sterile viral transport media consisting of DMEM supplemented with 3% v/v FBS, 0.02 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.7) and 0.25 mg/ml of each gentamicin and ampicillin, and stored at -80˚C until further processing.

On the day of necropsy, swabs (conjunctival mucosa, palatine cleft, infraorbital sinus and trachea) were taken from all birds prior to dissection. Separate instruments and workspaces

were used for opening the birds and the removal of TG. An entirely new set of instruments was used for each vaccine group. Instruments were cleaned and sterilised by application of 70% (v/v) ethanol and flaming between birds within a vaccine group. Workspaces were washed down after applying sodium hypochlorite solution and cleaned with 80% ethanol between vaccinated groups. Left and right TG collected from each bird 20 dpv were minced and divided into two fractions each and stored in 0.5 ml of sterile viral transport media (composition as above). One fraction was kept in wet ice and used for immediate TG co-culture (see below). The other fraction was immediately frozen in dry ice until permanent storage at -80˚C. Left and right TG collected from each bird on 21dpv were pooled and stored in 0.5 ml of sterile viral transport media at -80˚C until they were used for molecular detection of ILTV by polymerase chain reaction (PCR).

Co-culture of trigeminal ganglia

Co-culture of TG was performed according to the method described by Mostafa *et al.* [36] with modifications. Each TG fraction transported in wet ice on 20 dpv was added to a well in a twelve-well plate, containing a sub confluent (~80–90%) CEK monolayer and 1 ml of growth medium (DMEM supplemented with 5% v/v FBS, 2 μM L-glutamine, 2.5 μg/ml amphotericin B and 0.2 mg/ml of each ampicillin and gentamicin. Co-cultures were incubated at 37˚C in a humidified atmosphere with 5% (v/v) CO_2. Five hundred microliters of culture supernatants were collected daily and stored at -80˚C for subsequent testing for the presence of reactivated ILTV, and the same volume was replaced with fresh media. Depending on the integrity and viability of the CEK monolayer, TG tissues were transferred to a new monolayer every 5^{th} day and co-cultures were maintained for up to 15 days. Supernatants were analysed for the presence of ILTV DNA using nested PCR. Cultures negative for ILTV DNA, even at 10^{th} day post-explant, were given a heat shock at 43˚C for 3 hours before re-plating to provide an added stimulus for viral reactivation [36].

Molecular detection of ILTV DNA in swabs using UL15 qPCR

DNA was extracted from 200 μl of swab supernatants of conjunctival mucosa, palatine cleft, infraorbital sinus and tracheal swabs, or culture supernatants, using the PureLink Pro 96 viral RNA/DNA purification kit (Invitrogen, Carlsbad, California, United States) and the Corbett X-tractor Gene Robot (Corbett Life Science Pty Ltd, Sydney, Australia). For DNA extraction from TG collected on 21 dpv, Roche High Pure PCR template preparation kit (Roche diagnostics GmbH, Mannheim, Germany) was used according to manufacturer's instructions and DNA was eluted in 50 μl of elution buffer.

Quantitative polymerase chain reaction (qPCR) assay was used to quantify ILTV genome copy number (GCN) in all collected swab samples. Extracted DNA was subjected to qPCR targeting a 113 bp fragment in the UL15 gene of the ILTV genome, following the protocol by Mahmoudian *et al.* [37]. As this qPCR is convenient to apply to large numbers of samples, all collected swab samples were first screened for quantifiable ILTV DNA using this PCR. Selected negative swab samples were then further tested to qualitatively detect ILTV DNA using a more sensitive nested PCR which is more resource intensive to perform on a large scale.

Molecular detection of ILTV DNA in TG tissue and selected swabs using nested PCR

A nested PCR was used to test for the presence of ILTV DNA in TG collected on 21 dpv, TG co-culture supernatants and selected swabs collected on 21 dpv. Conjunctival mucosa, palatine cleft, infraorbital sinus and tracheal swabs were only tested for the presence of ILTV DNA

using the nested PCR if the swabs collected 21 dpv tested negative for ILTV DNA using the less sensitive UL15 qPCR, and if the TG sample from the same individual bird was positive for ILTV using the nested PCR. This nested PCR, the universal herpes virus (UHV) nested PCR, targets a conserved region of the herpes virus DNA polymerase gene [38]. Products amplified during the second round of this PCR were purified using QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Depending on the yield of purified DNA, amplicons were either directly subjected to sequencing or cloned into pGEM-T (Promega) following manufacturer's instructions before sequencing. Sequencing reactions were performed using Big Dye Terminator v3.1 (Life Technologies Corporation, Carlsbad, USA) according to manufacturer's instructions. Sample electrophoresis and sequencing was performed at the Centre for Translational Pathology, University of Melbourne. Geneious software version 9.1.3 [39] was used to analyse the sequencing data.

Statistical analysis

Microsoft Excel was used for log transformation of genome copy number (GCN) and Minitab statistical software version 18 (Minitab Pty Ltd, Sydney, Australia) was used to perform statistical analyses. Comparison of viral GCN was performed using one-way analysis of variance in conjunction with Dunnett's multiple comparison test. Comparisons of the proportion of ILTV DNA positives between groups was done using Fisher's exact test. P values ≤ 0.05 were considered statistically significant.

Results

In vivo study

None of the birds showed any clinical signs of ILT after vaccination. Re-titration of inoculum in CEK monolayers produced titres of $10^{3.70}$, $10^{3.94}$, $10^{5.08}$, and $10^{6.58}$ PFU/ml for Serva, A20, SA2 and ΔgG ILTV, respectively.

Detection of ILTV DNA in swab samples using UL15 qPCR

The presence of ILTV DNA in each swab sample collected per time point, was quantified using UL15 qPCR. Samples containing GCN of ≥ 200 per reaction were considered positive. The results from these assays are presented in Table 1. The highest proportion of positive swabs was detected at either 14 dpv (Serva) or day 4 dpv (all other vaccines). At 4 dpv, a significantly higher proportion of positive tracheal swabs were detected in birds vaccinated with SA2 (20/20) and A20 (18/20), compared to the other groups. At 4 dpv, the mean GCN in the SA2 and A20 vaccinated groups were significantly higher than the GCN in the groups of chickens vaccinated with Serva or ΔgG ILTV ($P < 0.001$), and the SA2 vaccinated group GCN were significantly higher than the A20 group ($P < 0.001$). At 14 dpv a significantly higher proportion of positive tracheal swabs were detected in birds vaccinated with Serva (5/20) compared to the other groups. There was no significant difference at 14 dpv in the GCN, between the different groups of birds.

At 20 or 21 dpv, the swabs collected from the palatine cleft accounted for the highest proportion of samples positive for ILTV DNA. Birds vaccinated with SA2 had the highest proportion of UL15 qPCR positive palatine cleft swabs (11/20), compared to all other vaccinated groups ($P = 0.001$). The ILTV GCN detected in SA2 vaccinated birds was also significantly higher than that observed in the other groups of vaccinated birds in this same anatomical site. The remaining sites of the URT that were tested for the presence of ILTV DNA yielded low proportions of positive samples. The only significant difference found at these other sites was a

Table 1. Proportion of ILTV positive swabs and genome copy numbers in samples collected from SPF chickens after eye-drop vaccination with Serva, A20, SA2, ΔgG ILTV, or mock inoculation with sterile media.

Group	4 dpv[‡]		14 dpv		20 or 21 dpv							
	Trachea		Trachea		Conjunctiva		Palatine cleft		Infraorbital sinus		Trachea	
	Proportion of positives	Mean Log_{10} GCN[¶] ± SD Log_{10}[#]	Proportion of positives	Mean Log_{10} GCN ± SD Log_{10}	Proportion of positives	Mean Log_{10} GCN ± SD Log_{10}	Proportion of positives	Mean Log_{10} GCN ± SD Log_{10}	Proportion of positives	Mean Log_{10} GCN ± SD Log_{10}	Proportion of positives	Mean Log_{10} GCN ± SD Log_{10}
Control	(0/20) [a]	2.30 ± 0.00[§ a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]
Serva ILTV	(2/20) [a]	2.36 ± 0.02 [a]	(5/20) [b]	2.50 ± 0.57 [a]	(1/20) [a]	2.36 ± 0.25 [a]	(3/20) [a, d]	2.36 ± 0.15 [a]	(0/20 [a]	2.30 ± 0.00 [a]	(1/20) [a]	2.31 ± 0.03 [a]
A20 ILTV	(18/20) [b]	3.85 ± 0.95 [b]	(1/20) [a]	2.31 ± 0.05 [a]	(2/20) [a]	2.40 ± 0.30 [a]	(5/20) [b, d]	2.35 ± 0.13 [a]	(1/20) [a]	2.31 ± 0.02 [a]	(2/20) [a]	2.35 ± 0.21 [a]
SA2 ILTV	(20/20) [b]	5.16 ± 0.34 [c]	(2/20) [a]	2.32 ± 0.06 [a]	(1/20) [a]	2.33 ± 0.12 [a]	(11/20) [b, e]	2.53 ± 0.30 [b]	(2/20) [a]	2.36 ± 0.23 [a]	(5/20) [b]	2.40 ± 0.18 [a]
ΔgG ILTV	(2/20) [a]	2.37 ± 0.25 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]	(0/20) [a]	2.30 ± 0.00 [a]

[a, b, c, d, e] Values marked with a different lowercase superscript character in each column were statistically significantly different ($p < 0.05$)

[§] The limit of detection using this qPCR was log_{10} (2.3) GCN per reaction. All samples that returned GCN values at or below this limit were determined to be negative and were assigned this GCN value for use in subsequent statistical analyses

[#] SD: Standard deviation

[‡] dpv: Days post vaccination

[¶] GCN: Genome copy number

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higher proportion of ILTV DNA positive tracheal swabs of SA2 vaccinated birds (5/20) compared to all other groups ($P = 0.047$). ILTV DNA was not detected at this time point in any of the mock-vaccinated chickens or in those vaccinated with ΔgG ILTV.

Detection of ILTV DNA in TG tissue, TG co-cultures and selected swab samples using UHV nested PCR

The TG collected during post-mortem examination were subjected to two different techniques for viral detection. Half of the birds in each group (n = 10) were culled on 20 dpv and their TG were co-cultured in CEK monolayers. The remaining birds (n = 10) were culled 21 dpv, and their TG were used in the molecular detection of ILTV genomes using the UHV nested PCR. ILTV DNA was detected in TG tissue using UHV nested PCR in 3/10 TG collected from the Serva vaccinated group, 4/10 TG from the ΔgG ILTV vaccinated group, 6/10 TG from the A20 vaccinated group and 10/10 TG from the SA2 vaccinated group (Table 2 and S1 Table). There was a significantly higher proportion of ILTV in the TG of the SA2 vaccinated group, compared with the Serva and ΔgG ILTV vaccinated groups ($P \leq 0.01$, Fisher's exact test). Sanger-sequencing confirmed the presence of ILTV DNA in 14/23 of the TG positive samples, all of which were collected from birds inoculated with SA2 or A20 ILTV.

In those individual birds where ILTV DNA was detected in the TG tissue using UHV nested PCR, but not in swabs collected at 21 dpv using UL15 qPCR, the swabs were re-tested with the UHV nested PCR. This was performed to identify birds that were actively shedding virus at these sites. A total of 3 birds in each of the Serva, A20, and SA2 groups, and 4 birds in the ΔgG ILTV group (Table 2 and supporting information S1 Table) had ILTV DNA in the TG without detectable virus shedding at any other sites and therefore were classifid as being latently infected without any concurrent lytic infection. Swab samples collected from birds in the mock-vaccinated group were tested in parallel with the other groups and all of them were negative for ILTV DNA. A summary of ILTV DNA detection results in TG and swabs using PCR at 21 dpv is presented in Fig 1. TG co-culture supernatants were tested up to 16 days post explantation using the UHV nested PCR, but viral DNA was not detected in any of the samples.

Table 2. Detection of ILTV DNA by quantitative and nested PCRs in swabs collected from the upper respiratory tract or trachea of SPF chickens after vaccination with Serva, SA2, A20 or ΔgG ILTV. Only results from birds that had detectable levels of ILTV DNA in their trigeminal ganglia are shown.

Group/Bird ID	UL15 qPCR Log_{10}*						UHV Nested PCR^				
	4 dpv‡ Trachea	14 dpv Trachea	21 dpv Eye	21 dpv Infraorbital sinus	21 dpv Palatine cleft	21 dpv Trachea	21 dpv Eye	21 dpv Infraorbital sinus	21 dpv Palatine cleft	21 dpv Trachea	21 dpv TG
Serva_NO†_3	-	2.48	-	-	-	-	-	-	-	-	+
Serva_7233	2.75	-	-	-	-	-	-	-	-	-	+
Serva_7235	-	-	-	-	-	-	-	-	-	-	+
A20_7247	2.76	-	-	-	-	3.23	NT Σ	NT	NT	NT	+
A20_NO_2	3.84	-	-	-	-	-	-	-	-	-	+
A20_7252	4.59	-	-	-	-	-	-	-	-	-	+
A20_7256	3.88	-	-	-	-	-	-	-	-	-	+
A20_7257	3.26	-	-	-	-	-	-	+	+	-	+
A20_7260	4.16	-	-	-	-	-	-	-	+	-	+
SA2_7262	5.10	-	-	-	2.65	2.79	NT	NT	NT	NT	+
SA2_7263	5.09	2.42	-	-	3.33	-	NT	NT	NT	NT	+
SA2_7264	5.03	-	-	-	2.40	-	NT	NT	NT	NT	+
SA2_7265	5.12	-	-	-	-	-	-	-	-	-	+
SA2_7266	5.30	-	-	-	2.85	-	NT	NT	NT	NT	+
SA2_7267	4.91	-	-	3.33	3.11	2.44	NT	NT	NT	NT	+
SA2_7272	4.71	-	-	-	-	-	-	-	-	-	+
SA2_7274	4.48	-	-	2.50	2.64	-	NT	NT	NT	NT	+
SA2_7275	5.17	-	-	-	-	-	-	-	-	-	+
SA2_7280	5.11	-	-	-	2.60	2.66	NT	NT	NT	NT	+
ΔgG_7281	-	-	-	-	-	-	-	-	-	-	+
ΔgG_7282	-	-	-	-	-	-	-	-	-	-	+
ΔgG_7289	-	-	-	-	-	-	-	-	-	-	+
ΔgG_7293	-	-	-	-	-	-	-	-	-	-	+

^ The UHV nested PCR was applied to swabs collected 21 dpv only if the UL15 qPCR did not detect ILTV DNA in any of the swabs collected 21 dpv, and only if the TG tissue was positive for ILTV DNA

‡ dpv: Days post vaccination

† NO: Bird lost its identification tag during the trial

Σ NT: Not tested with NPCR

* Copies of ILTV DNA per reaction

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Discussion

In this study, four ILTV vaccine strains were analysed for their ability to establish latent infection in chickens following eye-drop administration. The duration and sampling time points used in the current study were based on previous ILTV studies [1,20,40] and were selected to capture the replication kinetics of the vaccine strains. While vaccine doses used in this study were those defined by the manufacturer or similar, re-titration of these doses in CEK cells showed doses for the different vaccines ranged from $10^{3.70}$ (Serva) to $10^{6.58}$ (ΔgG) PFU/ml.

Tracheal swabs were collected on 4 dpv and 14 dpv to identify active viral replication and cessation of viral replication, respectively. There was a high proportion of ILTV-positive tracheal swabs in the SA2 and A20 vaccinated groups early after vaccination, which then decreased at later time points and a lower proportion of ILTV positive birds in the Serva and ΔgG vaccinated groups were detected early after vaccination, compared with the other

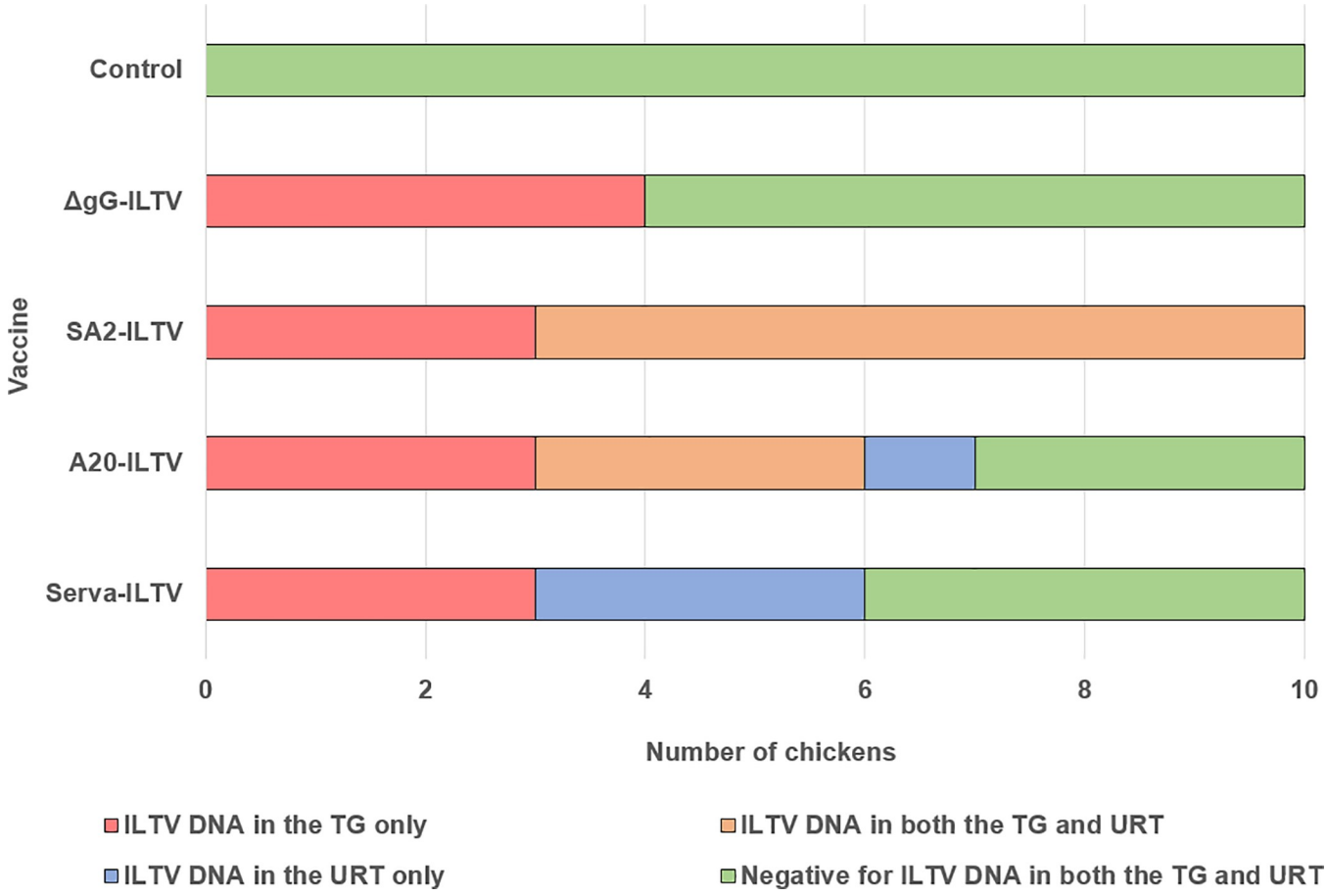

Fig 1. Summary of ILTV DNA detection results at day 21 post vaccination. The presence of ILTV DNA in the TG only suggests latent infection. The presence of ILTV DNA in the TG and URT suggests concurrent latent and lytic infection. The presence of ILTV DNA in the URT only suggests lytic infection.

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vaccinated groups. These results are largely consistent with previous studies [11,20,41], but the results from the Serva vaccinated group 4 dpv are in contrast to findings by Coppo *et al.* [19]. In Coppo *et al.*[19] study, a high proportion (1.0) of UL15 qPCR positive birds were detected 4 dpv following vaccination with a similar dose of Serva and using the same route of administration. Future studies may require the collection of samples from different anatomical sites (i.e. conjunctival swabs, palatine cleft swabs) at 4 dpv in order to better understand the replication characteristics of this virus after eye-drop vaccination.

Generally acute ILTV infections subside within 14 days post-infection and therefore final samples were collected at 20 or 21 dpv, as this is after the expected cessation of viral replication from the initial inoculation of virus. At the final time point, 26/80 (32.5%) of the vaccinated birds had viral DNA in at least one of the sites from which swab samples were collected (i.e. conjunctiva, palatine cleft, infraorbital sinus and trachea). This observation is consistent with a previous study that detected a high proportion of UL15 qPCR positives in tracheal swabs collected 21 dpv with the same ILTV strains, administered via the same route [20]. The high proportion of ILTV-positive birds in the SA2 vaccinated group is also consistent with previous results showing a high proportion (15/19) of ILTV DNA positive tracheas in chickens 21 dpv with SA2 [20]. Whether this DNA is coming from infectious viruses that are shed as a result of reactivation of latent viruses, re-infection of the respiratory tract with virus shed from other

birds, or whether this DNA is remnant DNA from vaccine viral replication after inoculation is currently unknown.

Latent ILTV could not be detected using the TG co-culture system implemented for this study. There are several possible explanations for this outcome, including: the division of the TG in half before co-culture (performed so that TG tissue could be directed towards different analytical methods), a potentially low number of neurones being infected after only one infection event, and dilution of the virus in the supernatant due to the sampling technique used at each time point during co-culture. In murine models of HSV-1 infection, approximately 5% of the neurones are latently infected [42] and among them, only a small number (1 in 2700 latently infected cells) have the capability of reactivation *in vivo* [43]. A similar phenomenon may occur during ILTV infection and future studies to examine ILTV latency may have to consider these limitations in the study design.

Viral DNA was detected in the TG tissue collected from 23/40 (57.5%) vaccinated birds 21 dpv. ILTV DNA was not detected anywhere else in 13 of these birds when tested using the UHV nested PCR. It is likely that these 13 birds represent latently infected animals without any concurrent lytic replication of virus occurring at other sites. Ten other birds had detectable ILTV DNA in TG but were also ILTV-positive in the URT or trachea. It is also possible that these birds may represent birds with latent infections that have been reactivated, as 9/10 of these birds showed cessation of viral active viral replication in trachea by 14 dpv, however re-infection with virus excreted from other birds in the group may also be possible. Although the trachea has been identified as a site of ILTV latency [32] it is unlikely that our approach of swabbing the trachea was able to detect latent virus at this site. The tracheal neurons are typically located in the adventitia and our swab sampling method only reaches the tracheal mucosa. It is also possible that chickens that have latent infections in their TG may not have latent infections in the trachea, and vice versa. It is likely that the route of inoculation and the site of primary replication play a role in the establishment of latent infections at each site.

Despite receiving a comparable dose of virus, viral DNA was detected in a higher proportion of birds vaccinated with SA2 (100%), compared to those vaccinated with ΔgG ILTV (40%). It is interesting to note that virus was detected in the TG of a small proportion of ΔgG ILTV vaccinated birds (not significantly different to the mock-vaccinated group) and also in the swab samples of a small proportion of ΔgG ILTV vaccinated birds. In contrast, SA2 was detected in the TG of all vaccinated birds and in the swab samples of most SA2 vaccinated birds. It is possible that a higher level of virulence in SA2 [1,32] is associated with greater viral persistence in the trachea and URT, and enhanced infection of sensory neurones and uptake by the TG, and vice versa for the ΔgG ILTV vaccine candidate. Studies of HSV-1 have shown that highly attenuated deletion mutants deficient in one or more critical genes are still capable of establishing latent infections [44–53]. Further, wild-type HSV-1 has been reported to establish latency in sensory ganglia that do not innervate the primary replication sites [54]. It would be interesting to investigate the association between ILTV strain-specific virulence and their potential to establish latency.

Nested PCR was used in the current study to detect viral DNA. This technique has been an important molecular tool for the detection of ILTV DNA in TG due to its high level of sensitivity [25,55–57]. However, detection of latency using PCR-based methods has two limitations: i) inability to differentiate between lytic and latent genomes and ii) incapability of predicting reactivation potential of the detected viruses in sites of latency. In murine models of HSV-1 infection, occasional viral transcriptional activation has been seen in a small number of latently infected neurones while the remaining majority are silent, resulting in molecular reactivations without clinical disease or viral shedding [58]. Ideally, approaches to better assess ILTV latent infections in TG would include a combination of TG explantation and co-culture together

with in-situ RT-PCR targeting latency associated transcripts (LATs). However, LATs associated with ILTV latency remain poorly understood [59,60] and *in vitro* TG explant reactivation systems are yet to be optimised.

This study has revisited an aspect of the pathogenesis of ILTV that has received little attention in recent years. The results from this study have confirmed results from early studies and have also added new information to our understanding of ILTV vaccine latency. The finding that attenuated ILTV vaccines differ in their capacity to establish latency may have practical relevance for control of ILTV in the field, as selecting vaccines that have a limited ability to establish latency may help to reduce the number of disease outbreaks that result from reactivation of latent vaccine virus. Future studies to establish consistent and reliable latency models of ILTV in the natural host and *in vitro* models of reactivation are warranted in prospects of future understanding of the pathogenesis of ILTV.

Supporting information

S1 Table. Detection of ILTV DNA by quantitative and nested PCRs in swabs collected from upper respiratory tract, trachea or trigeminal ganglia (TG) of SPF chickens at day 21 after eye-drop vaccination with Serva, SA2, A20, ΔgG ILTV or sterile media (control).
(DOCX)

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Supplementary Table 1: Detection of ILTV DNA by quantitative and nested PCRs in swabs collected from upper respiratory tract, trachea or trigeminal ganglia (TG) of SPF chickens at day 21 after eye-drop vaccination with Serva, SA2, A20, ΔgG ILTV or sterile media (control).

	UL15 qPCR Log_{10}				**Nested PCR^**				
Group/ Bird ID	Eye	Infraorbital sinus	Palatine cleft	Trachea	Eye	Infraorbital sinus	Palatine cleft	Trachea	TG
Control_7202	-	-	-	-	NT[Σ]	NT	NT	NT	-
Control_7203	-	-	-	-	NT	NT	NT	NT	-
Control_NO[†]_1	-	-	-	-	NT	NT	NT	NT	-
Control_7206	-	-	-	-	NT	NT	NT	NT	-
Control_7208	-	-	-	-	NT	NT	NT	NT	-
Control_7210	-	-	-	-	NT	NT	NT	NT	-
Control_7211	-	-	-	-	NT	NT	NT	NT	-
Control_NO_2	-	-	-	-	NT	NT	NT	NT	-
Control_7215	-	-	-	-	NT	NT	NT	NT	-
Control_7218	-	-	-	-	NT	NT	NT	NT	-
Serva_NO_1	-	-	2.75[*]	-	NT	NT	NT	NT	-
Serva_7224	-	-	-	-	NT	NT	NT	NT	-
Serva_7225	-	-	2.54	-	NT	NT	NT	NT	-
Serva_7226	-	-	-	-	NT	NT	NT	NT	-
Serva_7228	-	-	-	-	NT	NT	NT	NT	-
Serva_NO_2	-	-	-	-	NT	NT	NT	NT	-
Serva_NO_3	-	-	-	-	-	-	-	-	+
Serva_7233	-	-	-	-	-	-	-	-	+
Serva_7235	-	-	-	-	-	-	-	-	+
Serva_7238	-	-	2.82	-	NT	NT	NT	NT	-
A20_7244	-	-	-	-	NT	NT	NT	NT	-
A20_NO_1	-	-	-	-	NT	NT	NT	NT	-
A20_7247	-	-	-	3.23	NT	NT	NT	NT	+
A20_NO_2	-	-	-	-	-	-	-	-	+
A20_NO_3	-	-	-	-	NT	NT	NT	NT	-
A20_7252	-	-	-	-	-	-	-	-	+
A20_7256	-	-	-	-	-	-	-	-	+
A20_7257	-	-	-	-	-	+	+	-	+
A20_7258	3.31	-	-	2.40	NT	NT	NT	NT	-
A20_7260	-	-	-	-	-	-	+	-	+
SA2_7262	-	-	2.65	2.79	NT	NT	NT	NT	+
SA2_7263	-	-	3.33	-	NT	NT	NT	NT	+
SA2_7264	-	-	2.40	-	NT	NT	NT	NT	+
SA2_7265	-	-	-	-	-	-	-	-	+
SA2_7266	-	-	2.85	-	NT	NT	NT	NT	+
SA2_7267	-	3.33	3.11	2.44	NT	NT	NT	NT	+
SA2_7272	-	-	-	-	-	-	-	-	+
SA2_7274	-	2.50	2.64	-	NT	NT	NT	NT	+
SA2_7275	-	-	-	-	-	-	-	-	+
SA2_7280	-	-	2.60	2.66	NT	NT	NT	NT	+
ΔgG_7281	-	-	-	-	-	-	-	-	+
ΔgG_7282	-	-	-	-	-	-	-	-	+
ΔgG_7283	-	-	-	-	NT	NT	NT	NT	-

ΔgG_7284	-	-	-	-	NT	NT	NT	NT	-
ΔgG_7287	-	-	-	-	NT	NT	NT	NT	-
ΔgG_7289	-	-	-	-	-	-	-	-	+
ΔgG_7293	-	-	-	-	-	-	-	-	+
ΔgG_7295	-	-	-	-	NT	NT	NT	NT	-
ΔgG_7296	-	-	-	-	NT	NT	NT	NT	-
ΔgG_7300	-	-	-	-	NT	NT	NT	NT	-

^ The nested PCR was applied to swabs only in cases were TG tissue was positive for ILTV DNA

$^{\Sigma}$ NT: not tested with NPCR

† NO: bird lost its identification tag during the trial

* Copies of ILTV DNA per reaction

Chapter 4

Latency characteristics and systemic lymphocyte responses in specific pathogen free chickens long after intratracheal inoculation with vaccine or field strains of infectious laryngotracheitis virus

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Chapter 4

Latency characteristics and systemic lymphocyte responses in specific pathogen free chickens long after intratracheal inoculation with vaccine or field strains of infectious laryngotracheitis virus

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Abstract

Latency is an important feature of infectious laryngotracheitis virus (ILTV) yet is poorly understood. This study aimed to compare latency characteristics of vaccine (SA2) and field (CL9) strains of ILTV, establish an *in-vitro* reactivation system and examine lymphocyte responses during latency in specific-pathogen-free chickens. Birds were inoculated with SA2 or CL9 ILTV and then bled and culled at 21 or 35 days post

inoculation (dpi). Swabs (eye, palatine cleft, trachea) and tissues (including trigeminal ganglia; TG) were examined for ILTV DNA using PCR. Half of the TG, trachea and peripheral blood mononuclear cells (PBMC) were co-cultured with cell monolayers to assess *in-vitro* reactivation. Further, PBMC were stained using fluorochrome-conjugated monoclonal antibodies targeting cell surface markers before flow cytometric analysis. ILTV DNA was detected in the trachea of approximately 50% of ILTV-inoculated birds, whilst at 21 dpi, 2/7 CL9- and 1/6 SA2-infected birds had detectable ILTV DNA only in the TG. At 35 dpi, ILTV was detected only in the TG of 3/10 CL9- and 1/10 SA2-infected birds. Tracheal organ co-cultures from 3/10 CL9-infected birds and 7/10 SA2-infected birds were negative for ILTV DNA at cull but yielded quantifiable DNA within 6 days post explant (dpe). TG co-cultivation from 3/10 CL9-infected birds and 4/10 SA2-infected birds had detectable ILTV DNA within 6 dpe. Lymphocytosis due to B cells and decreased CD3+ cells occurred in SA2-infected birds, but no changes were correlated with the presence/absence of ILTV in the trachea. These results advance our understanding of ILTV latency and reactivation.

Key words: Chickens, infectious laryngotracheitis virus, latency, *in vitro* reactivation

Introduction

Herpesviruses are among the most successful pathogens affecting both humans and animals (Sehrawat, Kumar, & Rouse, 2018; Szpara et al., 2014). This success is largely

due to their capacity to establish latent infections following primary infection (Nicoll, Proença, & Efstathiou, 2012). Based on host range, genetic organization and replication strategies, the *Herpesviridae* family of viruses is divided into 3 subfamilies; *Alpha-*, *Beta-*, and *Gammaherpesvirinae* (Sehrawat, et al., 2018). The vast majority of known human and animal pathogens belong to the *Alphaherpesvirinae* subfamily and they typically establish latency in sensory ganglia (Szpara, Kobiler, & Enquist, 2010).

Gallid alphaherpesvirus 1 or infectious laryngotracheitis virus (ILTV) is an avian alphaherpesvirus that causes infectious laryngotracheitis (ILT), an acute respiratory tract infection of chickens, which may cause mild to severe disease (Kirkpatrick, Mahmoudian, Colson, Devlin, & Noormohammadi, 2006). Mortalities in infected flocks range between 5 - 70% depending on the virulence of the strain involved (Hidalgo, 2003). Similar to other herpesviruses, ILTV is capable of establishing latency and the principal sites of ILTV latent infection are the trigeminal ganglia (TG) (Williams et al., 1992) and the trachea (Bagust, Calnek, & Fahey, 1986). During latent infection, ILTV DNA has been detected in TG previously by several researchers (Chacón et al., 2015; Han & Kim, 2003; Hughes et al., 1991; Parra, Nuñez, Astolfi-Ferreira, & Ferreira, 2015; Williams, et al., 1992). Even though reactivation of latent ILTV from trachea has been demonstrated using a culture based approach previously (Bagust, et al., 1986) hitherto, no studies have been able to determine the location of

the latent ILTV in the trachea. Therefore, the location of latent ILTV genome in tracheal tissues is currently unknown.

Stressors leading to immune suppression can reactivate latent ILTV infections (Hughes, Gaskell, Jones, Bradbury, & Jordan, 1989) but the role of immune responses in the establishment of ILTV latency and reactivation is currently unknown. Studies on the well-characterised herpes simplex virus-1 (HSV-1) have shown a close association between host immune mechanisms and latency or reactivation (Decman, Freeman, Kinchington, & Hendricks, 2005; Sheridan, Knickelbein, & Hendricks, 2007; White, Suzanne Beard, & Barton, 2012). Reactivation of HSV-1 latent infections has been enhanced after depletion of CD8+ T cell responses and neutralisation of interferon-γ production (Liu, Khanna, Carriere, & Hendricks, 2001). Attenuated ILTV strains are also capable of reactivation from latency upon stressors (Hughes, et al., 1991).

Attenuated strains of ILTV are widely used as vaccines and they play an important role in protecting poultry flocks against ILT (García, Volkening, Riblet, & Spatz, 2013). In 1966 the first attenuated ILTV vaccine, SA2 was developed in Australia by serial passage of a low pathogenic ILTV field isolate in chicken embryos (Lee et al., 2011; Menendez, García, Spatz, & Tablante, 2014). Since then it has been widely used to control ILT in Australia, however, the high level of residual virulence of this vaccine has made it unsuitable for use in young chickens (Lee, et al., 2011; Lee et al., 2013).

Class 9 (CL9) ILTV is a virulent ILTV field strain that emerged in Australia after natural recombination between commercially available attenuated vaccines (Lee et al., 2012). Whole-genome sequencing and comparative sequence analyses have shown that the genome sequence of CL9 is a mosaic between the genome of the European Serva vaccine strain (PCR-RFLP class 7) and the Australian vaccine SA2, or the closely related Australian vaccine A20 (Lee, et al., 2012). ILTV strains classified as class 9 are currently the most prevalent ILTV field strains in the Australian state of Victoria (Agnew-Crumpton et al., 2016).

The capacity to establish latent infections, with subsequent reactivation later in life, is a known limitation associated with attenuated ILTV vaccine strains (Han & Kim, 2003; Hughes, et al., 1991) which may facilitate recombination events. Latency and reactivation of SA2 have been previously investigated *in vivo* as well as *in vitro* by performing TG or tracheal explant cultures (Bagust, 1986) however these culture techniques have not been replicated in recent times. The latency characteristics of the most prevalent strain in Victoria (CL9) have not been investigated previously. Further, the potential for *in vitro* reactivation of latent virus from PBMCs, or the systemic lymphocyte response during latency or reactivation of ILT, have not been previously investigated. The capacity of ILTV to establish latency in PBMC is unknown, but another member of the same viral subfamily (Gallid alphaherpesvirus 2, or Marek's disease virus) establishes latency in PBMC (Parcells, Arumugaswami, Prigge, Pandya,

& Dienglewicz, 2003). PBMCs could also potentially play a role in the systemic spread of ILTV (Calnek, Fahey, & Bagust, 1986; Rodríguez-Avila, Oldoni, Riblet, & García, 2007).

The current study aimed to establish a latency infection model and to better understand the latency and reactivation characteristics of ILTV in the natural host. Further, the systemic lymphocyte response during the latent stage infection was evaluated. The SA2 vaccine strain and the CL9 field strain of ILTV were used in this study to allow a comparison between a vaccine and virulent field strain. Understanding the latency characteristics of CL9 is important as this is a prevalent recombinant strain of ILTV that has shown improved fitness and a high level of virulence (Agnew-Crumpton, et al., 2016; Lee et al., 2015).

Materials and methods

ILTV strains

The SA2 and CL9 ILTV strains were used in this study. Poulvac® Laryngo SA2 (Zoetis) was obtained from the manufacturer and reconstituted according to the manufacturer's instructions. CL9 ILTV was propagated in the Leghorn male hepatoma (LMH) cells (Kawaguchi, Nomura, Hirayama, & Kitagawa, 1987) and growth medium

consisting of Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% v/v foetal bovine serum (FBS), 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, PH 7.7) and 50 µg/ml ampicillin. Both strains were titrated using plaque assays on LMH cells as previously described (Devlin, Browning, & Gilkerson, 2006) before eye-drop or intra-tracheal inoculation.

In vivo ILTV inoculation study

The *in vivo* study was conducted with the approval from the Animal Ethics Committee, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne (ethics identification number 1714129.1). One hundred one-day-old white Leghorn type specific-pathogen-free (SPF) chickens were randomly divided into 3 groups (40 birds in groups 1 and 2, and 20 birds in group 3) and wing-tagged for identification. The three groups were separately housed in negatively pressured Horsfall-Bauer-type isolator units at the Asia-Pacific Centre for Animal Health animal facility and provided with irradiated feed and sterilised water *ad libitum*.

Medium consisting of DMEM supplemented with 10% v/v FBS was used to dilute the ILTV strains to reach the desired viral titre. At 3 weeks of age, birds in group 1 were inoculated with SA2 ILTV strain at a dose of 10^3 plaque forming units (PFU) per bird. Similarly, birds in group 2 were inoculated with CL9 ILTV at a dose of 10^3 PFU/bird. All birds in group 3 were mock-inoculated with sterile media (DMEM supplemented

with 10% v/v FBS). Half of the dose was inoculated via eye-drop in 40 µL to each eye and the other half (80 µL) was inoculated directly into the trachea in each inoculated birds.

Birds were monitored for clinical disease after inoculation. Any birds showing signs of severe disease were euthanased to prevent suffering. At 21 days post inoculation (dpi), 6 birds from group 1, 7 birds from group 2 and 10 birds from group 3 were randomly selected, bled from the brachial vein and culled by exposure to halothane. At 35 dpi all remaining birds were bled and euthanased in a similar manner.

Sample collection

Approximately 1 mL of blood was collected from each bird into heparinized BD Vacutainer tubes (BD Biosciences) and stored on ice until processing for flow cytometry. At post-mortem examination, swab samples were collected from the conjunctiva of both eyes, the palatine cleft, and the trachea of each bird. All the swabs collected were placed in 1 ml viral transport medium (VTM; DMEM supplemented with 3% v/v FBS, 0.02 M HEPES (pH 7.7) and 0.25 mg/ml of each of gentamicin and ampicillin) and stored at -80° C until DNA extraction.

Tracheas and trigeminal ganglia (TG) for co-culture with cultured cells were collected only at 35 dpi from 25 randomly selected birds (n = 10 from each of groups 1 and 2,

and n = 5 from group 3). Trachea together with larynx were aseptically excised from the neck of each bird and placed in 50 mL centrifuge tubes (Falcon BD) containing 20 ml of VTM and stored on ice until further processing. Each bird was decapitated, and TG were aseptically removed. A section of brain tissue (from the olfactory lobe) immediately next to TG was collected from each bird to be used as a dissection contamination control. TG for co-culture were stored on ice until further processing. The TG collected from the remaining birds were snap frozen in dry ice and stored at -80° C until processing for molecular detection of ILTV DNA.

Peripheral blood mononuclear cells (PBMC) isolation

Whole blood from each bird was processed separately. One ml of whole blood was overlaid onto 1 ml of Ficoll-Paque PLUS (GE Healthcare) at room temperature. This was centrifuged (Allegra X-12R; Beckman) at 400 × *g* for 30 min at 18 °C without a brake. Using a transfer pipette, the white interphase containing mononuclear cells was aspirated and resuspended in 10 ml of chilled FACS buffer (1 mM EDTA, 25 mM HEPES pH 7.7, 1 % v/v FBS in phosphate-buffered saline (PBS)) before centrifugation at 800 × *g* for 5 min at 4 °C. The resulting cell pellet was resuspended in FACS buffer and centrifuged as above. The cell pellet was then gently resuspended in 200 µL FACS buffer. Visual inspection of PBMC was done by preparing a Diff-Quik™ (Microptic S.L.) stained smear. Viable cells were counted using Trypan blue exclusion (0.4 % w/v Trypan blue; Sigma-Aldrich) in a haemocytometer (Brightline; Hausser Scientific).

188

189 **PBMC staining for cell surface markers and flow cytometric analysis**

190 Fluorochrome-conjugated monoclonal antibodies used in this study include mouse anti-
191 chicken CD3-Aexa Fluor® 647 (clone CT-3), mouse anti-chicken CD8α-FITC (clone
192 CT-8) and mouse anti-chicken CD4-PE (clone CT-4), all from Southern Biotech. For
193 labelling, 100 µL PBMC suspension from each bird was incubated with 50 µL of anti-
194 CD3 (1/1000), anti-CD8α (1/1000) or anti-CD4 (1/1000) in a 96-well tissue culture
195 round-bottomed plate (Sarstedt) for 30 min on ice and in the dark. At the end of the
196 incubation, plates were centrifuged at 1500 × *g* for 5 min at 4°C. The supernatant was
197 decanted, and the pellet was washed with 150 µL FACS buffer. After two similar
198 washing steps, the pellet was resuspended in 250 µL FACS buffer and stored on ice in
199 the dark until flow cytometric analysis in a FACSVerse (BD Biosciences) flow
200 cytometer equipped with blue (488 nm) and red (640 nm) lasers. Data from a total of
201 10000 events were collected from each PBMC sample. The gating strategy used in this
202 study is shown in Figure 1.

203

204 **PBMC co-culture with LMH cells**

205 PBMC were co-cultured with LMH monolayers in 12-well tissue culture trays (Nunc).
206 The growth medium of the pre-established LMH monolayers was replaced with 1 ml of
207 a maintenance medium (MM; DMEM supplemented with 5% v/v FBS, 2 µM L-
208 glutamine, 2.5 µg/mL amphotericin B and 0.2 mg/ml of each ampicillin and

209 gentamicin). 100 µL of PBMC suspension (approximately 10^6 cells) from each bird
210 was added to a single well in a tissue culture tray. PBMC from each bird were
211 individually processed within a class II biological safety cabinet.
212
213 **Trigeminal ganglia co-culture with LMH cells**
214 TG co-culture with LMH cells was performed in 12-well tissue culture trays. Growth
215 medium of the pre-established LMH monolayers were replaced with 1 ml of MM. TG
216 from each bird were transferred aseptically into a sterile petri dish and were minced
217 using a sterile scalpel blade. TG pieces from each bird were then transferred using a
218 200 µl pipette tip into a single tissue culture well containing an LMH monolayer. TG
219 from each bird were individually processed within a class II biological safety cabinet.
220
221 **Tracheal organ co-culture with LMH cells**
222 The trachea from each bird was individually processed within a class II biological
223 safety cabinet. Each trachea was transferred to a plastic petri dish where adipose and
224 connective tissue surrounding the trachea were removed. The trachea was then divided
225 into three equal size segments: upper, middle and lower trachea. Four tracheal ring
226 slices (2-3 mm thick) were aseptically transected from each segment to be used for
227 tracheal organ co-culture with LMH cells in 12 well tissue culture trays. The rings from
228 each segment were cut from similar regions of each trachea; just below the larynx from
229 the upper tracheal segment, the centre of the middle tracheal segment and at the

proximal end of the lower tracheal segment. Separate wells were used to culture the tracheal rings from each segment. Co-cultures were established by removing the growth media from the sub-confluent LMH monolayers and adding 1 ml of MM to each well before the 4 tracheal rings were added.

Maintenance and sample collection from cultures

Tissue culture trays with PBMC, TG or tracheal co-cultures were incubated at 37°C in a humidified atmosphere of 5% v/v CO_2 in air for up to 12 days. From all cultures, 500 µL of culture supernatants were collected at 3-day intervals and stored at -80°C and a similar volume was replaced with fresh MM. In consideration of the integrity and viability of the LMH monolayers, TG tissues were transferred to a new sub-confluent monolayer every 6 days. When transferring to a fresh monolayer, all media in each well was collected. Culture supernatants collected during co-culture were stored at -80°C until further use. These supernatants were used for viral DNA detection by PCR and/or detection of viable viruses infecting a secondary monolayer.

DNA extraction and polymerase chain reactions

DNA was extracted from the VTM from swabs, co-culture supernatants, snap-frozen TG and the control brain tissue samples. DNA extractions were performed using 200 µL of swab suspension or culture supernatants, the whole TG or 0.5 cm^3 of the brain tissue as the starting material. MagMAX™ CORE Nucleic Acid Purification Kit

(Applied Biosystems™) together with the KingFisher™ Flex Purification System (Thermo Scientific™) were used for automated DNA extraction according to the manufacturers' instructions. Extracted DNA was eluted in 90 µL of elution buffer and subjected to the UL15 gene targeted quantitative PCR (UL15qPCR) and/or the UL15 gene targeted nested PCR (UL15NPCR). The approach was to quantify the viral copy numbers where possible, or alternatively to qualitatively detect ILTV DNA using the UL15NPCR. TG, control brain tissue, PBMC co-culture supernatants and TG culture supernatants were tested with UL15NPCR as low viral copy numbers (< 100) were expected. VTM from swabs and tracheal co-culture supernatants were subjected to UL15qPCR as quantifiable numbers of viral copies were expected.

UL15 gene targeted quantitative PCR (UL15qPCR)

Primers for this qPCR have been previously described (Mahmoudian et al., 2011). The modified protocol included the use of GoTaq® system (Promega, Madison, Wisconsin, USA) with 0.1 mM of each deoxyribonucleotide (dNTP), 2 mM magnesium chloride, 4 µM Syto9 (Invitrogen, Eugene, Oregon, USA), 0.8 µM of each primer, 0.5 U of GoTaq® DNA Polymerase (Promega), 2 µL of template DNA and nuclease-free water to reach the final reaction volume of 25 µL. For each run, a standard curve was generated using triplicates of 10^8 to 10^1 genome copy number/µL of a solution containing pGEM-T (Promega) bearing the 113 bp fragment of UL15 gene amplified from SA2 ILTV as the template. A Qubit fluorometer (Life Technologies) was used to

determine the concentration of pGEM-T containing the insert in stock solutions prior to ten-fold dilutions. The QIAgility Robot (QIAGEN) was used to prepare the standard serial dilution of the plasmid and dispensing the PCR master mix and template into Rotordiscs. Each dilution was generated in triplicate. The Rotor-Gene Q thermocycler (QIAGEN) was used for thermocycling and data acquisition. The reactions initially had 2 holds at 50 °C for 2 min and 95°C for 5 min. The reactions were subjected to 40 cycles of denaturation (95 °C for 5 sec), annealing (60 °C for 30 sec) and extension (72 °C for 30 sec) with fluorescence acquisition at the end of each extension step. Following another hold at 72 °C for 3 min and 95 °C for 1 min, high resolution melting analysis was done with temperature increase steps of 0.3 °C from 50 – 95 °C. Under these conditions, the linear range of detection was 10^8 to 10^2 genome copies per reaction, hence 100 copies per reaction was considered as the cut off value for this assay. Any amplicon with a deviated melting pattern to that of the standards was excluded and the viral copy numbers in each sample were calculated based on the standard curve generated by the Rotor-Gene Q software (QIAGEN).

UL15 gene targeted nested PCR (UL15NPCR)

This nested PCR was developed to address the limitations associated with UHVNPCR regarding low specificity and extended cycling time but maintaining high levels of analytical sensitivity. For this, a new pair of oligonucleotide primers were designed flanking the binding sites of the oligonucleotide primers described by Mahmoudian et

al. that amplify a 113 bp fragment of the UL15 gene coding sequence. This was done using the Primer3 application embedded within the Geneious 9.1 software. The new set of oligonucleotide primers, UL15NPCR F (5'-TCAGACTTGACAAACCCCCG-3') and UL15NPCR R (5'- AAGCGTGCATTGCCCCTATA -3'), amplify a 315 bp segment of the UL15 gene and were used in the first round of amplification. The second round of this nested PCR used oligonucleotide primers previously described (Mahmoudian, et al., 2011). In both PCR rounds, GoTaq® system (Promega, Madison, Wisconsin, USA) was used according to manufacturer's instructions. Amplification reaction mix consisted of 2 mM magnesium chloride, 0.1 mM of each dNTP, 0.5 μM of each forward and reverse primers, 0.5 U of Taq DNA polymerase and 5 μL of template DNA in a final reaction volume of 25 μl. The first round of PCR had a hot start at 95 °C, immediately followed by 45 cycles of denaturation at 95 °C for 30 sec, annealing at 60 °C for 30 sec and extension at 72 °C for 30 sec. Amplicons were further extended at 72 °C for 5 min. At the end of the first round, 5 ul of the PCR reaction was used as the template for the second round. The second round of amplification was performed using the same thermal profile as above, except for the annealing time which was reduced to 15 seconds in each cycle. All PCRs were performed in a BioRad T100™ thermocycler. At the end of the second round, PCR amplicons were resolved in a 2% agarose gel prepared in SYBR-safe and DNA fragments visualised in a ChemiDoc™ XRS+ imaging system (BioRad®).

Testing culture supernatant for viable viruses

Pre-established LMH monolayers with ~70% confluency were used. Growth media (GM- DMEM supplemented with 10% v/v FBS, 10mM HEPES (PH 7.5) and 50 µg/ml ampicillin) on LMH monolayers was removed and the monolayers were washed twice with PBS. 200 µL of each UL15qPCR positive culture supernatants were added to a single well on 12-well tissue culture tray. The culture plate was gently swirled to cover the monolayer with the culture supernatant.

The plates were incubated for 1h in a humidified atmosphere of 5% v/v CO_2 in air at 37 °C to allow viral adsorption and this was further facilitated by gentle swirling of the plates/flasks at 15-minute intervals. After 1h incubation, GM was added and re-incubated in same conditions for up to 72 h. The capacity of reactivated viruses to infect cells was assessed by observing cytopathic effects in LMH monolayers.

Data analysis

Microsoft® Excel (Version 16.16.2) was used for the log transformation of genome copy numbers (GCN) and Prism 5 for Windows version 5.03 (GraphPad Software, Inc.) was used to generate figures. Statistical analyses were performed using Minitab (Version 17) statistical software (Minitab Pty Ltd, Sydney, Australia). Fisher's exact test was used to compare proportions and one-way analysis of variance (ANOVA) in conjunction with Tukey's multiple comparison test were used to compare mean viral GCN between groups. During viral quantification, samples that returned GCN values at

or below the cut-off limit of the UL15qPCR (100 GCN) were considered negative. All qPCR negative samples were assigned the cut-off value for subsequent statistical analyses. FACS data analysis was performed using FlowJo software (V8). Percentages of PBMC populations were compared by two sample t-test using Minitab (Version 17) statistical software. In each statistical test a P value ≤ 0.05 was considered statistically significant.

Results

Mortalities

Survival percentage in each group is shown in Figure 2. Mortalities (sudden deaths or birds euthanased due to severe clinical disease) occurred up to 6 dpi in both CL9 and SA2 infected groups. The cumulative mortality percentages until 7 dpi were 32.5% and 35% in CL9- and SA2- infected groups, respectively. Mortalities were not significantly different between infected groups. Post-mortem examinations revealed severe haemorrhagic tracheitis in birds that died due to CL9 infection, whereas those that died due to infection with SA2 had diphtheritic plaques and muco-caseous plugs on the upper tracheal mucosal surface.

Viral copy numbers in conjunctival or upper respiratory tract (URT) swabs at the time of culling

The proportion of UL15qPCR positive swabs observed with each group and their mean log_{10} viral copy numbers at 21 dpi and 35 dpi are shown in Table 1. All the conjunctival and palatine cleft swabs collected from SPF chickens at 21 dpi with CL9 or SA2 ILTV were negative. qPCR positive tracheal swab samples were detected in 3/7 and 4/6 birds inoculated with either CL9 or SA2 ILTV, respectively. Neither the proportion of UL15qPCR positive tracheal swab samples nor the mean log_{10} GCN were significantly different between infected groups at this time point. Only birds inoculated with SA2 ILTV had U15qPCR-positive proportions and log_{10} GCN significantly higher than those of the mock inoculated group, which had no detectable ILTV DNA in any of the swab samples collected.

At 35 dpi all the conjunctival swabs and most of the palatine cleft swabs (96.2%) collected from both of the ILTV-inoculated groups were UL15qPCR negative. At this time point, 10/20 and 8/20 tracheal swabs collected from chickens inoculated with CL9 or SA2, respectively, were UL15qPCR positive. These proportions of UL15qPCR positive tracheal swab samples in both groups of birds were significantly higher than that of the mock-inoculated group of birds, which had no positive results. The log_{10} GCN detected in ILTV inoculated groups were not significantly different to each other or the mock inoculated birds.

No significant changes in proportions of UL15qPCR positive tracheal swab samples within each group of birds were observed between 21 and 35 dpi.

Molecular detection of ILTV DNA in TG

Table 2 shows the individual UL15 NPCR data for each TG and control brain tissue sample that were immediately snap-frozen after collection at 21 or 35 dpi. The proportions of UL15 NPCR positive TG collected from SPF chickens at 21 dpi with CL9 or SA2 ILTV were 2/7 and 5/6, respectively. At 35 dpi with CL9 or SA2 ILTV, the proportions of UL15 NPCR positive TG were 7/10 and 5/10, respectively. No statistically significant differences were observed between inoculation groups at each sampling time point or within each inoculation group between time points. None of the birds with UL15 NPCR positive TG had detectable ILTV DNA in the corresponding control brain tissue (Table 2). Interestingly, 7/19 (36.8%) of the ILTV-inoculated birds that had detectable ILTV DNA in their TG did not have quantifiable ILTV DNA in their URT swabs. In contrast, 6/33 (18.2%) of ILTV-inoculated birds that had quantifiable ILTV DNA in their URT swabs had no detectable ILTV DNA in TG tested using the UL15 NPCR (Table 2).

***In vitro* reactivation of latent ILTV by co-culture**

396 Supernatants collected from the TG co-culture of 3/10 and 4/10 birds inoculated with
397 CL9 or SA2 ILTV, respectively, were UL15 NPCR positive (Table 3). These
398 proportions were not significantly different. The majority (5/7) of the UL15 NPCR
399 positive samples were first detected at 6 days post explant (dpe). Additionally, 5/7
400 ILTV DNA positive TG co-culture supernatants were from birds that had no
401 quantifiable viral DNA in URT swabs at the time of culling at 35 dpi (birds 135, 145,
402 177, 180 and 189, Table 3). In addition, 5/20 ILTV-inoculated birds that had
403 quantifiable ILTV DNA in their URT swab samples at the time of euthanasia had no
404 UL15 NPCR positive results in their culture supernatant samples at any of the sampling
405 time points post explant (birds 136, 138, 140, 182 and 193; Table 3).
406
407 Upper and/or middle tracheal co-culture supernatants from 5/10 birds inoculated with
408 CL9 ILTV and all 10 of those inoculated with SA2 were UL15qPCR positive at one or
409 both sampling time points post explant (dpe) (Table 4). The majority (93.3%) of the
410 UL15qPCR positive co-culture supernatants were first detected positive at 3 dpe and
411 most (92.8%) of those co-cultures remained positive at 6 dpe. 3/5 and 7/10 of those
412 inoculated with CL9 or SA2 ILTV, respectively, which yielded UL15qPCR positive
413 co-culture supernatants at one or both sampling time points post explant, had
414 UL15qPCR negative tracheal swabs at the time of euthanasia at 35 dpi (highlighted in
415 Table 4). These birds may represent cases of potential *in vitro* reactivation of latent
416 ILTV infection in the trachea. In these cases, significantly higher $\log_{10}$ GCN were

417 detected in those originated from SA2-infected chickens compared to CL9-infected and
418 mock-inoculated chickens, at both time points.
419
420 Supernatant samples collected at 3 dpe from upper tracheal co-cultures prepared from
421 those inoculated with SA2 ILTV which underwent *in vitro* viral reactivation had
422 significantly higher (P = 0.005) mean $\log_{10}$ GCN (3.22) compared to those observed in
423 supernatant samples from similar co-cultures obtained from birds inoculated with CL9
424 ILTV (2.42) or the mock inoculated birds (2.00). Similarly, at 6 dpe, supernatant
425 samples collected from potentially reactivated upper tracheal co-cultures obtained from
426 birds inoculated with SA2 ILTV had significantly higher (P = 0.006) mean $\log_{10}$ GCN
427 (2.95) compared to those observed in supernatant samples from similar co-cultures
428 obtained from birds inoculated with CL9 ILTV (2.09) or the mock inoculated group
429 (2.00). In both ILTV-inoculated groups of chickens, the mean $\log_{10}$ GCN observed in
430 upper tracheal co-culture supernatants at 3 dpe were slightly higher than 6 dpe but the
431 difference was not significant. None of the lower tracheal co-culture supernatant
432 samples from ILTV-inoculated chickens were UL15qPCR positive at any sampling
433 time point. Similarly, none of the supernatant samples collected from tracheal
434 co-cultures prepared from the mock-inoculated group of chickens were UL15qPCR
435 positive at any time point.
436

Cytopathic effects on the LMH monolayer were not observed when UL15NPCR positive TG co-culture supernatants or UL15qPCR positive tracheal co-culture supernatants were sub-cultured on secondary LMH monolayers. None of the PBMC co-culture supernatants were positive for UL15NPCR in any of the sampling points tested.

Systemic lymphocyte response observed in the latent stage

Table 5 shows the mean percentages of total lymphocytes, CD3+ and CD3- cells observed in each group at 21 and 35 dpi. No significant difference was observed in total lymphocytes, CD3+ cells, CD3- cells or other cell sub-populations at 21 dpi. However, at 35 dpi significantly higher ($P < 0.02$) lymphocyte percentages were observed in both groups of ILTV-inoculated birds compared to mock inoculated birds. At this same time point, SA2 ILTV inoculated birds had significantly lower ($P < 0.02$) mean percentage of CD3+ cells (Table 5). This difference was explained by significantly lower mean percentages of both CD4+CD8- ($P < 0.02$) and CD4-CD8+ ($P < 0.03$) sub-populations, when compared to those inoculated with CL9 ILTV or mock inoculated birds (Table 6). In addition, a significantly higher ($P < 0.02$) mean percentage of CD3- cells was observed at 35 dpi in birds inoculated with SA2 ILTV compared to those inoculated with CL9 ILTV, or mock-inoculated birds (Table 5). This was explained by significantly higher mean percentages of CD4-CD8+ ($P < 0.001$) and CD4-CD8- ($P < 0.02$) sub-populations compared to those observed in CL9 ILTV or mock inoculated groups (Table 6).

458 Percentages of cells were further evaluated based on the positivity of UL15qPCR in
459 tracheal swabs by the time of euthanasia (Supplementary table 1). No changes in cell
460 percentages were shown to correlate with ILTV positivity in trachea.
461
462 **Discussion**
463 The experiment described here developed a TG co-culture system and reproduced a
464 tracheal organ co-culture (TOC) system to *in-vitro* reactivate latent ILTV. Further, it
465 compared the characteristics and lymphocyte response during latency established by a
466 virulent filed ILTV strain (CL9) and a moderately attenuated ILTV vaccine strain
467 (SA2).
468
469 The dose of the inoculum was chosen based on previous ILTV infection studies
470 (Kirkpatrick, et al., 2006; Lee, et al., 2015) assuming all inoculated birds would recover
471 from the infection. However, there were approximately similar levels of mortalities in
472 both CL9 (32.5%) and SA2 (35%) inoculated groups. This could potentially be
473 attributed to host factors, including the weight and size of the eggs or chicks, which in
474 turn may be related to the age of the laying flock of SPF birds, or incubation
475 conditions. Or could be related to host genetic factors. To date the relationship between
476 host genetics and susceptibility to ILTV infection is poorly understood (Coppo,
477 Hartley, & Devlin, 2013). It is possible that this relationship is similar to that observed
478 during infection with Marek's disease virus, another alphaherpesvirus that infects

chickens. Genetic variation in susceptibility to Marek's disease virus has been previously described in chickens (Kreager, 1998). Additionally, inoculating the virus at two primary replication sites (both conjunctiva and trachea) may have resulted in more severe clinical disease.

Approximately 50% of the inoculated birds had quantifiable viruses in trachea at both 21 or 35 dpi. According to the literature, acute ILTV infection in trachea subsides by 14 dpi (Rodríguez-Avila, et al., 2007). Therefore, quantifiable ILTV DNA observed after 21 dpi could be explained by the presence of residual ILTV DNA after cessation of primary replication, or horizontal transmission of infection from another bird, or from reactivation of latent ILTV infection. The observation of a similar pattern of detection at 35 dpi favours the latter alternative, as it seems unlikely that residual DNA would not be cleared by the host immune system this long after inoculation. This is further supported by the relatively high levels of ILTV DNA detected in the tracheal swabs collected from SA2 infected birds at 35 dpi. This long-term detection of ILTV in these groups of birds indicates that latency is established in a relatively high proportion of chickens and at any one time, long after infection, nearly half of the population has reactivated viruses in their trachea without any clinical signs of infection. This is likely a significant reason why ILTV is difficult to eradicate from an endemic area.

After three decades from the initial report (Bagust, 1986), the current study was able to reproduce the results obtained by Bagust *et al* using TOC and demonstrated the *in vitro* reactivation of ILTV infection from upper segments of the trachea of infected birds. Two thirds of these birds did not have detectable ILTV DNA in their trachea, as assessed by qPCR, at the time of euthanasia. Therefore, it is possible that these tracheal segments released ILTV from yet-to-be-identified sites of latency such as, neuronal cell body aggregations (tracheal ganglia), tracheal epithelial cells or both. The glossopharyngeal and vagus cranial nerves innervate the pharynx, larynx and cervical trachea. The proximal and distal ganglions of glossopharyngeal and vagus nerves have been identified as sites of neural cell bodies of the of the neurons that innervates the trachea (Williams, et al., 1992). Tracheal ganglia are also found embedded in the tracheal wall however the size, location and the nature of these ganglia limits their dissection and analysis in an experiment involving large number of birds. Even though alphaherpesviruses typically establish neuronal latency in sensory ganglia (Szpara, et al., 2010) there have been records of their capacity to establish latency in non-neuronal sites, for example corneal latency observed in HSV-1 (Fukuda et al., 2003; Kaye & Choudhary, 2006; Polcicova et al., 2005).

Neither Bagust (Bagust, 1986) nor our previous ILTV vaccine latency study (Thilakarathne et al., 2019) were able to detect ILTV release from TG explants *in vitro*, despite previous reports by Williams (Williams, et al., 1992) that TG is the main site of

latency for ILTV. In the current study, a limited number of TG co-culture supernatants had detectable ILTV DNA as assessed by the UL15NPCR. The proportions of positive culture supernatants were not significantly different between infected groups. It is likely that these UL15NPCR-positives represent cases of *in vitro* reactivation of latent ILTV from TG tissue because the majority (71.4%) of them did not have quantifiable ILTV DNA in their trachea at the time of euthanasia. The observation that these PCR-positive TG co-culture supernatants did not produce cytopathic effects when cultured on secondary LMH monolayers, raises the possibility that host cells were releasing either latent genomes without generating progeny viruses or non-infectious replication-incompetent progeny viruses.

Of the birds that had no qPCR detectable viruses in their conjunctival or URT swab samples by 35 dpi, only one bird exhibited *in vitro* reactivation in TG only (bird 135), whereas six birds had reactivation in trachea only (birds 142, 153,175, 176,185, and 186) and an additional four birds showed *in vitro* reactivation in both sites (birds 145, 177, 180, 189) (Tables 3 and 4). These results support the hypothesis that the trachea, as well as the TG, are important sites of latency for ILTV. Whether latency and reactivation share similar characteristics at each of these sites remains for further study.

The absence of ILTV DNA in the control brain tissue of individual birds helps to validate the detection of ILTV DNA in the TG from the same bird, showing that ILTV

DNA detected in TG is likely not contamination from the conjunctival or URT mucosae. Viral DNA detection by UL15NPCR in TG revealed no significant differences between the proportion of positives in inoculated groups at any sampling time point (21 or 35 dpi) or over the whole time period. Thus, this study provides evidence that latency observed in TG after 21 dpi is not substantially different from what is observed at 35 dpi under experimental conditions. Further, the results show that latent ILTV DNA can be readily detected by molecular methods compared to culture method which is expected since only a fraction of latently infected neurons will show reactivation under *in vitro* conditions (Bloom, 2016).

Interpretation of the results of peripheral blood lymphocyte response evaluation must proceed with care as the current study has several limitations. In a study conducted with five breeds of domestic chicken the relative percentage of lymphocytes as determined by the flow cytometry analysis has been ranged between 53 ± 2.5 to 74.99 ± 1.61(Bílková, Bainová, Janda, Zita, & Vinkler, 2017). However, in our study the percentage of lymphocyte recovered for 21 dpi as well as 35 dpi is lower than the above range. It is possible that some cells were lost during PBMC processing or during gating. This could have been prevented by using antibody targeting the panleukocyte cell surface marker CD45. Other possibilities for the observed variation include the difference in breed of chicken (Bílková, et al., 2017), age of the chickens (Beirão et al.,

561 2012) or the florescent label used (Fair, Taylor-McCabe, Shou, & Marrone, 2008),
562 compared to those used in the current study.
563
564 Flow cytometric analysis of PBMC revealed no distinct differences in cell populations
565 at 21 dpi, and this may be due to the small sample size. However, at 35 dpi both SA2
566 and CL9 inoculated groups showed lymphocytosis, compared to uninfected chickens.
567 Previous studies have shown ILTV causes lymphopenia which reaches peak around 5
568 dpi (Chang, Sculco, & Yates, 1977). It is possible that lymphocytosis follows the initial
569 lymphopenia during later stages of the infection, as evidenced by results from the
570 current study. Significant differences in cells positive for CD3+, CD3- or their
571 subpopulations were observed in SA2-inoculated birds but not in CL9-inoculated birds.
572 Differences observed in the pathology (haemorrhagic tracheitis versus diphtheritic and
573 suppurative tracheitis in CL9- and SA2-inoculated birds, respectively) may be a reason
574 for the significant differences in lymphocyte sub-populations observed. Lymphocytosis
575 observed in SA2-inoculated birds was due to a significant increase in CD3- cells, in
576 particular, CD3-CD4-CD8- (most likely B-cells) and a subpopulation of CD3-CD4-
577 CD8+ cells, possibly NK cells (Jansen et al., 2010). Further, SA2-inoculated birds
578 showed a significant decrease in CD3+ lymphocytes in peripheral circulation compared
579 to the CL9 or mock inoculated birds. This was explained by a significant decrease in
580 the single-positive CD4 and CD8 sub-populations. The decrease in these lymphocyte
581 sub-populations in peripheral blood may be associated with these cell subpopulations

582 being diverted to the infected tissues during infection with SA2. It is difficult to discern
583 if this observation is in any way associated with the high proportion of birds in which
584 latent ILTV infection of tracheal tissues was detected. The current study did not
585 examine histopathological changes in tracheal tissues or TGs, but previous studies have
586 shown that lymphocytes are an important component of the local immunological
587 response during acute infection of the trachea (Devlin et al., 2010). The
588 histopathological and immunological characteristics of latent ILTV infection in trachea
589 or TG are currently unknown and remain for further study.
590
591 In conclusion, ILTV latency characteristics and late systemic immune response for
592 ILTV could be a strain dependent property. Since the latency at 21 dpi is not
593 significantly different to latency at 35 dpi, the latency studies are more economical and
594 ethical to conclude at 21 dpi. The reproducible *in vitro* culture system developed here
595 will be useful for future latency investigations.
596
597 **Figure captions**

598 Figure 1. Gating strategy used to analyse PBMC

599 (A) The singlets gate created based on PBMC population, (B) the lymphocytes gate
600 created based on low forward scatter (FSC) and low side scatter (SSC) gate (C) the

601 gates created for CD3+ cells and CD3- cells (D) and (E) are the gates for the sub-
602 populations CD3+ and CD3- cells respectively.

603

604 Figure 2. Mortalities in SA2 and CL9 infected birds up to 7 days post infection (dpi).
605 There were no significant differences between infected groups.

606

607 **Supplemental information**

608 Supplementary table 1: Mean percentages of lymphocytes, CD3+, CD3- cells and their
609 subpopulations observed in peripheral blood collected from SPF chickens with or
610 without quantifiable ILTV DNA in trachea at 35 days post inoculation with SA2 ILTV,
611 CL9 ILTV or sterile media (mock).

612

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Research Highlights

- Following inoculation, latent ILTV infection was detected in a large proportion of chickens, irrespective of whether a field or vaccine strain was inoculated.
- *In vitro* reactivation of latent ILTV was readily detected in tracheal and trigeminal ganglia co-cultures using PCR.
- ILTV latency observed in SPF chickens at 21 days post infection was not substantially different to 35 days post infection.
- Lymphocytosis was observed in birds 35 days after infection with either the vaccine or field ILTV strain.

Figure 1

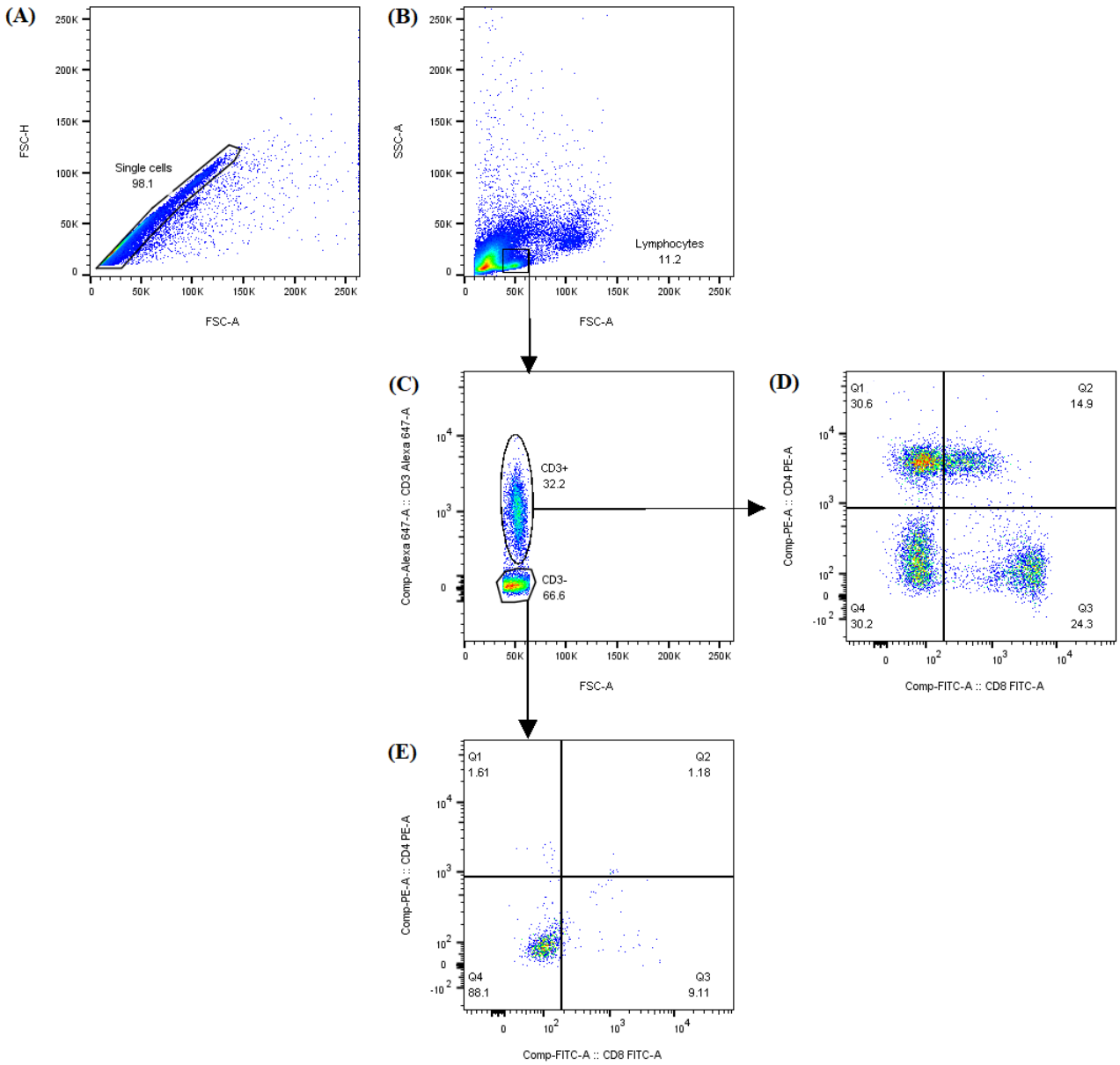
(A)
FSC-H
FSC-A
Single cells
98.1
(B)
SSC-A
FSC-A
Lymphocytes
11.2
(C)
Comp-Alexa 647-A :: CD3 Alexa 647-A
FSC-A
CD3+
32.2
CD3-
66.6
(D)
Comp-PE-A :: CD4 PE-A
Comp-FITC-A :: CD8 FITC-A
Q1
30.6
Q2
14.9
Q4
30.2
Q3
24.3
(E)
Comp-PE-A :: CD4 PE-A
Comp-FITC-A :: CD8 FITC-A
Q1
1.61
Q2
1.18
Q4
88.1
Q3
9.11

Figure 2

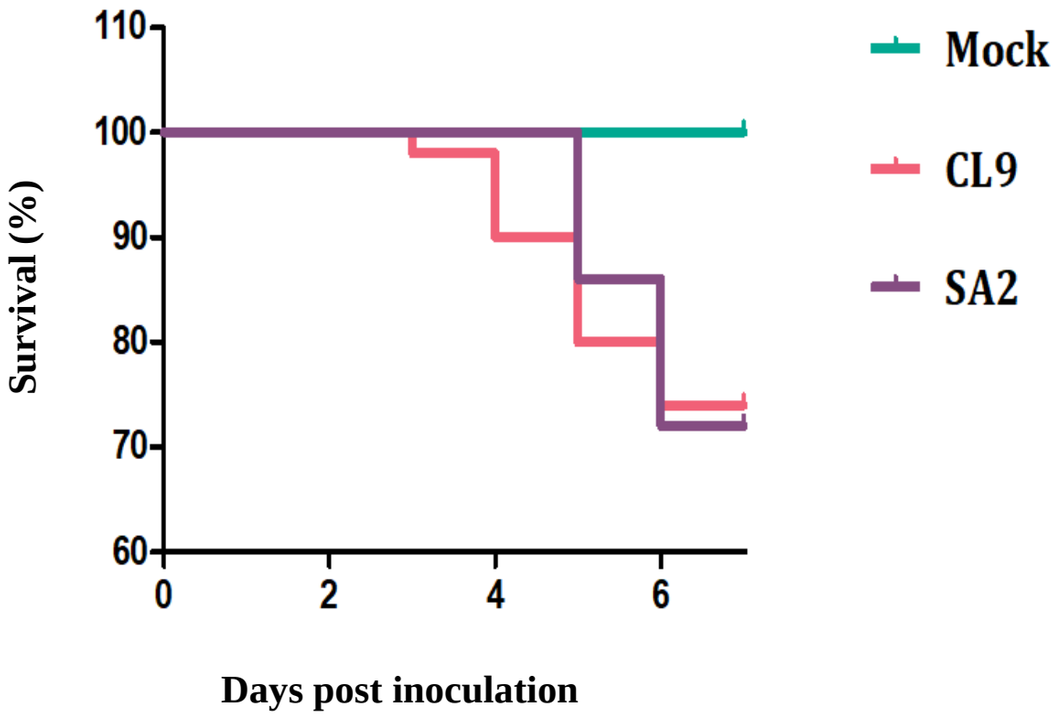
110
100
90
80
70
60
Survival (%)
0
2
4
6
Days post inoculation
Mock
CL9
SA2

Table 1. Proportion of ILTV positive samples and genome copy number as determined by UL15qPCR in swab samples collected from SPF chickens at 21 and 35 days post inoculation with CL9 or SA2 ILTV, or mock inoculated with sterile media.

	21 days post inoculation						35 days post inoculation					
	Conjunctiva		Palatine cleft		Tracheal swab		Conjunctiva		Palatine cleft		Tracheal swab	
	Proportion of positives	Mean $\log_{10}$ GCN[“]±SD[¥]	Proportion of positives	Mean $\log_{10}$ GCN±SD	Proportion of positives	Mean $\log_{10}$ GCN±SD	Proportion of positives	Mean $\log_{10}$ GCN±SD	Proportion of positives	Mean $\log_{10}$ GCN±SD	Proportion of positives	Mean $\log_{10}$ GCN±SD
Mock	(0/10) [a]	2.00 ± 0.00 [a]	(0/10) [a]	2.00 ± 0.00 [a]	(0/10) [a]	2.00 ± 0.00 [a]	(0/10) [a]	2.00 ± 0.00 [a]	(0/10) [a]	2.00 ± 0.00 [a]	(0/10) [a]	2.00 ± 0.00 [a]
CL9	(0/7) [a]	2.00 ± 0.00 [a]	(0/7) [a]	2.00 ± 0.00 [a]	(3/7) [ab]	2.30 ± 0.40 [ab]	(0/20) [a]	2.00 ± 0.00 [a]	(0/20) [a]	2.00 ± 0.00 [a]	(10/20) [b]	2.26 ± 0.32 [a]
SA2	(0/6) [a]	2.00 ± 0.00 [a]	(0/6) [a]	2.00 ± 0.00 [a]	(4/6) [b]	2.61 ± 0.48 [b]	(0/20) [a]	2.00 ± 0.00 [a]	(2/20) [a]	2.03 ± 0.11 [a]	(8/20) [b]	2.41 ± 0.64 [a]

[“] GCN: genome copy number
[¥] SD: standard deviation
[a, b] Values marked with the same superscript character in each column did not differ significantly ($P > 0.05$; Fisher's exact test was used to analyse the qPCR-positive proportions; one-way ANOVA with Turkey's comparison was used to analyse the GCN data).
[§]The limit of detection using this qPCR was $\log_{10}$ (2.0) GCN per reaction. All samples that returned GCN values at or below this limit were determined to be negative and were assigned this GCN value for use in subsequent statistical analyses

Table 2. UL15qPCR results for swab samples and UL15 nested PCR for control brain tissue and trigeminal ganglia collected from SPF chickens at 21 or 35 days post inoculation with CL9 or SA2 ILTV via the intra-tracheal route.

Group	Time of euthanasia	Bird Number	UL15 qPCR on swab samples			UL 15 nested PCR	
			Conjunctiva	Palatine cleft	Trachea	Control brain tissue	TG
Mock	21 dpi‡	94	-	-	-	-	-
		95	-	-	-	-	-
		96	-	-	-	-	-
		97	-	-	-	-	-
		101	-	-	-	-	-
		104	-	-	-	-	-
		108	-	-	-	-	-
		109	-	-	-	-	-
		111	-	-	-	-	-
		113	-	-	-	-	-
		98	-	-	-	-	-

	35 dpi	102	-	-	-	-	-
		105	-	-	-	-	-
		106	-	-	-	-	-
		110	-	-	-	-	-
CL 9	21 dpi	116	-	-	-	-	(+)
		119	-	-	2.62^	-	-
		123	-	-	-	-	-
		125	-	-	-	-	(+)
		127	-	-	2.99	-	-
		131	-	-	-	-	-
		132	-	-	2.51	-	-
	35 dpi	117	-	-	2.53	-	-
		120	-	-	2.10	-	(+)
		121	-	-	2.41	-	(+)
		122	-	-	2.91	-	(+)
		137	-	-	2.75	(+)	-
		143	-	-	-	-	(+)
		146	-	-	-	-	-
		147	-	-	2.41	-	(+)
		149	-	-	-	-	(+)
		151	-	-	-	-	(+)
	21 dpi	159	-	-	3.05	-	(+)
		161	-	-	2.95	-	(+)
		162	-	-	2.80	-	(+)
		163	-	-	-	-	-
		171	-	-	-	-	(+)

SA2		172	-	-	2.86	-	(+)
	35 dpi	155	-	-	2.65	-	(+)
		164	-	2.26	2.06	-	(+)
		166	-	-	-	-	-
		168	-	-	-	-	-
		178	-	-	2.98	-	(+)
		181	-	2.42	4.16	-	-
		183	-	-	-	-	-
		184	-	-	-	-	-
		190	-	-	-	-	(+)
		192	-	-	2.55	-	(+)

‡ dpi: Days post infection
^ Log_{10} copies per reaction
- Negative
(+) Positive

Table 3: UL15qPCR results for swab samples and UL15 nested PCR results for control brain tissue and culture supernatant collected during trigeminal ganglia co-culture with LMH cell monolayers

Group	Bird Number	UL15 qPCR on swab samples			UL15 Nested PCR				
					Control brain tissue	TG culture supernatants (Days post explant)			
		Eye	Palatine cleft	Trachea		D3	D6	D9	D12
Mock	99	-	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-	-
	103	-	-	-	-	-	-	-	-
	107	-	-	-	-	-	-	-	-
	112	-	-	-	-	-	-	-	-
CL 9	135	-	-	-	-	-	(+)	-	-
	136	-	-	2.30^	(+)	-	-	-	-
	138	-	-	2.87	-	-	-	-	-
	139	-	-	2.45	-	-	(+)	-	-
	140	-	-	2.37	(+)	-	-	-	-
	141	-	-	-	-	-	-	-	-
	142	-	-	-	-	-	-	-	-
	145	-	-	-	-	(+)	-	-	(+)
	152	-	-	-	-	-	-	-	-
	153	-	-	-	-	-	-	-	-
	175	-	-	-	-	-	-	-	-
	176	-	-	-	-	-	-	-	-
	177	-	-	-	-	-	(+)	-	-
	179	-	-	3.27	-	-	(+)	(+)	-
	180	-	-	-	-	-	(+)	-	-

SA2	182	-	-	3.29	-	-	-	-	-
	185	-	-	-	(+)	-	-	-	-
	186	-	-	-	(+)	-	-	-	-
	189	-	-	-	-	(+)	(+)	-	-
	193	-	-	3.27	-	-	-	-	-

^ Log_{10} copies per reaction
- Negative
(+) Positive

Table 4. UL15qPCR results of tracheal swab samples collected from SPF chickens at 35 dpi with CL9 or SA2 ILTV, or mock inoculated with sterile media, and UL15qPCR results of tracheal organ co-culture supernatant samples collected at days 3 and 6 post explant.

Group	Bird Number	Tracheal swab sample	Tracheal co-culture supernatant (Days post explant)					
			D3			D6		
			Upper trachea	Middle trachea	Lower trachea	Upper trachea	Middle trachea	Lower trachea
Mock	99	-	-	-	-	-	-	-
	100	-	-	-	-	-	-	-
	103	-	-	-	-	-	-	-
	107	-	-	-	-	-	-	-
	112	-	-	-	-	-	-	-
CL 9	135	-	-	-	-	-	-	-
	136	2.30^	2.36	-	-	-	-	-
	138	2.87	-	-	-	-	-	-
	139	2.45	-	-	-	-	2.03	-
	140	2.37	-	-	-	-	-	-
	141	-	-	-	-	-	-	-
	142	-	2.66	-	-	2.09	-	-
	145	-	2.32	-	-	2.13	-	-
	152	-	-	-	-	-	-	-
	153	-	2.27	-	-	2.06	-	-
	175	-	3.26	-	-	3.26	-	-
	176	-	3.47	2.05	-	3.25	-	-
	177	-	3.06	2.13	-	2.09	-	-

SA2	179	3.27	2.89	-	-	2.31	-	-
	180	-	3.28	-	-	3.21	-	-
	182	3.29	2.87	-	-	2.49	-	-
	185	-	2.66	-	-	2.32	-	-
	186	-	3.27	2.49	-	3.02	2.09	-
	189	-	3.51	-	-	3.53	-	-
	193	3.27	3.10	2.28	-	2.87	-	-

^Log_{10} copies per reaction
- Negative
Highlighted are potential *in vitro* reactivations

Table 5. Mean percentages of lymphocytes, CD3+ and CD3- cell populations observed in peripheral blood collected from SPF chickens at 21-and 35-days post inoculation with SA2 ILTV, CL9 ILTV or sterile media (mock).

Group	21 dpi‡			35 dpi		
	Lymphocyte (%€ ± SD¥)	CD3 + (% ± SD)	CD3 - (% ± SD)	Lymphocyte (% ± SD)	CD3 + (% ± SD)	CD3 - (% ± SD)
Mock	29.96 ± 5.44 [a]	61.00 ± 26.08 [a]	39.00 ± 26.08 [a]	34.13 ± 3.99 [a]	51.96 ± 12.10 [a]	48.04 ± 12.10 [a]
CL9	32.46 ± 12.22 [a]	44.17 ± 22.40 [a]	55.83 ± 22.40 [a]	40.90 ± 11.02 [b]	52.41 ± 23.31 [a]	47.58 ± 23.31 [a]
SA2	36.20 ± 12.28 [a]	37.5 ± 24.5 [a]	62.50 ± 24.5 [a]	44.44 ± 8.66 [b]	37.06 ± 15.54 [b]	62.94 ± 15.54 [b]

‡ dpi: Days post inoculation
€ %: Percentage
¥ SD: Standard deviation
[a,b] Values marked with the same superscript character in each column did not differ significantly (P > 0.05, Two sample T-test)

Table 6. Mean percentages of lymphocyte sub-populations within the CD3+ and CD3- lymphocyte populations as a percentage of total lymphocytes observed in peripheral blood collected from SPF chickens at 35 days post inoculation with SA2 ILTV, CL9 ILTV or sterile media (mock)

Group	CD3+				CD3-			
	CD4+CD8- (%GP† ± SD¥)	**CD4+CD8+ (%GP ± SD)**	**CD4-CD8+ (%GP ± SD)**	**CD4-CD8- (%GP ± SD)**	**CD4+CD8- (%GP ± SD)**	**CD4+CD8+ (%GP ± SD)**	**CD4-CD8+ (%GP ± SD)**	**CD4-CD8- (%GP ± SD)**
Mock	11.59 ± 4.02 [a]	2.38 ± 1.72 [a]	9.16 ± 4.50 [a]	28.85 ± 5.95 [a]	0.36 ± 0.11 [a]	0.02 ± 0.03 [a]	0.88 ± 0.46 [a]	46.77 ± 12.22 [a]
CL9	12.95 ± 8.74 [a]	2.84 ± 3.08 [a]	7.25 ± 4.14 [a]	29.36 ± 12.03 [a]	0.38 ± 0.24 [a]	0.02 ± 0.03 [a]	0.71 ± 0.46 [a]	46.49 ± 23.33 [a]
SA2	5.84 ± 3.42 [b]	1.20 ± 2.23 [a]	4.64 ± 3.19 [b]	25.38 ± 9.07 [a]	0.31 ± 0.20 [a]	0.01 ± 0.02 [a]	1.08 ± 0.40 [b]	61.54 ± 15.62 [b]

† %GP: Percentage to the grandparent (Lymphocytes)
¥ SD: Standard deviation
[a,b] Values marked with the same superscript character in each column did not differ significantly (P > 0.05, Two sample T-test)

Supplementary table 1: Mean percentages of lymphocytes, CD3+, CD3- cells and their subpopulations observed in peripheral blood collected from SPF chickens with or without quantifiable ILTV DNA in trachea at 35 days post inoculation with SA2 ILTV, CL9 ILTV or sterile media

				CD3+				CD3-			
Group	**Lymphocyte (%€ ± SD¥)**	**CD3- (% ± SD)**	**CD3+ (% ± SD)**	**CD4+ CD8- (%GP† ± SD)**	**CD4+ CD8+ (%GP ± SD)**	**CD4- CD8+ (%GP ± SD)**	**CD4- CD8- (%GP ± SD)**	**CD4+ CD8- (%GP ± SD)**	**CD4+ CD8+ (%GP ± SD)**	**CD4- CD8+ (%GP ± SD)**	**CD4- CD8- (%GP ± SD)**
Mock	34.13 ± 3.99 ^{a}	48.04 ± 12.10 ^{ac}	51.96 ± 12.10 ^{ac}	11.59 ± 4.02 ^{a}	2.38 ± 1.72 ^{a}	9.16 ± 4.50 ^{a}	28.85 ± 5.95 ^{a}	0.36 ± 011 ^{ab}	0.02 ± 0.03 ^{a}	0.88 ± 0.46 ^{ab}	46.77 ± 12.22 ^{ac}
CL9 ILTV+	43.30 ± 13.21 ^{ab}	54.93 ± 23.32 ^{abc}	54.07 ± 23.32 ^{ac}	11.45 ± 9.03 ^{ab}	2.40 ± 1.78 ^{a}	6.47 ± 3.81 ^{ab}	24.73 ± 11.15 ^{a}	0.40 ± 0.31 ^{b}	0.02 ± 0.03 ^{a}	0.81 ± 0.45 ^{ab}	53.72 ± 23.35 ^{abc}
CL9 ILTV-	38.76 ± 8.47 ^{ab}	40.24 ± 21.99 ^{a}	59.76 ± 21.99 ^{a}	14.44 ± 8.65 ^{a}	3.28 ± 4.05 ^{a}	8.03 ± 4.50 ^{a}	33.99 ± 11.57 ^{a}	0.35 ± 0.13 ^{b}	0.02 ± 0.04 ^{a}	0.61 ± 0.47 ^{b}	39.25 ± 22.08 ^{a}
SA2 ILTV+	43.75 ± 10.61 ^{b}	61.44 ± 19.85 ^{bc}	38.56 ± 19.85 ^{bc}	6.80 ± 4.85 ^{b}	1.34 ± 3.05 ^{a}	4.23 ± 4.47 ^{b}	26.21 ± 8.89 ^{a}	0.24 ± 0.12 ^{ab}	0.00 ± 0.00 ^{a}	0.88 ± 0.39 ^{ab}	60.31 ± 19.98 ^{bc}
SA2 ILTV-	44.89 ± 7.58 ^{b}	63.94 ± 12.80 ^{b}	36.06 ± 12.80 ^{b}	5.20 ± 2.02 ^{b}	1.11 ± 1.62 ^{a}	4.92 ± 2.17 ^{b}	24.82 ± 9.54 ^{a}	0.36 ± 0.23 ^{a}	0.01 ± 0.02 ^{a}	1.21 ± 0.36 ^{a}	62.36 ± 12.87 ^{b}

(mock).

€ %: Percentage
† %GP: Percentage to the grandparent (Lymphocytes)
¥ SD: Standard deviation
a, b Values marked with the same superscript character in each column did not differ significantly (P > 0.05, Two sample T-test)

Chapter 5

Pathogenesis and tissue tropism of natural field recombinants of infectious laryngotracheitis virus

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Pathogenesis and tissue tropism of natural field recombinants of infectious laryngotracheitis virus

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ABSTRACT

Infectious laryngotracheitis virus (ILTV) is an economically significant respiratory pathogen of poultry. Novel recombinant strains of ILTV have emerged in Australia during the last decade and currently class 9 (CL9) and class 10 (CL10) ILTV are the most prevalent circulating strains. This study conducted a comprehensive investigation of the pathogenesis of these two viral strains. Commercial broiler and specific pathogen free (SPF) chickens were inoculated with varying doses of CL9 or CL10 ILTV and subsequently evaluated for clinical and pathological signs of infection. While no difference in the levels of acute viral replication were observed across the different challenge doses, the severity of clinical signs, tracheal pathology and mortality were dose dependent. Both strains of virus persisted in the respiratory tract for up to 14 days post inoculation (dpi) and could be detected in the lung and feathers with sporadic detection in the liver, spleen or bursa. Given the prevalence of CL9 and CL10 in Australian poultry flocks, this study provides an important foundation for the development of diagnostic and therapeutic approaches for the detection and prevention of ILTV.

1. Introduction

Infectious laryngotracheitis virus (ILTV) causes acute upper respiratory tract disease in chickens (Bagust, 1986), and is associated with significant economic losses in the poultry industry worldwide (Blacker et al., 2011; Chin et al., 2009; Preis et al., 2013). Despite advances in biosecurity, diagnostics and vaccinology, ILTV remains an economic and welfare burden (Chin et al., 2009). Infectious laryngotracheitis (ILT) is associated with a range of clinical signs that vary in severity depending on the infecting strain, and is characterised by acute respiratory disease, conjunctivitis, reduced weight gain and death (Kirkpatrick et al., 2006a; Oldoni et al., 2009; Russell and Turner, 1983; Timurkaan et al., 2003).

In areas in which ILTV is enzootic, vaccination is used to protect flocks against disease (García et al., 2013). Currently, live attenuated vaccines are widely used, even though they have some limitations (Coppo et al., 2013). Attenuated vaccine viruses replicate in the host after inoculation (Coppo et al., 2012, 2011; Rodríguez-Avila et al., 2007) and can spread from bird to bird (Andreasen et al., 1989; Andreasen Jr et al., 1989; Coppo et al., 2011; Rodríguez-Avila et al., 2007), potentially regaining virulence (Guy et al., 1991). Attenuated ILTV vaccine strains can also establish a latent infection in vaccinated chickens and eventually reactivate (Han and Kim, 2003; Hughes et al., 1991). Shedding and transmission of vaccine strains may facilitate recombination between different ILTVs, including vaccine strains, and this can lead to the development of highly virulent field strains (Agnew-Crumpton et al., 2016; Lee et al., 2012). The concurrent use of Australian ILTV vaccine strains (SA2 or A20; class 1 genotype) and a European origin vaccine strain (Serva, class 7 genotype) led to the emergence of two new recombinant ILTV strains (class 8 and class 9; CL8 and CL9, respectively), one of which has replaced the previously predominant ILTV strain in the field (class 2 genotype) (Agnew-Crumpton et al., 2016; Blacker et al., 2011; Lee et al., 2015). More recently, a new ILTV strain (class 10 genotype; CL10) became predominant in New

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South Wales, Australia between 2013 and 2014. However, detection of CL10 has declined and the CL9 ILTV remains as the most prevalent strain in Australia. It is believed that these recombinant viruses are more virulent than previously dominant ILTV strains (Agnew-Crumpton et al., 2016).

Even though ILT is primarily a respiratory tract disease, different ILTV strains are known to exhibit tropism for different tissues (Kirkpatrick et al., 2006a; Oldoni et al., 2009), influencing the severity of the disease they cause, their rate of transmission and diagnosis of disease (McCall et al., 2016). As an example, the dissemination of ILTV into the feathers has been used for the diagnosis of ILTV (Davidson et al., 2009, 2016). This has enabled convenient collection of clinical samples, including multiple sampling from the same bird over time (Davidson, 2009; Davidson and Skoda, 2005). Therefore, investigations of tissue tropism and pathogenicity over the course of infection may have important implications for diagnosis and control of newly emerging ILTV strains.

This study aimed to examine the tissue tropism of CL9 and CL10 ILTV and to characterise the severity of tracheal pathology induced by these viruses. Additionally, we aimed to optimise a sensitive molecular diagnostic method for detecting these viral strains in the feathers of commercial broiler and specific pathogen free (SPF) layer-type chickens.

2. Materials and methods

2.1. Viruses and titres

Class 9 (GenBank accession number JN804827) and class 10 (GenBank accession number KR822401) ILTV were propagated and titrated in Leghorn Male Hepatoma (LMH) cells and were used as inocula to infect broiler and SPF chickens at different doses ($10^{2.0}$ - $10^{4.0}$ plaque forming units [PFU] per bird). The LMH cells were propagated and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % v/v foetal bovine serum (Sigma-Aldrich), 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.7), ampicillin (Sigma-Aldrich) at 50 µg/mL and amphotericin B (Astral Scientific) at 25 µg/mL. The same culture medium was employed for mock inoculation of negative control chickens. Following inoculation, both viruses were re-titrated in LMH cells to confirm the dose administered.

2.2. Chickens

Birds were housed at the Asia-Pacific Centre for Animal Health (APCAH) animal facility at The University of Melbourne. One hundred and five 28-day old commercial broiler chickens (Turi Foods Pty. Ltd.) and thirty 28-day old SPF hybrid white Leghorn-derived chickens (Australian SPF Services Pty. Ltd.) were housed in isolator units maintained under negative internal pressure. Birds were provided with filtered air and 14 h per day of artificial light. Water and irradiated feed were provided *ad libitum*.

2.3. Experimental design

The study was approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences at The University of Melbourne Project 1,513,669. On day 0, chickens were weighed and divided into nine groups of 15 birds each, with 7 groups of commercial broilers and 2 groups of SPF birds. On the same day, all birds in groups 2–9 were inoculated either CL9 or CL10 ILTV. Broiler birds in group 1 were inoculated with sterile LMH cell culture medium. Broiler birds in group 2, 3 and 4 were inoculated at a dose of $10^{2.0}$, $10^{3.0}$ and $10^{4.0}$ PFU/bird with CL9 ILTV respectively. Group 5 consisted of SPF birds that were inoculated with CL9 ILTV at a dose of $10^{4.0}$ PFU/bird. Broiler birds in group 6, 7 and 8 received CL10 ILTV at a dose of $10^{2.0}$, $10^{3.0}$ and $10^{4.0}$ PFU/bird respectively. SPF birds in group 9 received CL10 ILTV at a dose of $10^{4.0}$ PFU/bird. Half of each dose was administered via eye-drop (80 µl/bird) and the other half directly into the trachea (150 µl/bird).

Following inoculation, birds were monitored and assessed for 14 days post inoculation (dpi) for general health and clinical signs consistent with ILT. Monitoring was performed twice daily from day 1 to day 6 and daily thereafter. From 3 to 6 dpi, all birds were clinically scored, as these were the days that the most severe clinical signs of disease were expected. Scoring involved picking birds up individually for approximately 30 s to assess demeanour, respiratory signs and any signs of conjunctivitis. Demeanour was scored as 0 (normal) to 2 (severely depressed), respiratory signs were scored from 0 (none) to 4 (severe respiratory distress) and conjunctivitis was scored from 0 (none) to 2 (severe). At 4 dpi, tracheal swabs were collected from all birds, and at 7 dpi 10 birds from each group were euthanised by exposure to halothane and weighed. Necropsies were performed to assess the extent of tracheal pathology. The remaining birds (five per group) were monitored until 14 dpi, when they were euthanised, weighed, necropsied and sampled. If animals showed severe clinical signs at any stage during the study (0–14 dpi), they were euthanised by exposure to halothane.

2.4. Post-mortem sample collection

Separate workstations and sterilized instruments were used for sampling tissues. An entirely new set of instruments was used for sampling each inoculated group. Sterilization was performed by application of 70 % (v/v) ethanol and flaming between each tissue collection. Sodium hypochlorite solution was applied, and each workstation was washed and then cleaned with 80 % ethanol between the necropsies of different groups. At necropsy, conjunctival, palatine cleft, upper tracheal and cloacal swabs were collected from each individual bird. Three contour feather quills with visible feather pulp were collected from a wing of each bird prior to dissection and sampling of tissues in the coelomic cavity. Detailed necropsies were conducted and tissue samples approximately 0.5 cm^3 in volume were collected from the thymus, bursa, spleen, lung, liver and kidney of each bird. All samples were collected into 1 mL of sterile viral transport medium (VTM; DMEM supplemented with 3% v/v FBS, 0.01 M HEPES, pH 7.7, and gentamicin and ampicillin, each at 0.25 mg/mL) and stored at −80 °C until DNA extraction was performed. A 0.5 cm length of the mid-section of the trachea was also collected from each bird and immediately fixed in 10 % v/v formalin for histopathological examination. Trigeminal ganglia (TG) were collected at 14 dpi from all birds in five selected groups (mock inoculated, SPFs inoculated with CL9 and CL10, and broilers inoculated with CL9 and CL10 [$10^{4.0}$ PFU/bird]) for histopathological examination.

2.5. DNA extraction

The MagMAX CORE Nucleic Acid Purification Kit (Applied Biosystems), together with the KingFisher Flex Purification System (Thermo Scientific), were used according to the manufacturers' instructions for DNA extraction from swabs, tissues from visceral organs and feathers. Total DNA was extracted from 200 µL of the swab supernatant or a 0.5 cm^3 piece of thawed tissue sample and eluted in 90 µL of elution buffer. For feather pulp DNA extraction, 1 cm lengths of the quills from three feathers were used as the starting material. DNA was manually extracted from paraffin-embedded trigeminal ganglia using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's instructions and eluted in 50 µL of elution buffer.

2.6. Quantitative polymerase chain reaction (qPCR) assay

A modified version of a quantitative polymerase chain reaction

(qPCR) assay described previously (Mahmoudian et al., 2011) was used to determine the concentrations of ILTV genome (genome copy number; GCN) in all samples. In brief, 2.5 µL of 5X Green GoTaqFlexi Buffer (Promega, Madison, Wisconsin, USA), 0.1 mM of each deoxyribonucleotide, 2 mM magnesium chloride, 0.8 µM Syto9 (Invitrogen, Eugene, Oregon, USA), 0.8 µM of each primer, 0.05 U of GoTaq DNA Polymerase (Promega) and 1 µL of template DNA was added to a total volume of 12.5 µL. The template was either the DNA extracted from samples or pGEM-T (Promega) containing a 113-base pair (bp) fragment of the ILTV UL15 gene. Serial dilutions of the plasmid ($10^{8.0}$ to $10^{1.0}$ genome copy numbers [GCN]/µL) and the qPCR setup were performed using a QIAgility automated system (Qiagen), and incubation and data acquisition were performed in a Rotorgene 6000 thermocycler (Corbett Life Science Pty. Ltd., Sydney, Australia). The reactions were incubated at 50 °C for 2 min and 95 °C for 5 min, then through 40 cycles of denaturation (95 °C for 5 s), annealing (60 °C for 30 s) and extension (72 °C for 30 s), and finally at 72 °C for 3 min and 95 °C for 1 min. Reactions were then subjected to high-resolution melting curve analysis by increasing the temperature by 0.3 °C increments from 50 °C to 95 °C.

2.7. Testing for sample-specific inhibition of qPCR

DNA extracted from tissues was tested for the presence of qPCR inhibitors, as significant inhibition can be seen in blood rich organs (Schrader et al., 2012). DNA samples from five randomly selected extractions from each type of tissue from the negative controls (thymus, bursa, spleen, lung, liver, kidney and feathers) were pooled. Eight aliquots from each pool were diluted 0, 10, 100 or 1000-fold and each dilution was spiked with a serial dilution of the UL15 plasmid to yield concentrations of $10^{8.0}$ to $10^{2.0}$ copies per µL or with a similar volume of nuclease free water (negative control). All samples were tested in the qPCR assay described above.

2.8. Nested polymerase chain reaction (NPCR) and gel electrophoresis

A two-step nested PCR (NPCR) was used to detect ILTV DNA in feathers and trigeminal ganglia. The first step used a pair of primers - UL15-Ex forward (5′-TCAGACTTGACAAACCCCCG-3′) and UL15-Ex reverse (5′- AAGCGTGCATTGCCCCTATA-3′) - that amplified a 322 bp region of the ILTV UL15 gene. The first amplification reaction mix consisted of 5 µL of 5X Green GoTaqFlexi Buffer (Promega, Madison, Wisconsin, USA), 2 mM magnesium chloride, 0.1 mM of each dNTP, 0.5 µM of each of the forward and reverse primers, 0.05 U of Taq DNA polymerase, 8.25 µl of nuclease-free water and 5 µl of template DNA in a final reaction volume of 25 µL. Thermal cycling was performed in a BioRad thermocycler. Reactions were incubated at 95 °C for 5 min, then through 45 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s, with a final incubation at 72 °C for 5 min. Amplicons were held at 4 °C until gel electrophoresis was performed. Agarose (2% w/v) gels in SYBR Safe Buffer (Invitrogen) were used to separate PCR amplicons and a Molecular Imager ChemiDoc XRS + imaging system (BioRad) was used to visualise the DNA. Positive samples (reactions that yielded a 322 bp product) were not tested using the second round PCR.

For the second round of the NPCR, negative first round samples were diluted 1/10 with nuclease-free water. A 1 µL sample of a 1/10 dilution of the first round PCR was used as template for the second step of the NPCR. The reaction and the amplification conditions for the second round were similar to those for the first round, except that the primers used were; UL15a Forward (5′-TTGCTGTGCTATTTCGCGTG-3′) and UL15a Reverse (5′-GTAAATCGTTTAGTGCGGCAT-3′)(Mahmoudian et al., 2011).

2.9. ILTV high resolution melting curve and PCR-restriction fragment length polymorphism analysis

Ten selected ILTV NPCR positive feather DNA samples were submitted to the Asia-Pacific Centre for Animal Health (APCAH) Diagnostic Laboratory, University of Melbourne for PCR-high resolution melting curve analysis (PCR-HRM) and PCR-restriction fragment length polymorphism (PCR-RFLP) analysis for genotype classification (Agnew-Crumpton et al., 2016; Blacker et al., 2011; Kirkpatrick et al., 2006b).

2.10. Tracheal histopathology

Formalin-fixed tracheal sections were subjected to paraffin-embedding, sectioning, slide preparation, and haematoxylin and eosin (H&E) staining. The stained tracheal sections were examined blindly by two investigators and microscopic lesions were scored using a system described previously (Guy et al., 1990).

2.11. Statistical analysis

Prism 5 for Windows version 5.03 (GraphPad Software Inc.) was used to perform survival curves analyses. Minitab (Version 17) (Minitab Pty. Ltd., Sydney, Australia) was used for other statistical analyses. Comparisons of genome copy concentrations and weight gains were performed using one-way analysis of variance in conjunction with Tukey's multiple comparison test. The medians of the clinical scores and the tracheal lesion scores were compared using Mann-Whitney U tests. Comparisons of the proportion of ILTV DNA positives between groups were performed using Fisher's exact test. In each statistical test, $P \leq 0.05$ was considered significant.

3. Results

In this study, commercial broiler birds and SPF chickens were infected with varying doses of CL9 or CL10 ILTV. Due to study size limitations, SPF chickens were infected only with the highest inoculum dose ($10^{4.0}$PFU/bird) of either CL9 or CL10 and compared to broiler chickens. Results relevant to the nine groups included in this study are described below.

3.1. Clinical scores reflected the progression of clinical disease

Disease progression was monitored by individual clinical assessment. The median and the range of the clinical scores for demeanour, dyspnoea and conjunctivitis, together with the sum of the scores between 3 and 6 dpi are shown in Table 1. The clinical scores from 3 to 6 dpi clearly reflected the progression of clinical disease within the groups. The severity of the clinical signs was dose dependent, with the highest doses causing the more severe clinical signs. There was no significant difference in clinical scores between the birds infected with the same doses of CL9 or CL10 ILTV.

3.2. Tracheal viral genome concentration at 4 dpi did not differ between the groups

As the viral load in the primary replication site at the peak of infection may have an effect on disease severity, the viral genome concentration on tracheal swabs collected at 4 dpi were determined (Fig. 1A). Regardless of the ILTV strain administered or the dose used, the mean viral genome concentration did not differ between the groups, but there was a significant difference ($P < 0.001$) in the viral genome concentrations on swabs from the negative control group and each of the groups of birds inoculated with ILTV.

3.3. Mortalities were only reported in groups infected with the highest inoculum dose

As mortality is a measure of infection severity, the survival percentages across the groups were compared (Fig. 1B). Deaths and euthanasias because of severe clinical disease only occurred in the four

Table 1
The median and range of the clinical scores for broiler or SPF chickens between 3 and 6 days post-inoculation with different doses of CL9 or CL10 ILTV strains.

Group	Strain	Inoculum (PFU[†])	Median clinical score (range)						
			3 dpi[‡]				**4 dpi**		
			Demeanour	Dyspnoea	Conjunctiva	Total	Demeanour	Dyspnoea	Conjunctiva
Broiler	Negative		0(0)^{a}	0(0)^{a,e}	0(0)^{a}	0(0)^{a}	0(0–1)^{a}	0(0–1)^{a}	0(0)^{a}
Broiler	CL9*	$10^{2.0}$	0(0–1)^{a}	0(0–1)^{a}	0(0)^{a}	0(0–2)^{a}	1(0–1)^{b}	1(0–1)^{b,e}	0(0–1)^{b}
Broiler	CL9	$10^{3.0}$	0(0–1)^{a}	0(0–1)^{a,c,e}	0(0–1)^{a}	0(0–3)^{a,c,d}	0(0–1)^{b}	1(0–1)^{b,e}	0(0–1)^{a,b}
Broiler	CL9	$10^{4.0}$	0(0–1)^{a}	0(0–2)^{a,c,e}	0(0)^{a}	0(0–3)^{a,c,d}	1(1–2)^{c,d}	1(1–4)^{c}	1(0–2)^{c}
SPF	CL9	$10^{4.0}$	1(0–2)^{b}	2(0–4)^{b}	0(0–2)^{b}	3(0–8)^{b}	1(1–2)^{c,d}	1(1–4)^{c}	1(1–2)^{c}
Broiler	CL10‡	$10^{2.0}$	0(0–1)^{a,c}	0(0–1)^{a,c}	0(0–1)^{a}	0(0–2)^{a,c,d}	0(0–2)^{a}	0(0–2)^{a,d,e}	0(0–1)^{a}
Broiler	CL10	$10^{3.0}$	0(0–1)^{a,c}	1(0–1)^{c,d}	0(0–1)^{a}	1(0–3)^{c}	0(0–1)^{b}	0(0–2)^{d,e}	0(0–1)^{a,b}
Broiler	CL10	$10^{4.0}$	0(0–2)^{a,c}	1(0–4)^{d}	0(0–2)^{a,b}	1(0–8)^{d}	1(0–2)^{b,c}	1(0–4)^{c}	1(0–2)^{c}
SPF	CL10	$10^{4.0}$	1(0–2)^{b,c}	0(0–4)^{c,d,e}	0(0–2)^{a,b}	1(0–8)^{c,d}	1(0–2)^{c,d}	1(0–4)^{c}	1.5(0–2)^{c}

Group	Median clinical score (range)								
	4 dpi	**5 dpi**				**6 dpi**			
	Total	Demeanour	Dyspnoea	Conjunctiva	Total	Demeanour	Dyspnoea	Conjunctiva	Total
Broiler	0(0–1)^{a}	0(0–1)^{a}	0(0)^{a}	0(0)^{a}	0(0–1)^{a}	0(0)^{a}	0(0)^{a}	0(0)^{a}	0(0)^{a}
Broiler	2(0–3)^{b}	1(0–1)^{b}	1(0–2)^{b}	0(0–1)^{a,b,f}	2(0–4)^{b,e}	0(0–1)^{a}	0(0–2)^{b}	0(0–1)^{a}	0(0–4)^{b,f}
Broiler	2(0–3)^{b}	1(0–2)^{b,c,d}	1(1–2)^{b}	1(0–2)^{c,d,f}	3(1–5)^{c,e}	1(0–1)^{c,d}	1(1–2)^{c,d}	1(1–2)^{b,c,d,e}	3(1–4)^{c,d}
Broiler	3(2–8)^{c}	1(1–2)^{c,d,e}	2(1–3)^{c}	1.5(1–2)^{c,d,e}	5(3–7)^{d}	1(0–2)^{c,d,f}	1(0–2)^{b,c,d}	1(0–2)^{b,c,d,e}	3.5(0–5)^{c,d,g}
SPF	4(3–8)^{c}	2(1–2)^{d,e}	1(1–4)^{b,c}	2(1–2)^{d,e}	5(3–8)^{d}	2(1–2)^{e,f,g}	1(1–4)^{c,d}	2(1–2)^{b,c,e,f}	5(3–8)^{e,g}
Broiler	0(0–4)^{a}	1(0–2)^{b,c}	1(0–2)^{b}	0(0–1)^{a,b,f}	2(0–4)^{b,e}	0(0–1)^{c,d}	0(0–2)^{b}	0(0–1)^{a}	1(0–4)^{f}
Broiler	1(0–4)^{b}	1(0–2)^{b,c,d}	1(1–2)^{b,c}	0(0–2)^{b,c,f}	2(1–5)^{b,c,e}	0(0–2)^{c,d}	1(0–2)^{b,c}	1(0–2)^{b,d}	2(0–4)^{c,d,f}
Broiler	3(0–8)^{c}	1(0–2)^{b,c,d}	1(0–2)^{b}	2(0–2)^{d,e}	4(0–5)^{c,d,e}	1(0–2)^{d,e,f,g}	1(0–4)^{c,d}	2(0–2)^{b,c,e,f}	5(0–8)^{d,e,g}
SPF	4(0–8)^{c}	2(1–2)^{e}	1(1–3)^{b,c}	2(1–2)^{d,e}	5(3–6)^{d,f}	1(1–2)^{e,f,g}	1(1–4)^{c,d}	2(0–2)^{c,e,f}	4(3–8)^{e}

[†] PFU, plaque forming units.
[‡] dpi, days post inoculation.
*CL9, class 9 ILTV.
‡CL10, class 10 ILTV.
Values marked with the same superscript letter in the same column were not significantly different ($P > 0.05$, Mann − Whitney U test).

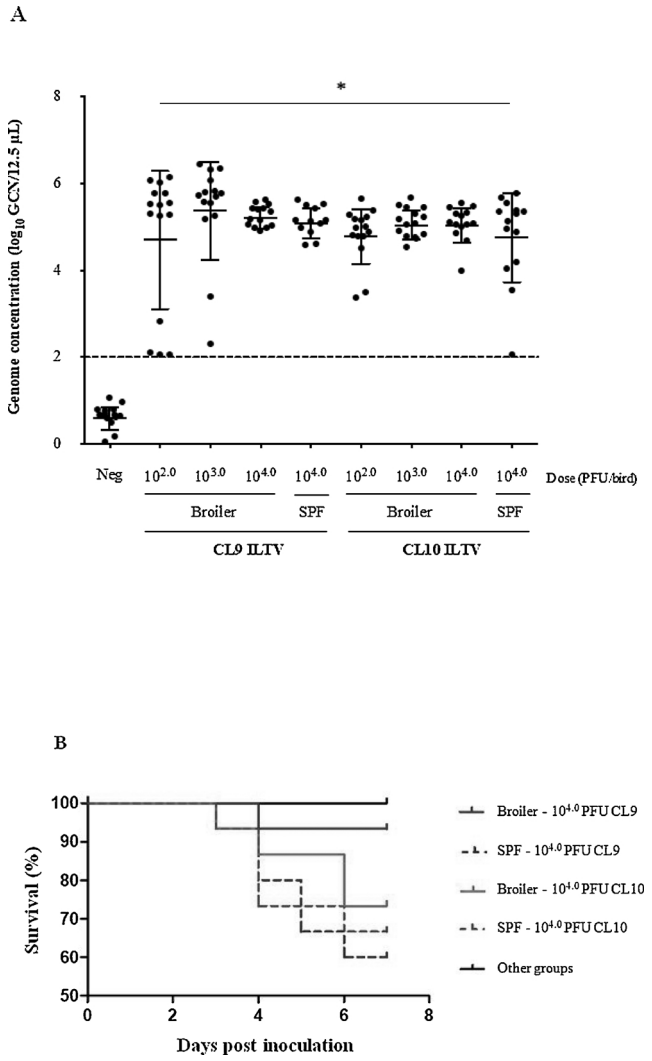

Fig. 1. (A) Scatter plot of log_{10} ILTV genome concentrations in tracheal swabs of SPF or broiler chickens infected with CL9 or CL 10 ILTV (and uninfected negative controls) at 4 dpi as determined by qPCR. The means and standard deviations are indicated by horizontal bars. The dashed line indicates the limit of detection of the assay (100 viral genome copies per reaction). Asterisks indicate a significant difference ($P < 0.05$, One-way ANOVA with Tukey's post hoc comparison) compared to the mock-inoculated group. There was no significant difference in viral genome concentrations between the different groups inoculated with ILTV ($P > 0.05$). PFU = plaque forming units, dpi = days post inoculation, SPF = specific pathogen free, CL = class, GCN = genome copy number per 12.5 µL.
(B) Survival curves for groups of SPF and broiler chickens inoculated with $10^{2.0}$-$10^{4.0}$ PFU/bird of CL9 or CL10 ILTV or cell culture medium. All mortalities occurred in the birds inoculated with $10^{4.0}$ PFU of CL9 or CL10 ILTV. Mortality rates did not differ significantly between the two strains of ILTV or the two types of chickens. SPF = specific pathogen free, CL = class.

groups inoculated with a dose of $10^{4.0}$ PFU of either CL9 (n = 7) or CL10 (n = 9) ILTV, but were seen in both broilers and SPF birds. The mortality rate after infection with CL10 ILTV did not differ significantly from that seen after infection with CL9 ILTV, in either broilers or SPF birds at the comparable dose ($P = 0.3295$ for broilers and $P = 1$ for SPF birds).

3.4. ILTV infection affected weight gain

Severe disease has been associated with lower weight gain rates

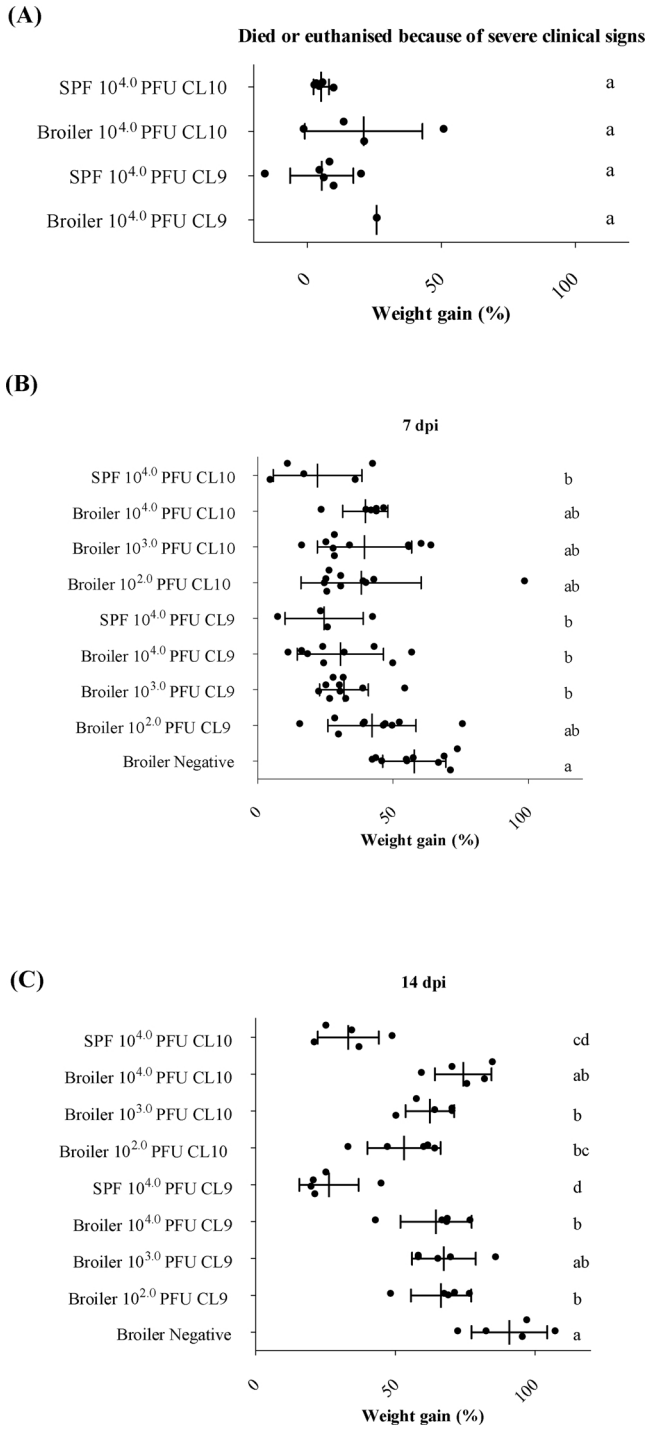

Fig. 2. Proportionate weight gain in groups of SPF or broiler chickens inoculated with $10^{2.0}$-$10^{4.0}$ PFU/bird of CL9 or CL10 ILTV or cell culture medium. (A) Birds that died of were euthanised because of the severity of their clinical signs; (B) birds that were euthanised at 7 dpi; (C) birds that were euthanised at 14 dpi. Means and standard deviations are indicated with horizontal bars. Weight gain was calculated by subtracting the weight at 28 days of age (day of inoculation) from the weight at the time of death or euthanasia. PFU = plaque forming units, dpi = days post inoculation, SPF = specific pathogen free, CL = class.

(Devlin et al., 2006; Kirkpatrick et al., 2006a; Oldoni et al., 2009). Therefore, proportionate weight gains for each group of birds were compared (Fig. 2). The results from birds that died or were euthanised

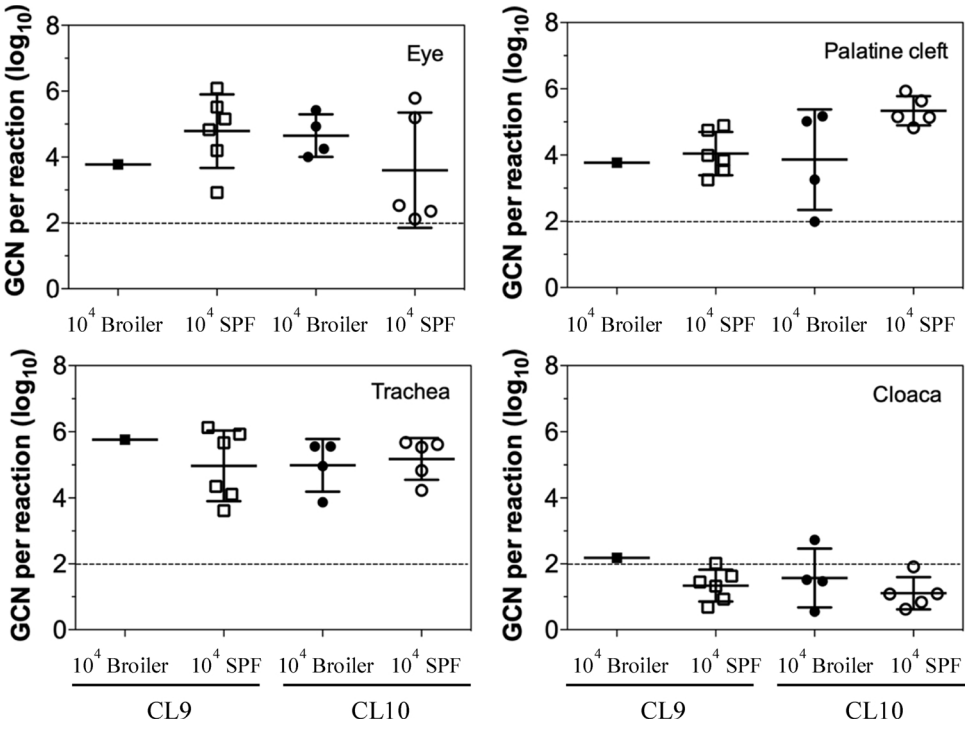

Fig. 3. Genome copy number (GCN) per reaction of ILTV detected in swabs taken from SPF or broiler birds that died or were euthanized due to severe signs. Lines and error bars show mean and SD. These birds were inoculated with $10^{4.0}$ PFU of CL9 or CL10 ILTV. There was no significant difference ($P > 0.05$) between the groups within each panel, as determined by One-way ANOVA with Turkey's comparison. The dashed line shows limit of detection for the PCR.

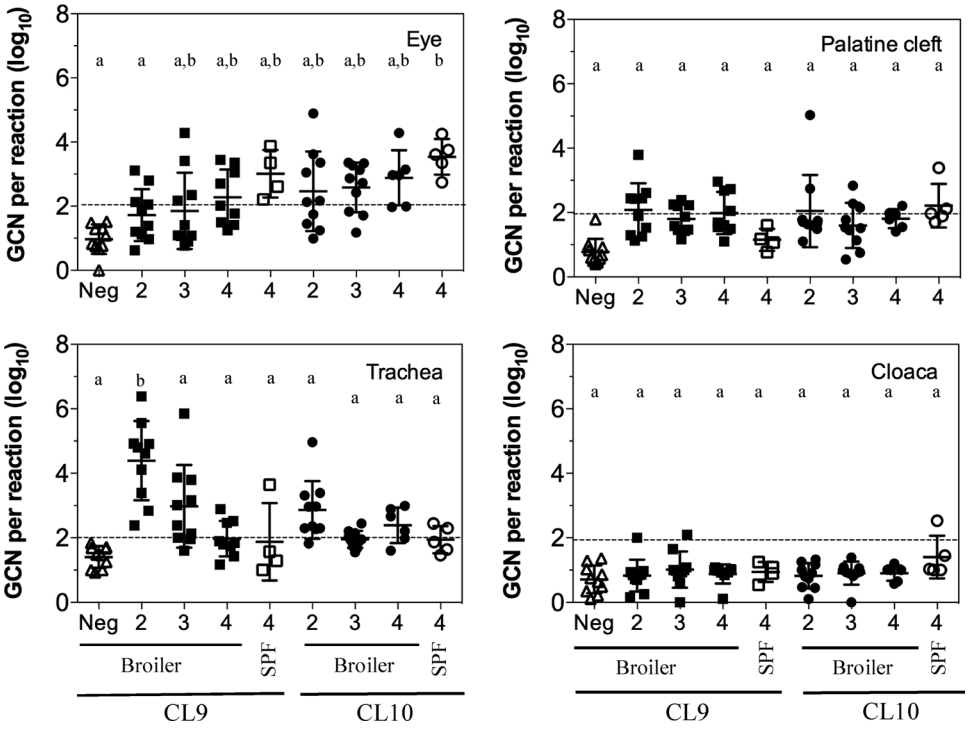

Fig. 4. Genome copy number (GCN) per reaction of ILTV detected in swabs taken at 7 dpi. Lines and error bars show mean and SD. Birds were inoculated with either media alone (Neg) or with $10^{2.0}$ (2), $10^{3.0}$ (3) or $10^{4.0}$ (4) PFU of CL9 or CL10 ILTV. Birds inoculated were either broilers or SPF birds as shown. Groups marked with the same lowercase superscript character in each panel were not statistically different ($P > 0.05$), One – way ANOVA with Turkey's comparison for GCN. The dashed line shows limit of detection for the PCR.

because of severe clinical signs are presented separately (Fig. 2A). When birds that died or were euthanised because of severe clinical signs were grouped by the dose they received, there was no significant difference in weight gain between the groups. At 7 dpi, broiler chickens infected with $\geq 10^{3.0}$ PFU of CL9 ILTV, and SPF birds inoculated with $10^{4.0}$ PFU of CL10 ILTV, had significantly lower weight gains than the mock inoculated group. At 14 dpi, all groups except the broilers inoculated with $10^{3.0}$ PFU of CL9 ILTV and the broilers inoculated with $10^{4.0}$ PFU CL10 ILTV had significantly lower weight gains than the mock inoculated chickens.

3.5. ILTV persisted in the respiratory tract and was detected in lung, liver, spleen and bursa

The ability of CL9 and CL10 ILTV to persist in primary replication sites and the capacity of virus to disseminate into visceral organs were evaluated by quantifying the viral genome load in swabs and tissues taken at different time points. The results from qPCR analysis of swabs and the mean $\log_{10}$ viral genome concentrations are shown in Figs. 3–5. These figures summarise data for birds that died or were euthanised because of severe clinical signs (Fig. 3), that were culled at 7 dpi (Fig. 4) or were culled at 14 dpi (Fig. 5).

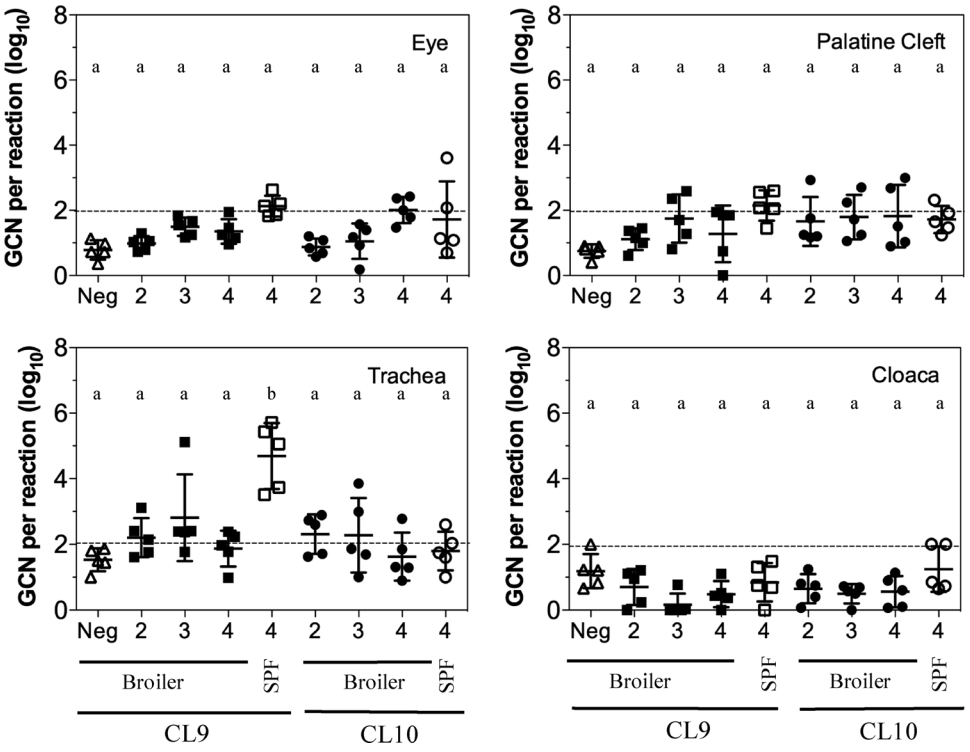

Fig. 5. Genome copy number (GCN) per reaction of ILTV detected in swabs taken at 14 dpi. Lines and error bars show mean and SD. Birds were inoculated with either media alone (Neg) or with $10^{2.0}$ (2), $10^{3.0}$ (3) or $10^{4.0}$ (4) PFU of CL9 or CL10 ILTV. Birds inoculated were either broilers or SPF birds as shown. Groups marked with the same lowercase superscript character in each column were not statistically different ($P > 0.05$, One−way ANOVA with Turkey's comparison for GCN. The dashed line shows limit of detection for the PCR.

Regardless of the viral strain or dose, all conjunctival and tracheal swabs collected from birds that died or were euthanised because of severe clinical signs were positive for ILTV by qPCR. The mean $\log_{10}$ GCN ranged from 3.6 to 4.79 per 12.5 µL in conjunctival swabs, and from 4.5 to 5.76 per 12.5 µL in tracheal swabs. Most of the palatine cleft swabs collected from these birds were qPCR positive, except for one broiler bird that received $10^{4.0}$ PFU of CL10. The mean $\log_{10}$ GCN on the palatine cleft swabs ranged from 3.77 to 5.34 per 12.5 µL. All groups that had mortalities or birds that were euthanised because of severe clinical signs, except the SPF group inoculated with $10^{4.0}$ PFU CL10 ILTV, had one cloacal swab that was qPCR positive (mean $\log_{10}$ GCN ~2.00 per 12.5 µL, Fig. 3).

At 7 dpi most of the swabs of ocular and respiratory sites of birds inoculated with ILTV were qPCR positive (Fig. 4). Of the swabs collected from 64 birds inoculated with ILTV that were euthanised at this time point, 62.5 % of the tracheal swabs, 60.9 % of the conjunctival swabs, 32.8 % of palatine cleft swabs and 4.7 % of cloacal swabs were qPCR positive. The swabs of the tracheas of broiler birds inoculated with $10^{2.0}$ PFU of CL9 ILTV had significantly higher ($P < 0.001$) viral genome concentrations than those collected from the other groups of birds inoculated with ILTV.

At 14 dpi a significantly lower ($P < 0.001$) number of swabs were positive by qPCR than were positive at 7 dpi (Fig. 5). Of the 40 inoculated birds euthanised at this time point, 55 % of tracheal swabs, 30 % of palatine cleft swabs and 20 % of conjunctival swabs were positive by qPCR. None of the cloacal swabs had detectable levels of ILTV DNA. Tracheal swabs of SPF birds inoculated with $10^{4.0}$ PFU of CL9 ILTV contained significantly greater concentrations of the ILTV genome ($P < 0.001$) than those from birds in the other inoculated groups. The same group also had a higher proportion of qPCR positive palatine cleft swabs.

DNA extracts from tissue samples contained a considerable level of qPCR inhibitors. The inhibitory effect was abrogated in the 1/100 dilutions of the extracted DNA. Therefore, a 1/100 dilution of the DNA extracts from tissues was used as the template for the UL15 qPCR assays (Mahmoudian et al., 2011). Tissues classified as positive and the concentrations of ILTV genome in the samples are shown in Table 2. ILTV DNA was only detected in liver, spleen and bursal tissue from birds inoculated with the highest dose of virus ($10^{4.0}$ PFU) and only in birds that died or were euthanised because of the severity of their clinical signs. Four liver samples (2 from birds inoculated with CL9 ILTV, and 2 from birds inoculated with CL10 ILTV) were positive for ILTV DNA. Two spleen samples (one from a bird inoculated with CL9 ILTV and one from a bird inoculated with CL10 ILTV) and two bursal samples (one from a bird inoculated with CL9 ILTV and one from a bird inoculated with CL10 ILTV) were also positive for ILTV DNA.

A total of 12 lung samples were positive for ILTV DNA. These included samples from birds inoculated with CL9 and CL10 ILTV, from birds that were inoculated with doses of $10^{2.0}$ PFU or $10^{4.0}$ PFU of virus, and from birds that died or were euthanised because of severe clinical signs, as well as birds that were culled at 7 dpi. ILTV DNA was not detected in the kidney or thymus of infected or mock inoculated birds.

3.6. ILTV DNA was detectable in feathers for up to 14 days

As feathers offer a convenient clinical sample, ILTV DNA detection in feather pulp was attempted. The proportions of feather samples that were ILTV positive, as determined using NPCR, are shown in Table 3. ILTV DNA in 100 % of the feather samples from infected birds that died or were euthanised because of the severity of their clinical signs, in 79.7 % of the feather samples from birds that were euthanised at 7 dpi, and in 45 % of the feather samples from birds that were euthanised at 14 dpi. At 14 dpi, the rate of detection of ILTV in feathers from birds that were infected with CL10 ILTV was significantly higher than the rate of detection in feathers from birds infected with CL9 ILTV.

ILTV detection and typing (PCR-RFLP) were attempted on DNA extracted from the feathers. However, the long-range conventional PCRs that targeted the TK, ICP4 and ICP18.5 genes could not amplify ILTV DNA from any of the 10 samples tested, so the PCR-RFLP assay could not be performed. PCR-HRM assays targeting the TK, ICP4 or

Table 2
Proportions of tissue samples found positive for ILTV and concentrations of ILTV genome in tissue samples collected from SPF or broiler chickens that died or were euthanased because of severe clinical signs during the course of the experiment or were euthanised at 7- or 14-days post inoculation.

Group	Strain	Inoculum (PFU[†])	Sampling time point	Thymus		Bursa		Spleen		Kidney		Liver		Lung		**Feather**	
				Proportion positive	Mean $\log_{10}$ GCN[«] ± SD[¥]	Proportion positive	Mean $\log_{10}$ GCN ± SD	Proportion positive	Mean $\log_{10}$ GCN ± SD	Proportion positive	Mean $\log_{10}$ GCN ± SD	Proportion positive	Mean $\log_{10}$ GCN ± SD	Proportion positive	Mean $\log_{10}$ GCN ± SD	Proportion positive	Mean $\log_{10}$ GCN ± SD
Broiler	Negative		DE[¤]	NA[δ]	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			7 dpi[‡]	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
Broiler	CL9*	$10^{2.0}$	DE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			7 dpi	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
Broiler	CL9	$10^{3.0}$	DE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			7 dpi	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(1/10)	4.07	(0/10)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
Broiler	CL9	$10^{4.0}$	DE	(0/1)	NA	(0/1)	NA	(1/1)	4.03	(0/1)	NA	(1/1)	4.10	(1/1)	4.41	(0/1)	NA
			7 dpi	(0/9)	NA	(0/9)	NA	(0/9)	NA	(0/9)	NA	(0/9)	NA	(2/9)	5.22 ± 0.08	(0/9)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
SPF	CL9	$10^{4.0}$	DE	(0/6)	NA	(1/6)	4.20	(0/6)	NA	(0/6)	NA	(1/6)	4.19	(2/6)	5.22 ± 0.69	(1/6)	5.02
			7 dpi	(0/4)	NA	(0/4)	NA	(0/4)	NA	(0/4)	NA	(0/4)	NA	(0/4)	NA	(0/4)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
Broiler	CL10[‡]	$10^{2.0}$	DE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			7 dpi	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(1/10)	4.37	(0/10)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
Broiler	CL10	$10^{3.0}$	DE	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
			7 dpi	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(0/10)	NA	(1/10)	4.26	(0/10)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
Broiler	CL10	$10^{4.0}$	DE	(0/4)	NA	(1/4)	4.00	(1/4)	4.75	(0/4)	NA	(1/4)	4.80	(2/4)	6.16 ± 1.14	(0/4)	NA
			7 dpi	(0/6)	NA	(0/6)	NA	(0/6)	NA	(0/6)	NA	(0/6)	NA	(0/6)	NA	(0/6)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
SPF	CL10	$10^{4.0}$	DE	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(1/5)	4.10	(2/5)	5.35 ± 1.85	(1/5)	4.27
			7 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA
			14 dpi	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA	(0/5)	NA

δ NA, not applicable.
[†] PFU, plaque forming unit.
[‡] dpi, days post infection.
[«] GCN, genome copy number.
[¥] SD, standard deviation.
[¤] DE, birds that either died or were euthanised because of severe clinical signs.
*CL9, class 9 ILTV.
[‡]CL10, class 10 ILTV.

Table 3
Proportions of feather samples found positive for ILTV DNA by nested PCR.

Group	Strain	Inoculum (PFU†)	Dead/ euthanised	7 dpi‡	14 dpi
			Proportion positive	Proportion positive	Proportion positive
Broiler	Negative		NA[δ]	(0/10)[a]	(0/5)[a]
Broiler	CL9*	$10^{2.0}$	NA	(5/10)[b]	(0/5)[a]
Broiler	CL9	$10^{3.0}$	NA	(8/10)[b]	(1/5)[a,c]
Broiler	CL9	$10^{4.0}$	(1/1)[a]	(6/9)[b]	(1/5)[a,c]
SPF	CL9	$10^{4.0}$	(6/6)[a]	(4/4)[b]	(3/5)[a,c,d]
Broiler	CL10[‡]	$10^{2.0}$	NA	(8/10)[b]	(1/5)[a,c]
Broiler	CL10	$10^{3.0}$	NA	(9/10)[b]	(5/5)[b,d]
Broiler	CL10	$10^{4.0}$	(4/4)[a]	(6/6)[b]	(4/5)[b,c,d]
SPF	CL10	$10^{4.0}$	(5/5)[a]	(5/5)[b]	(3/5)[a,c,d]

[δ]NA, not applicable (no birds died or were euthanised because of severe clinical signs in these groups).
†PFU, plaque forming units.
‡dpi, days post inoculation.
Values marked with the same superscript lowercase character in the same column were not significantly different (P > 0.05, Fishers exact test).

Table 4
The median (range) gross tracheal lesions scores for birds that died or were euthanised due to severe disease, and birds that were euthanised at 7 or 14 days post-inoculation with different doses of CL9 or CL10 ILTV strains.

Group	Strain	Inoculum (PFU†)	Dead/ euthanised		7 dpi‡		14 dpi	
			N[Θ]	Median score (Range)	N	Median score (Range)	N	Median score (Range)
Broiler	Negative			NA[δ]	10	0(0–1) [a]	5	0(0–1) [a]
Broiler	CL9*	$10^{2.0}$		NA	10	1.5(0–3) [b]	5	0(0–2) [a]
Broiler	CL9	$10^{3.0}$		NA	10	2(1–4) [b]	5	1(0–2) [a]
Broiler	CL9	$10^{4.0}$	1	1	9	3(0–3) [b]	5	1(0–3) [a]
SPF	CL9	$10^{4.0}$	6	3 (3 - 3)	4	2(1–2) [b]	5	0 (0 - 0) [a]
Broiler	CL10[‡]	$10^{2.0}$		NA	10	1.5(1–3) [b]	5	0(0–2) [a]
Broiler	CL10	$10^{3.0}$		NA	10	2(1–4) [b]	5	0(0–2) [a]
Broiler	CL10	$10^{4.0}$	4	2.5(1–3)	6	2.5(1–3) [b]	5	1(0–2) [a]
SPF	CL10	$10^{4.0}$	5	4 (4 - 4)	5	2(0–2) [b]	5	0(0–2) [a]

[Θ]N, number of available tracheas for scoring per group and per time point.
[δ]NA, not applicable (no birds died or were euthanised because of severe clinical signs in these groups).
†PFU, plaque forming units.
‡dpi, days post inoculation.
*CL9, class 9 ILTV.
[‡]CL10, class 10 ILTV.
Values marked with the same superscript lowercase character in the same column were not significantly different (P > 0.05, Mann − Whitney U test).

orfB-TK genes detected trace amounts of ILTV in all 10 samples, but ILTV typing based on HRM analyses of these products was inconclusive because of the low concentrations of the PCR products.

3.7. Histological changes were absent in trigeminal ganglia

Trigeminal ganglia, the main site of ILTV latency (Williams et al., 1992), are infected during the acute phase of disease (Oldoni et al., 2009). Therefore, in the present study TG from experimentally infected ILTV chickens were examined for the presence of histological lesions. Haematoxylin and eosin stained trigeminal ganglion sections from infected birds did not appear different histologically from that of mock infected birds. ILTV DNA could not be detected by NPCR in the trigeminal ganglia from ILTV- or mock-inoculated birds.

3.8. Gross tracheal lesions were dose dependent

Pathological changes caused by ILTV in the main replication site, the trachea, was evaluated by assessing gross tracheal lesions. The median and the range of the tracheal lesion scores for each group of birds are shown in Table 4. CL9 or CL10 inoculated SPF chickens that died or were euthanised because of severe clinical signs had higher median lesion scores than broiler chickens infected with CL9 or CL10. At 7 dpi all infected groups had significantly higher lesion scores than mock inoculated birds. At 14 dpi the median lesion scores of birds inoculated with ILTV did not differ significantly from that of the mock inoculated birds.

3.9. Histopathological tracheal lesions were more severe in broiler chickens

Pathological lesions were further studied by examining H&E stained tracheal sections. Examples of histopathological tracheal lesions seen in this study are shown in Fig. 6. The median and the range of the histopathological tracheal lesion scores are shown in Table 5. There were significant differences between the groups in the histopathological tracheal lesion scores at 7 dpi. At this time point, the median histopathological tracheal lesion score of broiler chickens inoculated with $10^{4.0}$ PFU of CL9 ILTV or $10^{4.0}$ PFU of CL10 ILTV were significantly higher (P = 0.026) than those of all other groups. At 14 dpi, the median histopathological lesion scores were lower than at 7 dpi in all the ILTV-inoculated groups. There were no significant differences between the groups at 14 dpi.

4. Discussion

This study has shown that the CL9 and CL10 Australian ILTV field isolates are highly pathogenic. Infection resulted in clinical disease characterised by reduced weight gain, pathological changes in the trachea and high mortality in birds that received the highest inoculum dose. Viral replication was detected in the trachea, the conjunctiva and the lung. Dose dependent differences were seen in the severity of clinical signs and tracheal pathology, but the concentrations of virus in the trachea were similar in all ILTV infected groups at 4 dpi. This is consistent with the study of Lee et al. (2015), which found that concentrations of ILTV in the trachea of infected broiler chickens were not affected by the dose with which the birds were inoculated.

Viral tropism was assessed by examining different sites and tissues for the presence of ILTV DNA using qPCR and NPCR. CL9 and CL10 ILTV could be detected in the primary replication sites (conjunctiva, palatine cleft and trachea) at 7 and 14 dpi. The concentrations of viral genome decreased from 7 to 14 dpi. This was consistent with a previous study that examined commercial broiler chickens infected with CL9 ILTV and found a decrease in tracheal viral load between 6 and 14 dpi (Lee et al., 2015).

In the study described here, CL9 and CL10 ILTV DNA was detected in a small number of lung samples (n = 12), and occasionally in liver (n = 4), spleen (n = 2) and bursal samples (n = 2). ILTV DNA was only detected in liver, spleen and bursal tissues of birds inoculated with the highest dose of virus ($10^{4.0}$ PFU) and only in birds that died or were euthanised because of the severity of their clinical signs. Severe clinical signs were also associated with high concentrations of ILTV genome in the conjunctiva and upper respiratory tract. The presence of qPCR inhibitors in the DNA extracts required the use of diluted DNA extracts (100-fold dilution) as PCR template. Thus, it is unlikely that ILTV DNA would be detected in tissues with a low viral load. A previous study detected the two Australian ILTV vaccine strains, A20 and SA2, in the trachea, lung and kidney for up to 28 days after administration of the vaccines in drinking water (Roy et al., 2015). Other ILTV strains have also been reported to disseminate to a range of organs during the acute phase of infection (Oldoni et al., 2009; Wang et al., 2013; Zhao et al., 2013). Further investigation is required to determine whether ILTV

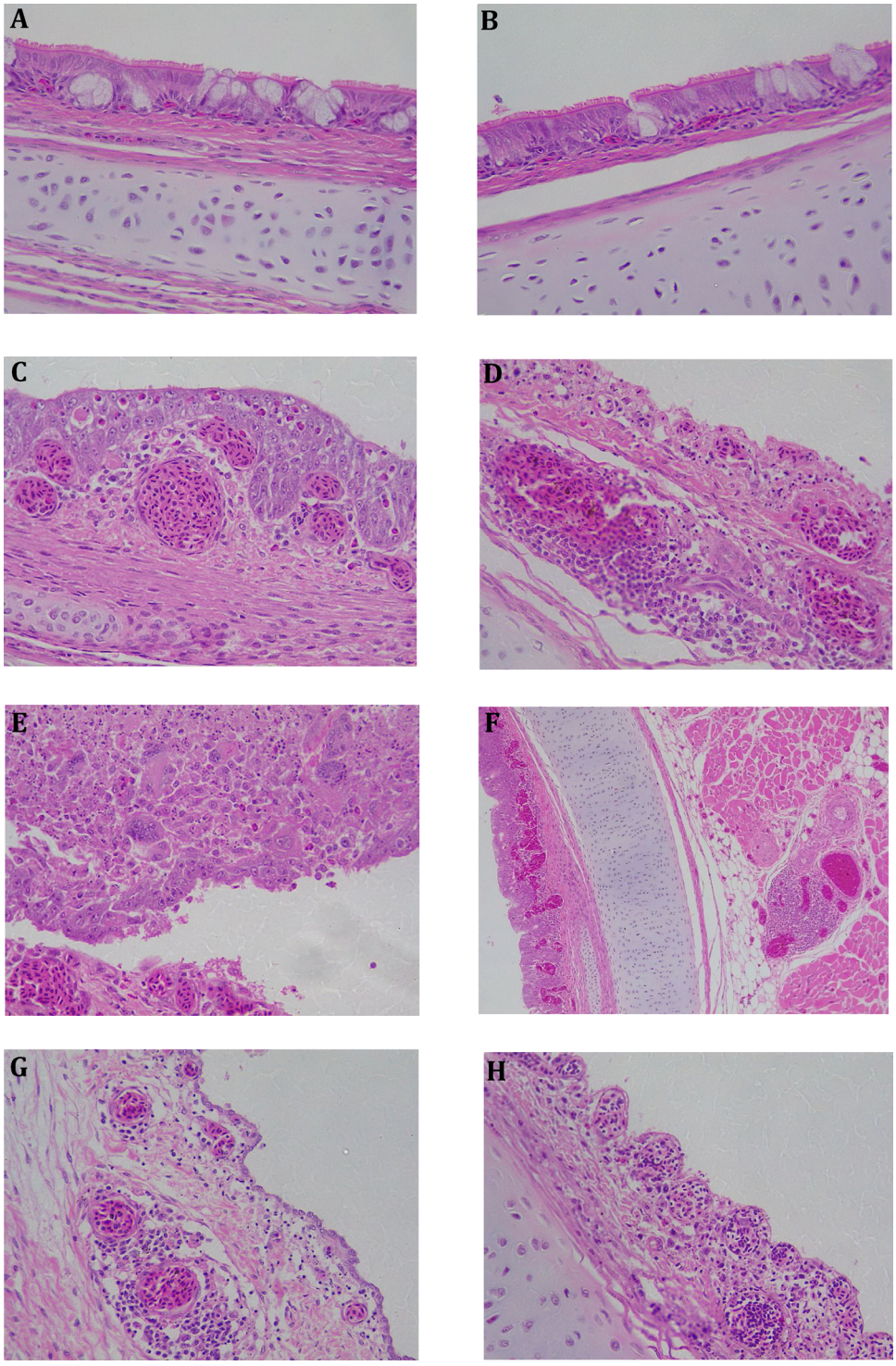

Fig. 6. Microscopic lesions observed in H&E stained tracheal sections. (A) Score 0, normal tracheal mucosa; (B) score 1, minimal changes in the tracheal mucosa with mild lymphocyte infiltration; (C) score 2, mild changes in the tracheal mucosa, including thickening, moderate lymphocytic infiltration and hyperaemia; (D) score 3, mucosal thickening due to marked lymphocytic infiltration and hyperaemia; (E) score 3, epithelial separation with the presence of numerous syncytia with intranuclear inclusion bodies; (F) score 3, cuffs of mononuclear cells outside the mucosa; (G) score 4, thickened, hyperaemic mucosa covered with a thin layer of basal cells; (H) score 5, absence of any epithelium and exposure of blood vessels to the tracheal lumen.

establishes a viraemia, which facilitates distribution to visceral organs and whether the presence of virus in these organs is a significant aspect of the pathogenesis of ILT.

This study evaluated the possibility of using feathers as a diagnostic sample for the detection of ILTV in chickens. Only two feather samples collected from SPF chickens that either died or were euthanised because of the severity of their clinical signs had concentrations of ILTV DNA that were quantifiable using the UL15 qPCR. Therefore, a more sensitive, UL15 NPCR was used to detect ILTV DNA in feathers. Vaccine and field ILTV strains have been detected in feathers in previous studies using both qPCR and NPCR (Davidson et al., 2009, 2018; Davidson et al., 2016). In these studies, the investigators were able to detect wild-type ILTV in feathers after inoculation and an ILTV vaccine strain for up to one month post-vaccination. An advantage of the UL15 NPCR is its high sensitivity and its capacity to detect ILTV DNA in feathers from a single bird, but this assay does not provide information about the genotype of the virus. It was not possible to genotype the extracted viral DNA using current methods, presumably because of the low quantity and/or quality of DNA extracted from this sample type.

Taken together, this study has shown that CL9 and CL10 ILTV cause severe clinical disease in chickens, characterised by severe conjunctivitis, moderate to severe dyspneoa, a depressed demeanour and

Table 5
The median (range) microscopic tracheal lesions score for birds that died or were euthanised due to severe disease, and birds that were euthanised at 7 or 14 days post-inoculation with different doses of CL9 or CL10 ILTV strains.

Group	Strain	Inoculum (PFU[†])	Dead/ euthanised		7 dpi[‡]		14 dpi	
			N[Θ]	Median score (Range)	N	Median score (Range)	N	Median score (Range)
Broiler	Negative			NA[δ]	10	1(1–2) [a]	5	1(1–2) [a]
Broiler	CL9*	$10^{2.0}$		NA	10	2(1–4) [a]	5	1(1–2) [a]
Broiler	CL9	$10^{3.0}$		NA	10	2(1–5) [a]	5	1(1–4) [a]
Broiler	CL9	$10^{4.0}$	1	3	9	3(1–5) [b]	5	1(1–2) [a]
SPF	CL9	$10^{4.0}$	5	2(2–3) [a]	4	2(1–3) [a]	4	1(1–3) [a]
Broiler	CL10[‡]	$10^{2.0}$		NA	10	2(1–3) [a]	5	1(1–3) [a]
Broiler	CL10	$10^{3.0}$		NA	10	2(1–4) [b]	5	1(1–4) [a]
Broiler	CL10	$10^{4.0}$	3	3(3–4) [b]	6	3(1–4) [b]	5	1(1-1) [a]
SPF	CL10	$10^{4.0}$	4	2(1–3) [ab]	5	2(1–3) [a]	5	2(1–4) [a]

[Θ] N: Number of available H&E stained sections with acceptable quality for scoring per group and per time point.
[δ]NA, not applicable (no birds died or were euthanised because of severe clinical signs in these groups).
[†]PFU, plaque forming units.
[‡]dpi, days post inoculation.
*CL9, class 9 ILTV.
[‡]CL10, class 10 ILTV.
Values marked with the same superscript lowercase character in the same column were not significantly different (P > 0.05, Mann – Whitney U test).

mortality. Further, this study showed that feathers could be used as a clinical sample for the detection of ILTV in commercial broiler flocks, although the viral DNA extracted from this sample could not be successfully genotyped. The results obtained in this study make an important contribution towards a better understanding of the pathophysiology of ILTV in chickens that may help to improve the diagnosis and control of this disease.

Data availability statement

All relevant data are within the manuscript and its supporting information files.

Declaration of Competing Interest

The authors have declared that they have no competing interests.

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Chapter 6 - General discussion

The avian alphaherpesvirus ILTV is a significant pathogen of poultry which is capable of entering its host via inhalation, ocular deposition or ingestion (49). Upon entry into a naïve host, ILTV replicates mainly in the conjunctiva and tracheal epithelium which are considered the primary replication sites for this pathogen (61). Replication of the virus within the host, and the host immune responses mounted against the virus, lead to clinical signs that typically develop around 4 days after inoculation (10, 83). During this acute phase of the infection it is thought there is a viremic phase which allows the virus to reach other tissues (107).

One aim of this thesis was to study ILTV pathogenesis by characterizing the tropism of two novel field recombinant strains of virus; class 9 and class 10 ILTV (2, 31) in their natural host (chapter 5). To achieve this, an *in vivo* experiment was conducted using commercial broiler and SPF chickens. Varying doses of the inoculums ($10^{2.0}$ - $10^{4.0}$ PFU per bird) were administered to both primary replication sites (eye and trachea) and the birds were observed for a period of 14 days. Both strains caused hemorrhagic tracheitis in chickens and the clinical signs, as reflected by median clinical scores, were at peak by 5 dpi. Both strains reduced the weight gain in broiler and SPF chickens; however, only the highest inoculum dose ($10^{4.0}$ PFU per bird) caused mortalities. Despite the differences in the dose of the initial inoculum between groups, both strains showed high levels of ILTV in the trachea by 4 dpi which was not significantly different between groups. Even though viral replication decreased over time, both strains persisted in upper respiratory tract for an extended period of time (14 dpi). Further, these ILTV strains showed dissemination to internal organs (lung, liver, spleen and bursa) and feathers. This is the first comprehensive investigation of the

pathogenesis and tissue tropism of these ILTV natural field recombinants. As this study included commercial broilers the findings are relevant to be applied to field situations. It would be interesting to investigate the host-virus interaction in immunised birds and the role of these respiratory viruses in non-respiratory tissues. Bagust et al. (108) have been able to isolate ILTV from lung, liver, brain and TG of chickens within 4 to 7 dpi. In a tissue tropism study starting from 5 dpi Wang et al. (102) have observed histopathological changes such as necrosis, inflammatory cell infiltration and oedema in all organs (throat, trachea, lung, cecum, kidney, pancreas, thymus and esophagus) that they detected qPCR positive. Taken together, these findings suggest pathology in non-respiratory sites may be a contributor to clinical disease and these non-respiratory sites can also produce infectious virus. In the present study, the qPCR assay was applied to diluted DNA extracts (1 in 100 dilution), therefore low copy numbers of ILTV may have remained undetected in some tissues. Future tissue tropism studies would need to be carefully designed to address the levels of inhibitors present in tissues.

Another important event that occurs during the acute phase, is virus infection of the termini of the sensory neurones that innervate the primary replication sites. This allows the virus to undergo retrograde transport to nerve cell bodies within the ganglion where the virus establishes a latent infection (288). Other than the sites of latency (TG (288) and trachea (289)) and some external factors that may cause reactivation (stressors such as on set of laying and re-housing (297, 298)), not much is known about the latent stage of ILTV infection. Therefore, this thesis also aimed to investigate several key aspects of ILTV latency. Latent herpesvirus genomes are not abundant at sites of latency, unless infection is in a stage of reactivation (211). Therefore in the current thesis, a

sensitive UL15 gene targeted nested PCR (UL15NPCR) was developed as an alternative to the routinely used universal herpesvirus nested PCR (299) with the objective of detecting low copy numbers of ILTV DNA in tissues (chapter 2). This UL15NPCR was successfully used in TG samples collected from commercial layers (chapter 2) as well as commercial broiler chickens (chapter 5). The UL15NPCR was also shown to be suitable for application to clinical samples (feather pulp) for the identification of flocks with a history ILTV exposure. In the future, the UL15NPCR could also be useful for identifying other sites of primary ILTV replication or latency. It may be that further investigations into ILTV tropism could guide the discovery of unidentified sites for primary ILTV replication and latency allowing investigations to be directed towards ganglia of neurones innervating those sites.

Reactivation from latency is an important phenomenon that is essential for the survival and evolution of herpesviruses. Studies reported in this thesis attempted to optimise culture methods for the *in vitro* reactivation of latent ILTV from TG and trachea. For the first time, this project reports a successful co-culture system to *in vitro* reactivate latent ILTV from TG (chapter 2 and chapter 4). Studies in this project used a modified version of an *ex vivo* reactivation method adopted in HSV research (252). HSV-1 *ex vivo* co-culture experiments have used cell culture monolayers that remain viable over extended periods of time (e.g. Vero cells maintained for 10-14 days) and have used detection of cytopathic effects on either the primary or a secondary cell monolayers to detect viral reactivation (238, 249, 252). In this thesis, a modified method was used to detect *in vitro* reactivation from TG. This approach detected reactivated viruses by testing the culture supernatant for ILTV DNA using PCR, rather than observing CPE in cell monolayers. Hence, the studies reported in this thesis were unable to demonstrate

the viability of the reactivated virions. It was noted that commercial layer birds had developed large TG at the time of sampling (chapter 2). Since these are long-lived birds, it is possible that they may have undergone repetitive episodes of reactivation cycles leading to self-infection and higher latent viral genomes in their TG. Despite this, the reactivated viruses did not cause cytopathic effects in primary or on secondary monolayers when tested (chapter 2). This TG co-culture system may be further optimised by using a supporting monolayer that has an extended lifespan and period of virus permissibility. However, such a cell line is currently unavailable in ILTV research.

The current project was able to study ILTV latency by reproducing a tracheal organ culture system that had not been published as in use in three decades (289), although the tracheal tissues were used not as an explant but in a co-culture system with LMH cell monolayers (chapter 2 and 4). Interestingly, we observed a higher number ($10^{2.0}$ - $10^{3.0}$ genome copy number per reaction) of reactivated virions being released from tracheal tissue, compared to TG (chapter 4). As the trachea contains a large number of permissive epithelial cells and is the primary site for ILTV replication which also promotes efficient horizontal transmission of virus between birds, it is possible that evolution may have favoured the trachea as a site of ILTV latency and maintenance of the virus in these populations over extended periods of time. However, whether this virus in the trachea represents persistent infection or true latent infection has not yet been determined. It would be interesting to investigate the nature of the ILTV genome (lytic or latent) and where it resides in the trachea during the latent stage of infection.

It has been hypothesized that the capacity of different viruses to establish or reactivate from latency may be related to the virulence of the virus. This was based on studies of

HSV-1 that demonstrated viral attenuation resulted in an impairment of the capacity of the virus to establish latency and/or reactivate from latency (252, 300). Further, it was hypothesized that there may be substantial differences in latency or reactivation characteristics observed at 21 dpi compared to 35 dpi, as previous HSV-1 studies have demonstrated that mouse models reach a stable latency after 28 days (156). Interestingly, the present study showed that following inoculation of SPF chickens with an attenuated vaccine ILTV (SA2) or a virulent field ILTV (CL9) no significant differences in latency characteristics between the ILTV strains at the two time points were observed (chapter 4). This is an important finding for future ILTV latency research in determining the endpoint of *in vivo* experiments. The same study showed that during the latent stage of infection the birds developed lymphocytosis, rather than the lymphopenia which has been observed during the acute stage of the disease (301). However, all the *in vivo* latency experiments described in this thesis used SPF chickens. It is known that the genetic background of SPF chickens differs from commercial layers or broilers (302, 303). This has effects on immunological responses which may alter their susceptibility to an infection as well as the tolerance of an infection. Maintenance of latency is a fine balance between viral factors, host factors (in particular immune responses of the host) and environmental factors (304). The genetic background of SPF birds used in these studies is different to that of the breeds used in poultry industry. The differences in the genetics of these birds are likely to influence the immune response of different breeds to ILTV. Further, these commercial breeds get exposed to frequent environmental stresses in the field. Therefore, ILTV latency in commercial chickens would be much more dynamic and hence the results presented here should be extrapolated with caution to field situations.

In the field, ILTV is controlled by implementing strict biosecurity measures together with vaccination of flocks largely with live attenuated vaccines (134). Establishment of latency and reactivation later in life is a limitation associated with live attenuated ILTV vaccines (298, 305). Results from this thesis showed that attenuated ILTV vaccines commonly used in Australia (SA2, A20 and Serva) differ in their capacities to establish latency in the TG of SPF chickens following eye drop vaccination (chapter 3) (306). The same study showed that at any given time, nearly half of the birds have quantifiable levels of ILTV DNA in their URT. This finding was consistent with the results from the study in which commercial layers were sampled and a large proportion of birds with reactivated A20 and/or SA2 ILTV in their URT were detected (chapter 2). These findings provide evidence for the role played by attenuated ILTV vaccines in ILTV epidemiology. Birds with reactivated infections may shed the virus to the environment, infecting other birds in the flock, particularly those that have been inadequately immunized (297, 298, 305). These attenuated strains may regain their virulence over serial *in vivo* passages leading to ILTV outbreaks (307). In some cases the same flock may encounter a different attenuated ILTV vaccine, and then replication of the two different strains within the same bird may result in viral recombination (14). Recent studies that have determined the full genome sequence of novel, virulent field strains of ILTV have shown these novel strains are a recombination product between two classes of attenuated ILTV vaccines (2, 14).

This thesis consisted of a number of separate *in vivo* studies. When the individual studies were compared with each other, a number of similarities were observed. Foremost, irrespective of whether the inoculating virus was an attenuated vaccine strain (chapter 2 and 3) or a virulent field strain (chapter 4), ILTV was capable of reaching

the TG, the main site of latency. Three weeks after vaccination or inoculation the ILTV DNA could be detected in TG (chapter 3 and 4) or in TG co-culture supernatants (chapter 2 and 4) using a sensitive NPCR. Moreover, at any given time post vaccination or inoculation, a considerable proportion of birds were found to positive for the presence of ILTV DNA in their trachea (chapter 2, 3, 4 and 5). The proximal one third of the trachea appeared to play a crucial role in viral shedding (chapter 2 and 4) but it was not possible to determine if this was due to reactivation of latent infection or persistent infection in deeper layers of the trachea.

A number of differences were also observed between the studies. When vaccine SA2 ILTV was inoculated via eye drop (chapter 3) approximately 50% of the birds were shedding the virus 21 days later, however ILTV DNA was mostly detected in the palatine cleft. In contrast when the same strain was inoculated to both eye and trachea (chapter 4) no ILTV DNA was detected in palatine cleft swabs at 21 dpi, however approximately 50% of the birds were shedding virus in the trachea. Future studies into viral dissemination after infection via different routes are warranted in order to understand these differences and the mechanisms underlying them. Another observed difference between studies was the inability to detect ILTV DNA in TG at 14 dpi using UL15NPCR (chapter 5) compared to the detection of ILTV DNA using the same assay at 21 or 35 dpi (chapter 4). A potential reason for this difference is the fractionation and use of paraffin embedded sections for DNA extraction in chapter 5 at 14 dpi, rather than whole fresh TG as was used in chapter 4. The use of whole fresh TG tissue is recommended for future studies where molecular detection of ILTV DNA is required.

At a time when many aspects of ILTV latency are poorly understood, and the tissue tropism of novel recombinant ILTV strains in Australia is unknown, this thesis enriches

the ILTV literature by unveiling some of the important features related to the latency and tissue tropism characteristics of Australian ILTV strains. Data that has been generated in this thesis is robust and relevant as the studies were conducted in the natural host species, rather than in laboratory animal models of herpesvirus infection. However, latency and tissue tropism characteristics of ILTV may also depend on other factors such as host genetics, level of immunization and the presence of environmental stressors. Therefore, future studies to examine the capacity of different ILTV strains to disseminate into tissues and to establish and reactivate from latency in vaccinated commercial chickens under field conditions are warranted. This thesis demonstrates the complex nature of ILTV tissue tropism and latency and highlights the need to perform further research to better understand and control this important disease.

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