Genomic recombination of infectious laryngotracheitis virus (ILTV) vaccines

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Abstract

Infectious laryngotracheitis virus (ILTV) is an alphaherpesvirus that causes acute respiratory disease in chickens. This disease causes economic loss in poultry industries worldwide and is a major concern for animal health and welfare. Although, ILTV vaccines are in use to control disease, biosecurity breaches and continuous evolution of the ILTV genome make outbreak prevention difficult. Genomic recombination plays a role in diversification of herpesvirus genomes and has been suggested to be an important alternative evolutionary mechanism in herpesviruses such as ILTV.

In previous studies of ILTV, two new genotypes of virulent field strains were shown to be independent recombinants derived from distinct attenuated commercial ILTV vaccines. These new strains became the dominant field viruses responsible for widespread and severe disease outbreaks in Australian poultry flocks. This project aimed to determine the frequency of these recombination events and the conditions that can facilitate recombination using *in vitro*, *in ovo* and *in vivo* studies. In these series of studies, a high-throughput and cost-effective method for detection of recombinant ILTVs was developed and was used to analyse the viruses generated under multiple infection conditions. The results from these analyses indicated that genomic recombination between vaccine strains of ILTV is a frequent event and takes place under a broad range of conditions. *De novo* mutation was detected in addition to genomic recombination.

The capacity of ILTV vaccines to allow secondary infection by another ILTV vaccine (i.e. superinfection) was investigated at both cellular and natural host levels. The result of this investigation showed that superinfection can occur after administration of ILTV vaccines,

providing the virus with ample opportunity for genomic recombination. The outcomes of these studies include advances in fundamental understanding about the factors that lead to ILTV recombination.

Declaration

This is to certify that

i. the thesis comprises only my original work towards PhD except where indicated in the Preface,

ii. due acknowledgement has been made in the text to all other material used,

iii. the thesis is fewer than 100,000 words in length, exclusive of tables, bibliographies and appendices.

Omid Fakhri

February 2020

Preface

All experimental work, data analysis and writing carried out for this PhD was undertaken by Omid Fakhri aside from the following cases. Management of the animals used in the *in vivo* studies was performed by June Daly, Jenece Wheeler and Pollob Shil. Samples for Sanger sequencing and whole genome sequencing for selected strains were processed by the Centre for Translational Pathology at The University of Melbourne, and Australian Genomics Research Facility, Australia.

Written works and the contributions of all persons involved in multi-authored publications

In total, three peer-reviewed papers have been published, accepted for publication or drafted for submission to a peer-reviewed journal as part of this degree, included in full in this thesis. The citations, and contribution of each author are listed below. Omid Fakhri contributed greater than 50% of the work in all cases.

1. **Fakhri O**, Hartley CA, Devlin JM, Browning GF, Noormohammadi AH, Lee SW. Development and application of high-resolution melting analysis for the classification of infectious laryngotracheitis virus strains and detection of recombinant progeny. *Arch Virol.* 2019;164(2):427-38.

OF developed experimental design, processed samples, conducted the experiments, analysed the data, and wrote the manuscript, CAH developed experimental design, revised, and reviewed the manuscript, JMD reviewed the manuscript, GFB reviewed the manuscript, AHN reviewed the manuscript, SWL developed experimental design, revised, and reviewed the manuscript.

2. **Fakhri O**, Devlin JM, Browning GF, Vaz PK, Thilakarathne DS, Lee SW, Hartley CA. Genomic recombination between infectious laryngotracheitis vaccine strains occurs under a broad range of infection conditions *in vitro* and *in ovo*. *PLoS ONE*. 2020;15(3):e0229082.

OF developed experimental design, processed samples, conducted the experiments, analysed the data, and wrote the manuscript, JMD reviewed the manuscript, GFB reviewed the manuscript, PKV reviewed the manuscript, DST reviewed the manuscript, SWL developed experimental design, and reviewed the manuscript, CAH developed experimental design, revised, and reviewed the manuscript.

3. **Fakhri O**, Devlin JM, Browning GF, Coppo MJ, Quinteros JA, Diaz-Méndez A, Lee SW, Hartley CA. Superinfection with and recombination of infectious laryngotracheitis virus vaccines in the natural host.

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OF developed experimental design, collected and processed samples, conducted the experiments, analysed the data, and wrote the manuscript, JMD reviewed the manuscript, GFB reviewed the manuscript, MJC collected samples, assessed clinical health of chickens, and reviewed the manuscript, JAQ collected samples, assessed clinical health of chickens, and reviewed the manuscript, ADM collected samples, assessed clinical health of chickens, and reviewed the manuscript, SWL developed experimental design, and reviewed the manuscript, CAH developed experimental design, collected samples, assessed clinical health of chickens, revised, and reviewed the manuscript.

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Omid Fakhri, Carol A Hartley, Joanne M Devlin, Glenn F Browning, Amir H Noormohammadi, Sang-Won Lee. Limited superinfection-exclusion in live attenuated infectious laryngotracheitis vaccines. *42nd Annual International Herpesvirus Workshop, 2017, Ghent, Belgium*

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Abbreviations

ANOVA	Analysis of variance
BoHV-1	Bovine herpesvirus 1
bp	Base pair
CAM	Chorioallantoic membrane
CDS	Coding DNA sequence
CEK	Chicken embryo kidney
CEO	Chicken embryo-origin
chCXCLi2	Chicken C-X-C motif ligand 2
cm	Centimetre
DIVA	Differentiation of infected from vaccinated animals
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
EHV-1	Equine herpesvirus-1
EHV-4	Equine herpesvirus-4
EHV-9	Equine herpesvirus-9
FBS	Foetal bovine serum
FPV	Fowlpox virus
GaHV-1	Gallid alphaherpesvirus-1
GaHV-2	Gallid alphaherpesvirus-2
GARD	Genetic Algorithm Recombination Detection
gB	Glycoprotein B
gC	Glycoprotein C
GCN	Genome copy numbers
gD	Glycoprotein D
gE	Glycoprotein E
gG	Glycoprotein G
gH	Glycoprotein H
gI	Glycoprotein I
gJ	Glycoprotein J
gK	Glycoprotein K
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
HCMV	Human cytomegalovirus
HR	Homologous recombination
HRM	High-resolution melting
HSV-1	Herpes simplex virus-1
HSV-2	Herpes simplex virus-2
HVEM	Herpesvirus entry mediator
HVT	Herpesvirus of turkeys
ICP	Infected-cell protein
IFN-α	Interferon alpha
IFN-γ	Interferon gamma

IL	Interleukin
ILT	Infectious laryngotracheitis
ILTV	Infectious laryngotracheitis virus
IR	Internal repeat region
ITPI	Intra-tracheal pathogenicity index
ITV	Intra-tracheal virulence
kbp	kilobase pairs
LATs	Latency associated transcripts
mAbs	Monoclonal antibodies
MAFFT	Multiple Alignment using Fast Fourier Transform
MDBK	Madin-Darby bovine kidney
MDV	Marek's disease virus
MICE	Mortality index for chicken embryos
mL	Millilitre
mM	Millimole
mut/nt/rep	Mutations per nucleotide, per genomic replication
nm	Nanometer
OBP-helicase	Origin binding protein-helicase
ORFs	Open reading frames
ori	Origin of DNA replication
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
pH	Potential of hydrogen
PI	Post infection
PRV	Pseudorabies virus
qPCR	Quantitative polymerase chain reaction
RDP	Recombination Detection Program
rpm	Revolutions per minute
SD	Standard deviation
SNP	Single nucleotide polymorphism
SPF	Specific-pathogen free
TCO	Tissue culture origin
TG	Trigeminal ganglia
TI	Time-interval
TR	Terminal repeat region
UL	Unique long
US	Unique short
USA	United States of America
VZV	Varicella-zoster virus
Y2H	Yeast two-hybrid
µL	Microlitre
µM	Micromole
$\mu m.s^{-1}$	Micrometre per second

Third-party copyright material

Below are the details of third-party materials used in this thesis for which permission for inclusion has been sought.

Third-party material citation	Location	Permission status
Fakhri O, Hartley CA, Devlin JM, Browning GF, Noormohammadi AH, Lee SW. Development and application of high-resolution melting analysis for the classification of infectious laryngotracheitis virus strains and detection of recombinant progeny. *Arch Virol.* 2019;164(2):427-38.	Chapter 2	Granted
Fakhri O, Devlin JM, Browning GF, Vaz PK, Thilakarathne DS, Lee SW, Hartley CA. Genomic recombination between infectious laryngotracheitis vaccine strains occurs under a broad range of infection conditions *in vitro* and *in ovo*. *PLoS ONE*. 2020;15(3):e0229082.	Chapter 3	Granted

Chapter 1. Review of the literature

1.1 Introduction

1.1.1 Overview of infectious laryngotracheitis (ILT)

Infectious laryngotracheitis is caused by infectious laryngotracheitis virus (ILTV; *Gallid alphaherpesvirus-1*; GaHV-1), a member of the *Alphaherpesvirinae* subfamily in the genus *Iltovirus*. Infectious laryngotracheitis virus is highly contagious and has horizontal transmission through generation and dissemination of respiratory aerosols (Bagust *et al.*, 2000, Devlin *et al.*, 2011). The birds that recover from acute infection will develop life-long latent infection in the central nervous system (trigeminal ganglia; TG) and trachea (Thilakarathne *et al.*, 2019a). The latent infection of ILTV is asymptomatic, however physiological and environmental stressors such as the onset of sexual maturity or the introduction of new birds to a flock can trigger the reactivation and shedding of virus in latently infected birds (Bagust *et al.*, 2000, Hughes *et al.*, 1989, Williams *et al.*, 1992). These reactivation events can lead to sub-clinical or clinical infection and may result in an outbreak of disease (Hughes *et al.*, 1989, Lee *et al.*, 2012, Thilakarathne *et al.*, 2019a).

1.1.2 General characteristics of viruses in *Herpesvirales* order

The members of the order *Herpesvirales* have linear double-stranded DNA (dsDNA) genomes ranging between 125 and 290 kilobase pairs (kbp) encoding between 70 and 170 open reading frames (ORFs) which are protected by an icosahedral capsid surrounded by a lipid bilayer viral envelope (Davison *et al.*, 2009). The genome of ILTV is

approximately 155 kbp and consists of unique long (UL), unique short (US) and repeat regions. The two repeat regions—internal (IR) and terminal (TR)—are located on either sides of US segment allowing the US segment to be assembled in any direction, and the ILTV genome to exist in two equimolar isomeric forms (Fuchs *et al.*, 2007, Thureen and Keeler, 2006, Slobedman *et al.*, 1999).

Although it has been hypothesised that all 122 species of *Herpesvirales* order are descended from a common ancestor (Davison, 2010), the genomic relationships between the three families (*Herpesviridae*, *Alloherpesviridae* and *Malacoherpesviridae*) are remote (McGeoch *et al.*, 2006). Members of the order can infect a wide range of hosts ranging from mammals, birds, reptiles (*Herpesviridae*), amphibians and fish (*Alloherpesviridae*) and invertebrates (*Malacoherpesviridae*). The most populated family of herpesviruses (*Herpesviridae* family—with 107 species currently classified) have been further classified into *Alpha-*, *Beta-* and *Gammaherpesvirinae* sub-families based on biological and genetic criteria with all currently known avian herpesviruses falling into the *Alphaherpesvirinae* sub-family (Pellett *et al.*, 2012). Despite their generally low DNA sequence similarity, a set of orthologous proteins have been identified among these three sub-families. Comparative analyses of all three herpesvirus sub-families was performed using the yeast two-hybrid (Y2H) system and suggested that protein interactions and protein functions may be more conserved than sequence (Fossum *et al.*, 2009).

Herpesvirus species are named after a taxon of the host that in its natural setting harbours the virus (Davison *et al.*, 2009). In general, each herpesvirus species is naturally considered to specifically infect a single species or group of related species with some exceptions. For instance, equine herpesvirus-1 (EHV-1), equine herpesvirus-9 (EHV-9),

and pseudorabies virus (PRV) lack strict host specificity and cause more severe pathology in animals that are not their natural host (Abdelgawad *et al.*, 2015, Pomeranz *et al.*, 2005). Natural infections of ILTV are limited to galliform birds (García *et al.*, 2013a). This restriction of hosts has facilitated the study of evolutionary relationships between herpesviruses and their respective hosts (McGeoch *et al.*, 2006). These studies have found evidence for coevolution of viruses and their host lineages over large evolutionary timespans by overlapping the phylogenetic trees of viruses and the hosts (McGeoch *et al.*, 2006, McGeoch *et al.*, 1995).

1.2 Molecular biology and pathogenesis of ILTV

1.2.1 Virus structure

Transmission electron micrographs of ILTV have revealed that it has a similar morphology to other herpesviruses (Cruickshank *et al.*, 1963, Davison *et al.*, 2009). The structure of ILTV consist of an icosahedral capsid that contains the DNA core and is surrounded by a proteinaceous layer called tegument which is contained by a phospholipid envelope as the outermost layer. (Davison *et al.*, 2009, Cruickshank *et al.*, 1963). The size of ILTV particle may vary between 200 and 350 nm depending on the amount of incorporated tegument protein (Granzow *et al.*, 2001). The envelope of ILTV has a range of viral glycoproteins on its surface which are homologues of the herpes simplex virus type 1 (HSV-1) glycoproteins that include glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN (Fuchs *et al.*, 2007). These glycoproteins are involved in different stages of virus replication cycle (e.g. gB has a role in viral entry (Poulsen and Keeler, 1997), or gI

impacts cell-to-cell spread (Devlin *et al.*, 2006b)) or have other functions such as evasion of host immune responses, as has been shown for gG (Devlin *et al.*, 2010).

1.2.2 Replication cycle

The general replication scheme used by herpesviruses is shown in Figure 1 (Harper, 2011). Much of the current knowledge of the replication of many herpesviruses, including ILTV, is understood through the conserved pathways described by the study of prototypic alphaherpesviruses such as HSV-1 and herpes simplex virus type 2 (HSV-2), PRV and varicella-zoster virus (VZV). Herpesviruses enter their host cells by the attachment of viral envelope glycoproteins to cell receptors, and after fusion of the viral envelope with the host cell plasma membrane release their nucleocapsid to the cytoplasm (Spear, 1993). In some herpesviruses (e.g. HSV-1, HSV-2, PRV and bovine herpesvirus 1: BoHV-1), this attachment begins as a result of the interaction between gC and gB in the virus envelope and any one of several entry receptors (Herold *et al.*, 1994, Herold *et al.*, 1991, Li *et al.*, 1996, Sawitzky *et al.*, 1990, Tal-Singer *et al.*, 1995, Spear, 2004). The roles of gC and gB in the attachment of ILTV have not been studied. However, studies have focused on immunologic aspect of gB and gC of ILTV given their potential as neutralising epitopes to be targeted by recombinant vaccines. The gB of ILTV is shown to be a major protective immunogen (Sun *et al.*, 2008) although the immune responses induced by gB or gC do not protect the chickens against virulent ILTV challenge (Kanabagatte Basavarajappa *et al.*, 2014).

Three types of entry receptors discovered to date include herpesvirus entry mediator (HVEM), nectin-1 and nectin-2, and in some cases specific sites in heparan sulphate (Spear, 2004, Spear *et al.*, 2000). In ILTV infection, there is evidence that the attachment and

fusion of ILTV is independent of heparan sulphate (Kingsley and Keeler Jr, 1999). Heparan sulphate independent infection has also been shown for HSV-1 and -2 which can use nectin-1 or HVEM as receptors (Spear, 2004). After receptor binding, entry occurs by fusion of the viral envelope with the host cell membrane (Fig 1). Alphaherpesviruses such as HSV-1 and -2 use a combination of fusion protein gB and the heterodimer gH/gL for virus-cell fusion (Cairns *et al.*, 2014). Following entry of the nucleocapsid into the cytoplasm, the viral tegument proteins interact with the microtubules and dynein motor protein complex of the cell to transport the viral nucleocapsid to the cell nucleus (Dohner *et al.*, 2002, Radtke *et al.*, 2010). Upon binding the nucleocapsid to nuclear pores, the dsDNA of the herpesviruses enters the nucleus of the host cell (uncoating stage) (Ojala *et al.*, 2000, Pasdeloup *et al.*, 2009).

Consistent with all other herpesviruses studied to date, gene transcription and replication processes of ILTV begin in a cascade-like manner (Caughman *et al.*, 1985, Mahmoudian *et al.*, 2012, Prideaux *et al.*, 1992). In 1992, Prideaux *et al.* classified the genes of ILTV into four types on the basis of the timing of their expression and their dependency on other gene products for their transcription (Prideaux *et al.*, 1992). This study, which was based on the previous works describing the gene expression of HSV-1 (Honess and Roizman, 1974), categorised the genes into α (immediate early), β (early), γ (late) types (Prideaux *et al.*, 1992). More recent works on the gene expression of human herpesvirus 6B (HHV-6B) (Oster and Hollsberg, 2002) and ILTV (Mahmoudian *et al.*, 2012, Mahmoudian *et al.*, 2013), have suggested that the gene expression of these viruses might function as a spectrum of six gene groups. To determine the expression kinetics of each gene, these studies used quantitative PCR to monitor the transcription of viral genes in

presence or absence of several inhibitor compounds which would inhibit functions such as *de novo* protein synthesis or viral DNA polymerase activity. The results showed that while many genes had transcription consistent with the original study (Prideaux *et al.*, 1992), some genes exhibited the features of both early and late genes, to suggest a more complex description of gene expression and regulation than previously classified (Mahmoudian *et al.*, 2012, Mahmoudian *et al.*, 2013).

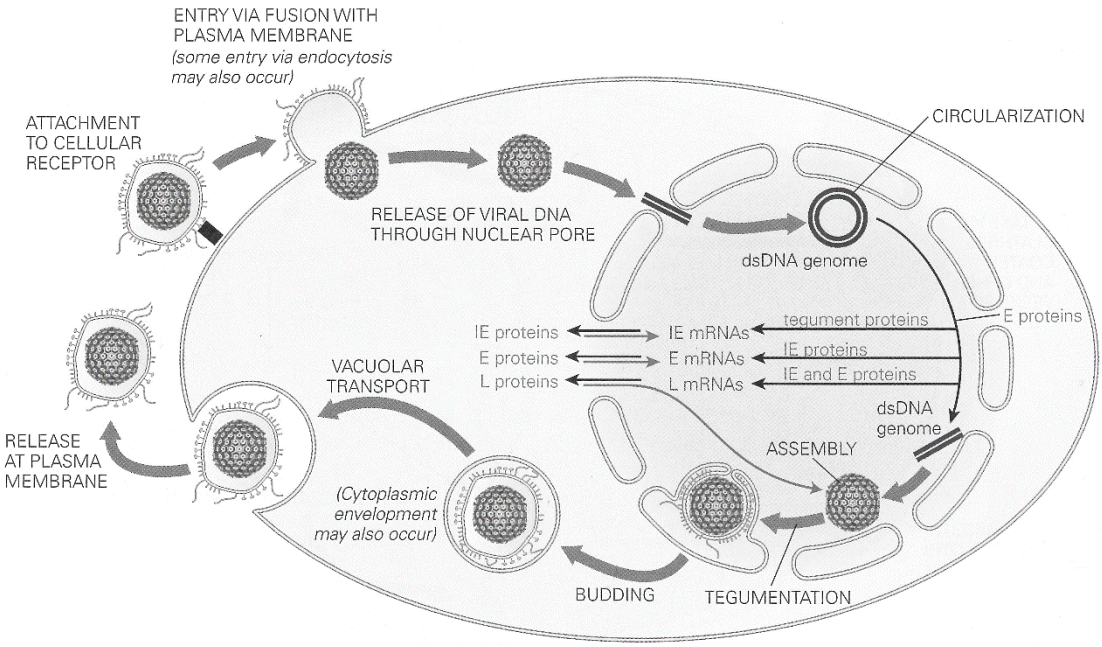


Figure 1 The schematic view of replication cycle of herpesviruses showing the classic stages of replication. Modified and adapted from Viruses: Biology, Applications, and Control by David Harper (2011) (Harper, 2011).

The replication of HSV-1 DNA involves both viral and host proteins (Weller and Coen, 2012) and is initiated at an AT-rich palindromic sequences known as origin of DNA

replication (ori) (Weller and Coen, 2012). Similar to HSV (Weller and Coen, 2012), three origins of replication are identified in ILTV—two in internal and terminal repeat regions (oriS) and one in unique long region (oriL) (Lee *et al.*, 2011a, Ziemann *et al.*, 1998a). In HSV-1 replication, the origin binding protein-helicase (OBP-helicase) (Elias and Lehman, 1988) and infected-cell protein 8 (ICP8) (Boehmer and Lehman, 1993) attach to the ori sequences and together with the viral DNA polymerase form a functional replisome (Marsden *et al.*, 1997, Monahan *et al.*, 1998). The herpesvirus DNA polymerase contains both polymerase and 3′–5′ proofreading exonuclease activities (Song *et al.*, 2004). The proofreading activity of this enzyme results in the mutation rate of an average of 1.8×10^{-8} mutations per nucleotide, per genomic replication (mut/nt/rep) as measured directly for HSV-1 only (Sanjuan *et al.*, 2010, Lu *et al.*, 2002, Drake and Hwang, 2005, Duffy *et al.*, 2008) which puts the herpesviruses on the lower end of mutation rate spectrum compared to other viruses (Peck and Lauring, 2018). The mutation rate of ILTV has not been measured directly.

The replication of herpesvirus DNA is not completely understood, however, it is known that it involves formation of concatemeric intermediates consisting of head-to-tail linked genomes (Bataille and Epstein, 1994, Weller and Coen, 2012). These high molecular weight DNA concatemers are further cleaved into unit-length genomes and packaged into preformed capsids (Scholtes and Baines, 2009, Deiss *et al.*, 1986). The process of concatemer formation has not been studied directly in ILTV. The remaining steps of replication of ILTV, that include the formation and release of infectious particles, are shown to be morphologically similar to other herpesviruses (Fuchs *et al.*, 2007, Granzow *et al.*, 2001, Mettenleiter, 2002, Guo *et al.*, 1993). Ultrastructural studies have shown that the

encapsidation of ILTV DNA occurs in the nuclei of infected cells before the nucleocapsid migrates to cytoplasm by budding at the inner nuclear membrane and fusion to the outer nuclear membrane (Granzow *et al.*, 2001, Guo *et al.*, 1993). This brings the naked ILTV nucleocapsid to the cytoplasm which then associates with relatively large amounts of tegument protein compared to other herpesviruses (Granzow *et al.*, 2001), prior to final step of maturation that involves being enveloped at the *trans*-Golgi network (Granzow *et al.*, 2001). This enveloped intracellular particle is infective at this stage before the release of ILTV by exocytosis of virus-containing vesicles (Guo *et al.*, 1993, Granzow *et al.*, 2001).

1.2.3 Acute infection and pathogenicity

Infectious laryngotracheitis (ILT) is an acute viral respiratory disease that was first reported in the United States of America in 1925 (May and Tittsler, 1925) and was identified for the first time in Australia in 1935 (Cover, 1996). Infectious laryngotracheitis continues to have a negative impact on the poultry industry due to the increased mortality, decreased egg production and reduced or delayed body weight gain, and predisposition of infected birds to other respiratory pathogens (García *et al.*, 2013b). The clinical signs of ILT can range from mild respiratory distress to conjunctivitis, nasal discharge, dyspnoea, and lethargy. In severe forms of the disease, clinical signs can include haemorrhagic tracheitis together with gasping, coughing, and haemoptysis (García *et al.*, 2013a). The morbidity and mortality rates of ILT depend on the virulence of circulating strains and predisposing factors, such as other respiratory infections (García *et al.*, 2013a).

Similar to other herpesviruses, lytic or acute infection with ILTV produces infectious virus and is the phase when the most clinical signs appear (García *et al.*, 2013a).

The clinical signs of the disease can vary from local signs such as ocular discharge, coughing, dyspnoea and gasping, to systemic signs such as decrease in egg production or weight gain (Coppo *et al.*, 2013a, García *et al.*, 2013a). The differential blood cell count of infected chickens shows lymphopenia and heterophilia between day 3 to 7 post infection (PI) which coincides with pyrexia seen at 4 to 6 days PI (Chang *et al.*, 1977). In macroscopic post-mortem examination of chickens infected with ILTV, conjunctivitis, involvement of the respiratory tract, especially larynx and trachea, and occlusion of the trachea by mucoid casts are prominent findings (García *et al.*, 2013a).

Several studies have investigated the chain of events that occur following the infection with ILTV on day-by-day basis (Hayashi *et al.*, 1985, Coppo *et al.*, 2018, Lee *et al.*, 2015, Chang *et al.*, 1977). These events eventually end by recovery or death of infected animals depending on several factors such as virulence of the virus and immune status of the chickens (Lee *et al.*, 2015). The course of ILTV infection with a field isolate can take up to 16 days and can be studied from both clinical aspect of the disease and microscopic tissue changes (Hayashi *et al.*, 1985). Microscopically, the first detectable effect of ILTV infection is the hypertrophy and hyperplasia of goblet cells responsible for mucous production in the trachea that occurs on day 1 PI (Hayashi *et al.*, 1985). From day 3 PI, intra-nuclear inclusion bodies form in the nucleus of epithelial cells which coincides with formation of syncytia by these cells (García *et al.*, 2013b, Hayashi *et al.*, 1985). This observation is followed by desquamation of epithelial cells on day 4 to 5 which exposes the connective tissue and is marked by the presence of serofibrinous, diphtheritic or haemorrhagic exudates in the lumen of infected tracheas visible in transverse sections (García *et al.*, 2013a, Hayashi *et al.*, 1985). By day 6 PI, severe congestion and infiltration

of inflammatory cells such as heterophils, lymphocytes and macrophages are detectable in the lamina propria of the respiratory mucosae (García *et al.*, 2013b, Hayashi *et al.*, 1985). The regeneration of tracheal epithelium begins around day 6 PI by appearance of microvillus-rich epithelial cells that continue to proliferate extensively around day 8 PI. The complete recovery of tracheal epithelium occurs by approximately day 16 PI as the ciliated columnar epithelial cell cover the mucosal surface and the histologic architecture returns to normal (Hayashi *et al.*, 1985). The clinical course of infection can differ between different strains of ILTV (García *et al.*, 2013a).

Immune responses to ILTV can be divided into immediate innate inflammatory responses and prolonged adaptive immune responses, through both cell-mediated and humoral components of the immune system (Coppo *et al.*, 2013a, Honda *et al.*, 1994a). The study of adaptive immune responses to ILTV infection has demonstrated that although neutralising antibodies are detectable in the serum as early as day 9 PI, the protection is independent of antibodies, and is through cell-mediated immunity (Fahey *et al.*, 1983, Honda *et al.*, 1994a, Honda *et al.*, 1994b). Several studies have shown that transferring spleen cells or peripheral blood leukocytes can protect chickens from ILTV infection, however, transferring hyper-immune sera or bursectomising chickens does not impact the immunity to ILTV (Honda *et al.*, 1994a, Fahey *et al.*, 1984, Robertson, 1977). Innate responses are observed earlier during infection and include production of pro- and anti-inflammatory cytokines such as interleukin-1β (IL-1β), IL-10 and interferon type I (IFN-α) which limit the replication of ILTV in the trachea (Vagnozzi *et al.*, 2018).

1.2.4 Latent infection

In herpesviruses, latent infection is when the genome of the virus is maintained within a host tissue without generation of infectious virions (Stevens, 1989). The latent phase of infection provides the virus with the advantage of evading the host immune responses and returning to lytic phase (i.e. reactivation) periodically during the life of the host when the microenvironment is suitable for the virus replication (e.g. during stress-induced immune system suppression) (Stevens, 1989). During the latent state, only latency associated transcripts (LATs) are detectable and are suggested to impact the balance between lytic and latent states (Waidner *et al.*, 2011). Latency is established after primary or acute infection and is a form of persistent infection where the viral genome is maintained only in a limited number of cells and has a highly limited expression of viral genes (Speck and Ganem, 2010). In alphaherpesviruses, following primary infection, the virus replicates within mucosal epithelial cells and enters sensory nerve terminals that innervate the primary replication sites. The virus genome is then transported to the nucleus of neuronal cells where it is assembled into nucleosomes in host chromatin structure (Deshmane and Fraser, 1989) and maintained as a circular episome during latency (Nicoll *et al.*, 2012, Efstathiou *et al.*, 1986).

Much of what we know about ILTV latency has been extrapolated from other alphaherpesviruses such as HSV-1 and -2 (Nicoll *et al.*, 2012, Thellman and Triezenberg, 2017). The ability of ILTV to establish latent infection was first described in 1986 by Bagust *et al.* (Bagust, 1986, Bagust *et al.*, 1986) and since then more evidence has shown that both wildtype and vaccine strains of ILTV can produce long term latent infections (Hughes *et al.*, 1991, Thilakarathne *et al.*, 2019a). Similar to other alphaherpesviruses,

there is evidence that ILTV establishes latent infection in TG (Chacón *et al.*, 2015, Oldoni *et al.*, 2009, Williams *et al.*, 1992). In addition to TG, one early study by Bagust (1986) and another recent study by Thilakarathne *et al.* (2019), detected reactivated ILTV vaccine strain in tracheal organ cultures of chickens vaccinated with modified live vaccines and suggested the trachea as another site of latency for ILTV (Bagust, 1986, Thilakarathne *et al.*, 2019b). Periodically, the virus can reactivate to resume its normal lytic cycle and traffic to the site of active infection (Nicoll *et al.*, 2012). Stressors such as vaccination, changes in housing and onset of sexual maturity can stimulate the reactivation of ILTV (Hughes *et al.*, 1991) and the reactivated virus can cause lytic infection, producing infectious virus particles. The precise mechanism of herpesvirus reactivation is not fully understood, but, in general, stimuli such as fever, microbial co-infection, stress, tissue injury, and respirable fibres have been found to trigger reactivation in different sub-families of herpesviruses (Stoeger and Adler, 2018). For instance, the reactivation of HSV-1 and HSV-2 is directly linked to neuronal stress response pathways (Cliffe and Wilson, 2017), which can be triggered by the stress hormones epinephrine and corticosterone (Ives and Bertke, 2017). Stress hormones are thought to support reactivation of HSV-1 and HSV-2 through suppression of parts of the immune system (Morey *et al.*, 2015) In addition, neurones that are infected with HSV-1 and HSV-2 express stress hormone receptors (such as glucocorticoid receptor, and the adrenergic α-2 and β-2 receptors) (Ives and Bertke, 2017), making these neurones more responsive to stress hormones. The *in vitro* treatment of HSV-1 infected neurones with epinephrine can increase the titres of virus released from these neurones compared to untreated infected neurones (Ives and Bertke, 2017). The role of these mechanisms in reactivation has not yet been investigated directly in ILTV. This

reactivation can result in either sub-clinical, self-limiting infections or clinical disease (Williams *et al.*, 1992). Either way, reactivation provides a source of infectious virus with the potential to be transmitted to new hosts. Knowledge about latent infection by ILTV is essential for the control of the disease since the reactivated live attenuated vaccines have the potential to be transmitted to new birds at an uncontrolled dosage which may result in clinical disease (Guy *et al.*, 1990, Pavlova *et al.*, 2010). Alternatively, the reactivated viruses may regain virulence (Guy *et al.*, 1991) or recombine with wild type ILTV or other vaccine strains and result in more virulent forms (Lee *et al.*, 2012).

1.3 Classification of infectious laryngotracheitis virus

The classification of ILTV has been based on genomic sequence variations, antigenicity and pathogenicity. The advantages and limitations of each approach is discussed in the following sections.

1.3.1 Classification of ILTV based on genomic variations (genotypes)

The overall genomic variation between ILTV strains and isolates that can range from 0.1% to 0.8% nucleotide sequence difference (Lee *et al.*, 2011a, García *et al.*, 2013b, Chandra *et al.*, 2012, Lee *et al.*, 2013, Loncoman *et al.*, 2017b). This level of variation is not uncommon among herpesviruses, for instance the overall inter strain variability is up to 0.5% for HSV-2 (Newman *et al.*, 2015, Burrel *et al.*, 2015) and up to 0.17% for VZV (Peters *et al.*, 2006), which are both less than the 3.8% reported previously for HSV-1 (Szpara *et al.*, 2014). The variable regions of ILTV genome are evenly distributed across the genome and at least one amino acid difference between the vaccine strains Serva ILTV and SA2 or A20 ILTV have been reported in at least 51 of the 79 predicted ORFs (Lee *et*

al., 2011a). The regions with these variations have been identified and studied by analysing the parts of genome after amplification by PCR or by sequencing techniques. The variability in ILTV genome has allowed identification and classification of different ILTV isolates. The sections below address the applications, limitations and advantages of each method.

1.3.1.1 Analysis of polymerase chain reaction (PCR) products

Since the introduction of polymerase chain reaction (PCR) in 1980s as a genetic analysis technique, several iterations of PCR-based methods alone or in combination with restriction fragment length polymorphism (PCR-RFLP) (Oldoni and Garcia, 2007, Kirkpatrick *et al.*, 2006, Graham *et al.*, 2000, Creelan *et al.*, 2006, Clavijo and Nagy, 1997, Chang *et al.*, 1997) have been developed for classification of ILTV strains and differentiation of vaccine and field strains (García *et al.*, 2013b). More recent studies have developed fluorescently labelled hydrolysis probes (Loncoman *et al.*, 2017a, Shil *et al.*, 2015) as high-throughput methods of classification and investigation of emerging types of ILTV.

Several research groups have developed methods to amplify regions of ILTV genomes to classify isolates using the variations in patterns produced by PCR-RFLP technique (Kirkpatrick *et al.*, 2006, Oldoni and Garcia, 2007, Chang *et al.*, 1997, Clavijo and Nagy, 1997, Creelan *et al.*, 2006, Graham *et al.*, 2000). This categorised ILTV isolates into ‘classes’ or ‘groups’. In 2006, Kirkpatrick *et al.* developed a method based on PCR-RFLP patterns to differentiate vaccine strains from field strains (Kirkpatrick *et al.*, 2006). In this method, the variations in sequence of four coding regions (US4/gG, UL23/TK, ICP4 and

ICP18.5) were used to classify the available strains/isolates into 5 classes (class 1 to 5) (Kirkpatrick *et al.*, 2006). This method has been extensively used by this group as a detection tool and to study the epidemiology of ILTV in Australia. When this method was used to analyse the viruses isolated from ILT outbreaks started in 2006, the isolates could not be classified as any of previously known 5 classes of ILTV, therefore, four new classes were defined (classes 6 to 9) to accommodate these new viruses (Blacker *et al.*, 2011). Another new class of ILTV (class 10) was also added to this list in a more recent study of viruses isolated from ILT outbreaks (Agnew-Crumpton *et al.*, 2016). The term 'class' throughout this thesis refers to the classes defined by Kirkpatrick *et al.* (Kirkpatrick *et al.*, 2006). In 2007, Oldoni and García developed a similar method to characterise the ILT viruses isolated in the United States and classified virus isolates into nine 'groups' (Oldoni and Garcia, 2007). Results obtained from this study clearly demonstrated that most of the commercial poultry isolates (17 of 22 isolates) were closely related to the vaccine strains.

The studies by Shil *et al.* (2015) and Loncoman *et al.* (2017) have used fluorescently labelled TaqMan® probes to detect ILTV and differentiate between strains (Loncoman *et al.*, 2017a, Simon-Loriere and Holmes, 2011). The assays were highly sensitive and highly specific, with a detection limit of 10 viral genome copies for each assay. Both assays were specifically designed for the purpose of each study and were not intended to be used as a classification tool. Nevertheless, the assay developed by Shil *et al.* could successfully differentiate the gG-deficient ILTV candidate vaccine strain of ILTV from ILTV strains that contain the gG gene and was suggested to have the potential for being used in a "Differentiating Infected from Vaccinated Animals" (DIVA) strategy for the control and eradication of ILT (Shil *et al.*, 2015). Also, the assay developed by Loncoman *et al.* (2017)

could successfully differentiate two Australian field strains of ILTV (CSW-1 and V1-99) which were used to study the recombination of ILTV and provided a tool for understanding of alphaherpesvirus recombination (Loncoman *et al.*, 2017a).

1.3.1.2 Sequencing methods (partial or whole genome sequencing)

Partial sequencing

Determining the partial or complete genome sequence of ILTV has also been used to more finely study and categorise the genomic variation among strains. Several method of targeted Sanger sequencing aiming to differentiate the ILTV isolates by targeting variations in the coding sequences have been described (Chacon and Ferreira, 2009, Ojkic *et al.*, 2006, Shehata *et al.*, 2013, Choi *et al.*, 2016). These methods parallel PCR-RFLP but sequence the targeted regions instead of using restriction digests and isolates are classified based on nucleotide similarities. For instance, in a study by Chacón and Ferreira (2009) two different regions of the infected-cell protein 4 (ICP4) gene of ILTV were amplified and sequenced. Phylogenetic analysis of the two regions was then performed and showed differences in the nucleotide and predicted amino acid sequences between field isolates and tissue culture-origin (TCO) and chicken embryo-origin (CEO) vaccine strains. Similarly, when Sheheta *et al.* (2013) sequenced only one of the two regions of the ICP4 gene targeted in previous study, they were able to classify the viruses isolated from outbreaks in Egypt into CEO or TCO vaccine-related isolates (Shehata *et al.*, 2013). In a similar approach Ojkic *et al.* (2006) sequenced and analysed parts of UL47 and gG genes and determined that both 'wild-type' and vaccine-derived viruses are involved in ILT cases in

Ontario province of Canada (Ojkic *et al.*, 2006). To maximise the resolution power of partial sequencing in differentiating ILTV variants, Choi *et al.* (2016) amplified and sequenced six variable genomic regions of ILTV (UL54, UL52, gB, ICP18.5, gJ and ICP4). This method was able to differentiate vaccine and field strains of ILTV originating from Australia, United States and China, and classify them into 14 different genotypes (Choi *et al.*, 2016).

Whole-genome sequencing

In more recent years, the wider availability and lowered cost of high throughput sequencing technology has led to full-length genome sequencing of more than 40 ILTV isolates from different origins (Chandra *et al.*, 2012, Kong *et al.*, 2013, Lee *et al.*, 2011a, Lee *et al.*, 2011b, Lee *et al.*, 2012, Spatz *et al.*, 2012, Piccirillo *et al.*, 2016, Schneiders *et al.*, 2018, Thureen and Keeler, 2006) and has contributed to a deeper understanding of ILTV evolution, taxonomy, recombination and epidemiology (Chandra *et al.*, 2012, Kong *et al.*, 2013, Lee *et al.*, 2011a, Lee *et al.*, 2011b, Lee *et al.*, 2012, Spatz *et al.*, 2012, Loncoman *et al.*, 2017b). The first complete genome sequence of ILTV (European CEO Serva vaccine; GenBank accession number: HQ630064) was published by Lee *et al.* in 2011 (Lee *et al.*, 2011b). The subsequent publication of the complete genome sequence of two Australian origin ILTV live attenuated vaccines (SA2 and A20 strains; GenBank accession numbers JN596962 and JN596963, respectively) showed that there are only two non-synonymous single nucleotide polymorphisms (SNPs) between these two Australian vaccine strains (Lee *et al.*, 2011b). It was hypothesised that these two SNPs were related to the lower virulence of A20 when compared to SA2 (Lee *et al.*, 2011b). One year later, Spatz *et al.* performed a comparative analysis to compare the full genome sequence of four

ILTV strains from the United States of America (USA) (one reference laboratory strain and three field strains) to full genome of Serva ILTV (Spatz *et al.*, 2012). They reported seven non-synonymous amino acid substitutions in UL27, UL1, UL10 and US8 genes encoding gB, gL, gM and gE, respectively, and three non-structural proteins (UL28; DNA packaging protein, UL5; helicase-primase, and ICP4; immediate early protein) that could differentiate between the Serva vaccine and the isolates circulating in the USA (Spatz *et al.*, 2012).

1.3.2 Classification of ILTV based on surface antigens of the virion (serotypes)

Although strains of ILTV can be differentiated genomically and phenotypically, virus-neutralization and cross-protection studies have shown only minor antigenic differences between them (Benton *et al.*, 1958, Russell and Turner, 1983, Shibley *et al.*, 1962b). The antigenic relationship between different strains of ILTV has been studied using reciprocal neutralization kinetics which is a sensitive serological method for studying the antigenic relationship among closely related viruses that measures the rate of virus neutralization by specific antisera (McBride, 1959, Russell and Turner, 1983). This method has been used for early differentiation of HSV-1 from HSV- 2 (Ashe and Scherp, 1963, Dowdle *et al.*, 1967) but could only show minor antigenic differences between ILTV strains which had significant differences in pathogenicity (Russell and Turner, 1983). Minor antigenic differences have also been reported for other herpesviruses including BoHV1 (Bagust, 1972), equine herpesvirus type 4 (EHV-4) (Kemeny, 1971), Marek's disease virus (Hamdy and Sevoian, 1973) and human cytomegalovirus (Andersen, 1972) using the same approach. Homologs of HSV-1 surface glycoproteins have been identified in the ILTV genome (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN) and are

considered to be responsible for stimulating humoral and cell-mediated arms of immune system (Thureen and Keeler, 2006). Veits *et al.* (2003) have shown that antibodies against gC and gJ of ILTV are reproducibly detected in sera of naturally or experimentally infected chickens. They reported that immunofluorescence analyses using monoclonal antibodies (mAbs) shows that the immunogenic region of these two glycoproteins are conserved in all tested ILTV isolates originating from different parts of the world (Veits *et al.*, 2003a) therefore, cannot be used for differentiation or classification of strains.

In addition, both early and recent studies have shown that different strains of ILTV with different level of virulence can induce cross immunity against each other despite their genetic distinctions (Russell and Turner, 1983, Benton *et al.*, 1958, Shibley *et al.*, 1962a, Lee *et al.*, 2014). Due to this close antigenic relationships between different strains and isolates of ILTV, it is not possible to use antigenicity alone as a classification method (Russell and Turner, 1983).

1.3.3 Classification of ILTV based on pathogenicity (pathotypes)

Different strains of ILTV can show significant differences in pathogenicity for poultry (Blacker *et al.*, 2011, Benton *et al.*, 1958, Shibley *et al.*, 1962b, Izuchi and Hasegawa, 1982, Hilbink *et al.*, 1987, Guy *et al.*, 1990, Coppo *et al.*, 2012b, Mahmoudian *et al.*, 2012, Agnew-Crumpton *et al.*, 2016). These variations can differentiate low virulence vaccine strains from highly virulent field strains (Guy *et al.*, 1990, Hilbink *et al.*, 1987, Izuchi and Hasegawa, 1982). The low virulence viruses generally cause subclinical to mild infection, in contrast to highly virulent strains that cause high morbidity and mortality in infected flocks (García *et al.*, 2013a, Blacker *et al.*, 2011). In order to systematically

classify the ILTV strains based on their virulence, several methods have been developed and applied (Guy *et al.*, 1990, Hilbink *et al.*, 1987, Izuchi and Hasegawa, 1982). These methods measure the virulence of ILTV strains either in chicken embryos (mortality index for chicken embryos; MICE) (Izuchi and Hasegawa, 1982) or in chickens (intra-tracheal pathogenicity index; ITPI (Guy *et al.*, 1990), and intra-tracheal virulence value; ITV-value (Hilbink *et al.*, 1987)). The mortality index for chicken embryos (MICE) is calculated by dividing the cumulative number of 11-day-old embryos dying within 7 days by the cumulative number of embryos surviving 7 days. The MICE values are well correlated with the pathogenicity's for chickens and it has been suggested that strains or isolates with MICE < 0.16 would have low or no pathogenicity for chickens, and those with MICE > 0.27 would be highly pathogenic (Izuchi and Hasegawa, 1982). The ITV-value method has a similar approach and is based on reporting the mortality rate in chickens after infection with each strain (Hilbink *et al.*, 1987). The other method (ITPI) has a different approach and factors in microscopic and macroscopic scoring of tracheal lesions in addition to mortality (Guy *et al.*, 1990).

These virulence-based classification methods preceded current molecular methods, where contemporaneous molecular methods (e.g. DNA restriction endonuclease) had failed to distinguish between 18 field outbreak isolates of ILTV, from vaccine strains (Guy *et al.*, 1989). It is important to note that some virulence-based studies use equal amounts of each virus when comparing the virulence, and some others would follow the manufacturer's instructions to prepare the vaccines therefore the amounts of each live vaccine can vary between groups. This variation is especially important since there are reports of vaccine strains causing up to 20% mortality when used in higher than recommended doses (Guy *et*

al., 1990, Pavlova *et al.*, 2010). Nevertheless, the results from these studies indicate that virulence of ILT field isolates was greater than that of attenuated vaccine viruses when equal doses are compared (Izuchi and Hasegawa, 1982, Guy *et al.*, 1990, Hilbink *et al.*, 1987). When these results were combined with the molecular analysis of vaccine and field strains, it was suggested that it is possible that some virulent field isolates originated from attenuated vaccine viruses through reversion to parental-type virulence, however, further investigation is needed to clarify the link between genomic changes and virulence (Guy *et al.*, 1991). The ability of attenuated vaccine strains to regain virulence after bird-to-bird passage continues to be a concern and an active area of research to date.

1.4 Control of ILT by vaccination

The initial attempts to control ILT dates back to 1934 when Brandly *et al.* administered non-attenuated ILTV via an intracloacal route to reduce the clinical signs of the disease (Brandly and Bushnell, 1934) or when Gibbs, in the same year, investigated brush vent application of tracheal scrapings from infected chickens to provide the industry with a means of protecting their flocks (Cover, 1996). Thereafter, it became apparent that ILT could be managed by a combination of vaccination and strict biosecurity measures (García *et al.*, 2013a). The vaccination of farmed poultry against ILT began in the late 1950's and early 1960's by the introduction of the first commercial live attenuated CEO vaccines in Australia (SA-2 strain introduced in 1966) and in the USA (Cover strain introduced in 1958 and Hudson strain in 1969) (García and Zavala, 2019). These attenuated vaccines were used to control ILT for many years, however, the residual virulence in many and increased virulence after bird-to-bird passages remained a concern in addition to the overriding issue of latent infection with live attenuated vaccines (Fuchs *et al.*, 2007, Guy *et*

al., 1991). Subsequently inactivated whole-particle and subunit vaccines were developed and were effective at producing serological response in chickens but had some disadvantages including the cost of production and the need to use large amounts of antigen to elicit an adequate antibody response (Burrell *et al.*, 2016). Thereafter, the quest for finding a safer vaccine against ILT continued and a third category of vaccines were successfully developed and tested—genetically engineered ILTV vaccines (Coppo *et al.*, 2013b, Fuchs *et al.*, 2007, García and Zavala, 2019). All vaccines against ILTV can diminish the clinical signs and the shedding of challenge viruses but generation of sterilising immunity and the ability to completely block the replication of the ILTV remains difficult to achieve (García *et al.*, 2013a, Vagnozzi *et al.*, 2012). Currently, live-attenuated vaccines and recombinant vaccines are widely used in the poultry industry worldwide (García, 2017).

1.4.1 Live-attenuated vaccines of ILT

The live-attenuated vaccines are the most effective (Coppo *et al.*, 2012a, Coppo *et al.*, 2012b, Fulton *et al.*, 2000, Han and Kim, 2003, Rodriguez-Avila *et al.*, 2008) and the most widely used type of ILT vaccine (García, 2017). There are two types of live-attenuated vaccines based on the procedure used for the attenuation of the virulent virus—CEO type which are attenuated via multiple passages in chicken embryos (Samberg *et al.*, 1971) and; the TCO vaccine which were attenuated by several passages on chicken kidney cells (Gelenczei and Marty, 1964, Izuchi *et al.*, 1984).

The first Australian CEO vaccine (SA2) against ILT was developed by attenuation of a field isolate (isolated from an outbreak in South Australia) in chicken embryos in 1966

(Pulsford *et al.*, 1956). Although attenuated compared to the original field isolate, the SA2 vaccine could still cause significant respiratory signs. In 1983, SA2 was further attenuated by passaging 20 times in chicken embryo kidney (CEK) cells to produce the A20 vaccine (Kirkpatrick *et al.*, 2006, Bagust and Mcgavin, 1995). In 2006, a third attenuated vaccine with European origin, known as NOBILIS® ILT or Serva ILTV, was registered in Australia for use in poultry flocks (Blacker *et al.*, 2011, APVMA, 2006). Despite the desired efficacy and cost-effectiveness of live vaccines, there are several drawbacks in regards to their use in poultry flocks including their potential to revert to virulent virus after *in vivo* passages (Guy *et al.*, 1991) and the possibility of recombination with other strains to form more virulent viruses (Lee *et al.*, 2012). Recent outbreaks of ILT in the United States, Australia, Europe and South America have been linked to live-attenuated vaccines (Blacker *et al.*, 2011, Menendez *et al.*, 2014).

Different routes of administration have been investigated in order to optimally deliver ILTV vaccines that can balance effective vaccination of individuals with cost effective methods for administering the vaccines to flocks containing thousands of birds. Vaccination via drinking water (Samberg *et al.*, 1971, Coppo *et al.*, 2012a, Coppo *et al.*, 2012b, Korsa *et al.*, 2015), eye drop or spray (Sinkovic and Hunt, 1968), orbit sinuses (Shibley *et al.*, 1962a), intranasal instillation (Benton *et al.*, 1958) and into feather follicles (Molgard and Cavett, 1947) have been studied and the results suggest the drinking water as the most applicable route for large-scale industry application (García and Zavala, 2019) with one drawback—non-uniformity of immunization in a flock due to unequal water consumption (García and Zavala, 2019, Robertson and Egerton, 1981). This non-uniformity

in protection against ILT, i.e. failure of vaccination, can potentially lead to *in vivo* passage of live-vaccines in unprotected birds (Guy *et al.*, 1991).

The diversity of vaccination strategies against ILTV increases as new vaccines are being developed. In general, the majority of flocks with long-lived birds are vaccinated against ILT multiple times to avoid the economic consequences of ILT outbreaks whereas the vaccination birds with short rearing period might only be practical in areas where ILT is deemed enzootic or an outbreak is anticipated (Dufour-Zavala, 2008, García, 2017).

1.4.2 Genetically engineered vaccines of ILT

Reverting to virulence by regaining the necessary genetic elements under field conditions remains a concern associated with ILTV vaccines. In order to address this issue, genetically engineered vaccines against ILT continue to be developed and are commercially available in some countries (Coppo *et al.*, 2013b, García and Zavala, 2019). These vaccines have been developed either by creation of deletion mutant of ILTV that lack virulence genes, or by generation of recombinant viral-vectored vaccines that express immunogenic antigens of ILTV (Coppo *et al.*, 2013b). Virally vectored recombinant vaccines use either fowlpox virus (FPV) (Davison *et al.*, 2006) or turkey herpesvirus (HVT) (Mebatsion *et al.*, 2008) to produce ILTV antigens. An example of a live fowlpox-vectored vaccine is Vectormune® that is developed to control both ILT and avian encephalomyelitis (Vectormune® FP-LT+AE, Biomune Co., Lenexa, KS, USA). This vaccine carries UL27 and UL32 genes of ILTV that encode gB and a DNA packaging protein respectively (Davison *et al.*, 2006). This vaccine is shown to prevent mortality, reduce clinical signs and improve weight gain however could not completely prevent the replication of challenge

virus (Guy *et al.*, 2010). This vaccine can be administered *in ovo* or via wing web injection (Davison *et al.*, 2006, Johnson *et al.*, 2010). When the vaccine is delivered *in ovo*, protection is less than that provided by conventionally attenuated TCO or CEO vaccines delivered via eye-drop or drinking water (Guy *et al.*, 2010). Another viral vectored vaccine that uses HVT to express ILTV antigens is Innovax®-ILT (Intervet International, NJ, USA) and is used to protect chickens against both ILTV and Marek's disease virus (MDV) (Mebatsion *et al.*, 2008). This live recombinant vaccine expresses two glycoproteins of ILTV (gD and gI) and is effective in preventing clinical signs of the disease (Esaki *et al.*, 2013). The level of protection established by this vaccine is also demonstrated to be lower than that of conventionally attenuated CEO and TCO vaccines (Guy *et al.*, 2010).

Another approach for the development of genetically engineered vaccines against ILT is generation of vaccine strains by the deletion of genes or parts of virulence related genes. A range of non-essential genes have been targeted and several vaccine candidates have been proposed based on this method. The deletion of each of these virulence-related genes, has led to attenuation of the virus by impairing a stage of ILTV replication; for instance viral entry (gB gene) (Poulsen and Keeler, 1997), DNA synthesis (UL0 gene) (Veits *et al.*, 2003b) or cell-to-cell spread (gI gene) (Devlin *et al.*, 2006b). After the demonstration of gG as a virulence factor in ILTV (Devlin *et al.*, 2006a), a gG-deficient ILTV vaccine candidate was developed. This vaccine candidate has performed equal to or better than commercial vaccines available in Australia in limiting the clinical signs, mortality rate and weight loss (Devlin *et al.*, 2007). This gG-deficient vaccine candidate was also shown to be less pathogenic than currently available commercial vaccine strains and a recent study has suggested that it might be less likely to establish latency in

vaccinated birds compared to other commercially available vaccines in Australia (Thilakarathne *et al.*, 2019a, Devlin *et al.*, 2007).

1.5 Genomic recombination: principles and implications

Genomic recombination (also known as genomic reshuffling) is a term used to describe the exchange of genetic material between organisms which results in generation of offspring with combinations of traits that are derived from either parent organisms (Carroll, 2001). Genomic recombination plays a major role in the evolution and adaptation of organisms by increasing the genetic diversity (Felsenstein, 1974, Muller, 1964, Mejia, 2012). If the genomic recombination is taking place between two identical or closely related organisms the term 'homologous recombination' (HR) is often used (Ma *et al.*, 2017). Homologous recombination is an especially important driving force in the evolution of organisms with more stable genomic material since the genetic diversity is less likely to be introduced to their genome through *de novo* mutations. The recombination of genomes happens during the process of replication as a mechanism that repairs damaged DNA to ensure the maintenance of genomic integrity (Amunugama and Fishel, 2012). In the case of viruses, the genomic material of two viruses infecting the same cell can result in genomic recombination and generation of progeny viruses that have genetic material from both parent viruses (Simon-Loriere and Holmes, 2011). The sections below provide more details around the genomic recombination in herpesviruses, specifically ILTV, and the consequences that this phenomenon has had on the control strategies against ILT.

1.5.1 Genomic recombination in alphaherpesviruses

The first evidence of recombination in an alphaherpesvirus was reported by Wildy (1955) (Ashe and Scherp, 1963, Dowdle *et al.*, 1967), who inoculated two easily differentiated strains of HSV[1] on to the chorioallantoic membrane (CAM) of embryonated eggs (Wildy, 1955). When they compared the type of lesions and the degrees of virulence of ten isolates resulting from that co-infection, they showed that some isolates had effects intermediate between the parent strains (Wildy, 1955). Later studies documented further evidence of intra- and interspecies recombination in a variety of alphaherpesviruses including HSV-1 and -2, PRV, and VZV (Christensen and Lomniczi, 1993, Norberg *et al.*, 2006, Norberg *et al.*, 2011, Ben-Porat *et al.*, 1982, Norberg *et al.*, 2007). These studies highlighted the clinical importance of recombination due to formation of novel strains with higher virulence. In addition to the clinical consequences of recombination, this phenomenon has also been suggested to significantly impact the evolution, adaptation, and genomic diversification of alphaherpesviruses (Thiry *et al.*, 2005). The evolutionary role of genomic recombination is generally more studied in viruses with dsDNA genome, like herpesviruses, since they are shown to have the lowest *de novo* mutation rate compared to viruses with other types of genomic material (1.8×10^{-8} mutations per nucleotide, per genomic replication) (Drake and Hwang, 2005, Duffy *et al.*, 2008).

1.5.2 Factors involved in recombination of herpesviruses

Although the factors influencing recombination in herpesviruses are not fully understood, it is evident that both viral and host proteins are involved in genomic

[1] The study by Wildy (1955) was published before two serotypes of HSV were differentiated therefore it is unclear which serotype was used.

recombination (Taylor and Knipe, 2004, Wilkinson and Weller, 2003). The role of factors involved in the replication of herpesvirus DNA are particularly important in recombination since the replication and recombination are closely linked (Wilkinson and Weller, 2003). On the virus side, the factors that are involved in virulence (Glazenburg *et al.*, 1994), tissue tropism (Theil *et al.*, 2003), latency (Maes *et al.*, 1997), and the homology of viral genomes (Yirrell *et al.*, 1992, Meurens *et al.*, 2004a) can influence virus replication, and thus the recombination of herpesvirus genomes. For example recombination in PRV is dose-dependent, with recombination only detectable when high doses of virus are used (Glazenburg *et al.*, 1994). In BoHV-1, co-infection with two closely related strains resulted in a diverse population of recombinants (Muylkens *et al.*, 2009).

On the other hand, when the host-related factors were studied, the role of immune responses or vaccination against herpesviruses in reducing the opportunities for recombination has been highlighted (Loncoman *et al.*, 2018a, Loncoman *et al.*, 2018b). For instance, given that high levels of replication is required for recombination to occur (Loncoman *et al.*, 2017c, Loncoman *et al.*, 2017b), there is evidence that vaccination against ILTV reduces both the number of recombinants and the genomic diversity of the new recombinants in chickens co-infected with field strains of ILTV (Loncoman *et al.*, 2018a, Loncoman *et al.*, 2018b). Other host-related factors that play a role in replication efficiency and thus recombination in other herpesviruses (e.g. HSV-1 and HSV-2) include the host DNA-damage response (DDR) pathway proteins (Shirata *et al.*, 2005). The DDR pathways are an antiviral response triggered in infected cells to limit replication by restricting the nuclear substructure and modifying the viral genome through epigenetic processes (Gu and Zheng, 2016, Turnell and Grand, 2012). Other herpesviruses, such as

HSV-1, HSV-2, VZV, EHV-1, BoHV-1 and PRV, use ICP0 as a countermeasure to inactivate DDR pathways (Gu and Zheng, 2016, Ziemann *et al.*, 1998b). As in MDV and HVT, no ICP0 homologs have been identified in ILTV and ILTV-specific UL0 and/or UL[−1] genes, which are positional homologues of the ICP0, have no amino acid sequence similarity with members of the ICP0 gene transactivator family (Ziemann *et al.*, 1998b). Therefore, the influence of DDR pathways on recombination in ILTV is yet to be studied.

1.5.3 Recombination detection methods

The methods for studying recombination in herpesviruses have evolved significantly in recent times especially through increased accessibility of sequencing methods over the past 10 years (Loncoman *et al.*, 2017c). The early methods of studying recombination would use distinguishable phenotypic characteristics to differentiate two similar viruses to determine whether the progenies of co-infection experiments were similar to parent viruses or if they exhibited new intermediate phenotypes. For instance, Wildy (1955) used two HSV strains which form severe and mild lesions on CAM to perform a co-infection study and considered the progeny viruses which caused moderate lesions as recombinants (Wildy, 1955). Later, the development of recombinant herpesviruses that encode different fluorescent proteins were generated and used to study recombination (Meurens *et al.*, 2004a, Hogue *et al.*, 2015). In these experiments the co-occurrence of two fluorescent proteins cloned in progeny virions was used as a readout for recombination and the importance of a high degree of genetic relatedness for successful interspecific recombination was revealed (Meurens *et al.*, 2004a).

The introduction of sequencing technologies, especially whole-genome sequencing methods, and improvement of computational and statistical analysis, has opened new

horizons for the study of herpesvirus recombination (Loncoman *et al.*, 2017c). Several bioinformatic software packages are currently available that comparatively analyse the whole-genome sequence of any given herpesvirus and search for evidences of recombination. Software like Recombination Detection Program (RDP) (Martin *et al.*, 2015), SplitsTree software (Huson and Bryant, 2006), the Genetic Algorithm Recombination Detection (GARD) program (Kosakovsky Pond *et al.*, 2006) and SimPlot software (Lole *et al.*, 1999) are commonly used to detect recombination based on nucleotide sequence variations. For instance, RDP uses a variety of 'recombination aware' phylogenetic tree construction and comparison tools for detecting and visualising possible exchanges of genome segments in sequence alignments of viral genomes (Martin *et al.*, 2015). Similarly, SimPlot software allows the analysis of multiple alignments of viral genomes, identifies the recombination breakpoints in the genome and generates similarity plot that indicate the similarity level to other viruses in the alignment at each nucleotide position (Lole *et al.*, 1999). The most thorough analysis in the current literature uses multiple methods of recombination detection in search for recombination events to increase the robustness and consistency of the results (Loncoman *et al.*, 2017c). Additionally, it is essential to interpret the output of these bioinformatic analysis tools in the context of each experiment including the relevant clinical, epidemiological and historical information, to reach a clear understanding of recombination events (Loncoman *et al.*, 2017c).

1.5.4 Genomic recombination in ILTV and generation of novel virulent strains

In 2012, Lee *et al.* (Lee *et al.*, 2012) performed whole-genome sequencing and comparative sequence analysis of two newly emerged virulent ILTV strains isolated from

outbreaks of the disease in Australia (Figure 2) (described as classes 8 and 9 ILTV by Blacker *et al.* (Blacker *et al.*, 2011)). These two strains had previously been hypothesized to represent sub-populations of viruses within the Serva vaccine, or to have arisen following *in vivo* passage of the Serva vaccine (Blacker *et al.*, 2011). When the sequences of these virulent field isolates were compared to that of previously sequenced vaccine strains, it was shown that although much of class 8 and 9 ILTV genomes were identical to a live attenuated vaccine newly registered in Australia (European-origin Serva ILTV), some parts of their genome was identical or almost identical to Australian-origin vaccines (SA2 and A20 ILTV) (Lee *et al.*, 2012). This finding indicated that the new field strains were novel, natural intra-species recombinants between the Australian origin and European origin vaccines (or viruses extremely closely related to these vaccines) (Lee *et al.*, 2012).

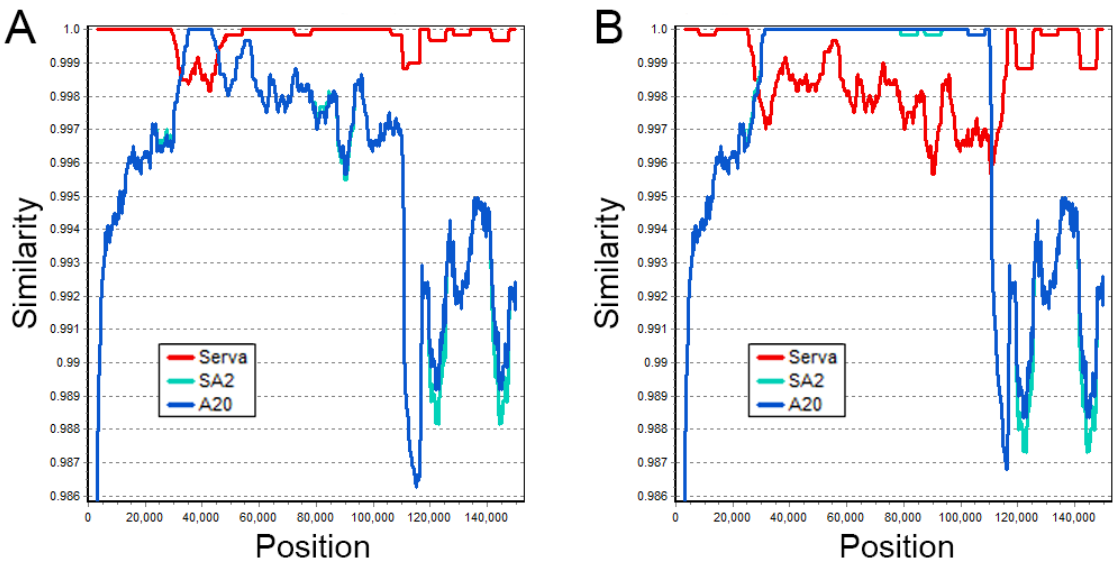


Figure 2. Similarity plots of the whole-genome sequences of the class 8 ILTV (A) and class 9 ILTV (B) with those of Serva, A20, and SA2 strains. The results indicate that the class 8 and 9 viruses were derived from Serva, A20 and SA2. This figure was originally published by Lee *et al.* (2012) and replotted using the genomic data from the same study (Lee *et al.*, 2012).

Phylogenetic and molecular epidemiological study of further ILTV strains revealed evidences of multiple recombination events in the unique long and repeat regions of these novel recombinants, but not in the unique short region. Most recombinants contained a pair of crossover points between two distinct lineages of ILTV, corresponding to the European origin and the Australian origin vaccine strains of ILTV. These results suggest that there are two distinct genotypic lineages of ILTV in Australia and that these commonly recombine in the field (Lee *et al.*, 2013). The formation of recombinant viruses documented by Lee *et al.* (2012) (Lee *et al.*, 2012), added to the concerns around ILTV vaccination with live vaccines which were previously focussed on the establishment of latent infection

(Hughes *et al.*, 1989, Hughes *et al.*, 1991) and reversion to virulence after *in vivo* passages (Guy *et al.*, 1991). Performing co-infection studies under a variety of conditions was suggested to facilitate the understanding of the major factors that lead to recombination in ILTV and provide a basis for advice to enhance the safety of current vaccination practices.

1.5.5 The role of latency and superinfection in recombination of alphaherpesviruses and ILTV

The reactivation of latent 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 (Bagust and Johnson, 1995), and may provide a potential source of virus for recombination with other circulating ILTV (Lee *et al.*, 2013, Thilakarathne *et al.*, 2019a). The reactivated viruses have the potential to be a parent of recombinant progeny either in the animal from which it was reactivated or by transmitting reactivated virus to other birds with prior or subsequent infections. Recently, Thilakarathne *et al.* (2019) have used a combined molecular and tissue culture-based approach to study latent ILTV in commercial chickens (Thilakarathne *et al.*, 2019b). This study provided strong evidence for reactivation and shedding of vaccine ILTV in commercial layer birds from known sites of latency, TG and trachea (Thilakarathne *et al.*, 2019b). Their findings supported the notion that multiple vaccine strains are frequently shed by latently infected and vaccinated birds (Thilakarathne *et al.*, 2019b).

Hosts with herpesvirus infection can be infected again with more herpesviruses, this is known as superinfection (Jarosinski, 2012). Superinfection can be studied at two levels, superinfection of host and superinfection of cells. Superinfection of host is when an already infected individual acquires a further infection at a later time and is marked by the presence

of two herpesviruses in a single sample from the same animal. On the other hand, superinfection of cells is when cells infected with a herpesvirus allow a secondary infection by another herpesvirus. The latter can provide the opportunity for two different viruses to replicate within a single cell which may lead to genomic recombination. The capacity of ILTV in superinfecting at both host and cellular level is important since it can provide a suitable environment for recombination by bringing the genome of two ILTV strains together, but this has not been investigated yet. Alphaherpesviruses can prevent a cell being infected by a second virus in a process known as superinfection exclusion (Meurens *et al.*, 2004b) which has not been studied previously for ILTV. Superinfection exclusion was first described in bacteriophages (Kliem and Dreiseikelmann, 1989) and then was reported as a common phenomenon in other viruses such as influenza virus (Huang *et al.*, 2008), poxviruses (Doceul *et al.*, 2010, Laliberte and Moss, 2014), flaviviruses (Schaller *et al.*, 2007, Zou *et al.*, 2009), alphaviruses (Karpf *et al.*, 1997), and herpesviruses (Meurens *et al.*, 2004b, Criddle *et al.*, 2016, Sloutskin *et al.*, 2014). In order to study superinfection in herpesviruses, the infection models (typically cells) are infected with two different viruses with a time-interval between infections also known as 'unsynchronized co-infection' as opposed to 'synchronized co-infection' with simultaneous infections. Most herpesviruses allow more superinfection when the time-intervals between the first and second infections are shorter. For instance, an interval of up to four hours between infections allowed *in vitro* recombination between BoHV-1 strains (Meurens *et al.*, 2004a) and *in vivo* recombination between PRV strains has been detected as soon as two hours after coinfection (Glazenburg *et al.*, 1994). In herpesviruses, superinfection exclusion is considered to occur mainly through a receptor interference mechanism involving the expression of gD (expressed with

early-late kinetics) (Campadelli-Fiume *et al.*, 1988, Campadelli-Fiume *et al.*, 1990, Stiles *et al.*, 2008), but it has also been shown to occur through gD-independent mechanisms (Criddle *et al.*, 2016). The gD-dependent mechanism of superinfection exclusion works by interaction between gD and cellular proteins, including nectin-1, to directly interfere with subsequent virion entry by inducing the endocytosis of cell surface receptors (Criddle *et al.*, 2016, Johnson and Spear, 1989, Stiles *et al.*, 2008). In HSV-1 and PRV, superinfection exclusion occurs by 2 hours after infection and does not coincide with the peak expression of gD in infected cells (Taylor *et al.*, 2012). On the other hand, gD-deleted PRV showed that the expression of gD was not required to establish superinfection exclusion of a secondary viral inoculum (Criddle *et al.*, 2016). Further experimentation is needed to elucidate the alternative mechanisms that may regulate the superinfection exclusion in herpesviruses (Criddle *et al.*, 2016).

The fact that two herpesviruses can infect a single cell may help explain the basis for recombination, but the role of superinfection, or superinfection exclusion, in this process is unclear. Little is known about the capacity for ILTV strains to allow superinfection, and the role of superinfection in enabling ILTV recombination has not been examined.

1.6 Aims and objectives

This project aims to study the factors that lead to recombination between strains of ILTV. In this way performing ILTV recombination studies in the natural host is a valuable experimental model for examining and understanding alphaherpesvirus infection and recombination more broadly. Using *in vitro*, *in ovo* and *in vivo* studies, this project aims to determine the frequency of these recombination events and the conditions that facilitate recombination. While *in ovo* studies can also be considered as *in vivo*, these 3 groups of studies have been used to reflect each of the systems relevant to the study of ILTV. In this thesis, the *in vivo* group refers to the infection of hatched chickens. The outcomes of the project will include a fundamental understanding of the factors that lead to ILTV recombination, as well as an understanding of how to use ILTV vaccines in the field more safely so that recombination between different vaccine strains can be avoided in the future.

Therefore, this study specifically aims to identify the biological conditions that result in ILTV recombination between two genetic lineages of ILTV. In order to conduct these experiments a molecular differentiation system was developed to differentiate recombinant progeny from the parental strains of ILTV. This differentiation method is presented in the **Chapter 2** of this thesis. The outcome of *in vitro* and *in ovo* co-infection and superinfection experiments under defined conditions is presented in **Chapter 3** of this thesis. Lastly, given that it would be reasonable to expect that perfectly synchronized co-infection with two vaccine strains is unlikely to happen under field conditions, the current study was aimed at investigating the possibility of superinfection in unsynchronized conditions with extended delays between the primary and secondary inoculums. This will be discussed in **Chapter 4** of this study.

Chapter 2. Development and application of high-resolution melting analysis for the classification of infectious laryngotracheitis virus strains and detection of recombinant progeny

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Supplementary material for this article is included in Appendix 1 of this thesis.

The following material has been included with permission from the journal.

Chapter 2

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ORIGINAL ARTICLE

Development and application of high-resolution melting analysis for the classification of infectious laryngotracheitis virus strains and detection of recombinant progeny

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Abstract

Live attenuated vaccines against infectious laryngotracheitis virus (ILTV) are widely used in the poultry industry to control disease and help prevent economic losses. Molecular epidemiological studies of currently circulating strains of ILTV within poultry flocks in Australia have demonstrated the presence of highly virulent viruses generated by genomic recombination events between vaccine strains. In this study, high-resolution melting (HRM) analysis was used to develop a tool to classify ILTV isolates and to investigate ILTV recombination. The assay was applied to plaque-purified progeny viruses generated after co-infection of chicken embryo kidney (CEK) monolayers with the A20 and Serva ILT vaccine strains and also to viruses isolated from field samples. The results showed that the HRM analysis is a suitable tool for the classification of ILTV isolates and can be used to detect recombination between ILTV vaccine strains *in vitro*. This method can be used to classify a broad range of ILTV strains to facilitate the classification and genotyping of ILTV and help to further understand recombination in these viruses.

Introduction

Infectious laryngotracheitis (ILT), caused by infectious laryngotracheitis virus (ILTV, species *Gallid alphaherpesvirus 1*), has an impact on the poultry industry worldwide [1]. Live attenuated vaccines are widely available and are often administered in the early life of commercial birds, followed by one or more rounds of revaccination in order to reach the desired level of immunity [2, 3]. Although the level of immunity developed by this vaccination procedure is adequate to protect the chickens from developing clinical disease (respiratory signs followed by reduced weight gain and egg production), the challenge virus is often able to replicate in the vaccinated birds [2, 4].

All of the ILTV strains form a single serotype [5], yet have shown a notable level of genotypic variation in different studies (0.1–0.8% nucleotide sequence difference) [6]. A number of different methods have been published to classify these viruses by genotype [7–10], including polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) [9–13], targeted sequencing [7, 8], and strain-specific fluorescent probe hydrolysis [14]. In all methods, multiple regions of the ~150-kbp genome are analyzed in order to more precisely differentiate genotypes. These methods have been used to classify ILT viruses to facilitate epidemiological studies and clinical diagnosis [15].

Intraspecific genomic recombination is considered an important driver in the evolution of ILTV and other herpesviruses [16] that have low synonymous nucleotide sequence substitution rates [17]. Genomic recombination requires coinfection with two or more strains, where replication of challenge virus in vaccinated chickens and reactivation of

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latent ILTV infection (either vaccine or field strains) can provide an environment that allows genomic recombination [18]. Intraspecific genomic recombination between vaccine strains of ILTV has been reported to occur in the field [18] and has been demonstrated for field strains under laboratory conditions [14, 18–20]. However, the recombination between modified live vaccine strains has not been studied in experimental settings.

High-resolution melting (HRM) analysis is a simple, rapid and cost-efficient method for genotyping of multiple single-nucleotide polymorphisms (SNPs) [21]. This method has been used in several pathogen identification procedures [22], as well as mutation scanning and diagnosis of genetic disorders in human genome research [23–25]. The extensive applications of this method demonstrate its wide potential to be used as an adjunct to current detection methods.

This study aims to develop an accurate and cost-effective HRM analysis that can be applied to the classification of known and emerging ILTV strains, and to study genomic recombination of ILTV vaccine strains under controlled *in vitro* conditions.

Materials and methods

Viruses

The eight ILTV isolates used in this study are listed in Table 1. The viruses (other than the commercial vaccine viruses) were plaque purified, propagated on primary CEK cells and re-typed by PCR-RFLP [9] prior to testing. In order to increase the titre of commercial vaccines, each vaccine was passaged twice on the chorioallantoic membranes (CAM) of specific-pathogen-free (SPF) embryonated eggs (Australian SPF Services, Woodend, Australia) using artificial air-sac inoculation [26]. The infected CAMs were extracted and thoroughly minced using a mortar and pestle.

Table 1 Viruses used in this study

Isolate	PCR-RFLP class [9]	GenBank accession number	Reference
NOBILIS® ILT (Serva)	7	HQ630064	MSD Animal Health
Poulvac® Laryngo A20	1	JN596963	Zoetis
ACC78	8	JN804826	[18]
Class 9	9	JN804827	[18]
Class 10	10	KR822401	[15]
SA2	1	JN596962	[6]
CSW-1	4	JX646899	[19]
V1-99	2	JX646898	[19]

The homogenates were allowed to sediment for 5 minutes at room temperature to let the larger fragments settle before the supernatants were collected. The harvested viruses were cultured on CEK monolayers. Viruses were harvested at 48 hours after infection of monolayers, and aliquots were stored at -80 °C.

Cell culture

CEK cells were prepared using kidneys harvested from 18-day-old chick embryos as described previously [27]. Monolayers of CEK cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 1% v/v foetal bovine serum (FBS), and 100 µg of ampicillin and 10 µg of amphotericin B per mL. All *in vitro* and *in ovo* experiments were performed on CEK cells or CAM from embryonated SPF hen eggs (Australian SPF Services, Woodend, Australia).

Coinfection of CEK monolayers

Primary CEK monolayers were infected with a mixture of Serva and A20 ILTV strains in 6-well tissue culture plates. After 1 hour of incubation at 37 °C, the inoculum was removed and 3 mL of fresh medium was added. The total multiplicity of infection (MOI) for the coinfection studies was 10. The progeny virus populations were collected by scraping the cells into culture supernatant 48 hours post infection when an extensive cytopathic effect was observed. Samples collected from this experiment were stored at -80 °C prior to virus isolation and plaque purification.

Virus isolation and plaque purification

Ten-fold dilutions of progeny viruses from an *in vitro* coinfection experiment were prepared and inoculated onto CEK monolayers in 6-well plates as described previously [14]. Following 1 hour of incubation at 37 °C, the inoculum was removed and the monolayers were overlaid with methylcellulose semi-solid medium containing 1% w/v methylcellulose in DMEM, with 1% v/v FBS, and 50 µg of ampicillin, 50 µg of gentamicin and 5 µg of amphotericin B per mL. After 48 to 72 hours of incubation at 37 °C in a humidified atmosphere of 5% v/v CO_2, 20 well-isolated plaques were picked using a micropipette guided under an inverted light microscope. Picked plaques were resuspended in 500 µL of DMEM, and each plaque was purified through three rounds of plaque purification before a final propagation step on CEK cells in 24-well plates. One freeze/thaw cycle was performed between rounds of plaque purification.

DNA extraction

Nucleic acid extraction was performed using a PureLink® Pro 96 viral RNA/DNA purification kit (Invitrogen, USA) and a QIAxtractor automated vacuum system. Purified DNA from 200-µL samples was eluted in 200 µL of pyrogen-free water (Milli-Q® Integral system, Darmstadt, Germany) and stored at -20 °C until testing. Pyrogen-free water, rather than the elution buffer supplied with the extraction kit, was used to avoid introducing any variation in HRM patterns.

HRM-based multiple-SNP ILTV genotyping assay

Assay conditions

HRM assays were developed to be used as a method of classification and genotyping ILTV. Following the alignment of full genome sequences of the virus isolates (Table 1), 662 SNPs were identified. These potential target regions were further analyzed *in silico* using uMelt software [28] in order to identify the regions with a detectable melting temperature (T_m) gap in their PCR products. Ultimately, six regions were selected based on their even distribution across the genome (in order to increase the chance of detecting recombinants), the ability to measure differences in their T_m values, and their ability to produce unique patterns for known/tested ILTV strains. Targeted sites included both transition and transversion mutations and resulted in either synonymous or non-synonymous changes (Table 2 and 3). The primers used for this assay are listed in Table 2. A unique single-nucleotide polymorphism (SNP) pattern was predicted for each strain based on the SNPs present in six genomic regions (Fig. 1A). These regions were also amplified and sequenced (Big Dye Terminator v3.1, Life Technologies) to confirm the presence of the targeted SNPs in these viruses.

For the HRM assay, all reactions were prepared using a QIAgility® automated system (QIAGEN). Reaction mixtures (20 µL) were prepared containing 2 mM $MgCl_2$, 200 µM each dNTP, 400 nM each forward and reverse primer, 8 µM SYTO® 9 (Thermo Fisher Scientific), 0.04 U of GoTaq® Flexi DNA Polymerase (Promega, Wisconsin, USA) per mL, Colorless GoTaq® Flexi Buffer (Promega, Wisconsin, USA), and 2 µL of DNA template. PCR amplification and the HRM assay were performed on a Rotor-Gene Q 2plex HRM instrument (QIAGEN Inc., MD, USA) in 100-tube rings (Rotor-Disc 100; QIAGEN, Australia).

The thermal cycling profile included 45 cycles of a denaturation step at 95 °C for 30 seconds, an annealing step at a primer-specific temperature (listed in Table 2) for 30 seconds, and 30 seconds of extension at 72 °C. A final extension step at 72 °C for 5 minutes was included before the melting step. The amplified products were incubated at 95 °C for 2 minutes before a renaturation step at 60 °C for 2 minutes. The HRM was performed in the temperature range of 70-95 °C with fluorescence acquisition at every 0.3 °C and 2-second ramps. Every reaction was performed in triplicate and in three independent experiments unless stated otherwise. Controls in each run included a no-template control and the appropriate reference-virus-positive controls (Table 1). In order to generate a reference for identification of possible mixed populations that were not plaque purified as expected, a 1:1 mixture of the two parent strains was also included in the experimental setup to identify samples containing more than one variant type.

Principal component analysis of the HRM output

The HRM data were processed using Rotor-Gene Q Series software (version 2.1.0 Build 9) and Rotor-Gene ScreenClust HRM software (version 1.10.1.3) [29] in supervised mode. Principal component analysis (PCA) was used to better differentiate the HRM profiles based on the sequence variations in the parent viruses (A20 and Serva). The ScreenClust software normalizes the pre- and post-melting fluorescence levels, generates residual plots, identifies two or three principal components (PC) in the normalized data, defines clusters based on the control samples, assigns unknown/test samples into these defined clusters, and reports the probabilities and typicalities of unknown samples belonging to known clusters based on posterior class probabilities [29]. When all of the unknown samples are classified, the covariance of each cluster is determined (represented by ellipsoids in Fig. 2b). The mean of the reference samples forms the center of the cluster. The test samples are classified into each cluster with posterior probabilities and typicality scores (Supplementary Table 1). These were determined for pure populations of each of the parent viruses as well as for the control samples containing the 1:1 premixed template of each parent virus. The PCA plots of each parent and the mixed populations showed distinct and non-overlapping clusters that made it possible to clearly differentiate each population (each of the two parents and the mixed population). A range of recent studies have used the ScreenClust software for further analysis alongside traditional HRM analysis in higher-throughput settings [30–34].

In silico analysis for classification of published ILTV strains

The full genome sequences of five additional ILTV strains were collected from GenBank (Table 4) to enable *in silico* analyses and to examine the potential for a wider application of the HRM-based genotyping to ILTV strains, including strains from other geographical regions. Using the methods previously established with the Australian ILTV isolates, a series of *in silico* amplifications was performed in Geneious 11.1.4 [35] on multiple regions of five ILTV strains that

Table 2 List of primers and selected target SNPs tested on Australian ILTV isolates

Target gene	Primer set (5'-3')	Annealing temp. (°C)	Amplicon length	T_m gap (°C) experimental [predicted[a]]	No. of SNPs in amplicon	Type of SNP (NS/S)[b]	SNPs							
							Serva	A20	CL8	CL9	CL10	SA2	CSW-1	V1-99
UL52	F: GGTCCGTTTCTAGCTGTTGG	49	130	0.4 [0.8]	2	TS (S)	A	G	A	A	A	G	G	G
	R: TTGCTCCCTTAAGTAGGTTACTTT					TV (NS)	T	G	T	T	T	G	G	G
UL27	F: CATGGTTGGGTCCCCTGG	50	186	0.4 [0.6]	2	TS (NS)	T	C	C	C	C	C	T	T
	R: CGCCAACTGATTGTATGGCA					TS (S)	A	G	G	G	G	G	G	G
UL36	F: AGATGTTTTAGTTCTGTGTGGGC	50	120	1.4 [1.3]	3	TS (NS)	T	C	T	C	T	C	T	T
	R: GGCTGCACTCCTCCAAGATC					TS (S)	T	C	T	C	T	C	T	T
						TS (NS)	T	C	T	C	T	C	C	C
UL8	F: ATTGATGGAAGAGGAAATGAAGCA	55	100	0.8 [1.2]	3	TS (NS)	T	C	T	C	T	C	T	T
	R: TTGTTTTCGCGGGTGTTAAGG					TV (NS)	C	A	C	A	C	A	C	C
						TV (NS)	C	A	C	A	C	A	A	A
IR	F: GTCAACGGGGCTAGATCATT	50	100	0.6 [0.9]	2	TS (n/a)	T	C	T	T	C	C	T	T
	R: ATTTACTTCTGGTTGCGTGCTC					TV (n/a)	T	A	T	T	A	A	T	T
US4	F: TTCGTGTTCGGGGTGGATAG	54	125	0.5 [0.5]	3	TV (NS)	C	A	C	C	A	A	A	A
	R: TCGAAATCAGGTATCCCGGC					TV (NS)	T	G	T	T	G	G	G	G
						TS (NS)	C	T	C	C	T	T	C	C
ORFB	F: CAGTGACGTTAATTTTACCGGGA	50	71	0.6 [1.1]	1	TS (NS)	T	C	T	T	T	T	T	T
	R: CAGCTCGAGAAATTGCAGCG													
US7	F: ACTGGAAATCACGTCTCCGC	50	76	1.5 [1.7]	3	TS (NS)	A	A	A	A	A	A	G	A
	R: GAATTGTAGCTTCGGGGCGA					TS (NS)	A	G	A	A	G	G	G	G
						TS (NS)	T	T	T	T	T	T	C	T

[a] Predicted T_m gap values were calculated using uMelt Batch software

[b] *TS* transition, *TV* transversion, *S* synonymous, *NS* non-synonymous

originated from the USA (Table 3) in order to obtain strain-specific PCR products. Four new sets of primers (Table 3) were designed using Primer3 software (v. 2.3.4) [36]. The products of *in silico* PCRs were imported to uMelt Batch software (v. 2.2) [28] to predict the melting characteristics of each product.

Results

Differentiation of closely related ILTV genotypes by HRM analysis of SNPs

An HRM panel (Table 2) targeting SNPs across six sites (UL52, UL27, UL36, UL8, IR and US4) was applied to A20 and Serva vaccine strains and the well-characterized recombinant class 8, 9 and 10 ILTV strains (Table 1). A schematic view of the SNP pattern is shown in Figure 1A. The melt curve analysis from each of these reactions (Fig. 1B) on A20 and Serva was consistent with that predicted by the uMelt software, particularly in regards to the melting temperature separation predicted for each of the sites. This HRM analysis of six genomic regions enabled differentiation of each of the vaccine and recombinant field strains of ILTV (Fig. 1). The results obtained between assays were reproducible and showed 0.02 to 0.03% coefficients of variation between runs.

Differentiation of parent from recombinant progeny viruses after recombination between Serva and A20 ILTV in coinfected primary cell cultures

The HRM panel targeting the same six sites (UL52, UL27, UL36, UL8, IR and US4) was applied to DNA extracted from plaque-purified viruses generated after a 1:1 coinfection of CEK monolayers with the A20 and Serva ILT vaccine strains (Fig. 2). Each tested region was classified as Serva-like or A20-like. Four isolates out of 20 tested samples were detected as mixed population of viruses and excluded from further analysis. The remaining 16 isolates were classified as either recombinant viruses or parent viruses (Fig. 2). In total, six different patterns of recombinants were detected, with no genotype(s) dominating amongst the recombinant progeny. Equal proportions of parent viruses and recombinant viruses were detected among the 20 progeny viruses tested (Fig. 2A).

Adaptation of the HRM panel for genotyping a broader range of ILTV isolates

Analysis of the six genomic regions across A20 and Serva genomes by HRM has shown the ability of this technology to build a picture of recombination across the ILTV genome, and this system was adapted to explore the potential for use

Table 3 List of primers and selected target SNPs for the classification of US isolates

Target gene	Primer set (5'-3')	Amplicon length	Predicted[a] T_m gap (°C)	No. of SNPs in amplicon	Type of SNP (NS/S)[b]	SNPs					
						Laryngo Vac	TCO IVAX	CEO TRVX	LT Blen	1874C5	
UL54	F: TGTTCTGGCCGGGTCTATTG R: TTGTGTATCCGCGACCAGAG	62	1	1	TS (NS)	T	T	C	C	T	
UL10	F: AAACCCTGCTTGCGGACTAA R: CTGGTGTTGATATCACTGGCCT	57	1.2	1	TS (NS)	T	C	T	T	C	
ICP4-2	F: ATCGTCGTTGTCGGTCTTCC R: CACGTAGTAATGGACAGGCGA	69	0.9	1	TS (NS)	T	T	T	C	T	
ICP4	F: CGGAGCACTTGCCGGTAC R: GTTGGCGGGAGATTCTTGGG	126	1.2	3	TS (S)	T	T	T	T	C	
					TS (NS)	A	A	A	A	G	
					TS (S)	T	T	T	T	C	
					Expected strain-specific HRM combination pattern[c]	AAAA	ABAA	BAAA	BABA	ABAB	

[a] Predicted T_m gap values were calculated using uMelt Batch software

[b] *TS* transition, *TV* transversion, *S* synonymous, *NS* non-synonymous

[c] In these 4-character HRM combination patterns, the letters represent the sequence type of UL54, UL10, ICP4 and a second region in ICP4 genes, respectively. The viruses with melting characteristics similar to those of the Laryngo Vac strain in each region are indicated by an "A" and otherwise by a "B"

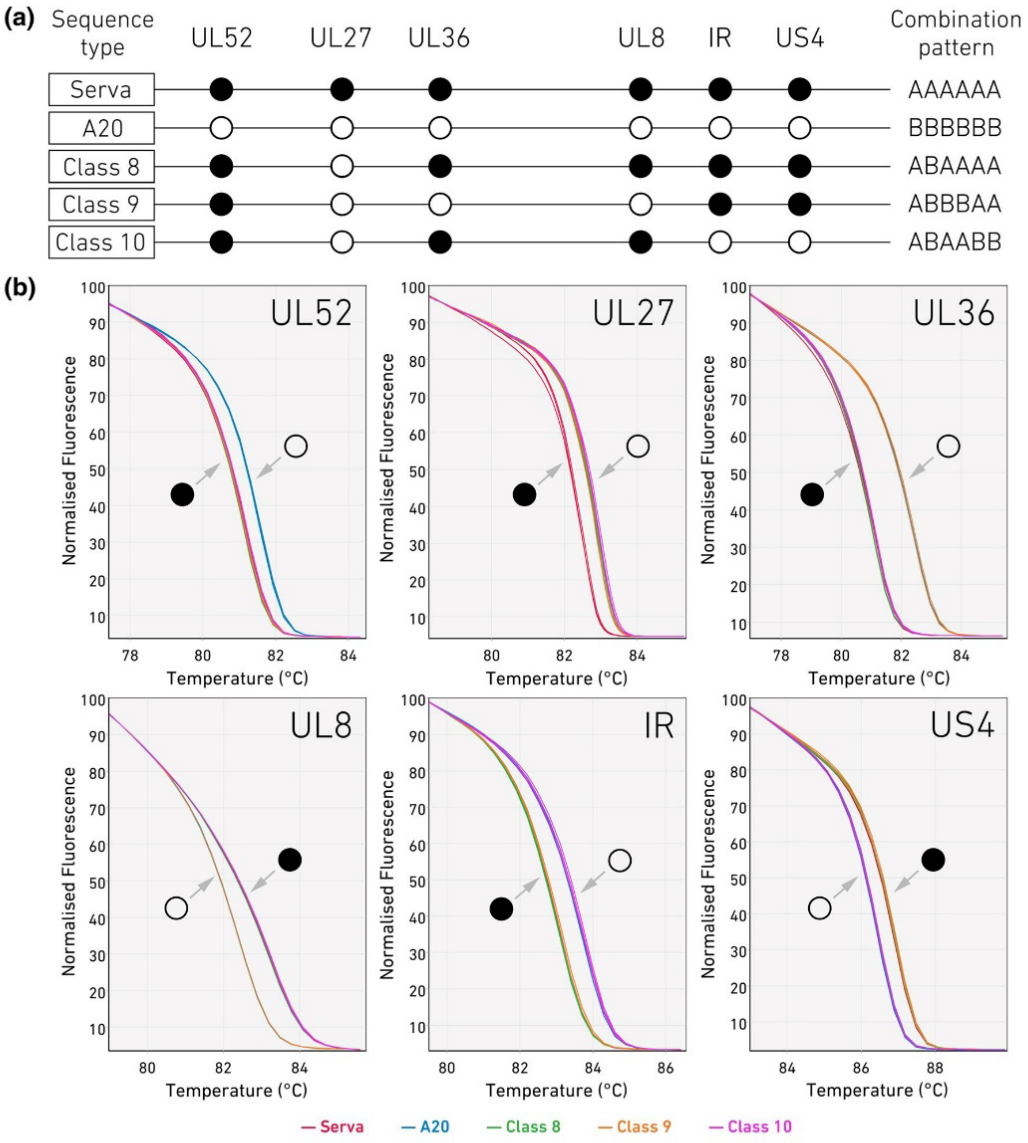


Fig. 1 HRM analysis of five fully sequenced ILTV isolates. (a) A schematic view of six target genomic markers with distinct melting characteristics showing the distribution of Serva-like (closed circles) and A20-like (open circles) SNPs in Serva, A20, class 8, 9 and 10 ILTV strains. (b) HRM plots showing the differentiation of Serva (red) and A20 (blue), along with the melt curves for class 8 (green), class 9 (orange) and class 10 (purple) ILTV strains by the six target genomic markers (UL52, UL27, UL36, UL8, IR and US4). Each sample was clustered into SNP identities that were either Serva-like (closed circles) or A20-like (open circles). The plots were generated by Rotor-Gene Q software. Each curve represents one replicate

in the genotypic classification of other ILTV isolates. To do so, the genomes of three more Australian strains (one vaccine [SA2] and two field strains [class 2 and 4]) were retrieved from GenBank and compared to the Australian ILTV isolates used previously in this study (Table 1). A panel of four SNPs was identified that would differentiate the three vaccine strains used in Australia (SA2, A20 and Serva) and five Australian genotypes (Table 1). This panel (Fig. 3A) includes two previously characterized regions (UL27 and UL36, Table 2, Fig. 1 and 2) and two new regions (ORFB and US7, Table 2, Fig. 3) predicted by uMelt to have a T_m gap of >1 °C. When this new panel of four sets of HRM primers (ORF B, UL27, UL36 and US7) was applied to extracted DNA from these viruses (Table 1), the actual

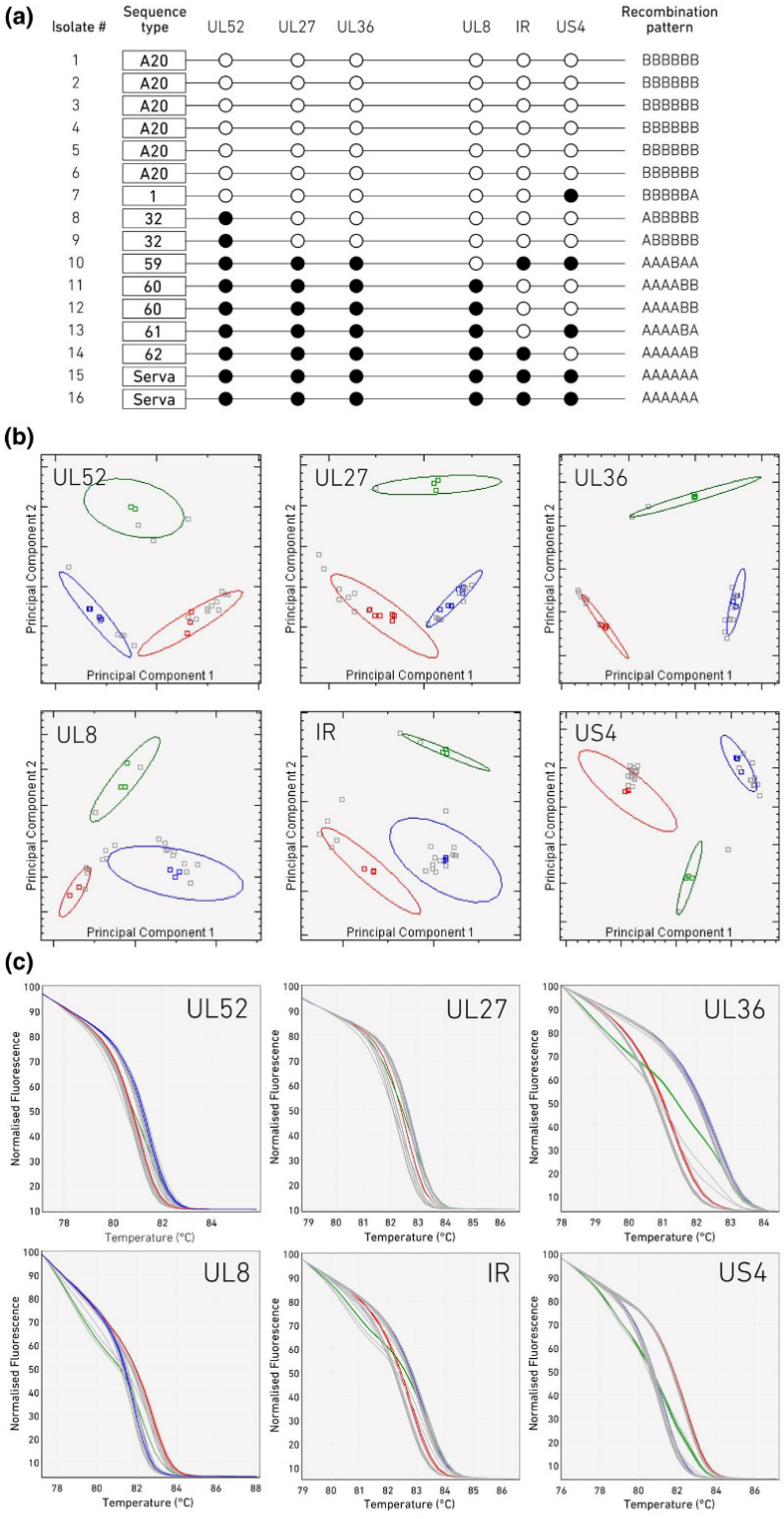


Fig. 2 HRM analysis of parent and progeny viruses generated two days after infection of CEK cells with a 1:1 mixture of Serva and A20 ILTV. (a) A schematic representation of SNP patterns in parent and progeny viruses is shown, along with (b) PCA cluster analysis and (c) HRM plots of the assays for each of the six genomic regions. (a) A schematic view of genotypes of plaque-purified parent and progeny viruses after coinfection. The closed circles represent the Serva-like SNPs, and open circles indicate A20-like SNPs. Each virus is genotyped as A20, Serva, or recombinant with a unique genotype code number (1 to 62) for each recombination pattern. (b) PCA cluster plots and (c) HRM plots show triplicate Serva (red) and A20 (blue) ILTV parent strains as references. A 1:1 mixture of Serva and A20 ILTV (green) are shown to identify mixed samples. Plaque-purified progeny viruses (grey squares/curves) are clustered into Serva-origin (red oval) SNPs, A20-origin SNPs (blue oval) or mixed populations (green oval) on PCA plots. Clusters are highlighted by ovals that represent the covariance of the classified samples in a two-PC-dimension plot. The samples that plot outside the ovals are classified into the closest cluster and receive a lower typicality score

Table 4 Viruses used in *in silico* classification of US isolates

Isolate	PCR-RFLP class [10, 37]	GenBank accession number	Reference
Laryngo Vac	IV	JQ083494	[38]
TCO IVAX	II	JN580312	[39]
CEO TRVX	IV	JN580313	[39]
LT Blen	IV	JQ083493	[38]
1874C5	VI	JN542533	[37]

HRM findings were used to generate a four-character combination code based on the melting characteristics of each of the target regions. Using these four sites, the genotype of the five distinct Australian field strains and the three vaccine strains used in Australia could be differentiated from each other (Fig. 3). Moreover, the SA2 and A20 vaccine strains could be differentiated from each other in one region (ORF B) despite their close relationship (sharing 99.9% nucleotide sequence identity) and both being classified by PCR-RFLP as class 1 viruses.

In silico exploration of HRM-based genotyping to ILTV strains from other countries

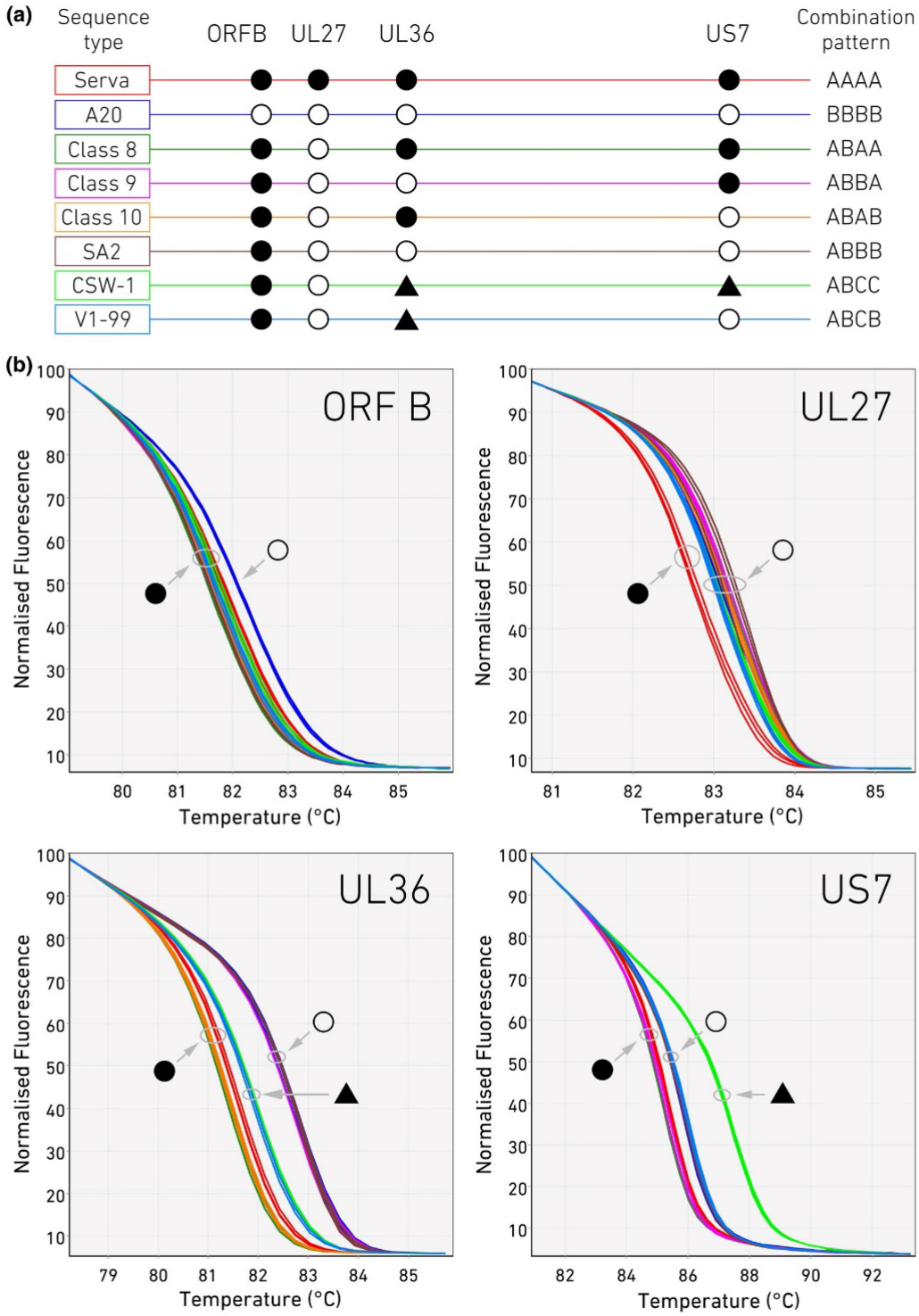
Given the consistency between the predicted and experimentally observed melting temperatures for HRM analysis, this method could be broadened as a means of genotyping viruses from different geographical regions. To illustrate this, similar *in silico* analysis of five US strains (Table 4) has identified four regions that could be used to differentiate US field and vaccine isolates (Table 3), suggesting the potential for the simple application of this technology to characterize a broad range of ILTV isolates across the world.

Discussion

In this study, we aimed to develop a reliable cost- and time-efficient method that can be applied to the classification and genotyping of ILTV strains and the identification of ILTV recombinant viruses. The majority of existing ILTV classification systems are based on identifying the sequence variations in representative regions of the genome that are first amplified by PCR. Sequence variation is identified in these regions by a range of methodologies including RFLP [9, 10], Sanger sequencing [7, 8] or fluorescently labelled TaqMan® probes [14, 40, 41]. Both the number of regions being analyzed and the method of analysis will influence the precision of the classification and differentiation of ILTV isolates. Although PCR-RFLP results are relatively easy to interpret, other studies have suggested the use of Sanger sequencing of one, two [7, 42–45] or multiple [8] region(s) of ILTV genome as an alternative to RFLP.

Fig. 3 The genotyping of eight Australian ILTV field and vaccine isolates using HRM analysis. (a) A schematic view of a panel of four SNPs with distinct melting characteristics showing the distribution of Serva-like (closed circles), A20-like (open circles) and other SNPs (closed triangle) used to differentiate Australian field strains in Serva, A20, class 2, 4, 8, 9 and 10 ILTV strains and (b) HRM plots comparing four target genomic markers (ORFB, UL27, UL36 and US7) in a panel of viruses including Serva (red) and A20 (blue) to SA2 (brown), class 2 (V1-99) (light blue), class 4 (CSW-1) (light green), class 8 (dark green), class 9 (pink) and class 10 (orange) ILTV isolates. Each sample was clustered into either Serva-like (closed circles), A20-like (open circles) or other (triangles) SNP identities. The plots were generated by Rotor-Gene Q software. Each curve represents one replicate

The HRM analysis used in the current study could differentiate field and vaccine strains of ILTV and classify them into distinct genotypes that had previously been determined by PCR-RFLP [9] and full-genome sequencing [6, 18, 19]. Australian ILTV classes 3, 4, 5 and 6 were not examined in this study, as no genomic sequences were available for these viruses to validate the results. The results of HRM analysis were reproducible and were in accordance with other methods; however, the successful use of this method relies on the precision of techniques used during sample preparation, DNA isolation, amplification and melting, and post-reaction analysis steps, as described in detail by Slomka *et al.* [21]. In order to overcome the variations introduced by different isolation methods and sample composition, a single type of DNA isolation kit was used for both test samples and the samples used as reference strains in this study. The commercial vaccine strains were passaged once in CEK cells so that the composition of the reference strains and test samples would be similar. Automated systems were used for extraction and PCR preparation to maintain the uniformity of samples and reactions. The sizes of amplicons for each target region was also kept short (<200 bp) where possible in order to obtain a higher T_m gap between variant types. The uMelt melting curve prediction software provided a reliable guide for selecting target SNPs based on the melting peaks. The predicted melting temperatures were close to experimental melting peaks with only slight differences (zero to 0.4 °C) in some regions. Therefore, when designing the second and third set of primers, the regions with a T_m gap of ≥1 °C were preferred. In order to use this system to classify viruses submitted from outbreaks of disease in the field it is likely that viruses from any clinical samples would also require processing (isolation and DNA extraction) using uniform techniques. It is conceivable that future assays could be developed and optimized for direct use on specific clinical samples (e.g., tracheal tissue or conjunctival swabs) after extensive evaluations using DNA extracted directly from clinical specimens. This would have time and


(a)
Sequence type
ORFB
UL27
UL36
US7
Combination pattern
Serva
A20
Class 8
Class 9
Class 10
SA2
CSW-1
V1-99
AAAA
BBBB
ABAA
ABBA
ABAB
ABBB
ABCC
ABCB
(b)
ORF B
UL27
UL36
US7
Normalised Fluorescence
Temperature (°C)

cost advantages and represents an important area of future work. The direct use of the HRM assay on clinical samples has been successfully applied to several pathogens in the past [46–49]. The potential of HRM in identifying mixed specimens has been reported to be primarily dependent on the quantity and proportion of the target DNAs in the mixture [50]. Current detection or genotyping methods for ILTV have not been able to rapidly identify the presence of mixed virus populations in laboratory or field samples. The potential of this method to identify mixed infections in these settings requires further exploration.

The HRM system developed in this study could also be used successfully to detect recombinant viral progeny. This represents the first time that HRM methods have been used to detect recombination in herpesviruses. Previous studies have shown that genomic recombination between Serva and A20 ILTV strains has occurred in the natural host and has resulted in field recombinants with higher levels of virulence [18]. The results of the current study showed that these two vaccine strains can recombine in coinfected (MOI = 10) CEK cells and generate a diverse pattern of recombinants. After this coinfection, recombinants accounted for 50% (8/16) of progeny viruses, and the highest proportion of an individual genotype (6/16) had an SNP profile identical to that of the A20 parent strain. The proportion of recombinants in the current study is consistent with the greater opportunity for coinfection of cells at this high MOI so that recombination can occur. These finding are consistent with findings from an *in vivo* coinfection study in SPF chickens using a mixture of CSW-1 and V1-99 ILTV strains delivered intratracheally, where 46.5% (in birds co-inoculated with 10^3 PFU of each virus) to 79.2% (in birds co-inoculated with 10^4 PFU of each virus) of isolated progeny viruses were identified as recombinants.

In conclusion, the HRM assay developed in this study was successfully applied as a high-throughput screening tool for recombinant detection and could be used to successfully classify different strains of ILTV. In these applications the assay was shown to be accurate, time-efficient and cost-efficient. The HRM genotyping method promises to be an important tool to facilitate the study of ILTV epidemiology and recombination in both experimental and field settings.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Chapter 3. Genomic recombination between infectious laryngotracheitis vaccine strains occurs under a broad range of infection conditions *in vitro* and *in ovo*

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RESEARCH ARTICLE

Genomic recombination between infectious laryngotracheitis vaccine strains occurs under a broad range of infection conditions *in vitro* and *in ovo*

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Abstract

Gallid alphaherpesvirus 1 causes infectious laryngotracheitis (ILT) in farmed poultry worldwide. Intertypic recombination between vaccine strains of this virus has generated novel and virulent isolates in field conditions. In this study, *in vitro* and *in ovo* systems were co-infected and superinfected under different conditions with two genomically distinct and commonly used ILTV vaccines. The progeny virus populations were examined for the frequency and pattern of recombination events using multi-locus high-resolution melting curve analysis of polymerase chain reaction products. A varied level of recombination (0 to 58.9%) was detected, depending on the infection system (*in ovo* or *in vitro*), viral load, the composition of the inoculum mixture, and the timing and order of infection. Full genome analysis of selected recombinants with different *in vitro* phenotypes identified alterations in coding and non-coding regions. The ability of ILTV vaccines to maintain their capacity to recombine under such varied conditions highlights the significance of recombination in the evolution of this virus and demonstrates the capacity of ILTV vaccines to play a role in the emergence of recombinant viruses.

Introduction

Herpesviruses are an important cause of disease in a wide range of animal species, including production and companion animals. Infectious laryngotracheitis virus (ILTV; *gallid alphaherpesvirus 1*) is an alphaherpesvirus that causes acute respiratory tract disease in domestic poultry and results in significant economic losses worldwide [1, 2]. The economic losses caused by ILTV are due to the mortalities it causes, as well as its impact on the growth rate of broiler chickens, on egg production in layer farms, and on susceptibility to other respiratory pathogens [1]. Live attenuated vaccines are effective in reducing mortality, clinical signs [2, 3] and

controlling the outbreaks of disease [4]. Several recombinant or virally vectored vaccines are also in use or development and include ILTVs with deletions of virulence-related open reading frames (ORFs) [5, 6], and fowl-pox virus (FPV) [7] and turkey herpesvirus (HVT) [8, 9] expressing specific ILTV glycoprotein genes.

Recombination makes a significant contribution to the evolution, diversity and fitness of herpesviruses, particularly in the face of the low rates of point mutation seen in these viruses [10, 11]. Genomic recombination between ILTV strains is common [12–15], and has had a significant impact on currently circulating viruses [15]. *In vitro* and *in vivo* co-infection studies in birds with field strains has revealed recombinational hotspots in the ILTV genome, with specific genomic regions having a higher frequency of recombination events [16]. These studies have shown that recombinant diversity is highest at peak virus replication [16]. Host factors such as vaccination-induced immune responses are also suggested to exert a selective pressure on the emergence of recombinant progeny [17].

The importance of genomic recombination in ILTV was first highlighted with the discovery that two new virulent field strains (class 8 and class 9 ILTV) were recombinants independently derived from distinct attenuated ILTV vaccine strains (Australian origin SA2/A20 and European origin Serva ILTV) [12]. Recombination events between these vaccine strains resulted in viruses of higher fitness that became the predominant field strains in Australia and largely replaced the previously dominant class 2 genotype viruses [15]. Recombination requires a host cell to be simultaneously infected with 2 different virus strains. While simultaneous co-infection of cells or animals can occur under experimental conditions, simultaneous co-infections would not necessarily be expected to occur frequently under field conditions. Instead, some delay between infection with the first and second strain may be more likely. Little is known about the capacity for ILTV strains to allow superinfection, and the role of superinfection in enabling ILTV recombination has not been examined.

The aim of this study was to identify the conditions that lead to recombination between ILTV vaccine strains by performing *in vitro* and *in ovo* co-infection and superinfection experiments under defined conditions. To identify recombinant progeny, we have previously developed a differentiation system based on a panel of high-resolution melting (HRM) curve profiles of the parent isolates [18]. The parental viruses used in this study were the two distinct genetic lineages of ILTV vaccine virus strains, A20 and Serva. In previous studies, these or similar viruses have been found to be the parental viruses from which the virulent recombinant field viruses (class 8 and class 9) have been derived [12].

Material and method

Cell and virus culture

Primary chick embryo kidney (CEK) cells were prepared using kidneys harvested from 18-day-old chick embryos as previously described [19]. The use of embryonated eggs was approved by the Animal Ethics Committee of The University of Melbourne (ethics approval number 1814492.1). Monolayers of CEK cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), 1% v/v foetal bovine serum (FBS), 100 μg ampicillin/mL and 10 μg amphotericin B/mL. All *in vitro* studies were performed with CEK cells, and *in ovo* experiments were performed by inoculation of the chorioallantoic membrane (CAM) of embryonated, specific-pathogen-free (SPF) hybrid white Leghorn-derived chicken eggs (Australian SPF Services, Woodend, Australia).

The vaccine strains NOBILIS® ILT (Serva, GenBank accession number HQ630064) and Poulvac® Laryngo (A20, GenBank accession number JN596963) were plaque purified as

described previously [13], propagated on CEK cells and re-typed by PCR-RFLP [20] prior to co-infection experiments. To obtain the high-titred virus stocks required for these studies, each vaccine was passaged twice on the CAM of SPF embryonated eggs [21]. The infected CAMs were extracted and thoroughly homogenised using a mortar and pestle. The homogenates were allowed to sediment for 5 minutes at room temperature to let the larger fragments settle before the supernatants were collected. The CAM supernatants, allantoic fluid (AF) and CEK culture supernatants were aliquoted and stored at -80˚C until further use. Viruses were titrated by plaque assay on CEK monolayers [13].

Growth kinetics (multi-step growth curve, entry kinetics and kinetics of plaque development) of Serva and A20 strains of ILTV in CEK cells were measured as described previously [22]. Briefly, for multi-step growth curve, CEK cells in 24-well plates were infected with a MOI of 0.001 and cells and supernatant harvested at the indicated time points. Virus growth was quantified in combined cell lysate and supernatant after DNA extraction (PureLink® Pro 96 viral RNA/DNA purification kit, Invitrogen, USA) using a UL15-specific qPCR, as described by Mahmoudian *et al.* (2011) [23]. The mean and standard deviation of triplicate measurements performed in each of 3 independent experiments were compared by Student's *t*-test within GraphPad Prism (v. 6.07).

To determine whether Serva and A20 ILTV differ in their entry kinetics, an experiment was performed as described by Mundt *et al.* (2011) [24] with some modifications. Briefly, the CEK monolayers in 6-well plates were chilled to 4˚C on ice for 10 minutes, and 500 μL of pre-chilled Serva or A20 inoculum (MOI = 0.0005) were added to each well for adsorption of viral particles on the cell surface. The plates were kept at 37˚C (i.e. temperature shift) for 5, 15, 30, 45 and 60 minutes, then the excess inoculum was removed, extracellular virus inactivated with 0.2 M citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) before further washing and the addition of a final 5 mL of 1% w/v methylcellulose in DMEM to each well. The plates were further incubated at 37˚C for 72 hours. Viral entry into cells at different time points were calculated by dividing the number of plaques detected at each time point by the number of plaques formed after an incubation period of 60 minutes and reported as percentages. The mean and standard deviation from three independent experiments were then compared using Kruskal-Wallis test followed by Dunn's multiple comparisons test within GraphPad Prism (v. 6.07).

The capacity of Serva and A20 ILTV to spread from cell to cell was also compared by examining the kinetics of plaque development in CEK cells. The plaque area of 13 to 22 individual plaques photographed at different time points were measured using ImageJ (version 1.50i) [25], and compared using Student's *t*-test as described previously [22].

Progeny viruses were isolated using ten-fold dilutions of AFs, CAMs, and homogenates of cells and supernatant fluids (SNFs) inoculated onto CEK monolayers in 6-well plates as described previously [18, 22, 26].

DNA extraction

Nucleic acid extraction was performed using the PureLink® Pro 96 viral RNA/DNA purification kit (Invitrogen, USA) and the Corbett Robotics X-tractor Gene automated vacuum system (Corbett Life Science, Australia). Purified DNA from 200 μL samples was eluted in 200 μL of pyrogen-free water (Milli-Q Integral system, Germany) and stored at -20˚C until it was assayed. Pyrogen-free water, rather than the elution buffer supplied with the extraction kit, was used to reduce variation in HRM patterns [27]. For full genome sequencing, viral DNA was extracted from purified nucleocapsids using a High Pure PCR Template Preparation kit (Roche, Germany) according to the manufacturer's instructions.

Co-infection and superinfection of CEK monolayers and superinfection of CAMs with a mixture of Serva and A20

For co-infection experiments, CEK monolayers in 6-well tissue culture plates were inoculated with a mixture of the Serva and A20 ILTV strains. Inoculating viruses were mixed prior to infection at the multiplicities of infection (MOI) and ratios shown in Table 1. After 1 hour of incubation at 37˚C, the inoculum was removed, and the monolayer was washed three times with 5 mL of PBS, then 3 mL of fresh medium was added. At 48 hours post infection (hpi), extensive cytopathic effect (CPE) was observed, and the cells were scraped into the culture supernatant. Samples collected from these experiments were stored in 1 mL aliquots at -80˚C until plaque purification was performed, as described previously [13].

In the superinfection study, CEK cells were infected with either the Serva or A20 strain and then, after a range of incubation times, infected with the heterologous virus. After removal of the growth medium from the cells, the first inoculum was added and allowed to adsorb for one hour at 37˚C. Prior to removal of the inoculum, extracellular virus was inactivated by washing for one minute in 0.2 M citrate buffer, and then washing several times with growth medium before further incubation of the cells in 3 mL of DMEM until the 2nd inoculation. The 2nd inoculation was performed at 2, 4, 6 or 8 hours after the first (Table 1), using the same procedure as was used for the first inoculation. For the 1:1 simultaneous infection control for this part of the study, the inoculating viruses were premixed prior to inoculation onto the monolayers, and removal of the inoculum and washing with the citrate buffer were performed after one hour as described above. The total MOI used for the superinfection study was 5.

Table 1. Summary of inocula used and the samples collected.

Infection model	Total MOI	Serva to A20 ratio	Delay between infections (hours)	First inoculum	Sample collected[a]
CEK cells	10	1:1	0	n/a	Cells & SNF
		1:4	0	n/a	Cells & SNF
		4:1	0	n/a	Cells & SNF
	5	1:1	0	n/a	Cells & SNF
		1:1	2	Serva	Cells & SNF
		1:1	4	Serva	Cells & SNF
		1:1	6	Serva	Cells & SNF
		1:1	8	Serva	Cells & SNF
		1:1	2	A20	Cells & SNF
		1:1	4	A20	Cells & SNF
		1:1	6	A20	Cells & SNF
		1:1	8	A20	Cells & SNF
Embryonated eggs	$10^{4.5}$ pfu/embryo	1:1	0	n/a	CAM
			0	n/a	AF
		1:2	0	n/a	CAM
			0	n/a	AF
		2:1	0	n/a	CAM
			0	n/a	AF
		1:10	0	n/a	CAM
			0	n/a	AF
		10:1	0	n/a	CAM
			0	n/a	AF

[a] SNF: supernatant fluid, CAM: chorioallantoic membrane, AF: allantoic fluid

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For the *in ovo* co-infection studies, a total of $10^{4.5}$ plaque forming units (pfu) of the two vaccine strains at varying ratios (Table 1) were inoculated onto each CAM. After inoculation, eggs were incubated at 37˚C in a humidified egg incubator (Maro, South Korea) and at 48 hpi the AF and CAM samples were collected separately [28].

Identifying recombinant progeny viruses using HRM-based multiple-single nucleotide polymorphism (SNP) ILTV genotyping assay and recombination analysis

Any detection of recombinant progeny was used as a readout of co-infection or superinfection of cells. In order to detect recombinant viruses, individual viruses were cultured and plaque purified. In all experiments, progeny viruses were plaque purified on CEK monolayers in 6-well plates as previously described [13]. At least 20 progeny viruses were randomly picked per infection condition and plaque purified three times prior to recombination analysis using the HRM panel.

The analysis of isolates was performed using a technique we have described previously [18]. Briefly, the DNA extracted from plaque purified isolates was tested using a panel of 6 PCR-HRM assays targeting 6 sites (UL52, UL27, UL36, UL8, IR and US4) that differ between the A20 and Serva genomes. To confidently assign genotype to the progeny viruses, this assay is used to examine only plaque purified populations of virus. Therefore, any that was not purified as expected and showed a mixed SNP/HRM profile in any of the 6 sites were considered as a mixed population and were excluded from further analyses. Following the PCRs, a 6-character genotype pattern code was applied to each isolate as an identifier. When the HRM curve of an isolate matched Serva in a region, the letter A was used as the identifier, therefore a pure Serva parent genotype pattern code is AAAAAA. If the HRM curve matched A20 the letter B was incorporated into the genotype pattern code, therefore a pure A20 parent genotype pattern code is BBBBBB. Recombinant viruses were those identified as having a mixture of Serva and A20 genomic regions, with a mixture of A and B regions in their 6-character genotype pattern code (e.g. ABBAAB). All possible genotype pattern codes (64 codes) were also given a number (genotype code 0 to 63) for ease of referencing [18].

Full genome sequencing

One representative isolate from each genotype pattern code (33 patterns isolated in total) was screened to look for possible variations in their plaque sizes on CEK cells. Plaque areas were measured using ImageJ (version 1.50i) [25] calibrated using a stage micrometre [22]. Eight isolates were selected for full genome analysis based on their HRM genotype and their plaque size. These isolates were further cultured by passage on CEK cells in 175 cm^2 tissue culture flasks with 50 mL of growth medium. The viruses were harvested 72 hours after infection, when extensive CPE was observed. The cells were scraped into the growth medium, homogenized by pipetting and stored in 50 mL aliquots at -80˚C. A freeze-and-thaw step was included in order to maximise the release of viruses from cell debris. Viral nucleocapsids were purified from the lysate as described by Atherton *et al.* [29].

Library preparation and sequencing. Extracted DNA samples were sent to the Australian Genome Research Facility (AGRF) for library preparation and whole-genome sequencing. The libraries were prepared using Nextera XT Index kits with no insert size selection. The paired-end sequences were determined using an Illumina MiSeq sequencing machine with a Nano flow cell and 300 cycles. The total number of reads and the read lengths are shown in Table 2.

Genome assembly of progeny viruses. Viral genome assembly using the Illumina read data was performed using the Geneious 11.1.4 sequence analysis package [30]. The paired-end

Table 2. Full genome reads and assembly metrics for the 8 isolates selected for full genome sequenced in this study.

Isolate number	Genome size (bp)	Total reads	Mapped reads (after trimming)	Breadth of coverage of reference* (%)	Mean depth of coverage (number of reads)
29	152,643	327038	23,405	99.90	21.0
109	152,627	309310	27,202	99.90	24.5
138	152,660	338586	40,504	99.90	36.6
157	152,663	329770	70,534	99.98	63.6
237	152,713	273274	27,759	99.90	24.7
238	152,717	317580	33,116	99.99	29.8
319	152,686	301232	38,651	99.90	34.0
358	152,600	314154	88,048	100.00	79.3

* Serva ILTV strain, GenBank accession number HQ630064, was used as the reference

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Table 3. Comparison of plaque areas in CEK cells of 8 recombinant viruses at 48 hpi with those of Serva and A20 parental strains.

Strain or isolate	Genotype code	Genotype pattern code	Mean plaque area ± SD (mm^2) [a]	GenBank accession number
A20	0	BBBBBB	0.72 ± 0.21	JN596963
Serva	63	AAAAAA	0.58 ± 0.25	HQ630064
29	57	AAABBA	0.10 ± 0.03 [#]	MK894996
109	57	AAABBA	1.06 ± 0.34 [#, †]	MK894997
138	57	AAABBA	1.17 ± 0.46 [#, †]	MK894998
157	39	ABBAAA	1.34 ± 0.44 [#]	MK894999
237	17	BABBBA	0.88 ± 0.35 [‡]	MK895000
238	24	BAABBB	0.17 ± 0.07 [#]	MK895001
319	0	BBBBBB	0.36 ± 0.22 [¥]	MK895002
358	63	AAAAAA	0.56 ± 0.19	MK895003

[a] Comparisons were performed using a one-way ANOVA followed by Tukey's test for pairwise comparisons within GraphPad Prism (v. 6.07); We considered p values of <0.05 to be statistically significant

[#] Significant difference with Serva and A20

[†] Significant difference with #29

[‡] Significant difference with Serva

[¥] Significant difference with A20

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short reads generated for each isolate were trimmed with a quality threshold Phred score of 30 and the Nextera adapters were removed using the BBDuk 37.64 tool (Brian Bushnell within Geneious 11.1.4). The trimmed reads were then assembled by mapping the raw reads to the published Serva ILTV genome (Genbank accession HQ630064) as a guide using Geneious Mapper in the "Map to Reference" tool with "Medium Sensitivity" and up to 5 reiterations for fine tuning the assembly. Following the assembly, consensus sequences were generated using read quality as threshold. The total number of reads, mapped reads, breadth of coverage, average read length, and mean depth of coverage for each isolate are shown in Table 2. The full genome sequences of these isolates are available in the GenBank database under the accession numbers (Table 3).

Identification of recombination events. After aligning the recombinant whole genome sequences to the parental sequences, recombination events were initially detected by visually/manually reviewing the sequences for known SNPs within the Geneious viewer tool, and the exchanged regions of the genome were marked as Serva-like or A20-like. In addition, the 8

viruses sequenced in this study were individually aligned to the Serva and A20 reference genomes using the MAFFT aligner [31] within Geneious and sequence similarity plots of these sequence trios were generated using SimPlot version 3.5.1 [32]. The recombination events detected in these sequence trios were then verified using the Recombination Detection Program version 4 (RDP4, version beta 4.95) [33] (S1 Table). In SimPlot, the sliding window size was set to 6000 bp to scan the genome in 200 bp steps and F84 (maximum likelihood) was used as the distance model. A manual reviewing step was included to ensure that the recombination detection algorithms had not overlooked small fragment exchanges due to the high sequence identity (99.2%) between the parental strains. For the visual/manual detection of recombination in the aligned sequences, the full genome sequence of each recombinant was aligned to those of Serva and A20, and the disagreements were highlighted after setting the Serva genome as the reference sequence. Following this, the regions in recombinants that contained Serva-like SNPs were annotated as Serva-like, and those that contained A20-like SNPs were annotated as A20-like.

Results

Growth kinetics of the Serva and A20 ILTV strains

Given the close association between replication and recombination [16], the *in vitro* growth characteristics of both parental viruses used in this study were compared. Both the Serva and A20 strains had similar entry kinetics into CEK cells, with no significant difference between them at any time point (Fig 1A).

In the multistep growth curve comparison, both A20 and Serva plateaued to similar maximal titres at 48 to 72 hpi, although cultures of Serva had significantly higher genome concentrations ($p < 0.05$, Student's *t*-test) than the A20 strain in the exponential growth phase (Fig 1B). A20 produced significantly larger plaques ($p < 0.05$, Student's *t*-test) than Serva at 72 and 96 hpi, although the plaque sizes did not differ significantly at 48 hpi and 120 hpi (Fig 1C).

Recombinant progeny are produced under a broad range of infection conditions

The titres of ILTV recovered from co-infected CEK cells, superinfected CEK cells, CAMs and AFs are shown in Fig 2. A summary of these results for co-infection/superinfection of CEK monolayers and eggs is shown in Fig 2, with the proportion of recombinants calculated by considering any progeny that did not have a 6-character genotype pattern code matching the parental strains (AAAAAA or BBBBBB) as recombinant.

In ovo co-infections

In co-infected eggs, recombinant progeny were detected under all conditions tested. In these experiments, the ratio of parental viruses in the inoculum varied from equal proportions to a 10:1 excess of one of the parental strains. Between 47 and 50% of the progeny of the 1:1 co-infections were recombinants, and a 2:1 ratio of the parental viruses still resulted in a high proportion of recombinant progeny (31.5–59%). However, when a 10:1 ratio of the parental viruses was inoculated, differences in the proportion of recombinant progeny were seen, depending on which parental virus was included at a higher concentration. In eggs that were inoculated with 10 times more Serva than A20, a considerable reduction in the proportion of recombinant progeny was seen ($\leq$ 6.5% recombinants), while when 10 times more A20 was inoculated, a reduction in the proportion of recombinants was not detected (37–43.5% recombinants). The ratio of recombinants to parental viruses (Serva and A20 combined) was similar

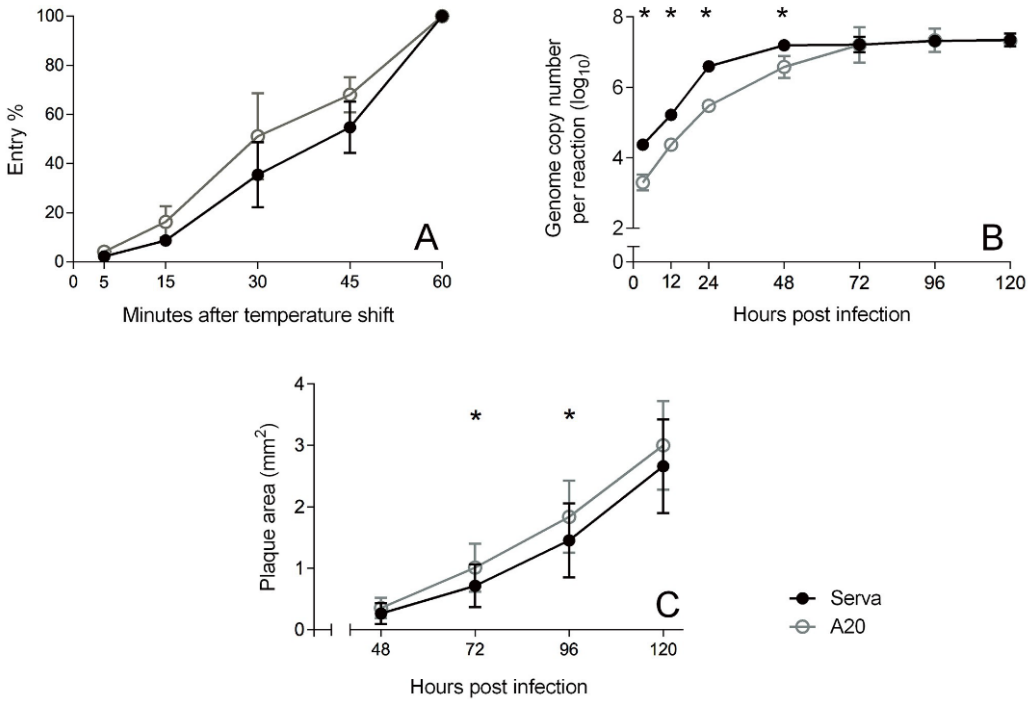


Fig 1. Growth kinetics of Serva (closed circles) and A20 (open circles) strains of ILTV in CEK cells. (a) Entry kinetics of Serva and A20 in CEK cells. The mean and standard deviation from 3 independent experiments are shown. No significant difference between the two different viruses was detected at any time point (b) Multi-step growth curve of Serva and A20 in CEK cells infected with a MOI of 0.001. The mean and standard deviation of triplicate measurements performed in each of 3 independent experiments are shown; * indicates $p < 0.05$ (Student's *t*-test). (c) Kinetics of plaque development by the Serva and A20 strains of ILTV in CEK cells. The mean and standard deviation of the plaque area (mm^2) of 13 to 22 individual plaques at each time point are shown; * indicates $p < 0.05$ (Student's *t*-test).

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regardless of the site of recovery (CAM or AF) (Fig 2). The genotype pattern codes of the recombinant progeny detected in these co-infected eggs are shown in Fig 3.

Co-infection of CEK cells

The influence of the ratio of the parental viruses on the proportion of parent and recombinant viruses after simultaneous co-infection was also examined in CEK cells. Recombinant progeny were detected in all experiments, regardless of the ratio of each infecting parental strain. In these experiments, the highest proportion of recombinant progeny (50%) was detected after co-infection with a 1:1 ratio of the two viruses, and the proportion decreased when the ratio of parental viruses was less balanced (4:1 or 1:4). Regardless of which parental virus was in excess, the proportion of recombinant progeny was similar at 18.5–20% (Fig 2). The genomic patterns of the recombinant progeny detected after CEK co-infection are shown in Fig 4.

Superinfection of CEK cells

A co-infection control was included in this experiment to enable a direct comparison (Fig 2). The profile of the progeny viruses from the co-infection control was notable for the absence of

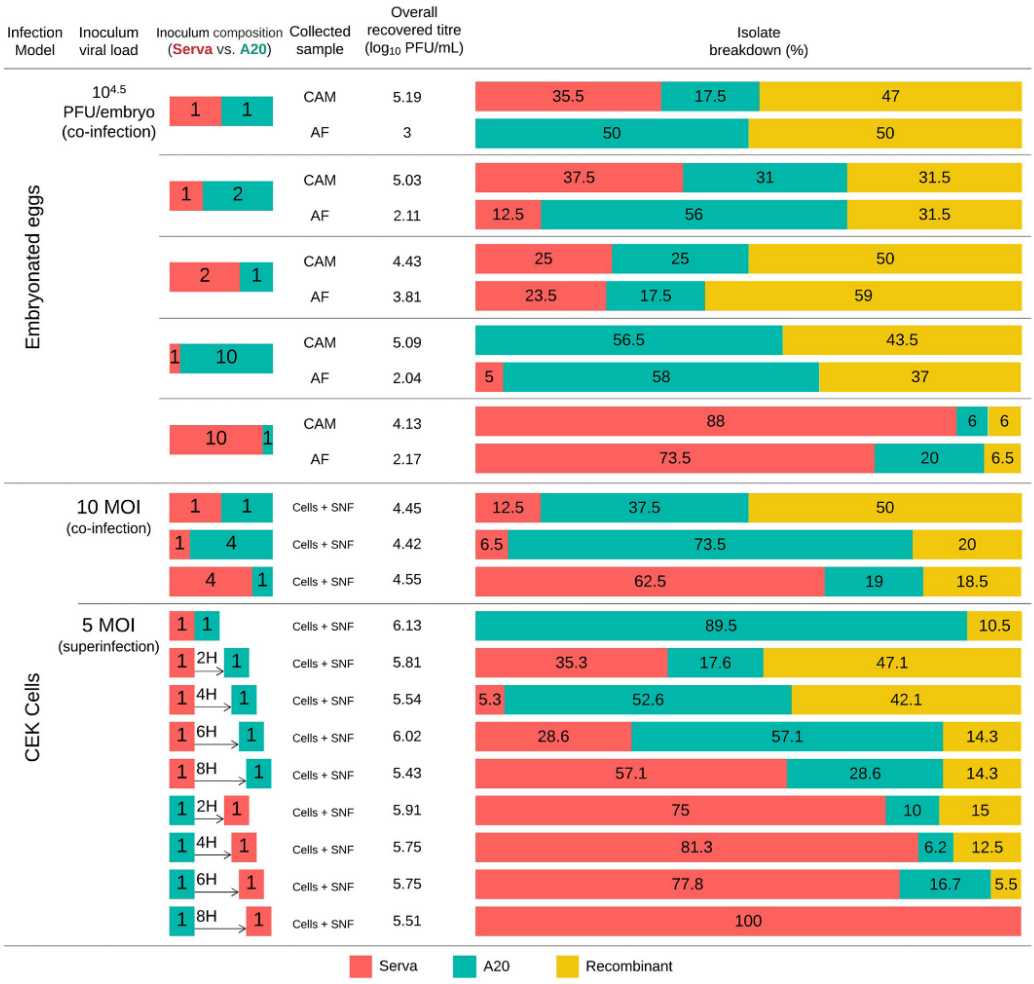


Fig 2. Summary of results from co-infection and superinfections *in ovo* and in CEK cultures. Proportions of parental strains or recombinants in both the inoculum and progeny viruses are shown as red (Serva), green (A20), or yellow (recombinant) blocks. The isolates from co-infection/superinfection experiments on CEK cells were obtained from a combination of the cell lysate and the supernatant fluid (Cell + SNF) from the infected CEK monolayers. The isolates from the co-infection experiment in embryonated eggs were obtained from CAM homogenates or AF, which were collected separately. The overall titre of virus recovered from each infection condition is also shown.

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any detectable Serva strain among the progeny, with 89.5% of the progeny the A20 parent and 10.5% recombinants.

The MOI of 5 used in these experiments should have been sufficient to infect >95% of the cells according to the predictions based on the Poisson distribution [34], so the presence of any genomic region derived from the second virus was considered an indication that superinfection had occurred. The results of the superinfection experiments showed that ILTV-infected cells can be superinfected with a second ILTV strain. The second infecting strain was detected

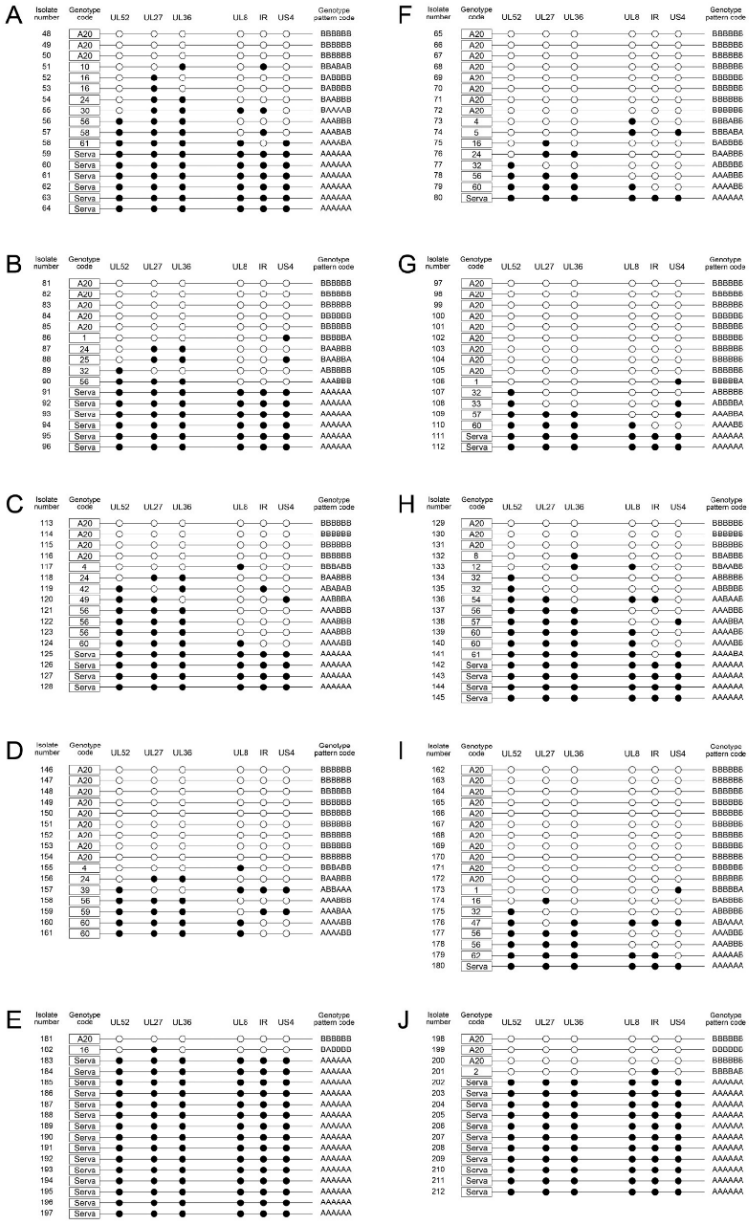


Fig 3. Schematic view of SNP distribution, showing the composition of the progeny population collected from the CAM (left column = A, B, C, D and E) and AF (right column = F, G, H, I and J) of co-infected chicken embryos 72 hours after inoculation. Five eggs were inoculated per group and the viruses were isolated from 3 or 4 eggs (pooled samples) that did not show any signs of embryonic death. The panels represent each ratio of infecting parent viruses (Serva:A20) as 1:1 (A, F), 1:2 (B, G), 2:1 (C, H), 1:10 (D, I) or 10:1 (E, J). The closed circles indicate Serva–like SNPs and

open circles indicate A20 like SNPs. Each row represents a virus that was genotyped as A20, Serva, or a recombinant with a unique genotype code (1 to 62) for each genotype pattern code.

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among the progeny virions in all cases, even when the second inoculation occurred 8 hours after the first (Fig 2). Recombinant progeny were detected in all superinfections, except when cells were superinfected with the Serva strain eight hours after primary inoculation with A20. However, as all the progeny virions were the second infecting strain (Serva), the observations were still consistent with successful superinfection. The highest proportion of recombinant progeny was detected when there was a 2- to 4-hour gap between infections, with Serva as the initial infecting virus (42.1 to 47.1%). The genomic patterns of the recombinant progeny detected after CEK co-infection are shown in Fig 5.

Genotype pattern of the recombinant progeny

With few exceptions (Figs 3E, 3J and 5H), different infection conditions resulted in isolation of recombinant viruses representing more than one genotype. The recombinants detected in these studies had 33 unique genotypes based on the HRM analyses (Fig 6). Of these 33 genotypes, 17 were detected only once, while 16 were isolated on more than one occasion, either within the same group or from different co-infection conditions. In CEK cells, the most abundant recombinant genotype pattern among the progeny was genotype code 60 (AAAABB), while in eggs genotype code 56 (AAABBB) was the most abundant. In general, isolates derived

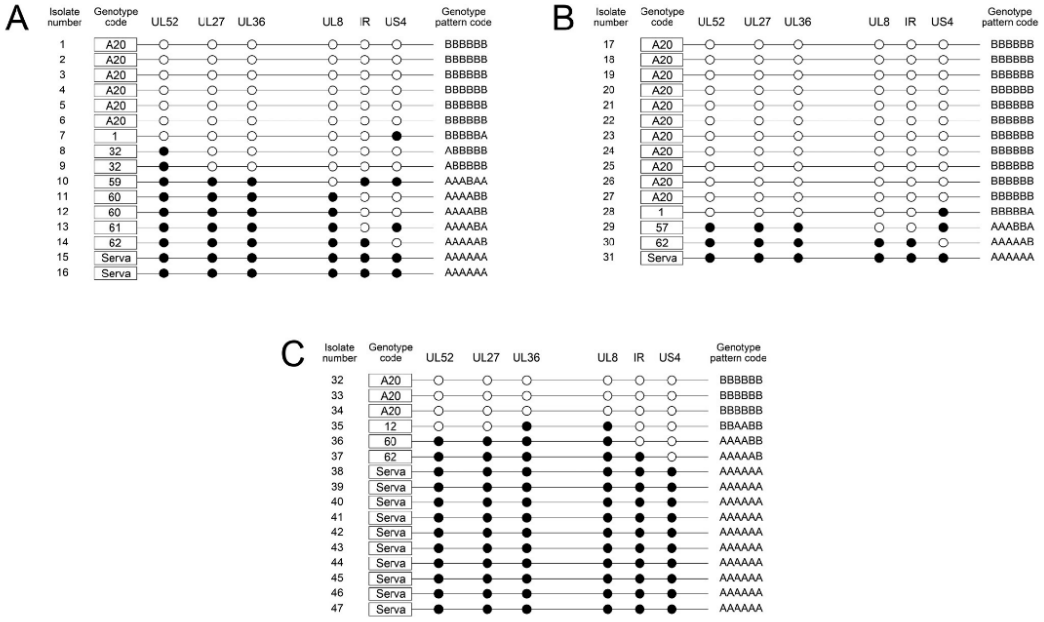


Fig 4. The composition of the progeny population collected from CEK cells co-infected with A20 and Serva ILTV at a total MOI of 10. The distribution of Serva-like (closed circles) and A20-like (open circles) SNPs in each isolate is shown. The isolates were collected from (A) 1:1 co-infection, (B) 1:4 (1 part Serva, 4 parts A20) co infection, and (C) 4:1 (4 parts Serva, 1 part A20) co infection.

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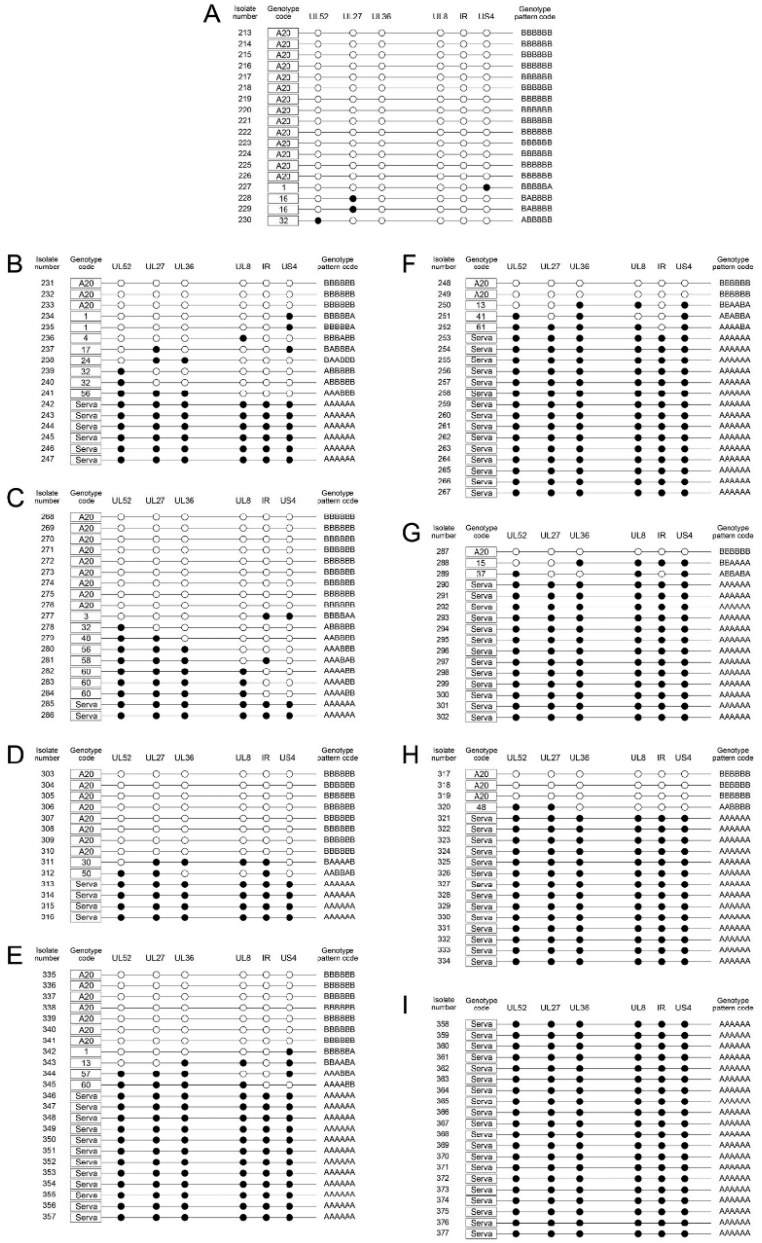


Fig 5. Schematic view of SNP distribution showing the composition of the progeny population collected from CEK cells (A) co-infected at a MOI of 5 with Serva and A20, or (B-I) superinfected with either Serva or A20. The left panels (B, C, D, E) show primary infection with Serva, while the right panels (F, G, H, I) show primary infection with A20. The incubation period between primary and secondary infection was 2 (B, F), 4 (C, G), 6 (D, H) or 8 (E, I) hours. The samples were collected at 72 hpi. The closed circles indicate Serva-like SNPs and open circles indicate

A20 like SNPs. Each row represents a virus that was genotyped as A20, Serva, or a recombinant, with a unique genotype code number (1 to 62) for each genotype pattern code.

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from multiple recombination events were recovered less frequently than those that were consistent with a single recombination event.

Full genome sequencing of progeny isolates

Eight progeny viruses were selected for full genome sequence analysis to more finely map the recombination events. Viruses were chosen for sequencing based on their genotype pattern code and differences in plaque sizes in CEK cells (Table 3). These included 3 isolates with the genotype pattern code AAABBA (isolates 29, 109 and 138) that had differing plaque sizes and 4 isolates with different genotype pattern codes and a range of plaques sizes derived from the superinfection experiments (isolates 237, 238, 319 and 358). Two isolates were also selected that had genotype pattern codes identical to the parental strains (isolate 319 was A20-like and isolate 358 was Serva-like by HRM). Isolate 319 formed significantly smaller plaques than the A20 parent, but the Serva-like candidate 358 formed plaques that were similar in size to those formed by the Serva strain (Table 3).

The full genome sequences of the 8 isolates were mostly consistent with results from the HRM genotyping assay, with each of the 8 viruses having the same SNPs that were identified by HRM, with one exception.

Isolates 29, 109 and 138 (genotype pattern code AAABBA). The US4 region of isolate 29 was Serva-like in the full genome sequence analysis, not A20-like, as detected by the HRM analysis. This event was miscategorised because of a different change in the amplicon that resulted in a change in the T_m of the amplicon to one similar to that seen with amplicons from A20 (Table 4, Isolate 29, position 131365). Therefore, while isolate 29 was classified as AAABBA by HRM analysis, the correct genotype code for this isolate based on the full genome sequence would be AAABBB (see top panel, Fig 7). Similarity plot analyses of the full genome sequences of these isolates detected one further recombination event in isolates 29 and 138,

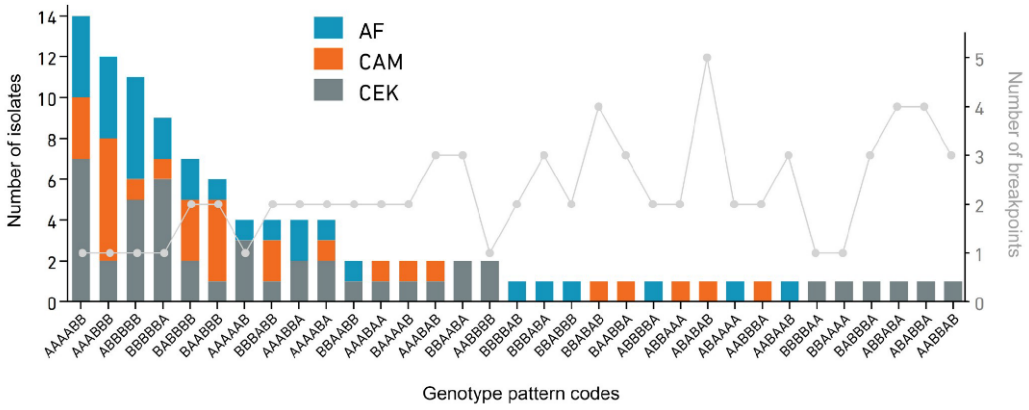


Fig 6. Number of isolates with each genotype pattern code (left y axis) and the origin of each isolate, together with the number of recombination breakpoints on the right y axis. Isolates resulting from multiple recombination events were obtained less frequently. The negative correlation between the number of recombination events in a recombinant virus and the abundance of those recombinants in samples was statistically significant (Spearman's correlation, r_s = -0.55, 95% confidence interval = -0.7579 to -0.2490, two-tailed p value = 0.0008).

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Table 4. Summary of polymorphisms in ILTV isolates sequenced in this study.

Isolate number (genotype pattern code)	Origin of isolate	Original position in sequenced isolate	Gene involved	Polymorphism type	Nucleotide/codon change	Amino acid change
29 (AAABBA)	CEK cells co-infected with 10 MOI (1:4)	10809	UL54	SNP (transition)	ATA–ATG	I–M
		17348	UL51	SNP (transition)	CAC–CAT	None
		55545	UL36	SNP (transition)	CGC–CAC	R–H
		69827	UL39	SNP (transversion)	AAA–AAC	K–N
		75368	UL43	SNP (transition)	ACA–ATA	T–I
		79996	UL20	SNP (transition)	GAT–GAC	None
		131365	US4	SNP (transition)	TCT–CCT	S–P
109 (AAABBA)	AF of 1:2 co-infection	58644	UL36	SNP (transition)	AAG–AAA	None
		109522	UL0	Insertion (tandem repeat)	(C)6–(C)7	Frame Shift
138 (AAABBA)	AF of 2:1 co-infection	58705	UL36	SNP (transition)	AAG–AAA	None
		59226	UL36	SNP (transition)	GTG–ATG	V–M
		120488	Intergenic	SNP (transversion)	A–C	n/a
		131933	US4	SNP (transition)	ACG–GCG	T–A
157 (ABBAAA)	CAM of 1:10 co-infection	15128	UL52	SNP (transition)	TGC–TGT	None
		72497	UL41	SNP (transversion)	GCA–GAA	A–E
237 (BABBBA)	CEK cells re-inoculated w/ A20 2 hours after Serva inoculation	109582	UL0	Insertion (tandem repeat)	(C)6–(C)7	Frame Shift
238 (BAABBB)	CEK cells re-inoculated w/ A20 2 hours after Serva inoculation	26366	ORFB CDS	SNP (transversion)	CAA–CAT	Q–H
319 (BBBBBB)	CEK cells re-inoculated w/ Serva 6 hours after A20 inoculation	21656	UL46	SNP (transition)	GCC–GCT	None
		90066	UL15	SNP (transition)	ACA–GCA	T–A

Each recombinant isolate was aligned with the Serva and A20 genomes and variations* unique to the isolate, and not present in either parent, are shown.

* Variations from A20 and Serva genomes were called using "Find variations/SNPs" tool within Geneious (version 11.1.4).

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but not in isolate 109 (Fig 7). Isolate 29 has a significantly smaller plaque size than the 2 other isolates (109, 138) with the AAABBA genotype pattern code (Table 3) and compared to the parental sequences, each of these isolates had a range of SNPs in the core genes that resulted in disruptive frame shifts and amino acid changes (Table 4).

Isolates 157, 237, 238. Full genome sequencing was consistent with the HRM recombinant genotyping of these viruses and no further recombination events were detected by Simplot analysis of these recombinant viruses. A small number of changes were detected in these isolates compared to the parental sequences (Table 4 and Fig 7).

Isolate 319. This isolate was sequenced because it had the same HRM genotype pattern code as the A20 parent but formed significantly smaller plaques in CEK cells. The full genome sequence was consistent with the HRM genotyping, and the genome sequence of isolate 319 was identical to that of A20, except for 2 SNPs. Only one of these SNPs resulted in a nonsynonymous mutation, from T_{195} to A_{195} in the UL15 gene product. This encodes the DNA packaging terminase subunit 1, which is the ATPase domain of the large terminase subunit [32]. While T_{195} is not a conserved amino acid among all herpesvirus UL15 homologues, it is conserved among the UL15 homologues in each of the 40 full-genome sequences of ILTV currently available in public repositories.

Discussion

Recombination is a common event in ILTV infections [12, 14, 16, 17] and the results of this study support and extend previous studies by demonstrating the resilience of ILTV

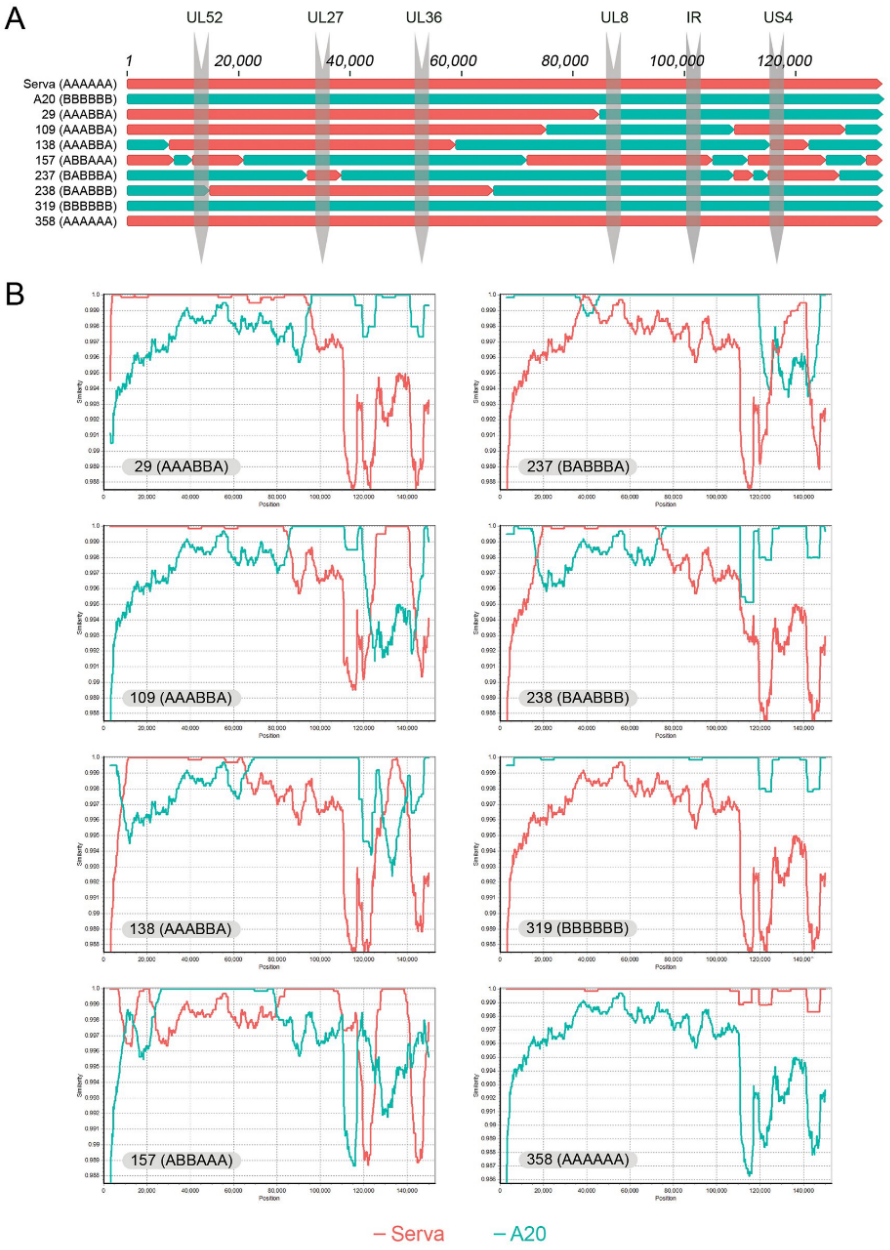


Fig 7. Schematic view of the genomic composition of the isolates sequenced in this study (A) and sequence similarity plots (B) of isolate #29, #109, #138, #157, #237, #238, #319 and #358 with those of Serva and A20. The plots were generated using SimPlot v3.5.1 set to scan the sequences with 6000 bp sliding windows, which were moved across the alignments in 200 bp steps.

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recombination under a broad range of infection conditions. Here we have shown that the A20 and Serva vaccine strains can recombine in both CEK cells and chicken embryos, generating intertypic recombinants under a wide range of conditions. Recombination was detected whether or not there was an imbalance in the ratio of parental viruses in both *in vitro* and *in ovo* co-infection models and could also be detected when there was a significant (up to 8 hour) delay between the primary and secondary infections. These findings are consistent with prior studies showing the production of a high proportion of recombinant progeny under both laboratory and field conditions by a range of different ILTV strains [12, 16].

In most cases, optimal conditions for recombination appeared to occur when equal amounts of both parental viruses were inoculated, with a delay after inoculation of the A20 strain of 4 hours or less. Under these conditions, 42 to 50% of the progeny viruses were genotyped as recombinants. The ratio of viruses that were typed as parental strains in the progeny virus population rarely reflected the composition of inoculum. For instance, no Serva-like virus was isolated from the samples that were collected from the AF of eggs inoculated with equal amounts of the Serva and A20 strains. Analysis of a much larger number of progeny viruses would be required to determine if any Serva parent could be detected among this population, however the detection of recombinants shows that while infection by Serva has occurred, this appears to have been outcompeted by A20 and the recombinant viruses.

The presence of any recombinant viruses is indicative of co-infection or superinfection by the second strain. In this study, both parental strains clearly had considerable capacity to superinfect CEK cells and superinfection occur up to 8 hours after the first virus had been inoculated, for either of the viruses. When there was an 8-hour delay and the Serva strain was the superinfecting virus, no recombinant viruses were detected and Serva was the only type detected in the progeny. Alphaherpesviruses have been shown to be able to prevent a cell being infected by a second virus (superinfection exclusion) [35], but this has not been studied previously for ILTV. Superinfection exclusion is considered to occur mainly through a receptor interference mechanism involving the expression of glycoprotein D (gD) [36–38], but has also been shown to occur through gD-independent mechanisms [39]. In herpes simplex virus 1 and pseudorabies virus (PRV), superinfection exclusion occurs by 2 hours after infection and does not coincide with the expression of gD in infected cells. The timing of gD translation in ILTV has not been studied, but transcripts are detected as early as 1 hpi and it has been classified as an early/late gene [40]. The coding sequence of gD in Serva differs from that of A20 by seven SNPs. Six of these 7 SNPs are non-synonymous. The high number of non-synonymous mutations in this gene, despite the similarities in superinfection exclusion by these two strains seen in the study described here, might indicate that this gene does not have a crucial role in controlling superinfection. In this study, superinfection occurred up to 8 hours after primary infection, so it appears that superinfection exclusion is unlikely be a significant factor in ILTV infection. Other alphaherpesviruses that are controlled using live attenuated vaccines (e.g. PRV and bovine herpesvirus-1) also have the capacity to establish superinfection *in vitro* or *in vivo*, and to generate recombinant viruses that cannot be effectively controlled using the conventionally used attenuated vaccines [41–44].

The generation of recombinant ILTV viruses in the field has resulted in the spread of viruses with increased fitness [12, 14, 15]. The range of infection conditions in this study generated recombinant viruses with 33 unique genotype pattern codes. The analysis of the genotype pattern codes highlighted the strong negative correlation between the number of recombination events in an isolate and the abundance of those isolates. As shown in Fig 6, the most abundant genotype pattern codes were those that resulted from a single recombination event (single recombinants). Isolates with genotype codes indicating multiple recombination

events were rarely isolated more than once, consistent with results from prior recombination studies on other ILTV strains [16], and on bovine herpesviruses [45].

Recombination could help to maintain relatively high diversity among populations of ILTV in different host environments. Full-genome sequence analysis was used to analyse recombination events in finer detail. These analyses supported the results of the HRM assay and identified additional variations that were not detected in the HRM assay (Table 4). These variations included SNPs, insertions/deletions and substitutions. Some of these led to protein sequence changes (Table 4), which could potentially affect the growth of the virus at most stages of viral replication cycle. The isolates with an identical HRM assay type (genotype code 57) had significantly different plaque sizes (Table 3), which may be a result of multiple point mutation differences between these isolates, as listed in Table 4. In addition, one isolate that was detected as a parent-like (A20-like) virus (isolate 319) had a significantly different plaque size compared to the reference A20 strain. This isolate had only one non-synonymous change, in a region that codes for a protein involved in packaging of DNA (UL15).

The existence of field viruses that are recombinants between similar vaccine strains [12] is consistent with the ability of ILTV to establish co-infection and superinfection *in vitro* and *in ovo*, although this has yet to be tested experimentally in chickens. This resilience further establishes the importance of strict biosecurity and the application of good vaccination practice on poultry farms, in particular the importance of use of only one type of vaccine per flock. The finding that a relatively high proportion of commercial layer birds are shedding ILTV vaccine at any given time [46] is a strong indication that ILTV vaccine strains will be important in the evolution of ILTV in the field. Recombination could occur after infection of vaccinated chickens with wild-type strains of ILTV following a biosecurity breach; or the infection of vaccinated chickens with reactivated latent ILTV following the stress of re-vaccination. The use of multiple vaccines could further enhance the genomic diversification of ILTV in these scenarios. Future investigations are required to help determine the behaviour of ILTV when infecting the natural host.

Supporting information

S1 Table. Recombination breakpoint analysis of ILTV isolates sequenced in this study, generated by RDP4 software.
(DOCX)

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Writing – review & editing: Joanne M. Devlin, Glenn F. Browning, Paola K. Vaz, Carol A. Hartley.

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Chapter 4. Superinfection with and recombination of infectious laryngotracheitis virus vaccines in the natural host

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Chapter 4

Superinfection with and recombination of infectious laryngotracheitis virus vaccines in the natural host

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Chapter 4

Summary

Infectious laryngotracheitis virus (ILTV, *Gallid alphaherpesvirus 1*) causes severe respiratory disease in chickens and has a major impact on the poultry industry worldwide. Live attenuated vaccines are widely available and are administered early in the life of commercial birds, often followed by one or more rounds of revaccination, generating conditions that can favour vaccine recombination. Better understanding of the factors that contribute to the generation of recombinant ILTVs, including the survival and transmission characteristics of the recombinant viruses, will inform the safer use of live attenuated herpesvirus vaccines. This study aimed to examine the parameters of infection that allow superinfection and may enable the generation of recombinant progeny. In this study, specific-pathogen free (SPF) chickens were inoculated with two genetically distinct live-attenuated ILTV vaccine strains with 1 to 4 days separating the first and second vaccinations. After infection, viral genomes were detected in tracheal swabs in all groups, with the lowest concentrations detected in swabs collected from chickens in which the interval between inoculations were 4 days. Superinfection of the host was defined as the detection of the virus that was inoculated last, and this was detected in tracheal swabs from all groups. Virus could be isolated from swabs at a limited number of time-points, and these further illustrated superinfection of the birds as recombinant viruses were detected among the progeny. This study has demonstrated superinfection at both the cellular and host level and is consistent with our previous findings that recombination events occur under a very broad range of infection conditions. The occurrence of superinfection after unsynchronised infection with multiple viruses, and subsequent recombination, highlight the importance of using only one type of vaccine per flock.

Chapter 4

Introduction

Infectious laryngotracheitis (ILT) is an acute viral upper respiratory tract disease of chickens, pheasants, and peafowl [1]. The clinical signs can range from conjunctivitis, nasal discharge, dyspnoea, and lethargy to haemorrhagic tracheitis, gasping, coughing, and expectoration of bloody mucus [1]. Several genotypes of ILTV have been identified using various methods, including polymerase chain reaction combined with restriction fragment length polymorphism analysis (PCR-RFLP) [2-5], PCR followed by high-resolution melting (HRM) curve analysis [6], and sequencing of the whole genome [7-12] or of targeted regions of the genome [13-16]. Genetically distinct attenuated vaccine strains of ILTV (Serva, GenBank accession number HQ630064; A20, GenBank accession number JN596963; and SA2, GenBank accession number JN596962) have recombined in the field and formed several novel and virulent recombinant viruses [17] that have become the dominant ILTV genotypes across several poultry producing areas in Australia [18]. It has been hypothesised that vaccination of the same chickens with different vaccine strains at different times resulted in these recombination events [17] and that recombination might be enabled by latent infections with ILTV vaccine strains [19]. Reactivation of ILTV from latency as a result of environmental stressors (for instance, a second round of vaccination) could hypothetically create the opportunity for both coinfection and superinfection, infection with more than one virus at the same time, and secondary infection of a cell or animal that is already infected. ILTV can both co-infect [20, 21] and superinfect chicken cells *in vitro* [Fakhri *et al.*, in press]. When a single cell is infected with two different strains of virus at the same time, the genome of these viruses can recombine to create diverse recombinant viral progeny [22].

Some ILTV vaccination protocols recommend more than one round of vaccination to achieve a high level of immunity [23]. In some cases, different strains of live attenuated ILTV vaccines may be used at different stages in the life of a flock. When birds are younger, they may be vaccinated with highly attenuated vaccines. Less attenuated vaccines may then be used when the birds are older to induce greater durations of immunity [23]. The use of multiple strains of vaccine in the same bird can contribute to genomic recombination between different strains of ILTV [17].

The potential for infection of birds with two vaccine viruses administered asynchronously with extended delays between the primary and secondary inoculations and the effect that the period between the primary and secondary inoculations can have on superinfection, co-infection and recombination between the ILTV vaccines are important to understand. *In vivo* investigation of these interactions can help to inform the development of vaccination protocols that minimise the risk of genomic recombination. The current study aimed to investigate the potential for superinfection and ILTV recombination to occur in the host by performing primary and secondary vaccinations separated by varying time-intervals (TI) in specific-pathogen-free (SPF) chickens.

Materials and Methods

Experimental infection and sample collection

This study was performed with the approval of the Animal Ethics Committee of The University of Melbourne (Approval No. 1714128.1) following the Australian Code for the Care and Use of Animals for Scientific Purposes [24]. One hundred and twenty 21-day-old specific-pathogen-free (SPF) chickens (SPF Australia, Woodend) were randomly allocated

into separate groups housed in 8 Horsfall-Bauer-type isolators maintained under negative pressure using HEPA-filtered air and provided with sterilized food and water *ad libitum*. The animals were vaccinated twice with two different vaccines (NOBILIS ILT (Serva) and Poulvac Laryngo A20). The vaccines were administered to each bird via both ocular (½ commercial dose in 30 µL of vaccine diluent) and intratracheal (½ commercial dose in 300 µL of vaccine diluent) routes at both vaccination times. Depending on the group, there was a TI of 1, 2, 3 or 4 day(s) between vaccinations. The chickens in Groups one to four were vaccinated with Serva vaccine first and received the A20 vaccine second, while the chickens in Groups five to eight received the A20 vaccine first and Serva vaccine second (Table 1). Vaccines were prepared by reconstituting a freeze-dried pellet of each vaccine in commercial vials using the recommended vaccine diluent (Sterile diluent 2B8720, Merial Select). One thousand commercial doses of each vaccine were prepared and stored in 1 mL aliquots at -80ºC until use. On vaccination days, the prepared inocula were transferred on ice to the isolators. To ensure the transport of the vaccines did not compromise the viability of the viruses and to ensure the correct dose was delivered, excess inocula were transferred back to the laboratory for retention dose titration in chicken embryo kidney cells (CEKs), as described previously [25]. These samples were kept on ice at all times. The animals were monitored three times a day for any clinical signs of disease or any adverse events. Five age-matched naïve chickens were introduced to each isolator four days after the second vaccine was administered, at the time at which the peak of replication of the second virus was expected [26, 27]. Adding naïve birds at this time-point would be expected to provide an opportunity for transmission of any newly generated viruses to these in-contact chickens.

Tracheal swabs were collected using sterile swabs with cotton heads and aluminium shafts (COPAN Diagnostics, USA) every 48 hours, starting at 2 days after the second vaccination. The swabs were placed in 15 mL centrifuge tubes (BD Falcon) containing 1 mL of viral transport medium (Dulbecco's modified Eagle medium with 1% v/v foetal bovine serum, 50 μg ampicillin/mL, 50 μg gentamicin/mL and 5 μg amphotericin B/mL). On day 8 after the second vaccination, birds were euthanised by exposure to halothane. Necropsies were performed, tracheal swabs were taken, and upper tracheal tissue sections were collected in 10% v/v neutral buffered formalin solution (Sigma-Aldrich) for histopathological examination. Tracheal sections were stained using haematoxylin and eosin, lesions were scored as described previously [28], and the means of three scores were reported.

Quantification of ILTV in tracheal swabs

Following the collection of samples in the animal facility, tracheal swabs were transferred to the laboratory on ice. The tubes containing the swabs were vortexed individually and the swabs were then discarded. The medium (~ 900 μL) was divided into 3 equal volumes. One volume was retained as an individual bird sample and stored at -80°C, one was used for DNA extraction and quantification of virus, and the third was pooled with other samples from the same group of birds collected at the same time-point. Samples from in-contact birds were pooled separately. Samples from chickens that had to be euthanised or that died suddenly prior to the planned time for euthanasia were collected separately and were not pooled with the samples from rest of the birds in that group. Nucleic acid extraction was performed using the PureLink® Pro 96 viral RNA/DNA purification kit (Invitrogen, USA) and the X-tractor automated vacuum system (Corbett, Australia). Purified DNA from 200 μL of the extract from each swab was eluted in 200 μL of pyrogen-free water (Milli-Q®

Integral system, Germany) and stored at -20°C until it was tested. Viral genome copy numbers (GCN) were quantified using a UL15-specific qPCR, as described by Mahmoudian *et al.* [29] with one modification – the SYBR Green dye was replaced with SYTO™ 9 green fluorescent nucleic acid stain (ThermoFisher, Australia). This PCR detected both the A20 and Serva strains of ILTV.

Differentiation of parental viruses by indel PCR

Superinfection of each animal was determined by detection of the virus in the secondary inoculum in any tracheal swab. Primers were designed to differentiate the Serva and A20 strains by targeting a 32 bp insertion/deletion site in the unique long region of the ILTV genome. In this reaction, a single pair of primers (forward, 3'-ACTTCCACTGACCGGCCTA-5', and reverse, 3'-GTGTGCAGCAGTGAACTTGG-5') were designed to amplify a 156 bp product from strain A20 (nucleotides 4,591 to 4,746 of GenBank accession JN596963) and a 124 bp product from Serva ILTV (nucleotides 4,363 to 4,486 of GenBank accession HQ630064). PCR products from tracheal swabs were differentiated by electrophoresis through 2% w/v agarose gels and visualisation using SYBR Safe (Invitrogen) and a ChemiDoc MP imaging system (Bio-Rad, Australia).

Identification and genotyping of ILTV isolates from tracheal swabs

Samples of the tracheal swab extract (~300 μL) at each time-point from each bird in a group were pooled, vortexed and stored at -80°C. Three dilutions (undiluted, diluted 1 in 2, and diluted 1 in 10) of each sample pool were cultured in CEK cells and 20 well-isolated plaques were picked from each pool. These viruses were further purified through three rounds of plaque purification, as described previously [20]. DNA extracted from plaque

purified isolates was genotyped using the HRM-based multiple-SNP genotyping assay [6] to enable these isolates to be classified as parental or recombinant viruses.

Statistical analysis

Microsoft Excel was used for $\log_{10}$ transformation of GCNs and GraphPad Prism version 8.3.1 for Windows (GraphPad Software, San Diego, California USA) was used to perform statistical analyses. The comparisons of viral GCNs were performed using one-way analysis of variance in conjunction with Tukey's multiple comparisons test. The tracheal histopathology scores were compared using the Kruskal-Wallis test and Dunn's multiple comparisons test. Comparisons of proportions of chickens in which superinfection was detected were performed using Fisher's exact test. Two-tailed p values ≤ 0.05 were considered significant.

Results

Viral replication and tracheal histopathology

Figure 1 shows the mean GCN of ILTV in individual tracheal swabs from the directly inoculated birds in each group. Significantly ($p < 0.05$) lower concentrations of virus were detected at the first sampling time-point in birds that had a four-day gap between the primary and secondary inoculations than in the birds in the groups in which the gap between inoculations was one or two days (Figure 1D). Figure 2A shows the GCNs of ILTV in the tracheas of all chickens on the day of necropsy, with the mean $\log_{10}$ concentration of ILTV similar in directly inoculated chickens across all groups ($p > 0.05$). The GCNs in the tracheas of seven chickens that were euthanised or died suddenly before completion of the experiment are also shown. Four of these chickens (red symbols

encircled in the oval in Figure 2A) were examined individually for detection of recombination events.

In-contact birds were added 4 days after the second inoculation and were exposed to the inoculated birds for 4 days in total. These birds were sampled only once, at the last sampling time-point of each group. Figure 2B shows the GCNs of ILTV detected in these birds at this time-point. ILTV genome was detected in most of the in-contact birds in all groups except Group 4 (in-contact with chickens that were vaccinated with Serva strain first and strain A20 4 days later) (Figure 2B). Birds in contact with birds in the groups with the shortest interval between vaccinations (1 day) had significantly higher GCNs of ILTV than birds in the groups with longer intervals between vaccination (Figure 2B; G1 versus G3, G4, G7 and G8).

No significant difference ($p > 0.05$) was detected between the median scores for histopathological lesions in upper tracheal sections of the different groups (Table 1). Back titration of the reserved inoculum demonstrated that the expected concentration of each vaccine was maintained during transport (data not shown).

Superinfection was detected in all groups

An indel PCR capable of differentiating the two ILTV strains used in this study was used to detect ILTV superinfection in chickens. This PCR was applied to DNA extracted from each swab from each bird at each time-point to detect and differentiate each of the viruses that were inoculated. Superinfection of the host was considered to have been detected when the indel PCR could detect the vaccine strain that was inoculated second. The number of birds found to be superinfected varied between groups and sampling time-points. At least one

chicken was detected with superinfection in all groups (Figure 3). The highest proportions of superinfected birds were in groups in which the administration of the vaccines was separated by 1 day (Figure 3). To investigate the effect of the order of vaccination on superinfection, the proportions of chickens that were superinfected in Group 1 at each time-point were compared to the proportions of chickens that were superinfected in Group 5 at the same time-point. A significant difference was only detected at the first sampling time-point (Fisher's exact test, $p < 0.05$) (Figure 3). In general, there was less superinfection as the interval between vaccinations increased, except for the birds in group 8 (Figure 3B; TI=4 days, inoculated with A20 then Serva) in which superinfection was detected at all time-points.

Recombination of ILTV occurs in superinfected chickens

While superinfection could be detected by applying the indel PCR directly to DNA extracted from the tracheal swabs, detection of recombinant viruses required virus isolation and plaque purification. Viruses could be isolated and purified from Groups 1, 2, 3, 5 and 6 at one or two sampling time-points (Figure 4) but virus could not be isolated from Groups 4, 7 or 8 at any time-point of or from any of the in-contact birds (data not shown). Isolated and plaque purified viruses were subjected to PCR-HRM analysis targeting six regions of the viral genome in order to detect recombination. Between 18 and 22 plaques were isolated and purified from pooled tracheal swab samples (one pool was prepared per group per time-point). Recombinant viruses were detected in Groups 1, 3, 5 and 6 (Figure 4). The abundance of recombinant viruses ranged from 0% (Figure 4C and 4E) to 23.8% (Figure 4F). The highest number of recombinants were detected at the second sampling time-point

(4 days after receiving the second vaccine) in groups in which the time interval between the two vaccinations was shortest (Figure 4B and 4F).

The HRM assay on pooled samples showed that only isolates with the Serva strain profile were detected in Group 5 at the first sampling time-point (2 days after receiving Serva as the second vaccine) (Figure 4E). However, at a later time-point (4 days after receiving Serva as the second vaccine, Figure 4F), the A20 strain predominated (Figure 4F).

Severe clinical signs of ILT and mortalities

The survival rate of the chickens in this experiment ranged from 80 to 100%, with birds that were culled because of severe clinical signs regarded as mortalities. Seven of 120 chickens were euthanized or died suddenly prior to the end of the experiment (red symbols, Figures 1 and 2A). These chickens were all directly inoculated. Five of these seven chickens had a higher ILTV GCN at the time of euthanasia and presented with more severe clinical signs of ILT than the other birds in the same group. To further investigate the ILTV viruses in these chickens, 24 to 29 plaque-purified isolates from each chicken were analysed using the HRM assay. These viruses could be isolated from four of five of the chickens that showed severe signs of ILT (Figure 2A, encircled). A considerable proportion of the viruses isolated from these birds were recombinants (3.7% to 37.5%) (Figure 5). Ten different recombination patterns were detected among these isolates. One of these recombination patterns (genotype 13) was consistently isolated from all four chickens (Figure 5, isolates number 519, 548, 549, 550, 584 and 601—all highlighted with grey shading).

Discussion

Unless birds are deliberately and simultaneously vaccinated with two distinct attenuated vaccine strains, perfectly synchronized co-infection of birds with two vaccine strains is unlikely to occur under field conditions. The design of the experiment described here may simulate field conditions in which vaccinated flocks accidently become infected with a second vaccine strain after primary vaccination (for example, by spread of vaccine virus from a nearby farm) or where flocks are deliberately re-vaccinated using a different vaccine strain. In both scenarios recombination could occur during primary replication of the vaccine viruses in the host, or potentially after reactivation of latent vaccine virus [17]. The influences of the TI between primary and secondary infection with different strains of ILTV, and the order in which different strains are inoculated, have previously been studied using cell culture techniques. These previous studies detected a limited capacity to prevent superinfection (i.e. superinfection exclusion) in cell culture (Fakhri *et al.*, 2020). In the study described here, the TIs between primary and secondary infection were chosen to mimic the time required for a latent virus to reactivate and traffic to the site of active replication. Studies on other neuroinvasive herpesviruses from the sub-family *Alphaherpesvirinae* - herpes simplex virus type 1 (HSV-1) and pseudorabies virus (PRV) - have reported an average axonal transport velocity of approximately 1 $\mu m.s^{-1}$ following infection [30]. At this speed, the reactivated virus would require approximately 2 hours to reach the conjunctiva (approximately 1 cm) and 40 hours to reach the trachea (approximately 15 cm between the trigeminal ganglia and the middle of trachea). Therefore, a series of time intervals, ranging from 24 to 96 hours, were tested in this study.

Both vaccines could establish superinfection in chickens under all the conditions tested (Figure 3). Similarly, a range of studies on other alphaherpesviruses, including HSV-1 [31], human cytomegalovirus (HCMV) [32] and Marek's disease virus (MDV, *gallid herpesvirus 2*, *GaHV-2*) [33, 34], have also reported the frequent occurrence of superinfection in the natural host. The studies on the superinfection of chickens with different MDV strains have similarly evaluated the effect of short and long TIs between the first and second inoculations on superinfection, and showed that a significantly higher proportion of chickens were superinfected at shorter TIs (approximately 55% of chickens, TI=1 day) than at a longer TI (approximately 5%, TI=13 days) [33]. The same pattern was seen in the study described here, with significantly higher numbers of chickens were detected as superinfected in groups that had the shortest TI between inoculations (1 day) than in groups with a longer TI (4 days; Figure 3).

Recombination was also more commonly detected when the TI between vaccinations was the shortest (1-day interval, Figure 4B and 4F). The recombination detected in the current study could have been a result of either superinfection or co-infection of cells because of the ongoing replication of both vaccine strains, regardless of the TI between inoculations. This is consistent with our previous *in vitro* studies using the same two ILTV vaccine strains in primary chicken embryo kidney cells, which showed that superinfection and generation of recombinants occurred up to 8 hours after the first virus had been inoculated, irrespective of which strain was inoculated first (Fakhri *et al.*, 2020). Similarly, in another *in vitro* study, TIs of 2 to 6 hours between successive inoculations of Madin-Darby bovine kidney (MDBK) cells with different strains of bovine herpesvirus 1 (BoHV-1) resulted in superinfection and generation of recombinants [35]. Under *in vitro* superinfection

conditions, it is possible to infect the entire cell population at a high multiplicity of infection and remove or inactivate the primary virus before addition of the secondary inoculum. Therefore, the formation of recombinants can be confidently attributed to superinfection, rather than co-infection. In contrast, in the natural host, it is not possible to infect all permissive cells synchronously or to remove extra-cellular viruses from the tissue, so it is likely that cells would be infected stochastically [34]. Studies on superinfection with MDV in chickens have faced a similar challenge, although the presence of two distinguishable viruses in the same cell was confirmed using sequencing techniques or differential expression of fluorescent proteins [33, 34]. Intravital imaging techniques may be helpful in future investigations in order to confirm the occurrence of superinfection at the cellular level by enabling accurate determination of the exact time of virus entry into each cell.

Previous studies have shown that some recombinant viruses can have enhanced levels of virulence [36]. Analysis of the viruses isolated from birds with severe clinical signs during this study have shown that: (i) at least one recombinant was isolated from all the birds with severe disease; (ii) one specific genotype (genotype code 13) was common to each of these birds; and (iii) one of the euthanized chickens in Group 5 (Figure 5C) had the highest proportion of recombinants observed in this study (9 recombinants out of 24 isolates), as well as the highest diversity of recombination patterns (9 different genotypes). The increased severity of clinical signs could be due to the higher virulence or fitness of newly formed recombinant viruses in these chickens, which can be further investigated by comparing the *in vivo* growth kinetics and transmissibility of these recombinant progeny. The isolation of a recombinant virus (isolate number 495, Figure 4F) with an SNP pattern

identical to that of a virulent field virus (Class 8 ILTV, GenBank accession number JN804826) that was formed by recombination between attenuated ILTV vaccines [17] will enable hypotheses about ILTV evolution to be tested, although this experimentally generated recombinant needs to be compared with the field isolate using whole-genome sequencing and phylogenetic analysis.

The decreasing concentrations of ILTV detected over the time course of the experiment (Figure 1) is consistent with the self-limiting nature of infection with ILTV vaccine strains [26, 27] and virulent field isolates [36] after inoculation with a single strain. After experimental infection, ILTV starts replicating in the epithelial cells of the upper respiratory tract, peaking at 4-6 days after inoculation [3, 37]. The concentration of ILTV in the trachea declines after day four and ILTV is cleared completely by day 20-28 after inoculation [26]. In the study described here, the first samples were collected 2 days after the second inoculation, which was between 3 days (Groups 1 and 5) and 6 days (Groups 4 and 8) after the first virus was inoculated. Without exception, each group had the highest concentrations of ILTV in the trachea at this first sampling time-point (Figure 1). Some differences in viral replication and transmission were associated with the order of vaccination. A lower concentration of ILTV was seen when Serva was used as the first vaccine (Figure 1), except at one time-point (Figure 1B, day 8 of experiment). This difference in concentration was significant at two time-points (asterisks in Figure 1). The reason for this is not clear, but it could be related to differences in the replication patterns of these two vaccines, as has been demonstrated for other strains of ILTV [27, 38].

Transmission of ILTV to in-contact chickens was significantly higher in birds that were exposed to birds with the shortest interval between vaccinations when Serva was used as

the first vaccine (Group 1, Figure 2B). The greater transmission in Group 1 was consistent with the high viral titre detected in directly inoculated birds in this group. However, greater level of transmission was not seen in groups that received A20 first (Figure 2B). The reason for this difference is unclear, but it has been shown previously that different genotypes of ILTV may have different patterns of transmission and reach peak concentrations in the trachea at day 6 to 8 after exposure to inoculated chickens [21, 27] and the birds in this study were only monitored up to day 4 after exposure.

In this study, the second vaccine was inoculated into a different host environment to the first vaccine, because of the immune response and tracheal tissue damage initiated by the first vaccine [38-40]. Several studies have shown that the changes to the tracheal epithelium following ILTV infection range from minimal pathology with scattered focalized intranuclear inclusion bodies at day one post infection to complete structural destruction of the epithelium by day five post infection [38, 40]. This loss of epithelial cells in the trachea may be an explanation for the significant reduction in concentration of ILTV when the TI between vaccinations increased from 1 to 4 days, as the intact epithelium may allow ample viral replication of the second vaccine when it is delivered 1 or 2 days after the primary vaccination, and the reduced number of epithelial cells limiting the replication of the second vaccine when it is delivered 4 days after primary vaccination.

The role of the host immune response in inhibiting viral replication also needs to be considered. Immune responses to ILTV can be differentiated into immediate innate inflammatory responses and prolonged adaptive immune responses, through both cell-mediated and humoral components of the immune system [39, 41]. Innate immune responses observed at early time-points of infection include production of inflammatory

cytokines, chemokines and interleukins, including chicken CXC ligand 2 (chCXCLi2), interleukin (IL)-1β, IL-10 and type I interferon (IFN-α), as well as infiltration of leukocytes into infected tissues [40, 42]. These can be detected as early as 4 days after inoculation [39, 40, 43]. Similarly, upregulation of IFN-γ production in the trachea can be detected as early as 4-5 days post infection in naïve birds [40, 43]. These responses may be induced by the first vaccination and affect the replication of the second vaccine, particularly when the TI between the first and second vaccination is longer. Further studies to investigate the immunological responses during superinfection are required to better understand their influence on viral replication.

The results of this study accord with our observations from previous *in vitro* and *in vivo* studies [21, 44-46]. Analyses of ILTV field strains [18, 36, 47] have shown how frequently recombination occurs in ILTV, presumably under a very broad range of infection conditions. The widespread occurrence of superinfection after unsynchronised infection with multiple virus, and subsequent recombination, highlight the importance of following good vaccination practice on poultry farms, and in particular the importance of using only one type of vaccine per flock.

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Table 1. Histopathology scores of birds after direct inoculation with Serva and A20 ILTV vaccines with a time-interval between vaccinations, or after contact with dual vaccinated birds.

Group	1st inoculum	2nd inoculum	Days between inoculations	Days between 1st or (2nd) inoculation and collection of tracheal samples[1]	Median tracheal histopathology scores (range)[1]	
					Inoculated	In-contact (4 days post exposure)
1	Serva	A20	1	9 (8)	2.5 (2–4)	0 (0–5)
2	Serva	A20	2	10 (8)	3 (1–5)	1 (0–5)
3	Serva	A20	3	11 (8)	2 (1–5)	0 (0–5)
4	Serva	A20	4	12 (8)	2 (1–5)	0 (0–1)
5	A20	Serva	1	9 (8)	3 (1–5)	5 (0–5)
6	A20	Serva	2	10 (8)	2 (1–5)	1 (0–2)
7	A20	Serva	3	11 (8)	2.5 (1–5)	5 (0–5)
8	A20	Serva	4	12 (8)	1.5 (1–2)	0 (0–5)

[1] No significant difference was detected using Kruskal-Wallis tests and Dunn's multiple comparisons test ($p > 0.05$).

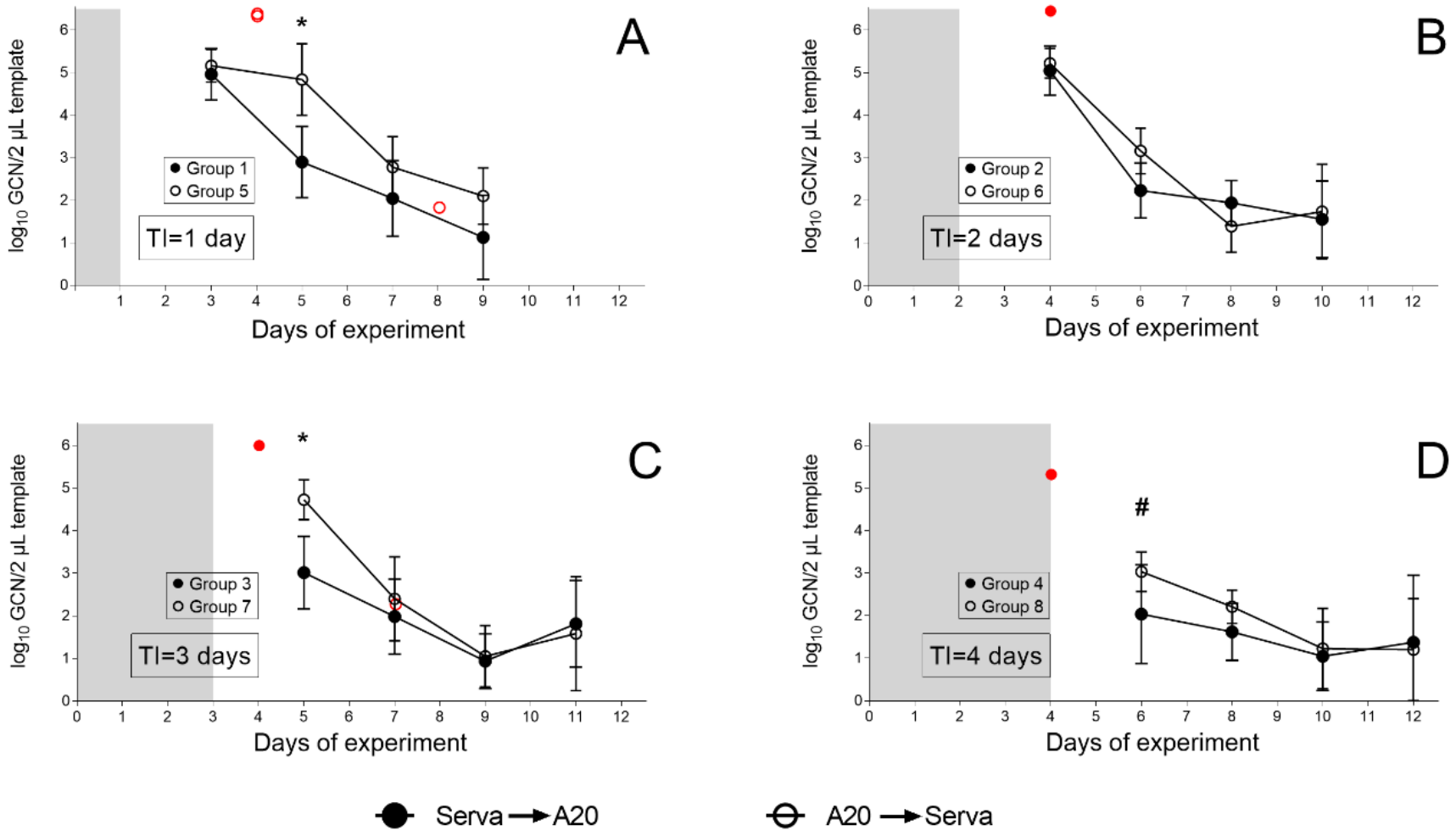

A
B
C
D
log10 GCN/2 µL template
Days of experiment
Group 1
Group 5
TI=1 day
Group 2
Group 6
TI=2 days
Group 3
Group 7
TI=3 days
Group 4
Group 8
TI=4 days
*
*
#
Serva → A20
A20 → Serva

Figure 1. GCNs of ILTV in tracheal swabs collected from directly inoculated birds. The time-interval (TI) between the first and second vaccination in each group is shown by the grey shading. The secondary vaccine was administered with a TI of (A) 1, (B) 2, (C) 3 or (D) 4 day(s) following the primary vaccination by both ocular and intra-tracheal routes. The closed circles indicate groups that were inoculated with Serva strain first and strain A20 second, while the open circles indicate groups inoculated with strain A20 first and Serva strain second. Mean GCNs of 10 chickens are plotted, with error bars indicating the standard deviation. Asterisks indicate time-points at which the GCN concentrations differed significantly between the two groups ($p < 0.05$, one-way ANOVA and Tukey's multiple comparisons test). Single data points in red indicate the GCNs of ILTV in chickens that were euthanised or died suddenly before the experiment end-point (the sample collection time-point for these chickens may differ from that of the rest of their group).

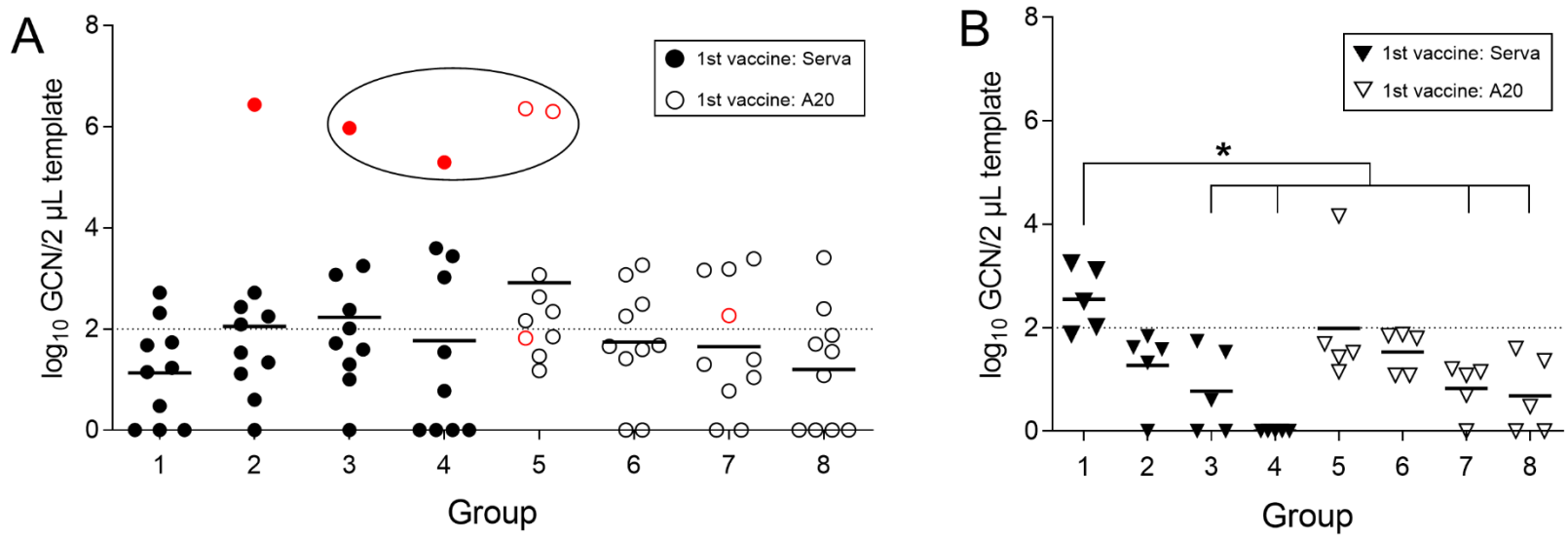


Figure 2. Abundance of ILTV genome in final tracheal swabs of (A) directly inoculated birds and (B) in-contact birds 8 days after secondary inoculation. Each closed circle represents one chicken inoculated with Serva vaccine first and A20 vaccine second, while open circles represent chickens vaccinated with A20 vaccine first and Serva vaccine second. Triangles represent in-contact chickens. Horizontal lines in both panels indicate the mean values and the asterisk in panel (B) indicates values that were significantly different (one-way ANOVA and Tukey's multiple comparisons test; $p < 0.05$). The red symbols represent the chickens that were euthanised or died suddenly before the completion of the experiment. Dotted lines indicate the lower limit of detection for this qPCR (< 100 genome copies) [29]. Samples below this were regarded as negative.

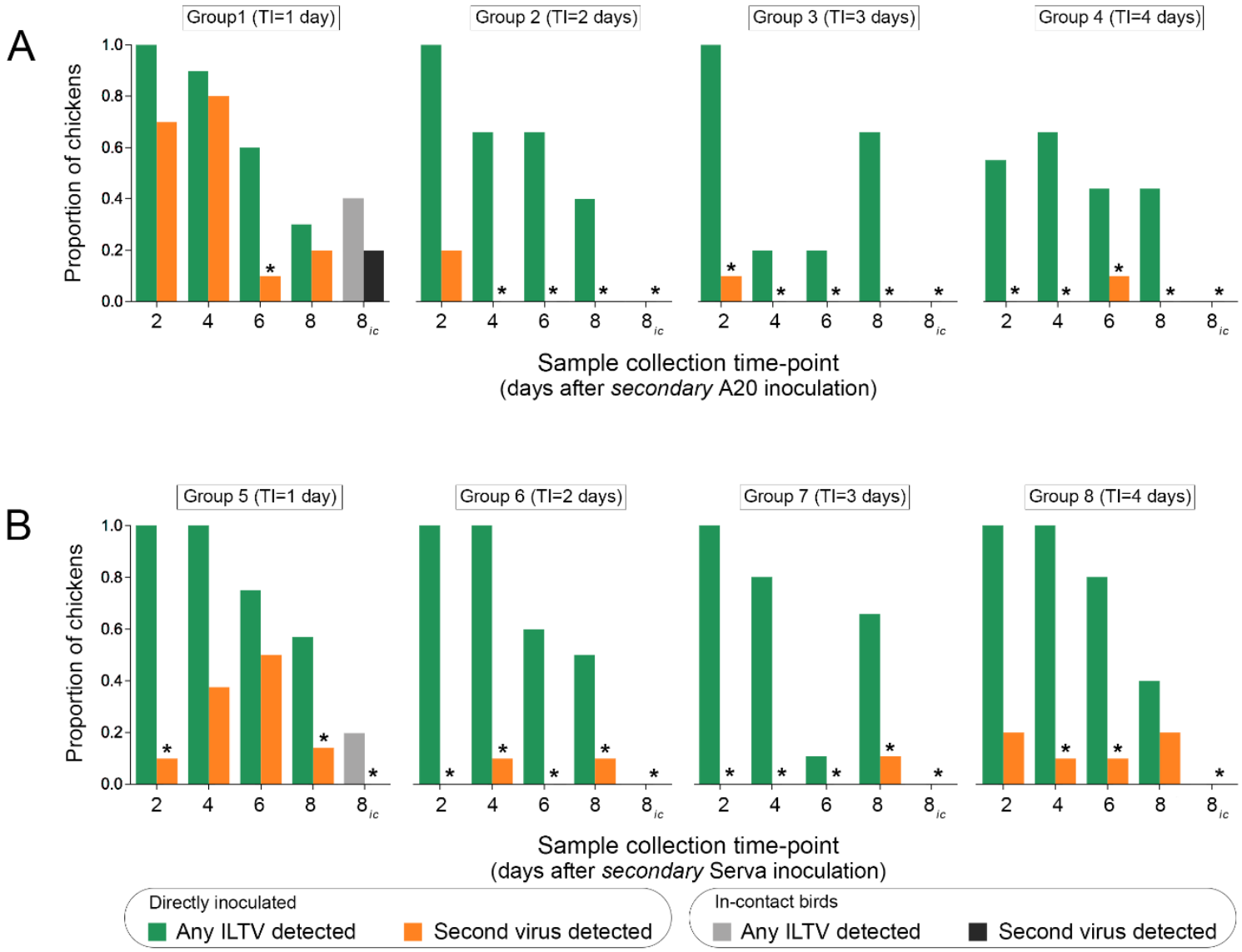

A
Group1 (TI=1 day)
Group 2 (TI=2 days)
Group 3 (TI=3 days)
Group 4 (TI=4 days)
Proportion of chickens
1.0
0.8
0.6
0.4
0.2
0.0
2
4
6
8
8 ic
Sample collection time-point
(days after secondary A20 inoculation)
B
Group 5 (TI=1 day)
Group 6 (TI=2 days)
Group 7 (TI=3 days)
Group 8 (TI=4 days)
Proportion of chickens
Sample collection time-point
(days after secondary Serva inoculation)
Directly inoculated
Any ILTV detected
Second virus detected
In-contact birds
Any ILTV detected
Second virus detected

Figure 3. Proportion of birds in which superinfection was detected. The UL indel PCR assay, which differentiates the Serva and A20 strains, was applied to DNA extracted from individual tracheal swab samples from directly inoculated and in-contact birds. Each column indicates the proportion of birds in that group in which any ILTV or the secondary inoculum was detected. Panel (A) shows the results for groups that received the Serva strain first, while panel (B) shows the results for groups that received the A20 strain first. The proportions for the in-contact birds (n=5) are shown separately (8_{ic}). Groups marked with an asterisk had a significantly lower proportion of chickens in which superinfection was detected than in Group 1 at the first sampling time-point (Fisher's exact test, $p < 0.05$).

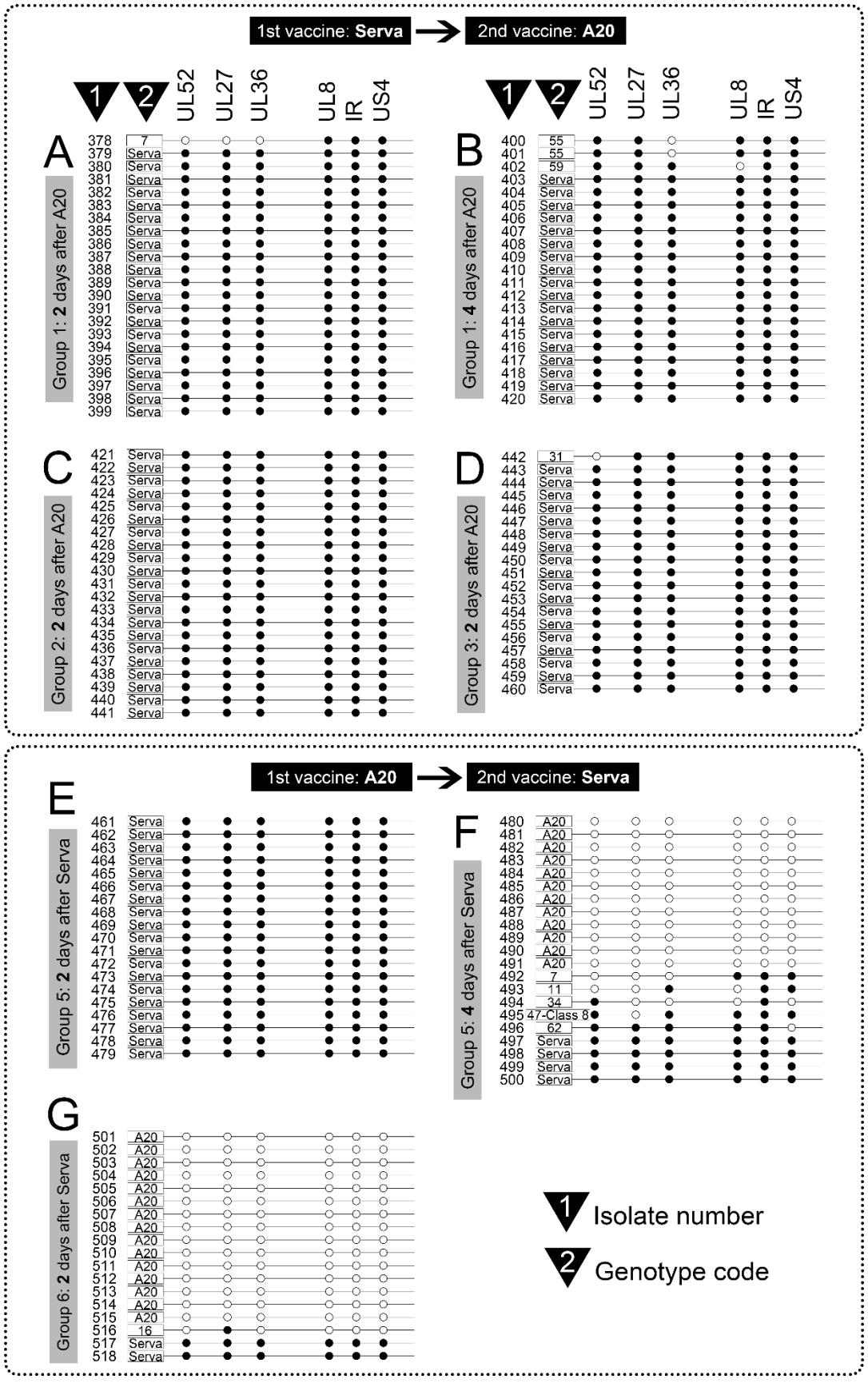

1st vaccine: Serva
2nd vaccine: A20
1st vaccine: A20
2nd vaccine: Serva
1 Isolate number
2 Genotype code
UL52
UL27
UL36
UL8
IR
US4
A
Group 1: 2 days after A20
B
Group 1: 4 days after A20
C
Group 2: 2 days after A20
D
Group 3: 2 days after A20
E
Group 5: 2 days after Serva
F
Group 5: 4 days after Serva
G
Group 6: 2 days after Serva

Figure 4. Schematic view of SNP patterns of individual viruses isolated from tracheal swabs collected from chickens inoculated with Serva and A20 ILTV. Each string of connected circles represents one isolate. The closed circles represent Serva-like SNPs and the open circles represent A20-like SNPs detected using the HRM curve analysis (six genomic markers were tested for each isolate). The viruses in panels A-D were isolated from chickens vaccinated with Serva first and A20 second, while those in panels E-G were isolated from chickens vaccinated with A20 first and Serva second. Only samples from Groups 1 (two time-points, A and B), 2 (C), 3 (D), 5 (two time-points, E and F) and 6 (G) could be analysed using this method as viruses could not be cultured from the other groups and time-points.

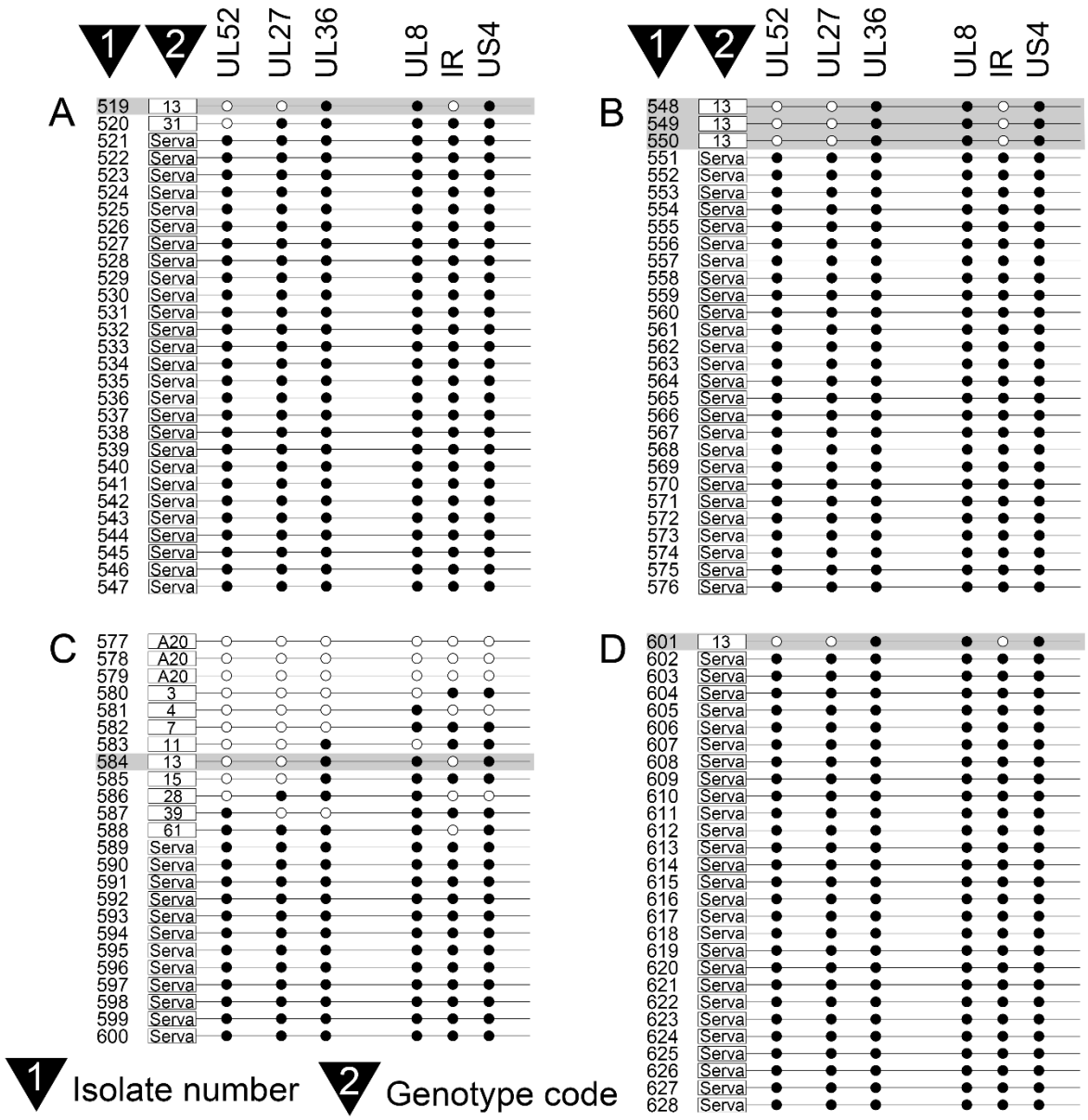


Figure 5. Schematic view of SNP patterns of individual viruses isolated from tracheal swabs collected from chickens with severe clinical signs of ILT. These chickens were from three different groups. Chicken A was from Group 3, chicken B was from Group 4, and chickens C and D were from Group 5.

Chapter 5. General discussion

The studies described in this thesis examined several infection conditions to determine whether it is possible to influence recombination by merely changing the quantity of viruses involved or the timing of multiple infections. After reviewing the literature and considering the advantages and disadvantages of existing ILTV recombinant detection methods, a novel cost- and time-efficient method was developed to use in this study. This was based on high-resolution melting curve analysis and could reproducibly detect all known ILTV recombinant strains (Fakhri *et al.*, 2019). This method was also applicable to the classification of vaccine and wild-type strains of ILTV and has been applied successfully to detection of another recently detected field recombinant of ILTV in Australia (Sabir *et al.*, 2019).

During this study, the high-resolution melting curve analysis method was used to investigate the genetic composition of progeny viruses that were produced after multiple ILTV infections in a range of infection models (*in vitro*, *in ovo* and *in vivo*). The results from these experiments, as discussed in the third and fourth chapters of this thesis, showed that recombination between ILTV vaccine strains occurs across a broad range of conditions and that recombinant viruses were generated under all of the conditions tested. These findings are consistent with prior studies demonstrating that a high proportion of progeny viruses were recombinant after co-infection with a range of different ILTV strains under both laboratory and field conditions (Lee *et al.*, 2012, Loncoman *et al.*, 2017b). Although previous work has shown that live vaccines can reduce the prevalence of recombination (Loncoman *et al.*, 2018a, Loncoman *et al.*, 2018b), the level of genomic recombination

observed in the all infection systems investigated in this study highlighted the fact that live vaccines appear unable to completely prevent ILTV recombination. Given that vaccination against ILTV relies on live vaccines, regular monitoring using tools similar to the HRM-based method described in the Chapter 2 or full genome sequencing may improve the assessment of these risks.

The other interesting finding in this study was the capacity of ILTV to superinfect permissive cells and the natural host of ILTV, chickens. Unsynchronized infection with two different strains can also result in recombination of ILTV. This finding would be helpful in explaining the detection of natural recombination under field conditions, as unless birds are deliberately vaccinated with two distinct attenuated vaccine strains, it would be unlikely that closely synchronized co-infection with two vaccine strains would occur in the field. The superinfection study was designed to infect the natural host with extended delays between the inoculations with the primary and secondary virus strains to mimic the time that prototypic alphaherpesviruses, such as HSV-1, require for reactivation from latency and transport to sites of lytic infection. The shortest time-interval tested (1 day) resulted in the highest level of host superinfection and recombinant generation which was similar to the results of superinfection studies shown for HSV-1, PRV or MDV (Antinone and Smith, 2010).

Genomic recombination helps to generate diversity in the genome of ILTV by placing different mutations along the genome in novel combinations, which may improve the fitness of the virus (Loncoman *et al.*, 2018a, Loncoman *et al.*, 2018b). For instance, a virus with improved transmissibility can be considered more fit than the parental strains if it can outcompete the parental strains and become dominant in a population, as has been reported

for field recombinants of ILTV in Australia (Agnew-Crumpton *et al.*, 2016, Lee *et al.*, 2013, Lee *et al.*, 2012, Lee *et al.*, 2015). These viruses, which resulted from recombination between vaccine strains, grew to higher titres in cell culture and embryonated eggs, and could cause more severe tracheal pathology and greater weight loss than previously known field strains (Lee *et al.*, 2015). Similarly, the studies described in this thesis found that recombinant ILTVs can have enhanced growth rates in cell culture. These advantages could result from *de novo* mutations, in addition to recombination. In the studies described in this thesis, ILTV was shown to have the capacity to accumulate *de novo* mutations after co-infection in cell culture or in chorioallantoic membranes. These mutations included SNPs, insertions/deletions and substitutions that resulted in protein sequence changes and could potentially explain the differences between recombinant ILTVs in their cell-to-cell spread *in vitro*. The link between *de novo* mutations and recombination remains to be determined in future studies.

The continued widespread use of live attenuated herpesvirus vaccines emphasises the need to improve our understanding of the factors that contribute to the evolution of herpesviruses. In addition to ILTV, live attenuated vaccines are also used to control other herpesviruses, including VZV (Takahashi *et al.*, 1974), PRV (Luo *et al.*, 2014) and BoHV-1 (Castrucci *et al.*, 2002), and herpesviruses, such as herpesvirus of turkeys (HVT), are also used as vectors to deliver vaccine antigens from other pathogens (Loncoman *et al.*, 2017c, Kapczynski *et al.*, 2015). Pseudorabies virus (PRV) is controlled with a live attenuated vaccine, Bartha-K61 (Luo *et al.*, 2014), which has recombined with endemic strains and formed new variants (Ye *et al.*, 2016). The Bartha-K61 vaccine cannot induce protection against this new variant of PRV (Luo *et al.*, 2014). VZV is the only human herpesvirus

controlled using live attenuated vaccines (Norberg *et al.*, 2006) and has a long history of natural genomic recombination (Peters *et al.*, 2006, Norberg *et al.*, 2015). Although recombination events have been detected in this virus, currently there is no evidence to show that vaccine strains of VZV have recombined with wild-type strains (Norberg *et al.*, 2015). The potential for ongoing recombination in this virus and thus for evolution of novel virulent strains, should be monitored as vaccination with live VZV vaccines continues.

The studies described in this thesis were focused on investigating the occurrence of recombination in those members of the ILTV population that could replicate in primary cell cultures. Although this approach has it merits, as it analyses the infectious (transmissible and growable) progeny, it also has limitations as it does not analyse the whole population of progeny viruses. Culture and purification of the entire population of viruses is constrained by the limitations of available culture techniques, and even if it was possible to culture and purify all the virions, analysis of defective virus particles would still be excluded. Therefore, future research on the analysis of recombination in ILTV should include methods that could analyse the entire viral population, such as single molecule real-time (SMRT) sequencing techniques. These platforms have the potential to be low-cost, low-input, high-throughput alternatives to the methods used previously for recombinant analysis. The recent advances in these sequencing approaches have been helpful in analysing entire viral populations in environmental and clinical samples (Sauvage *et al.*, 2018, Warwick-Dugdale *et al.*, 2019). A similar approach may shed some light on the genomic dynamics of ILTV during and after recombination events and offer new perspectives on recombination in this virus. Single-molecule sequencing methods may also provide us with a clearer understanding of the genomic regions that are more common sites

of recombination and perhaps the role different genetic elements in the recombination process and how they regulate it. The current challenge is that the error rate of high-throughput, long-read sequencing technologies (from both PacBio and Oxford Nanopore) ranges between 5 and 10% (mainly insertion-deletion errors) (Weirather *et al.*, 2017). Therefore, these technologies alone do not provide a sufficient level of accuracy to confidently identify SNPs in closely related viruses such as the strains used in the studies described here (which had 99.2% nucleotide sequence identity). This is especially challenging in mixed viral populations, but a combination of data from more accurate short-reads with these long-reads may be useful in addressing this issue.

The combination of findings presented in this thesis provides some support for the premise that genomic recombination of ILTV vaccines happens frequently and that novel variants of this virus can emerge via both recombination and *de novo* mutation. The approaches to control of ILTV need to accommodate the constant generation of variants of this virus, and the potential for vaccines to contribute to generations of these variants.

The studies described in this thesis have enhanced our knowledge about genomic recombination between ILTV vaccines. Vaccines have been used to help control infectious laryngotracheitis for many decades but a number of limitations have been recognised including, most recently, the potential for vaccine recombination (Coppo *et al.*, 2013a, García, 2017). The outbreaks of ILT and emergence of novel virulent strains of ILTV in Australia despite vaccination changed the narrative about control and management of this disease (Lee *et al.*, 2012). Prior to the emergence of novel virulent ILTV strains in the field, the strategies to control the disease would focus on finding a balance between the protective immunity and the level of pathogenicity induced by different vaccines, so the use of

multiple vaccines with different levels of pathogenicity (and hence inducing differing durations of immunity) was justified (Fulton *et al.*, 2000). Genomic analyses of the novel ILTV strains demonstrated the role of recombination between two attenuated vaccine strains in the genesis of these outbreaks and raised concerns around the concurrent use of different live vaccines in flocks (Lee *et al.*, 2012). Further investigations revealed a long history of genomic recombination between ILTV strains circulating in poultry farms and suggested scenarios underlying these events (Lee *et al.*, 2013). Recombination between ILTV genomes requires concurrent replication of two strains of ILTV in the nucleus of an infected cell. To date, there is no evidence of the ILTV genome recombining with other viral species, so the scenarios proposed to explain ILTV recombination are limited to multiple infection with different strains of ILTV - intraspecies recombination (Lee *et al.*, 2013, Loncoman *et al.*, 2017c). Vaccination against ILTV plays a central role in potential scenarios that result in the replication of two ILTV strains in the same cell. An example of a scenario that can result in recombination is when, after a biosecurity breach, wild-type strains of ILTV infect vaccinated chickens, or when the stress of re-vaccination reactivates a latent ILTV infection in vaccinated chickens. Under these circumstances, two strains of ILTV can have the opportunity to infect a host cell at the same time, and their genomes may recombine during this co-infection. The finding that a relatively high proportion of commercial layer birds are shedding ILTV vaccine at any given time (Thilakarathne *et al.*, 2019b) is a strong indication that vaccine strains of ILTV can be important in recombination and subsequent evolution of ILTV in the field.

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Appendices

Appendix 1. Supplementary Table 1 for Chapter 2: Probability and typicality scores for each tested strain/isolate. Generated by ScreenClust software.

					Probabilities[d]		
Reaction #	Sample label[a]	HRM Target region	Cluster[b]	Typicality[c]	Serva-like	A20-like	Mixed
1	Serva	UL52	Serva-like	0.874196	1.0000	0.0000	0.0000
2	Serva	UL52	Serva-like	0.435242	1.0000	0.0000	0.0000
3	Serva	UL52	Serva-like	0.602155	1.0000	0.0000	0.0000
4	A20	UL52	A20-like	0.986018	0.0000	1.0000	0.0000
5	A20	UL52	A20-like	0.822288	0.0000	1.0000	0.0000
6	A20	UL52	A20-like	0.982501	0.0000	1.0000	0.0000
7	Mixed std	UL52	Mixed	0.646321	0.0000	0.0000	1.0000
8	Mixed std	UL52	Mixed	0.646321	0.0000	0.0000	1.0000
9	P1	UL52	Serva-like	0.923305	1.0000	0.0000	0.0000
10	P2	UL52	A20-like	0.490563	0.0000	1.0000	0.0000
11	P3	UL52	Serva-like	0.216705	1.0000	0.0000	0.0000
12	P4	UL52	A20-like	0.265993	0.0000	1.0000	0.0000
13	P5	UL52	Serva-like	0.156532	1.0000	0.0000	0.0000
14	P6	UL52	Mixed	0.682577	0.0000	0.0000	1.0000
15	P7	UL52	A20-like	0.209699	0.0000	1.0000	0.0000
16	P8	UL52	Mixed	0.267606	0.0000	0.0000	1.0000
17	P9	UL52	Serva-like	0.749664	1.0000	0.0000	0.0000
18	P10	UL52	A20-like	0.031533	0.0000	1.0000	0.0000
19	P11	UL52	Serva-like	0.684698	1.0000	0.0000	0.0000
20	P12	UL52	Serva-like	0.45652	1.0000	0.0000	0.0000
21	P13	UL52	Serva-like	0.811807	1.0000	0.0000	0.0000
22	P14	UL52	Serva-like	0.331768	1.0000	0.0000	0.0000
23	P15	UL52	Serva-like	0.347001	1.0000	0.0000	0.0000
24	P16	UL52	Serva-like	0.042221	0.9590	0.0410	0.0000
25	P17	UL52	Serva-like	0.495575	1.0000	0.0000	0.0000
26	P18	UL52	A20-like	0.31982	0.0000	1.0000	0.0000
27	P19	UL52	A20-like	0.583388	0.0000	1.0000	0.0000
28	P20	UL52	Mixed	0.355839	0.0000	0.0000	1.0000
29	Serva	UL27	Serva-like	0.972047	1.0000	0.0000	0.0000
30	Serva	UL27	Serva-like	0.791332	1.0000	0.0000	0.0000
31	Serva	UL27	Serva-like	0.847056	1.0000	0.0000	0.0000
32	A20	UL27	A20-like	0.447492	0.0000	1.0000	0.0000
33	A20	UL27	A20-like	0.895133	0.0000	1.0000	0.0000
34	A20	UL27	A20-like	0.487201	0.0000	1.0000	0.0000
35	Mixed std	UL27	Mixed	0.572407	0.0000	0.0000	1.0000
36	Mixed std	UL27	Mixed	0.572407	0.0000	0.0000	1.0000
37	Mixed std	UL27	Mixed	0.572407	0.0000	0.0000	1.0000
38	P1	UL27	Serva-like	0.675542	1.0000	0.0000	0.0000
39	P2	UL27	A20-like	0.103159	0.0000	1.0000	0.0000
40	P3	UL27	A20-like	0.348297	0.0000	1.0000	0.0000
41	P4	UL27	A20-like	0.290435	0.0000	1.0000	0.0000
42	P5	UL27	Serva-like	0.241356	1.0000	0.0000	0.0000
43	P6	UL27	A20-like	0.74021	0.0000	1.0000	0.0000
44	P7	UL27	A20-like	0.066363	0.0000	1.0000	0.0000
45	P8	UL27	A20-like	0.40193	0.0000	1.0000	0.0000
46	P9	UL27	Serva-like	0.146722	1.0000	0.0000	0.0000
47	P10	UL27	A20-like	0.581781	0.0002	0.9998	0.0000
48	P11	UL27	Serva-like	0.195983	1.0000	0.0000	0.0000
49	P12	UL27	Serva-like	0.05165	1.0000	0.0000	0.0000

50	P13	UL27	Serva-like	0.750359	1.0000	0.0000	0.0000
51	P14	UL27	A20-like	0.290546	0.0000	1.0000	0.0000
52	P15	UL27	Serva-like	0.309781	1.0000	0.0000	0.0000
53	P16	UL27	A20-like	0.263484	0.0075	0.9925	0.0000
54	P17	UL27	Serva-like	0.393293	1.0000	0.0000	0.0000
55	P18	UL27	A20-like	0.454944	0.0000	1.0000	0.0000
56	P19	UL27	A20-like	0.674516	0.0000	1.0000	0.0000
57	P20	UL27	Mixed	0.391625	0.0000	0.0000	1.0000
58	Serva	UL36	Serva-like	0.391854	1.0000	0.0000	0.0000
59	Serva	UL36	Serva-like	0.991152	1.0000	0.0000	0.0000
60	Serva	UL36	Serva-like	0.24128	1.0000	0.0000	0.0000
61	A20	UL36	A20-like	0.863102	0.0000	1.0000	0.0000
62	A20	UL36	A20-like	0.836366	0.0000	1.0000	0.0000
63	A20	UL36	A20-like	0.991428	0.0000	1.0000	0.0000
64	Mixed std	UL36	Mixed	0.981401	0.0000	0.0000	1.0000
65	Mixed std	UL36	Mixed	0.665945	0.0000	0.0000	1.0000
66	Mixed std	UL36	Mixed	0.496606	0.0000	0.0000	1.0000
67	P1	UL36	Serva-like	0.409389	1.0000	0.0000	0.0000
68	P2	UL36	A20-like	0.165872	0.0000	1.0000	0.0000
69	P3	UL36	A20-like	0.711315	0.0000	1.0000	0.0000
70	P4	UL36	A20-like	0.730578	0.0000	1.0000	0.0000
71	P5	UL36	Serva-like	0.609391	1.0000	0.0000	0.0000
72	P6	UL36	A20-like	0.068476	0.0000	1.0000	0.0000
73	P7	UL36	A20-like	0.937196	0.0000	1.0000	0.0000
74	P8	UL36	Mixed	0.266283	0.0000	0.0000	1.0000
75	P9	UL36	Serva-like	0.386923	1.0000	0.0000	0.0000
76	P10	UL36	A20-like	0.168485	0.0000	1.0000	0.0000
77	P11	UL36	Serva-like	0.57955	1.0000	0.0000	0.0000
78	P12	UL36	Serva-like	0.546459	1.0000	0.0000	0.0000
79	P13	UL36	Serva-like	0.697461	1.0000	0.0000	0.0000
80	P14	UL36	A20-like	0.391483	0.0000	1.0000	0.0000
81	P15	UL36	Serva-like	0.131539	1.0000	0.0000	0.0000
82	P16	UL36	A20-like	0.198419	0.0000	1.0000	0.0000
83	P17	UL36	Serva-like	0.28274	1.0000	0.0000	0.0000
84	P18	UL36	A20-like	0.208802	0.0000	1.0000	0.0000
85	P19	UL36	A20-like	0.620022	0.0000	1.0000	0.0000
86	P20	UL36	Mixed	0.270988	0.0000	0.0000	1.0000
87	Serva	UL8	Serva-like	0.90979	1.0000	0.0000	0.0000
88	Serva	UL8	Serva-like	0.90979	1.0000	0.0000	0.0000
89	A20	UL8	A20-like	0.998374	0.0000	1.0000	0.0000
90	A20	UL8	A20-like	0.99121	0.0000	1.0000	0.0000
91	A20	UL8	A20-like	0.991341	0.0000	1.0000	0.0000
92	Mixed std	UL8	Mixed	0.517471	0.0000	0.0000	1.0000
93	Mixed std	UL8	Mixed	0.515335	0.0000	0.0000	1.0000
94	Mixed std	UL8	Mixed	0.772424	0.0000	0.0000	1.0000
95	P1	UL8	A20-like	0.250836	0.0001	0.9999	0.0000
96	P2	UL8	A20-like	0.529484	0.0000	1.0000	0.0000
97	P3	UL8	A20-like	0.733539	0.0000	1.0000	0.0000
98	P4	UL8	A20-like	0.880882	0.0000	1.0000	0.0000
99	P5	UL8	A20-like	0.272311	0.7835	0.2165	0.0000
100	P6	UL8	Mixed	0.403909	0.0000	0.0000	1.0000
101	P7	UL8	A20-like	0.365511	0.0000	1.0000	0.0000
102	P8	UL8	Mixed	0.333997	0.0000	0.0000	1.0000
103	P9	UL8	Serva-like	0.529324	0.9992	0.0008	0.0000
104	P10	UL8	A20-like	0.643233	0.0000	1.0000	0.0000
105	P11	UL8	A20-like	0.13947	0.0000	1.0000	0.0000
106	P12	UL8	A20-like	0.291954	0.0000	1.0000	0.0000
107	P13	UL8	Serva-like	0.547609	0.9997	0.0003	0.0000
108	P14	UL8	A20-like	0.260149	0.0000	1.0000	0.0000

109	P15	UL8	Serva-like	0.177541	0.9999	0.0001	0.0000
110	P16	UL8	A20-like	0.865039	0.0000	1.0000	0.0000
111	P17	UL8	A20-like	0.002252	0.0000	1.0000	0.0000
112	P18	UL8	A20-like	0.565292	0.0000	1.0000	0.0000
113	P19	UL8	A20-like	0.976643	0.0000	1.0000	0.0000
114	P20	UL8	Serva-like	0.198413	0.9979	0.0021	0.0000
115	Serva	IR	Serva-like	0.805854	1.0000	0.0000	0.0000
116	Serva	IR	Serva-like	0.9917	1.0000	0.0000	0.0000
117	Serva	IR	Serva-like	0.924779	1.0000	0.0000	0.0000
118	A20	IR	A20-like	0.981401	0.0000	1.0000	0.0000
119	A20	IR	A20-like	0.992494	0.0000	1.0000	0.0000
120	A20	IR	A20-like	0.999352	0.0000	1.0000	0.0000
121	Mixed std	IR	Mixed	0.612439	0.0000	0.0000	1.0000
122	Mixed std	IR	Mixed	0.448517	0.0000	0.0000	1.0000
123	Mixed std	IR	Mixed	0.666369	0.0000	0.0000	1.0000
124	P1	IR	Serva-like	0.570965	1.0000	0.0000	0.0000
125	P2	IR	A20-like	0.467544	0.0000	1.0000	0.0000
126	P3	IR	A20-like	0.990447	0.0000	1.0000	0.0000
127	P4	IR	A20-like	0.968079	0.0000	1.0000	0.0000
128	P5	IR	Serva-like	0.298821	1.0000	0.0000	0.0000
129	P6	IR	A20-like	0.007253	0.0000	1.0000	0.0000
130	P7	IR	A20-like	0.234166	0.0000	1.0000	0.0000
131	P8	IR	Mixed	0.501741	0.0000	0.0000	1.0000
132	P9	IR	Mixed	0.306662	0.0000	0.0000	1.0000
133	P10	IR	A20-like	0.833773	0.0000	1.0000	0.0000
134	P11	IR	A20-like	0.480631	0.0000	1.0000	0.0000
135	P12	IR	Serva-like	0.133808	1.0000	0.0000	0.0000
136	P13	IR	A20-like	0.702349	0.0000	1.0000	0.0000
137	P14	IR	A20-like	0.350133	0.0000	1.0000	0.0000
138	P15	IR	A20-like	0.623835	0.0000	1.0000	0.0000
139	P16	IR	A20-like	0.730398	0.0000	1.0000	0.0000
140	P17	IR	Serva-like	0.158997	1.0000	0.0000	0.0000
141	P18	IR	A20-like	0.71158	0.0000	1.0000	0.0000
142	P19	IR	A20-like	0.894707	0.0000	1.0000	0.0000
143	P20	IR	A20-like	0.001484	0.0000	1.0000	0.0000
144	Serva	US4	Serva-like	0.993494	1.0000	0.0000	0.0000
145	Serva	US4	Serva-like	0.994084	1.0000	0.0000	0.0000
146	Serva	US4	Serva-like	0.999797	1.0000	0.0000	0.0000
147	A20	US4	A20-like	0.835162	0.0000	1.0000	0.0000
148	A20	US4	A20-like	0.95849	0.0000	1.0000	0.0000
149	A20	US4	A20-like	0.982227	0.0000	1.0000	0.0000
150	Mixed std	US4	Mixed	0.572407	0.0000	0.0000	1.0000
151	Mixed std	US4	Mixed	0.572407	0.0000	0.0000	1.0000
152	Mixed std	US4	Mixed	0.572407	0.0000	0.0000	1.0000
153	P1	US4	Serva-like	0.311066	1.0000	0.0000	0.0000
154	P2	US4	A20-like	0.241988	0.0000	1.0000	0.0000
155	P3	US4	A20-like	0.63635	0.0000	1.0000	0.0000
156	P4	US4	A20-like	0.599478	0.0000	1.0000	0.0000
157	P5	US4	Serva-like	0.612067	1.0000	0.0000	0.0000
158	P6	US4	A20-like	0.745149	0.0000	1.0000	0.0000
159	P7	US4	A20-like	0.731094	0.0000	1.0000	0.0000
160	P8	US4	Mixed	0.565137	0.0000	0.0000	1.0000
161	P9	US4	Serva-like	0.991598	1.0000	0.0000	0.0000
162	P10	US4	A20-like	0.937948	0.0000	1.0000	0.0000
163	P11	US4	A20-like	0.81826	0.0000	1.0000	0.0000
164	P12	US4	A20-like	0.861941	0.0000	1.0000	0.0000
165	P13	US4	Serva-like	0.24958	1.0000	0.0000	0.0000
166	P14	US4	A20-like	0.751248	0.0000	1.0000	0.0000
167	P15	US4	A20-like	0.134296	0.0000	1.0000	0.0000

168	P16	US4	A20-like	0.334642	0.0000	1.0000	0.0000
169	P17	US4	Serva-like	0.192503	1.0000	0.0000	0.0000
170	P18	US4	A20-like	0.109779	0.0000	1.0000	0.0000
171	P19	US4	Serva-like	0.40864	1.0000	0.0000	0.0000
172	P20	US4	Mixed	0.000748	0.0000	0.0000	1.0000

[a] Known (control) samples were labelled as Serva, A20 or Mixed standard (1:1 mixture of Serva and A20). The viruses isolated from progeny population after co-infection were labelled P1 to P20.

[b] Samples are identified as either Serva-like, A20-like or Mixed SNP identity and clustered accordingly.

[c] The typicality measures how well a sample falls within the cluster distribution in which it has been classified.

[d] The probability of each sample fitting into a cluster. The samples are called into the cluster that has the highest probability.

Appendix 2. Supplementary Table 1 for Chapter 3

S1 Table. Recombination breakpoint analysis of ILTV isolates sequenced in this study, generated by RDP4 software

Recombination event number	Isolate number	Exchanged segment position		*p* value of methods by which breakpoint was detected in RDP4								
		In alignment	Relative to Serva	RDP	GENECONV	Bootscan	MaxChi	Chimaera	SiSscan	PhylPro	LARD	3Seq
1	29	928–94394	909–94124	8.21E-98	4.18E-97	1.63E-34	1.73E-42	5.42E-43	NS	NS	NS	1.11E-16
2	109	84978–122578	84709–122265	2.53E-62	1.05E-63	4.70E-55	3.94E-34	8.45E-34	NS	NS	NS	1.11E-16
3	109	144702–152025	144332–151616	5.61E-37	1.52E-49	8.66E-46	6.48E-17	3.12E-16	NS	NS	NS	1.11E-16
4	138	8766–66662	8500–66393	1.81E-67	1.15E-66	1.69E-62	1.04E-30	1.76E-30	NS	NS	NS	1.11E-16
5	138	130265–138154	129939–137798	1.45E-45	1.32E-45	1.48E-45	1.33E-13	1.71E-13	NS	NS	NS	1.11E-16
6	157	23846–81159	23571–80881	4.79E-44	1.68E-39	4.76E-44	6.48E-23	4.49E-23	NS	NS	NS	1.11E-16
7	157	142414–149663	142040–149266	8.01E-22	7.60E-17	3.88E-18	1.03E-18	8.38E-18	NS	NS	NS	1.11E-16
8	157	117468–125491	117184–125166	4.88E-30	7.45E-22	2.29E-16	1.20E-19	8.08E-18	NS	NS	NS	1.11E-16
9	157	9787–13504	9512–13229	2.90E-23	6.49E-23	2.91E-23	1.26E-08	1.24E-08	NS	NS	NS	8.77E-15
10	237	129824–144268	129497–143901	5.21E-51	1.20E-48	1.64E-18	6.70E-20	5.15E-20	NS	NS	NS	2.22E-16
11	237	122974–126790	122660–126466	8.54E-25	4.42E-24	1.02E-21	6.18E-08	6.69E-07	NS	NS	NS	3.52E-14
12	237	36752–43529	36483–43260	NS	6.12E-08	6.45E-09	4.69E-02	4.51E-02	NS	NS	NS	3.81E-04
13	238	16966–74234	16700–73965	4.80E-75	4.43E-76	1.14E-16	2.56E-27	3.41E-27	NS	NS	NS	2.22E-16
14	238	113903–114416	113631–114144	6.11E-32	1.10E-30	6.11E-32	2.11E-10	2.03E-10	NS	NS	NS	2.22E-16

NS No significant *p* value was recorded for this recombination event using this method. The highest acceptable *p* value was set to 0.05 and the sizes of the scanning windows were set to 30-200 bases depending on the method used.