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

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ABSTRACT

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 recombination between vaccines. Better understanding of the factors that contribute to the generation of recombinant ILTVs 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 the natural host. In this study, 120 specific-pathogen free (SPF) chickens in 8 groups were inoculated with two genetically distinct live-attenuated ILTV vaccine strains with 1–4 days interval between the first and second vaccinations. After inoculation, viral genomes were detected in tracheal swabs in all groups, with lowest copies detected in swabs collected from the groups where the interval between inoculations was 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 timepoints, and these further illustrated superinfection of the birds as recombinant viruses were detected among the progeny. This study has demonstrated superinfection at host level and shows recombination events occur under a very broad range of infection conditions. The occurrence of superinfection after unsynchronised infection with multiple viruses, and subsequent genomic recombination, highlight the importance of using only one type of vaccine per flock as the most effective way to limit recombination.

1. Introduction

Infectious laryngotracheitis (ILT) is an acute viral respiratory 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]. Moreover, viral 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) might create the opportunity for coinfection or superinfection. ILTV can both coinfect [20,21] and superinfect chicken cells *in vitro* [22]. 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 [23].

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Some ILT vaccination protocols recommend more than one round of vaccination to achieve a high level of immunity [24]. In some cases, different strains of 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 improve the duration of immunity [24]. The use of multiple strains of vaccine in the same bird can contribute to genomic recombination between different vaccine strains of ILTV [17].

Recombination is commonly detected in *in vitro*, *in ovo* and *in vivo* systems after simultaneous co-infection of different ILTV strains [22,25,26]. In the field, birds may be exposed at different times to different ILTV strains either by vaccination, reactivation from latency or exposure to field strains such that simultaneous co-infection may be less likely to occur. This study investigates 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. Understanding the potential for superinfection can help to inform the development of vaccination protocols that minimise the risk of genomic recombination that might generate novel virulent strains.

2. Materials and methods

2.1. 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 [27]. One hundred and twenty 21-day-old specific-pathogen-free (SPF) chickens (Australian SPF Services Pty. Ltd.) 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 on separate occasions with each of two different vaccines known as Serva (NOBILIS ILT, Batch No: 1627721) and A20 (Zoetis Poulvac Laryngo A20; Batch No: 116742).

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. 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 −80 °C until use. On vaccination days, the prepared vaccines 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 vaccine inoculum was transferred on ice back to the laboratory to validate their titres on chicken embryo kidney cells (CEKs), as described previously [28]. Back titration of the reserved inoculum confirmed that the expected amount of each vaccine virus was given to these birds ($10^{2.6}$ PFU/bird Serva and $10^{3.4}$ PFU/bird A20).

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). The animals were monitored three times a day for any clinical signs consistent with ILT 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 [29,30]. 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 naïve chickens.

Tracheal swabs were collected using sterile swabs with cotton heads and aluminium shafts (COPAN Diagnostics, USA) every 48 h, 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/mL ampicillin, 50 μg/mL gentamicin and 5 μg/mL amphotericin B). 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 [31], and the mean of three scores was reported.

2.2. 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 three 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.* [32] 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.

2.3. Differentiation of parental viruses by insertion/deletion (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′-GTGTGCAGCAGT GAACTTGG-5′) were designed to amplify a 156 bp product from strain A20 (nucleotides 4591 to 4746 of GenBank accession JN596963) and a 124 bp product from Serva ILTV (nucleotides 4,363 to 4486 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).

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 ($p > 0.05$).

2.4. 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, 1/2 and 1/10 dilutions) 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. This assay identifies A20- or Serva-specifc SNPs at 6 sites across the genome [6]. Each possible combination of the A20 or Serva specific SNPs in recombinant viruses have been assigned a numbered genotype code that reflects their arrangement of the SNPs in their genome and are shown in figures alongside the SNP pattern of the genome.

2.5. 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.

3. Results

3.1. Viral replication and tracheal histopathology

The mean GCN of ILTV in individual tracheal swabs from the directly inoculated birds in each group were compared (Fig. 1). 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 (Fig. 1D and Supplementary Table 1). Fig. 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 Fig. 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 (Fig. 2B). 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) (Fig. 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 (Fig. 2B; Group 1 versus Group 3, Group 4, Group 7 and Group 8).

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).

3.2. Superinfection was detected in all conditions

An indel PCR capable of differentiating the two ILTV strains was used to identify the virus strains in swabs. 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 determined to occur when the indel PCR detected the vaccine strain that was inoculated second. The number of birds that were superinfected varied between groups and sampling times, but at least one chicken was superinfected in each group. (Fig. 3). Groups were compared to determine the influence of the TI or vaccination order on the presence of superinfection (Fig. 3 and supplementary Table 2). Regardless of which vaccine was administered first, birds that had the shortest TI (1 day) had a significantly higher proportion of superinfected birds compared to all other TIs, except for when Serva was the second vaccine after a TI of 4 days (Group 5 vs Group 8). The order of vaccination had no effect on superinfection, regardless of the TI (Fig. 3 and supplementary Table 2).

3.3. Recombination of ILTV occurs in superinfected chickens

While superinfection of the host 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 (Fig. 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. Isolated and plaque purified viruses were subjected to PCR-HRM analysis targeting six regions of the viral genome in order to detect recombination [6]. 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

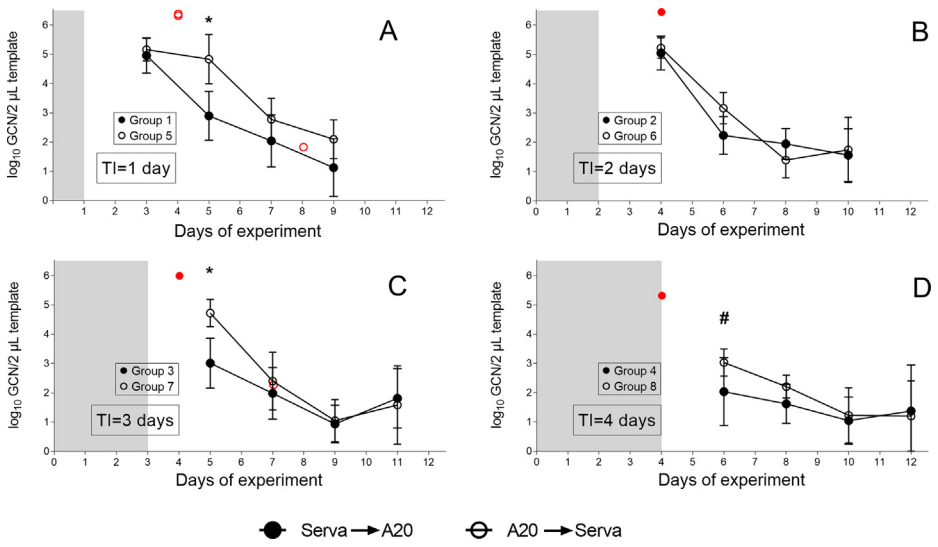

Fig. 1. GCNs of ILTV in tracheal swabs collected from directly inoculated birds. The time-interval (TI) between the first and second vaccination in each group is shown by the grey shading. The second vaccine was administered with a TI of (A) 1, (B) 2, (C) 3 or (D) 4 day(s) following the primary vaccination 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 each group 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). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

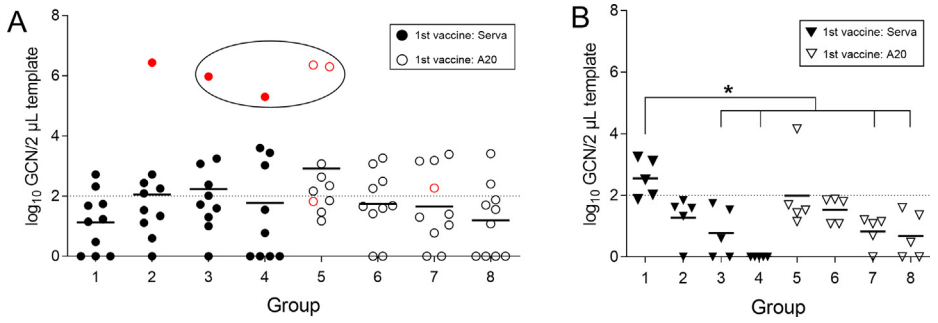

Fig. 2. Abundance of ILTV genome in final tracheal swabs of (A) directly inoculated birds and (B) in-contact birds 8 days after secondary inoculation. Results show GCN of tracheal swabs from individual birds in each group on the day of necropsy. Circles (A) show results from directly inoculated birds and triangles (B) show in-contact birds. Closed shapes show results from groups receiving Serva first and A20 second, and open shapes show results from groups receiving A20 first and Serva second. Horizontal lines in both panels indicate the mean values for each group 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]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Groups 1, 3, 5 and 6 (Fig. 4). The abundance of recombinant viruses ranged from 0% (Fig. 4C and E) to 23.8% (Fig. 4F, Group 5 TI 1 day, sampled 4 days after Serva as 2nd vaccine).

Birds in group 5 that had one day TI between A20 (1st) and Serva (2nd), showed that only the Serva virus was detected at the first sampling time-point (2 days after receiving Serva as the second vaccine, Fig. 4E). However, 2 days later the A20 strain predominated (Fig. 4F) alongside recombinant viruses with 5 distinct HRM profiles in the same group.

3.4. Severe clinical signs of ILT and mortalities

Seven of 120 chickens were euthanized or died suddenly prior to the end of the experiment (red symbols, Figs. 1 and 2A). These chickens were all directly inoculated. Five of these seven chickens had a high ILTV GCN detected in tracheal swabs 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 birds, 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 (Fig. 2A, encircled). The proportion of the recombinant viruses isolated from these birds was from 3.7% to 37.5% (Fig. 5). Ten different recombination patterns were detected among these isolates. One of these recombination patterns (genotype 13) was consistently isolated from all four chickens (Fig. 5, isolates number 519, 548, 549, 550, 584 and 601—all highlighted with grey shading).

4. Discussion

Unless birds are deliberately and simultaneously vaccinated with two distinct attenuated vaccine strains, synchronized co-infections of birds with two vaccine strains is unlikely to occur under field conditions. Asynchronous infection might occur when vaccinated flocks become infected with a second vaccine strain

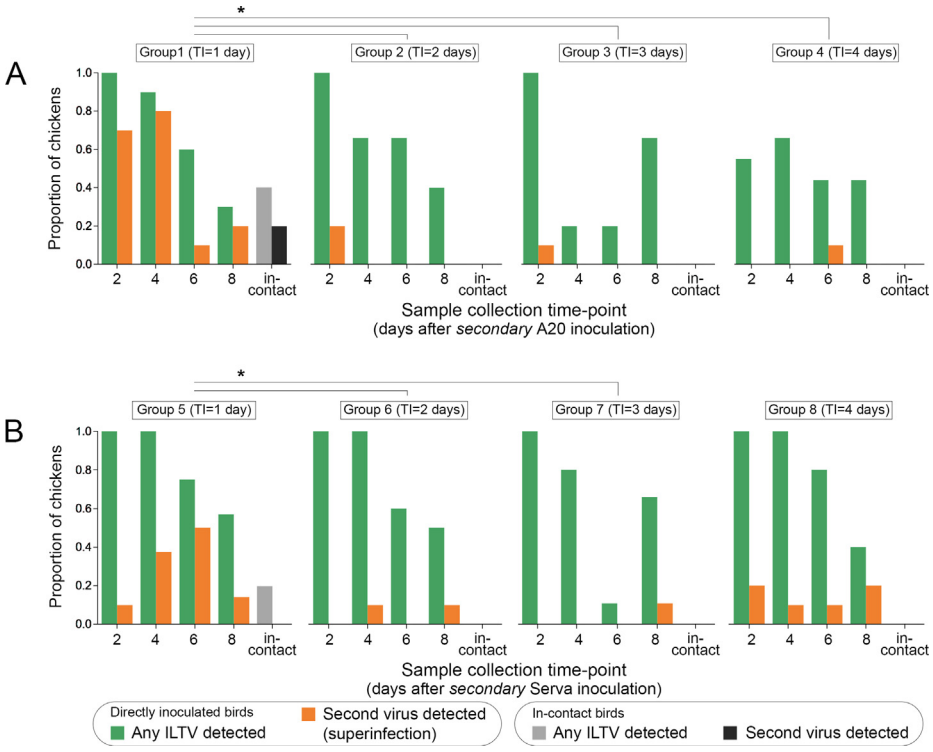

Fig. 3. Proportion of birds in which superinfection was detected. The indel PCR assay can differentiate the Serva and A20 strains and was applied to DNA extracted from individual tracheal swab samples from directly inoculated and in-contact birds. Green bars show the proportions of birds in each group where any ILTV was detected and orange shows the proportion of birds where the secondary inoculum was detected (superinfected). The top panel (A) shows the results for groups that received the Serva strain first, while the bottom 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 in grey (total ILTV) or black (detection of second inoculum). Asterisks shows where a significant difference is detected between the group with a 1 day time interval and all other groups in the same panel (Fisher's exact test, $p < 0.05$, see supplementary Table 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 shown that ILTV has a limited capacity to prevent superinfection *in vitro* (i.e. superinfection exclusion) [22]. 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 [33]. At this speed, the reactivated virus would require approximately 2 h to reach the conjunctiva (approximately 1 cm) and 40 h 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 h, were tested as the time intervals in this study.

Both vaccines could establish superinfection in chickens under all the conditions tested (Fig. 3). Similarly, a range of studies on other herpesviruses, including HSV-1 [34], human cytomegalovirus (HCMV) [35] and Marek's disease virus (MDV, *gallid herpesvirus 2*, *GaHV-2*) [36,37], 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) [36]. A similar pattern was seen in the study described here, with significantly higher numbers of chickens superinfected in groups with the shortest TI between inoculations (1 day) than in groups with a longer TI (2 and 3 days for either vaccine order and 4 days when A20 was given after Serva; Fig. 3).

Recombination was also more commonly detected when the TI between vaccinations was the shortest (1-day interval, Fig. 4B and F). The recombination detected in this study could occur after simultaneous coinfection or asynchronous infection (superinfection) 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 h after the first virus had been inoculated, irrespective of which strain was inoculated first [22]. Similar findings have also been published for of bovine herpesvirus 1 Madin-Darby bovine kidney [38]. 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. Under these conditions, the formation of recombinants can be confidently attributed to superinfection, rather than simultaneous co-infection. In contrast, it is not possible to infect all permissive cells synchronously or to remove extra-cellular viruses from the tissue *in vivo,* so it is likely that cells would be infected stochastically [37]. Studies on superinfection with MDV in

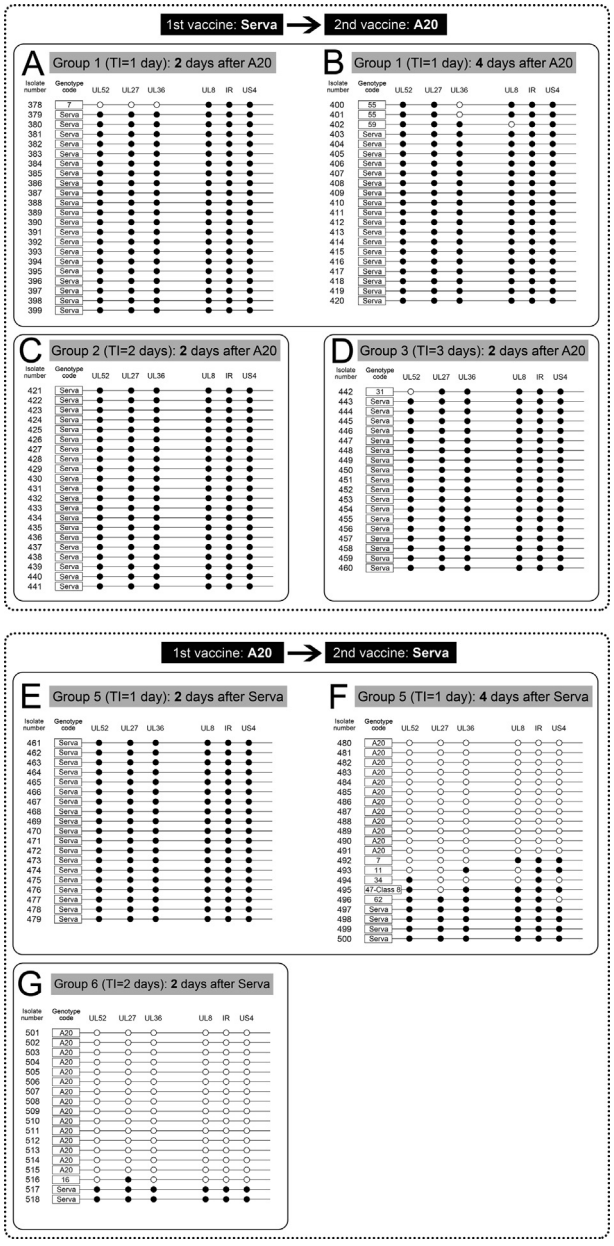

Fig. 4. Schematic view of SNP patterns of individual viruses isolated from tracheal swabs collected from chickens inoculated with Serva and A20 ILTV. Each string of connected circles represents one isolate. The closed circles represent Serva-like SNPs and the open circles represent A20-like SNPs detected 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. These samples from Group 1 (two time-points, panels A and B), Group 2 (C), Group 3 (D), Group 5 (two time-points, panels E and F) and Group 6 (G) could be analysed using this method as these were the only groups from which viruses could be isolated. Each possible pattern of recombination has been given a unique genotype code number [6] and these are also shown for each isolate.

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 [36,37]. However, the detection of recombinant viruses in our study is consistent with coinfection of individual cells within the host by both ILTV vaccine strains.

Previous studies have shown that some recombinant viruses can have enhanced levels of virulence [39]. Analysis of the viruses isolated from birds with severe clinical signs during this study have shown that: (i) at least one recombinant virus 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 (Fig. 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 higher virulence of newly formed recombinant viruses in these chickens, and this requires further investigation of *in vivo* growth kinetics and transmissibility of these recombinant progeny. One of the recombinant viruses detected (isolate number 495, Fig. 4F) had an SNP pattern identical to that of a virulent field recombinant virus (Class 8 ILTV, GenBank accession number JN804826 [40]). Whether the recombinant detected in the current study is identical to the virulent class 8 recombinant field virus generated from A20-like and Serva-like viruses [17] requires further investigation by whole-genome sequencing and phylogenetic analysis.

The decreasing concentrations of ILTV detected over the time course of the experiment (Fig. 1) is consistent with the self-limiting nature of infection with ILTV vaccine strains [29,30] and virulent field isolates [39] after inoculation with a single strain. After experimental infection, ILTV starts replicating in the epithelial cells of the respiratory tract, peaking at 4–6 days after inoculation [3,41]. The concentration of ILTV in the trachea declines after day four and ILTV is cleared completely by day 20–28 after inoculation [29]. 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 (Fig. 1). These higher titres at the early time-points are consistent with increased transmission of ILTV to in-contact chickens that were exposed to birds with the shortest interval between vaccinations when Serva was used as the first vaccine (Group 1, Fig. 2B). The group with shedding the lowest levels of virus (Group 4, Fig. 1) did not transmit virus to any in-contact birds.

In this study, the second virus was inoculated into a different host environment to the first virus, because of the immune response and tracheal tissue damage initiated by the first virus [42–44]. 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 [42,44]. 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 [43,45]. Innate immune responses observed at early time-points of infection include production of inflammatory cytokines, chemokines and interleukins, including CXC inflammatory chemokine ligand 2 (chCXCLi2), interleukin (IL)-1β, IL-10 and type I interferon (IFN-α), as well as infiltration of leukocytes into infected tissues [44,46]. These can be detected as early as 4 days after inoculation [43,44,47]. Similarly, upregulation of IFN-γ production in the trachea can be detected as early as 4–5 days post infection in naïve birds [44,47]. These responses may be induced by the first

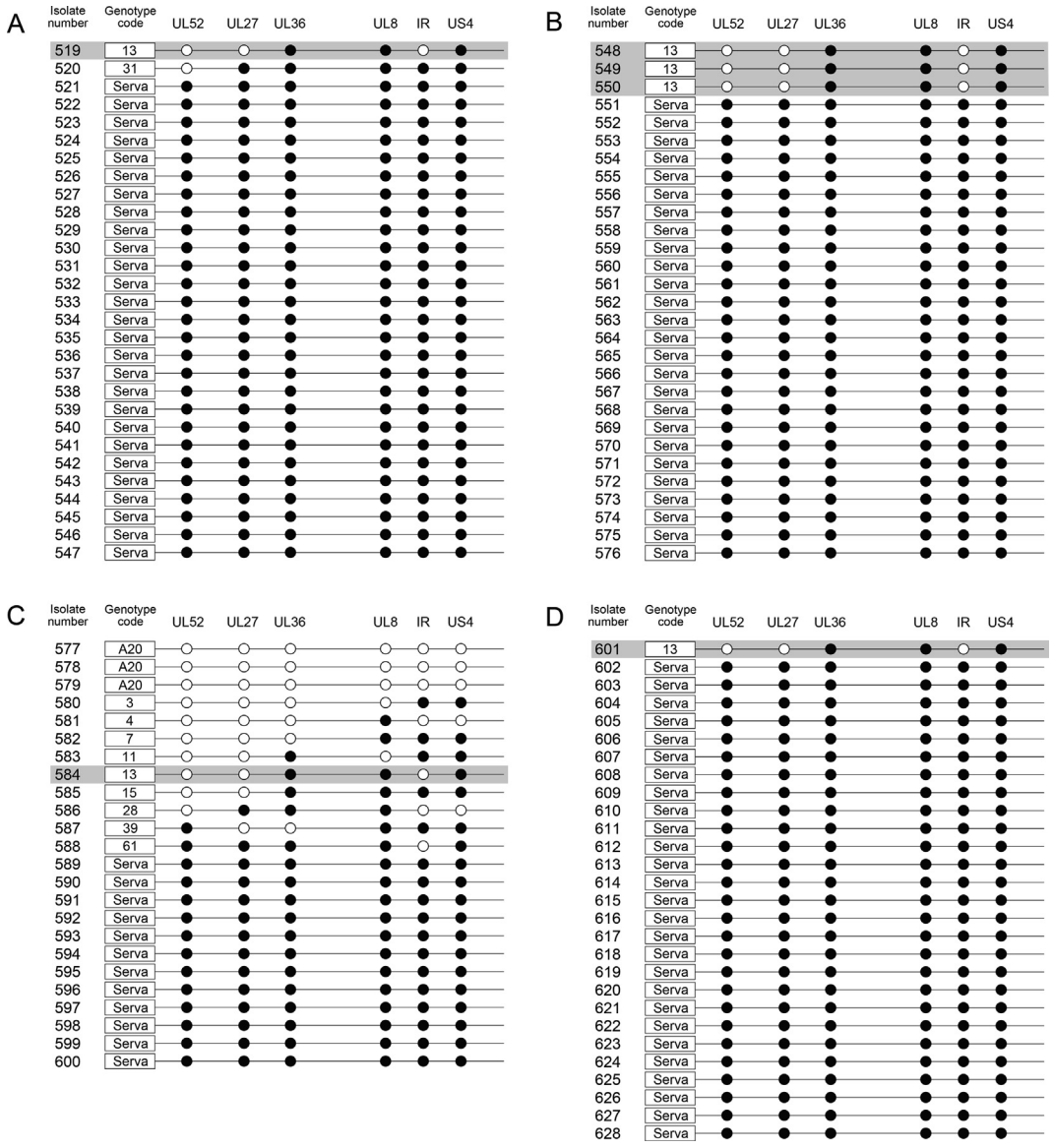

Fig. 5. Schematic view of SNP patterns of individual viruses isolated from tracheal swabs collected from chickens with severe clinical signs of ILT. Each string of connected circles represents one isolate. The closed circles represent Serva-like SNPs and the open circles represent A20-like SNPs detected using the HRM curve analysis (six genomic markers were tested for each isolate). Viruses were isolated from the swabs taken at necropsy from chickens in three different groups. Chicken A was from Group 3 (1 day after A20 as the 2nd vaccine), chicken B was from Group 4 (1 day after A20 as the 2nd vaccine), and chickens C and D were from Group 5 (3 days after Serva as the 2nd vaccine). Each possible pattern of recombination has been given a unique genotype code number [6] and these are also shown for each isolate.

vaccination and affect the replication of the second vaccine, particularly when the TI between the first and second vaccination is longer.

The results of this study accord with our observations from previous *in vitro* and *in vivo* studies [6,21,25,26]. Analyses of ILTV field strains [18,39,48] 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.

CRediT authorship contribution statement

Omid Fakhri: Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Joanne M. Devlin:** Conceptualization, Validation, Resources, Writing - review & editing, Supervision. **Glenn F. Browning:** Conceptualization, Resources, Writing - review & editing, Supervision. **Mauricio J.C. Coppo:** Investigation, Writing - review & editing. **José A. Quinteros:** Investigation, Writing - review & editing. **Andrés Diaz-Méndez:** Investigation, Writing - review & editing. **Sang-Won Lee:** Conceptualization, Validation, Methodology, Writing - review & editing, Funding acquisition, Supervision. **Carol A. Hartley:** Conceptualization, Validation, Investigation, Writing - review & editing, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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