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MICROBIAL ECOLOGY

Genetic Diversity of Infectious Laryngotracheitis Virus during *In Vivo* Coinfection Parallels Viral Replication and Arises from Recombination Hot Spots within the Genome

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ABSTRACT Recombination is a feature of many alphaherpesviruses that infect people and animals. Infectious laryngotracheitis virus (ILTV; *Gallid alphaherpesvirus 1*) causes respiratory disease in chickens, resulting in significant production losses in poultry industries worldwide. Natural (field) ILTV recombination is widespread, particularly recombination between attenuated ILTV vaccine strains to create virulent viruses. These virulent recombinants have had a major impact on animal health. Recently, the development of a single nucleotide polymorphism (SNP) genotyping assay for ILTV has helped to understand ILTV recombination in laboratory settings. In this study, we applied this SNP genotyping assay to further examine ILTV recombination in the natural host. Following coinoculation of specific-pathogen-free chickens, we examined the resultant progeny for evidence of viral recombination and characterized the diversity of the recombinants over time. The results showed that ILTV replication and recombination are closely related and that the recombinant viral progeny are most diverse 4 days after coinoculation, which is the peak of viral replication. Further, the locations of recombination breakpoints in a selection of the recombinant progeny, and in field isolates of ILTV from different geographical regions, were examined following full-genome sequencing and used to identify recombination hot spots in the ILTV genome.

IMPORTANCE Alphaherpesviruses are common causes of disease in people and animals. Recombination enables genome diversification in many different species of alphaherpesviruses, which can lead to the evolution of higher levels of viral virulence. Using the alphaherpesvirus infectious laryngotracheitis virus (ILTV), we performed coinfections in the natural host (chickens) to demonstrate high levels of virus recombination. Higher levels of diversity in the recombinant progeny coincided with the highest levels of virus replication. In the recombinant progeny, and in field isolates, recombination occurred at greater frequency in recombination hot spot regions of the virus genome. Our results suggest that control measures that aim to limit viral replication could offer the potential to limit virus recombination and thus the evolution of virulence. The development and use of vaccines that are focused on limiting virus replication, rather than vaccines that are focused more on limiting clinical disease, may be indicated in order to better control disease.

KEYWORDS herpesvirus, recombination, replication, diversity, SNP genotyping assay, vaccine, infectious laryngotracheitis virus (ILTV), recombination hot spot, gallid herpesvirus 1, ILTV

Received 12 July 2017 **Accepted** 12 September 2017

Accepted manuscript posted online 22 September 2017

Citation Loncoman, CA, Hartley CA, Coppo MJC, Vaz PK, Diaz-Méndez A, Browning GF, García M, Spatz S, Devlin JM. 2017. Genetic diversity of infectious laryngotracheitis virus during *in vivo* coinfection parallels viral replication and arises from recombination hot spots within the genome. Appl Environ Microbiol 83:e01532-17. https://doi.org/10.1128/AEM.01532-17.

Editor Harold L. Drake, University of Bayreuth

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Infectious laryngotracheitis virus (ILTV; *Gallid alphaherpesvirus 1*) is an alphaherpesvirus that causes mild to severe respiratory disease in chickens. The virus causes major economic losses due to mortality and decreases in weight gain and egg production in poultry industries worldwide (1). Recombination between different strains of ILTV has been recognized as a problem for the poultry industry, particularly because natural recombination between attenuated vaccine strains of ILTV has been shown to generate virulent viruses (2). The importance of recombination as a mechanism of viral genome diversification and evolution in ILTV and other alphaherpesviruses is increasingly being recognized (3). Viruses can acquire genetic changes through several mechanisms, including point mutation and recombination, with the latter particularly important in many alphaherpesviruses (4–6). Viruses that belong to the order *Herpesvirales* have double-stranded linear DNA genomes and have complex viral DNA replication machinery, comprising a DNA polymerase with a highly efficient proofreading capacity, resulting in very low rates of spontaneous mutation (7–9). Therefore, rather than random changes or point mutations throughout the genome, recombination is considered to be a major evolutionary driving force enabling many alphaherpesviruses to persist, evolve and eventually become more virulent over time (2, 10, 11).

Four types of recombination have been described, based on the structure of the crossover site: site-specific recombination, transposition recombination, nonhomologous or illegitimate recombination, and homologous recombination (12). Homologous and illegitimate recombination, specifically intraspecific recombination, are the most commonly described recombination mechanisms in alphaherpesviruses (12). Intraspecific recombination under *in vitro* conditions has been extensively described for several alphaherpesvirus species, including *Human alphaherpesvirus 1*, also known as herpes simplex virus 1 (HSV-1) (13), *Bovine alphaherpesvirus 1*, also known as bovine herpesvirus 1 (BoHV-1) (14), *Human alphaherpesvirus 3*, also known as varicella-zoster virus (VZV) (15), *Felid alphaherpesvirus 1*, also known as felid herpesvirus 1 (FeHV-1) (16) and *Suid alphaherpesvirus 1*, also known as pseudorabies virus (PRV) (17). Full-genome sequencing and sequence analysis are the most suitable methods for detecting and describing alphaherpesvirus recombination events that occur under natural (field) conditions, but even nowadays these techniques have their limitations, including intensive labor and costs. In contrast, under experimental conditions, other techniques to detect recombination are still more efficient and cost-effective, as well as more suitable for testing large numbers of viruses (14, 18).

We have recently described the development and validation of a TaqMan single nucleotide polymorphism (SNP) genotyping assay to study recombination under experimental conditions in specific-pathogen-free (SPF) chickens (18), the natural host of ILTV. Use of the natural host to study recombination may reveal aspects of herpesvirus biology that may not be apparent in studies that utilize laboratory animal models of infection (19). The aim of this study was to apply this ILTV SNP genotyping assay to examine *in vivo* recombination between two ILTV strains over time. This was performed in order to describe recombination patterns and to examine genetic diversity among the viral progeny during the course of an ILTV infection. Additionally, we aimed to identify any recombination hot spots in the ILTV genome by performing full-genome sequencing of selected recombinants and analyzing them along with other ILTV genomes that were already available from different geographical regions, including Asia, Australia, Europe, and the United States.

RESULTS

Bird survival rates and virus genome quantification. Groups of chickens were coinoculated with the V1-99 and CSW-1 strains of ILTV at two different doses or were inoculated with only either V1-99 or CSW-1. The survival rates in groups of birds that were inoculated only with low or high doses of V1-99 ILTV were around 70% and 45% throughout the experiment, respectively (Fig. 1). Groups that received only CSW-1 had 100% survival rates throughout the experiment (Fig. 1). In the coinoculated groups, the

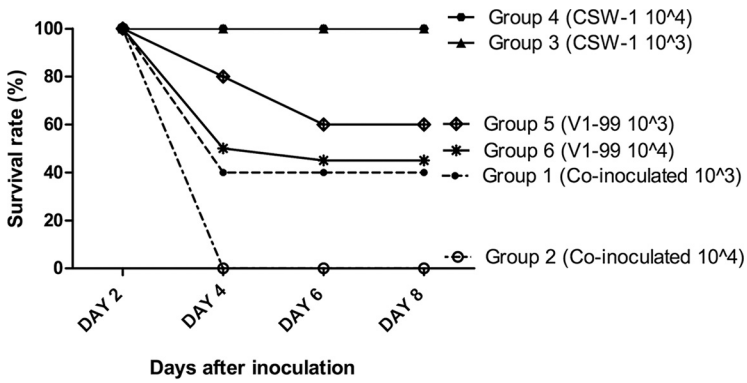

FIG 1 Survival rates within six groups of SPF chickens following intratracheal inoculation with either low (10^3 PFU) or high (10^4 PFU) doses of CSW-1 or V1-99 ILTV or coinoculation with the two viruses at the same doses. All birds inoculated only with CSW-1 survived. The group inoculated with a low dose of only V1-99 (group 5) had an 80% survival rate at day 4 and then a survival rate of 60% at days 6 and 8. Meanwhile, in the group inoculated with a high dose of V1-99 (group 6), a survival rate of 45% was seen at day 4, and the survival rate was 40% at days 6 and 8. Group 1 (birds coinoculated with a low dose of both viruses) had a survival rate similar to that of the group that received the single high dose of V1-99. None of the birds in group 2 (coinoculated with a high dose of both viruses) survived beyond day 4.

survival rates were 40% and 0% at 4 days after coinoculation with low and high doses of virus, respectively (Fig. 1).

In the high-dose-coinoculated group, virus could be isolated from only 5 of the 10 birds at both 2 and 4 days after coinoculation (birds A, B, C, D, and E), so tracheal samples from these birds were used to compare virus replication and diversity across days 2 and 4. Significantly higher $\log_{10}$ viral genome copy numbers (means $\pm$ standard deviations) were detected at day 4 than at day 2 (4.94 $\pm$ 0.648 versus 3.02 $\pm$ 1.11, respectively; $P = 0.0008$, Mann-Whitney test) (Fig. 2E and F). The samples collected from the five birds in this group from which virus could be isolated at only one time point (4 days after infection) were used to verify the performance of SNP genotyping assay, and these results have been previously reported (18). None of the birds survived in the high-dose-coinoculated group after day 4 (Fig. 1), so no further sampling was possible in this group.

In the low-dose-coinoculated group, those animals with high viral genome copy numbers at days 2 and 4 (birds F, G, H, I, and J) (Fig. 2A and B) did not survive, so samples could not be obtained at days 6 and 8. Therefore, we compared virus replication and diversity at days 2 and 4 in birds F, G, H, I, and J and then compared virus replication and diversity at days 6 and 8 in the remaining birds (K, L, and M) (Fig. 2C and D). In this group (low-dose-coinoculated group), significantly higher $\log_{10}$ genome copy numbers were detected 4 days after coinoculation than 2 days after coinoculation (3.77 $\pm$ 1.91 versus 2.22 $\pm$ 1.22, respectively; $P = 0.045$, Mann-Whitney test). Log_{10} genome copy numbers did not differ significantly at 6 days and 8 days after coinoculation (3.375 $\pm$ 1.097 versus 3.825 $\pm$ 1.338, respectively; $P = 0.343$, Mann-Whitney test).

Characterization of progeny viruses. Up to 20 progeny viruses were isolated from each coinoculated chicken, and each was plaque purified before viral DNA extraction. Six SNPs across the length of each of the genomes of the progeny viruses were identified as being of either CSW-1 or V1-99 origin using the SNP genotyping assay interface within the Stratagene Mx3000P quantitative PCR (qPCR) software system, as previously described (18). The genotyping assay detected different patterns of recombination at 2 and 4 days after coinoculation with the high dose of viruses (Fig. 3) and also at 2, 4, 6, and 8 days after coinoculation with the low dose of viruses (Fig. 4). In total, 43 (67%) of the 64 possible genotype patterns were detected among the viral progeny of the two coinoculated groups. In the group of birds that were coinoculated with the high dose of viruses, 19 and 24 genotype patterns were detected at 2 and 4 days postinoculation, respectively (Table 1). In the group of birds that were coinocu-

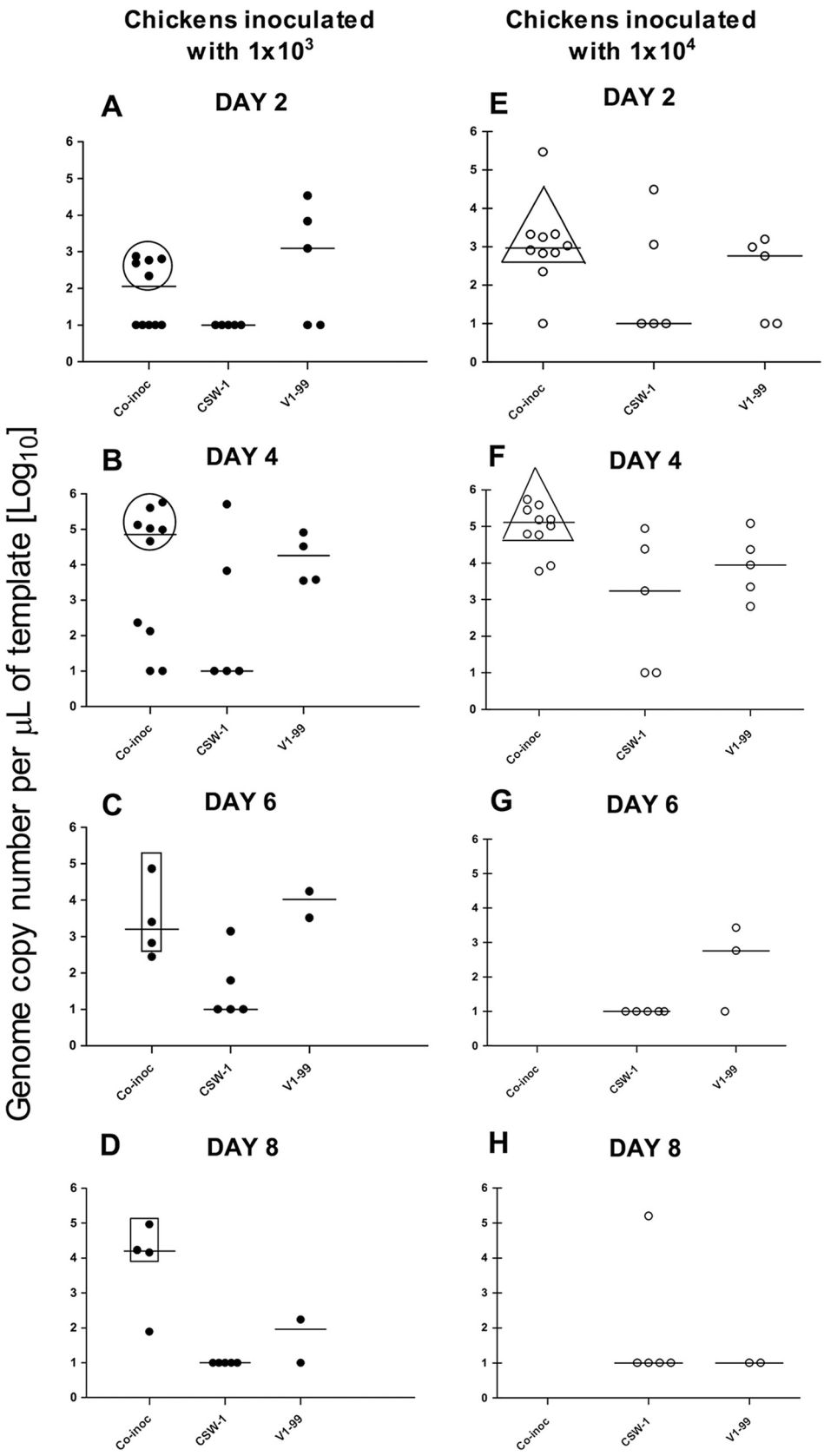

FIG 2 Replication of ILTV in SPF chickens based on genome copy numbers in tracheal swabs measured using qPCR. Medians are indicated by lines for each group. Birds were inoculated with either CSW-1 or V1-99 or coinoculated (co-inoc) with 10^3 PFU (low dose) (A to D) or with 10^4 PFU (high dose) (E to H) of the V1-99 and

(Continued on next page)

lated with the low dose of viruses, 14, 26, 15, and 9 genotype patterns were detected at 2, 4, 6, and 8 days postinoculation, respectively (Tables 2 and 3). Some of these genotype patterns were detected consistently over time in both low- and high-dose-inoculated groups. In the singly inoculated groups, no recombination was detected within the 10 viruses that were isolated and purified from each infected chicken at day 4 from the groups inoculated with the higher viral dose of 1×10^4 PFU/ml (see Fig. S1 in the supplemental material).

Viral-diversity analysis. The higher level of viral replication in the low-dose-coinoculated group at day 4 than day 2 (Fig. 2A and B) coincided with a higher level of viral diversity, as assessed using Renyi diversity profiles (Fig. 5A) and as indicated by significantly higher values for diversity as measured by richness (0), Shannon-Weaver (1), 1/Simpson (2), 1/Berger Parker (infinite [Inf]), and Hill (0.25, 0.5, 4, 8, 16, 32, and 64) scales, as well as evenness values at these time points (see Table S1 in the supplemental material). The Renyi diversity profile was higher at day 6 than at day 8 (Fig. 5B); however, the diversity measurements were not significantly different between these days (see Table S2). In the high-dose-coinoculated group, the Renyi diversity profile was higher at day 4 than at day 2 (Fig. 5C), coinciding with the higher levels of viral replication at day 4 than day 2 in this group (Fig. 2E and F). Significant differences were detected only in the Shannon-Weaver (1), 1/Simpson (2), and Hill (4) values for these days (see Table S3).

Full-genome sequencing and identification of recombination hot spots. Six progeny viruses were selected for full-genome sequencing and analysis (see Table S4) in order to identify all recombination events and compare these to those detected with the SNP genotyping assay and to examine the distribution of recombination breakpoints along the genome. These recombination patterns were also searched for among 35 other full ILTV genome sequences of isolates from Asia, Australia, Europe, and the United States in order to identify recombination hot spots in ILTV from distinct geographical regions. In addition to the distribution of recombination breakpoints detected by RDP4, reticulate network and phi test analyses were performed as previously described (20) (see Fig. S2).

The six SNPs targeted by the TaqMan SNP genotyping assay were confirmed by full-genome analysis. Additional events were detected outside the genome regions targeted by the TaqMan assay. The full genomes of the two genotype pattern 9 viruses shared 99.9% pairwise sequence identity. Between these two genotype pattern 9 viruses, 11 SNPs were detected within genes UL[−1], US5, US6, US7, US8, and US9, located between bp 110000 and bp 140000 in the aligned sequences. Two SNPs were identified in the protein IF and UL49 genes located between bp 8000 to bp 18000 in the aligned sequences, and two SNPs were identified in the UL43 and UL17 genes between bp 74000 and bp 86000. When CSW-1 was used as the reference strain, a total of 55 polymorphisms were found in the six viruses sequenced in this study (see Table S5 in the supplemental material).

A high number of recombination breakpoints were identified in two locations extending from bp 20000 to bp 50000 and from bp 106000 to bp 137000 in the six genome sequences from viruses isolated in this study (Fig. 6B). When the full-genome sequences from other ILTV isolates from varied geographical regions were examined, the recombination hot spots detected in those genomes were consistent with those found in the six genomes sequenced from the *in vivo* study (Fig. 6C, D, and E). Recombination events within genes UL[−1] and ICP4 were detected in ILTV sequences

FIG 2 Legend (Continued)

CSW-1 strains of ILTV. (A and B) Low-dose-coinoculated birds F, G, H, I, and J (in the circles) had higher numbers of viral genome copies at day 4 than at day 2 ($P < 0.05$, Mann-Whitney test). These birds did not survive to day 6. (C and D) Low-dose-coinoculated birds K, L, and M (in the rectangles) did not have significantly different numbers of viral genome copies between day 6 and day 8 ($P > 0.05$, Mann-Whitney test). (E and F) High-dose-coinoculated birds A, B, C, D, and E (in the triangles) had higher numbers of viral genome copies at day 4 than at day 2 ($P < 0.05$, Mann-Whitney test). (G and H) High-dose-coinoculated birds did not survive to days 6 and 8.

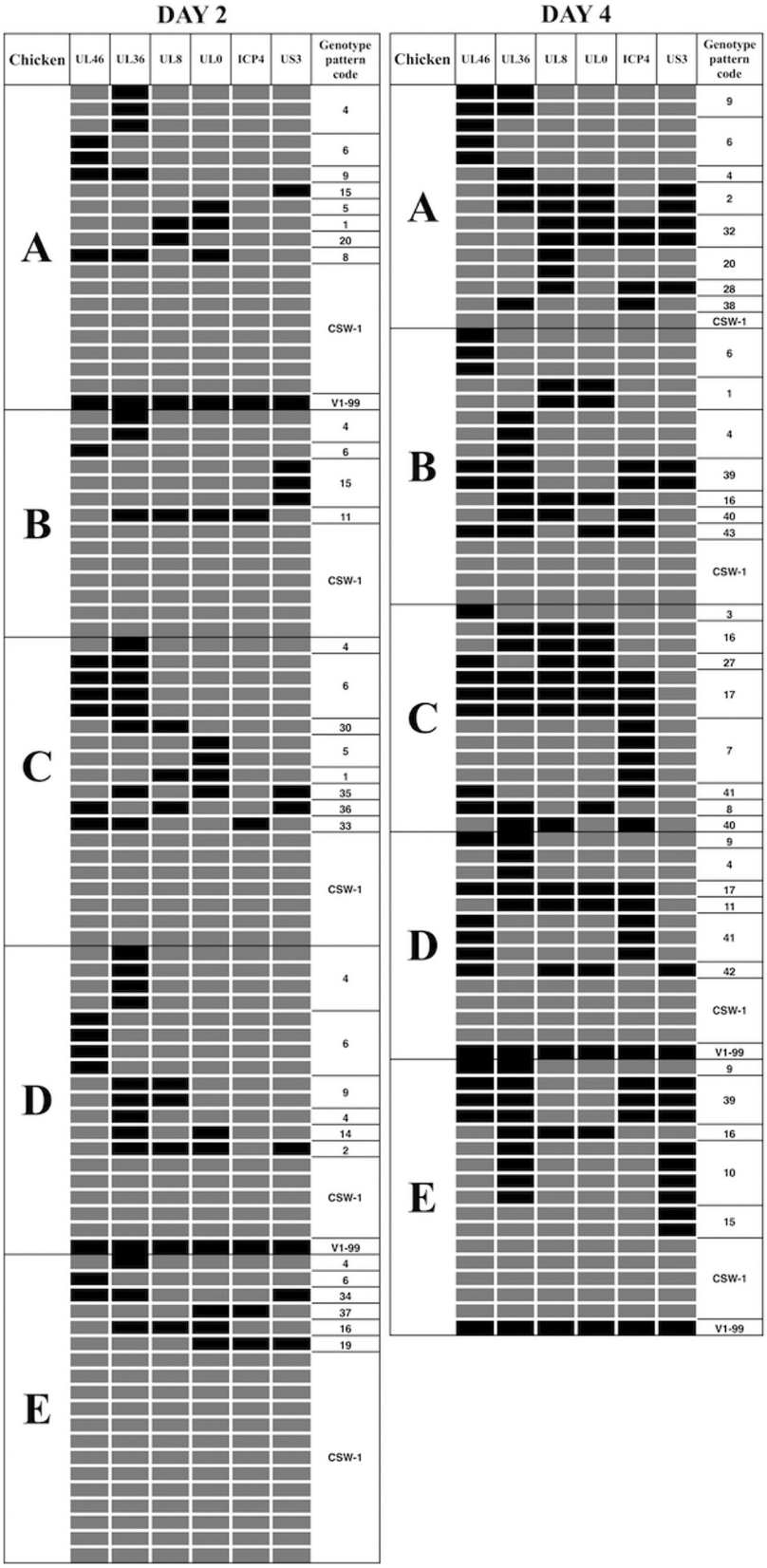

FIG 3 Recombination patterns, as determined using SNP genotyping, in progeny viruses isolated 2 and 4 days after coinoculation of SPF chickens with 10^4 PFU of each of CSW-1 and V1-99 ILTV. Each row corresponds to a virus isolate, with the CSW-1 SNPs indicated by gray boxes and the V1-99 SNPs by black boxes. Each distinct recombination pattern was given a unique genotype code (final column).

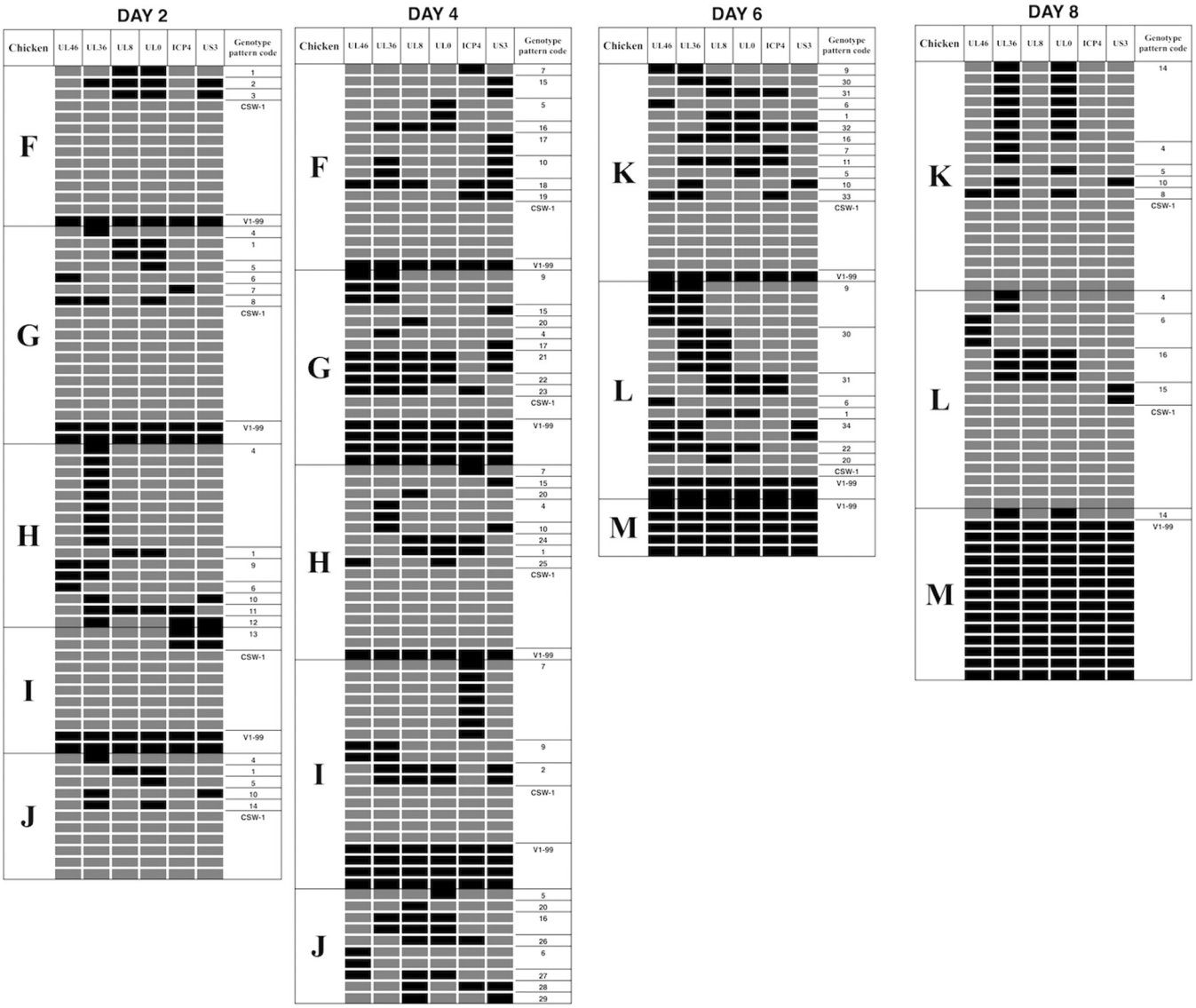

FIG 4 Recombination patterns, as determined using SNP genotyping, in progeny viruses isolated 2, 4, 6, and 8 days after coinoculation of SPF chickens with 10^3 PFU of each of CSW-1 and V1-99. Each row corresponds to a virus isolate, with the CSW-1 SNPs indicated by gray boxes and the V1-99 SNPs by black boxes. Each distinct recombination pattern was given a unique genotype code (final column).

from Australia and the United States and in our *in vivo* recombinants (Table 4). Recombination events among the Asian ILTV strains were detected at sites similar to those detected in the Australian strains, with recombination breakpoints found in genes UL4, UL3.5, UL28, UL29, UL30, UL31, UL32, UL33, and UL34 (Table 4). Only one recombination breakpoint was detected and confirmed by several methods in the ILTV sequences from Europe (Italy) (Table 4). Hence, because of the low number of recombination events in the European group, no recombination hot spots were detected.

DISCUSSION

Our results show that higher levels of diversity in the recombinant progeny occurred at the same time as higher levels of virus replication (as measured by viral titers). A direct relationship between viral replication and recombination diversity would have important implications for disease control, as it suggests that measures taken to reduce viral replication, such as administration of vaccines to limit virus replication following challenge (21), may be able to reduce the diversity of recombinant viruses, thus potentially reducing the capacity of ILTV to evolve to higher levels of virulence. Vaccines are used

TABLE 1 Genotypes of viruses detected in high-dose-coinoculated (10^4 PFU) chickens at 2 and 4 days after coinoculation[a]

Day 2			Day 4		
Genotype pattern code	Chicken(s) from which virus was isolated	No. (%) of isolates	Genotype pattern code	Chicken(s) from which virus was isolated	No. (%) of isolates
4	A, B, C, D, E	12 (13.2)	4	A, B, D	3 (3.9)
6	A, B, C, D, E	12 (13.2)	6	A, B	6 (7.8)
9	A, D	3 (3.3)	9	A, D, E	4 (5.2)
15	A, B	4 (4.4)	15	E	2 (2.6)
1	A, C	2 (2.2)	1	B	2 (2.6)
20	A	1 (1.1)	20	A	2 (2.6)
8	A	1 (1.1)	8	C	1 (1.3)
11	B	1 (1.1)	11	D	1 (1.3)
2	D	1 (1.1)	2	A	2 (2.6)
16	E	1 (1.1)	16	B, C, E	4 (5.2)
19	E	1 (1.1)	32	A	2 (2.6)
34	E	1 (1.1)	28	A	1 (1.3)
37	E	1 (1.1)	38	A	1 (1.3)
14	D	1 (1.1)	42	D	3 (3.9)
33	C	1 (1.1)	39	B, E	5 (6.5)
36	C	1 (1.1)	40	B, C	2 (2.6)
35	C	1 (1.1)	43	B	1 (1.3)
30	C	1 (1.1)	3	C	1 (1.3)
5	A, C	3 (3.3)	27	C	1 (1.3)
CSW-1	A, B, C, D, E	40 (44)	17	C, D	4 (5.2)
V1-99	A, D	2 (2.2)	7	C	4 (5.2)
			41	C, D	4 (5.2)
			4	A, B, D	1 (1.3)
			10	E	4 (5.2)
			CSW-1	A, B, D, E	14 (18.2)
			V1-99	D, E	2 (2.6)

[a]For day 2, there were a total of 42 (46.2%) parental viruses and 49 (53.8%) recombinants, for a total of 91 (100%) viruses; for day 4, there were a total of 16 (20.8%) parental viruses and 61 (79.2%) recombinants, for a total of 77 (100%) viruses.

regularly in poultry industries to control ILT. As previously described, most vaccines reduce, but do not prevent, ILTV replication (as measured by viral titers) after challenge, due to cell-mediated immune responses that develop in the host in response to vaccination (21). Many vaccine efficacy studies examine ILTV replication after challenge, but some focus only on clinical signs of disease and mortality (21, 22). Furthermore, studies to meet vaccine registration requirements remain focused mainly on mortality and clinical signs of disease (23). Expanding standard vaccine characterization studies to include examination of a vaccine's capacity to reduce viral replication after subsequent challenge (as measured by viral titers), and potentially viral recombination, would help to further control disease caused by this virus. Similar approaches could be taken for other attenuated live vaccines that are used to control alphaherpesviruses, such as FeHV-1, BoHV-1, and PRV, in veterinary medicine (24, 25) and for varicella-zoster virus (VZV) vaccines in human medicine (26), as these viruses all have capacity for recombination (3).

In this study, a high proportion (67%) of viruses detected following *in vivo* ILTV coinoculation were recombinants. This is consistent with findings from an HSV-1 study that found that 59% of progeny viruses from the cornea and 74% of progeny viruses from the trigeminal ganglia of mice coinoculated with two strains of HSV-1 were identified as recombinants using restriction fragment length polymorphism (RFLP) analysis (13). Additionally, *in vitro* studies have also shown abundant recombination following alphaherpesvirus coinoculation, although lower proportions of recombinants are typically detected in cell culture than in *in vivo* models. For example, approximately 26% of progeny viruses obtained after coinoculation of two HSV-1 strains into cultured cells were identified as recombinants using RFLP analysis (13), and 13% of the viral progeny obtained after coinoculation of two VZV strains into cell culture were identified

TABLE 2 Genotypes of viruses detected in low-dose-coinoculated (10^3 PFU) chickens at 2 and 4 days after coinoculation[a]

Day 2			Day 4		
Genotype pattern code	Chicken(s) from which virus was isolated	No. (%) of isolates	Genotype pattern code	Chicken(s) from which virus was isolated	No. (%) of isolates
1	F, G, H, J	5 (7)	1	H	1 (1.2)
2	F	1 (1.4)	2	I	2 (2.4)
4	G, H, J	11 (15.5)	4	G, H	3 (3.7)
5	G, J	2 (2.8)	5	F, J	3 (3.7)
6	G, H	2 (2.8)	6	J	2 (2.4)
7	G	1 (1.4)	7	F, H, I	9 (10.9)
9	H	2 (2.8)	9	G, I	5 (6.1)
10	H, J	2 (2.8)	10	F, H	3 (3.7)
8	G	1 (1.4)	15	F, G, H	4 (4.8)
3	F	1 (1.4)	16	F, J	3 (3.7)
11	H	1 (1.4)	17	F, G	3 (3.7)
12	H	1 (1.4)	18	F	1 (1.2)
13	I	2 (2.8)	19	F	1 (1.2)
14	J	1 (1.4)	20	G, H, J	3 (3.7)
CSW-1	F, G, I, J	33 (46.5)	21	G	2 (2.4)
V1-99	F, G, I	5 (7)	22	G	1 (1.2)
			23	G	1 (1.2)
			24	H	1 (1.2)
			25	H	1 (1.2)
			26	J	1 (1.2)
			27	J	1 (1.2)
			28	J	1 (1.2)
			29	J	1 (1.2)
			CSW-1	F, G, H, I	19 (23.2)
			V1-99	F, G, H, I	10 (12.1)

[a]For day 2, there were a total of 38 (53.5%) parental viruses and 33 (46.5%) recombinants, for a total of 71 (100%) viruses; for day 4, there were a total of 29 (35.4%) parental viruses and 53 (64.6%) recombinants, for a total of 82 (100%) viruses.

as recombinants by RFLP analysis (15). Similarly, 10% to 21% of the viral progeny obtained after coinoculation of an FeHV-1 wild-type strain and two vaccine strains were identified as recombinants by restriction endonuclease digestion of PCR products (16). More recently a TaqMan SNP genotyping assay was developed to better detect

TABLE 3 Genotypes of viruses detected in low-dose-coinoculated (10^3 PFU) chickens at 6 and 8 days after coinoculation[a]

Day 6			Day 8		
Genotype pattern code	Chicken(s) from which virus was isolated	No. (%) of isolates	Genotype pattern code	Chicken(s) from which virus was isolated	No. (%) of isolates
6	K, L	2 (4.7)	6	L	3 (5.6)
5	K	1 (2.3)	5	K	1 (1.9)
10	K	1 (2.3)	10	K	1 (1.9)
9	K, L	5 (11.6)	14	K, M	8 (15)
30	K, L	5 (11.6)	4	K, L	2 (3.7)
31	K, L	3 (7)	8	K	1 (1.9)
1	K, L	2 (4.7)	4	K, L	2 (3.7)
32	K	1 (2.3)	16	L	3 (5.6)
16	K	1 (2.3)	15	L	2 (3.7)
7	K	1 (2.3)	CSW-1	K, L	17 (31)
11	K	1 (2.3)	V1-99	M	14 (26)
33	K	1 (2.3)			
34	L	2 (4.7)			
22	L	1 (2.3)			
20	L	1 (2.3)			
CSW-1	K, L	7 (16.2)			
V1-99	K, L, M	8 (19)			

[a]For day 6, there were a total of 15 (34.9%) parental viruses and 28 (65.1%) recombinants, for a total of 43 (100%) viruses; for day 4, there were a total of 31 (57.4%) parental viruses and 23 (42.6%) recombinants, for a total of 54 (100%) viruses.

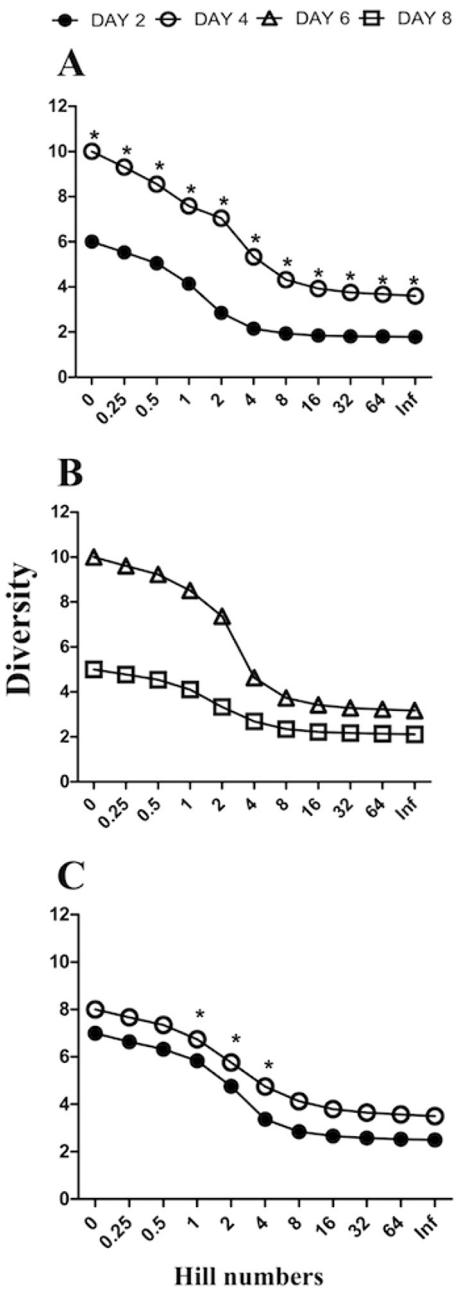

FIG 5 Renyi diversity profiles showing levels of diversity on the *y* axis and diversity measures on the *x* axis. Separate plots are shown for recombinants isolated 2 and 4 days after coinoculation with 10^3 PFU of CSW-1 and V1-99 (A), recombinants isolated 6 and 8 days after coinoculation with 10^3 PFU of CSW-1 and V1-99 (B), and recombinants isolated 2 and 4 days after coinoculation with 10^4 PFU of CSW-1 and V1-99 (C). Renyi profiles contain 11 diversity measurements, including richness (*x* axis value = 0), Hill values (*x* axis values = 0.25, 0.5, 4, 8, 16, 32, 64), Shannon-Weaver (*x* axis value = 1), 1/Simpson (*x* axis value = 2), and 1/Berger Parker (*x* axis value = Inf) values. One community can be regarded as more diverse than another if all its Renyi diversity measurements are higher (28). Asterisks indicate *P* values of <0.05 in paired Student's *t* test analyses on individual diversity measures, which were performed as additional statistical analyses.

and describe recombination between two closely related BoHV-1 strains in cell culture. Around 40% of the viral progeny were found to be recombinants using this assay (14), but the assay has not been applied to study recombination between the BoHV-1 strains *in vivo*. Previous application of the ILTV TaqMan SNP genotyping assay to viruses isolated from SPF chickens 4 days after coinfection with 10^4 PFU of CSW-1 and V1-99

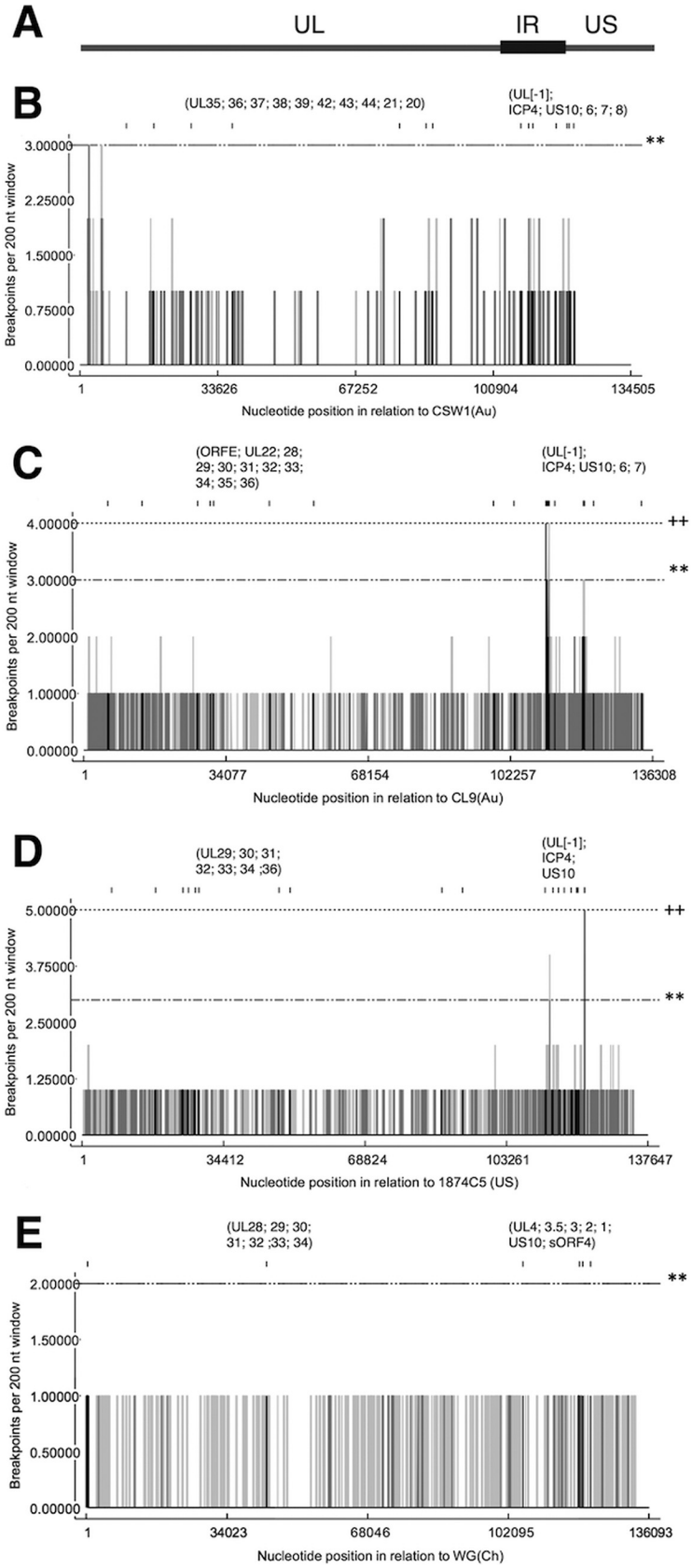

FIG 6 Detection of recombination hot spots in genome sequences. (A) Schematic representation of ILTV genome encompassing unique long (UL), internal repeat (IR), and unique short (US) regions; (B) the 6 ILTV isolates derived from the *in vivo* study; (C) ILTV isolates originating from Australia ($n = 8$); (D) ILTV isolates

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found that 74% of progeny viruses were recombinants (18). This is consistent with our finding in this study that 79% of the progeny viruses were identified as recombinants 4 days after high-dose coinoculation. In both our present study and the previous *in vivo* study of ILTV recombination (18), SPF chickens were used as experimental animals. Future studies to examine ILTV recombination in commercial chickens would be advantageous, as differences in responses to infection have been noted between different breeds and lines of poultry (1).

The use of the ILTV SNP genotyping assay to detect recombination following coinoculation of SPF chickens has helped to measure viral diversity over time during the course of acute clinical disease (up to 8 days after coinoculation). Diversity is a product of the number of categories that can be differentiated and the proportions or relative abundances of the objects in each category (27). One community can be regarded as more diverse than another if all Renyi diversity values are higher (28). Our Renyi diversity analyses found that diversity was higher at day 4 than at day 2, regardless of the viral dose used to inoculate the chickens (Fig. 5A and C). However, further analyses of these results found that only those coinoculated with the lower dose had significant differences between day 4 and day 2 in every diversity measure (Fig. 5A; see also Table S1 in the supplemental material). This may indicate that a higher viral coinoculation dose may increase recombinant diversity at day 2 (Fig. 5C) but not necessarily at day 4, which is the peak of viral replication.

In both coinoculated groups some virus genotype pattern codes were detected in multiple birds at both days 2 and 4 after inoculation (Tables 1 and 2), as well as at days 6 and 8 after inoculation (Table 3). The detection of such recombinants in multiple birds over time may indicate that these recombinant viruses have fitness advantages that allow them to persist and be transmitted to other birds. Alternatively, these recombinants may have arisen from multiple independent recombination events, perhaps indicating that the regions of the genomes involved in these events are more prone to recombination. Some data to support the latter possibility were evident in the whole-genome sequence analyses. For example, viruses with genotype pattern codes 2 and 6 had recombination events between the UL46 and UL36 genes, which extend from approximately bp 20000 to 50000. These recombination breakpoints match with a recombination event previously detected in a study of Chinese ILTV strains, in which the K317 vaccine strain was determined to have arisen as a result of recombination between the Serva vaccine strain and virulent WG field strains (29) (Table 4). Recombination in this genome region (Table 4) has also been detected previously in Australian ILTV field isolates (2, 30).

Full-genome analysis of selected recombinants isolated from the *in vivo* study and ILTV full-genome sequences that were available in GenBank from Asia, Australia, Europe, and the United States (see Table S6) enable better characterization and interrogation of regions in the ILTV genome with a higher frequency of recombination events (recombination hot spots). Two genome regions were identified as hot spots in the recombinants isolated from the *in vivo* study and in isolates from Australia and the United States. These hot spots were located in the region from bp 20000 to 50000 and the region from bp 120,000 to 140000. Recombination hot spots were not identified in the Asian or the European isolates, although some recombination breakpoints were detected (Table 4). Our results are consistent with those obtained with HSV-1, in which recombination hot spots have been identified within the inverted repeat regions of the genome (31–33). The region from bp 20000 to 50000 has not been found to be a recombination hot spot in alphaherpesviruses in previous studies. Further research is

FIG 6 Legend (Continued)

originating from the United States ($n = 18$); (E) ILTV isolates originating from Asia ($n = 3$). The vertical lines indicate recombination breakpoints per 200-nucleotide (nt) window in each analyzed sequence, as detected with 95% confidence (gray) or 99% confidence (black). Horizontal lines indicate the limits for global hot spot detection, indicated at a level of 95% confidence (**) or 99% confidence (++).

TABLE 4 Recombination breakpoint analysis of full ILTV genome sequences and field isolates from Asia, Australia, and the United States and *in vivo* recombinants

Origin of isolates	Breakpoint (in alignment), 99% CI[a]		Gene(s) at the beginning of the breakpoint location[b]	Gene(s) at the end of the breakpoint location[b]	Possible viruses involved in recombination event[c]	Method(s) by which breakpoint was detected in RDP4
	Beginning	End				
Asia (China)	106423–109054	125644–129391	UL4, UL3.5, UL3, UL2, UL1	US10, sORF4	R, WG; M, unknown; m, K317	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	43560–45099	45674–54215	UL28, UL29	UL29, UL30, UL31, UL32, UL33, UL34	R, K317; M, SERVA; m, WG	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
Australia	27228–28747	112173–113630	ORFB, ORFC	UL[−1]	R, CL9; M, SERVA; m, A20	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	114752–115584	139504–140702	No gene between UL[−1] and ICP4	US6, US7	R, CL10; M, ACC78; m, SA2	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	30261–31965	51062–60138	ORFE, UL22	UL30, UL31, UL32, UL33, UL34, UL35, UL36	R, CL10; M, SERVA; m, A20	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	134126–137622	Undetermined	US6, US7	Undetermined	R, CL9; M, unknown; m, V1-99	RDP, GENECONV, MaxChi, Chimaera, SiScan, 3SEQ
	113596–114071	120150–124890	No gene between UL[−1] and ICP4	US10	R, CL9; M, unknown; m, CSW-1	RDP, MaxChi, Chimaera, SiScan, 3SEQ
	111500–113586	113598–126007	No gene between UL[−1] and ICP4	ICP4, US10	R, C10; M, CSW-1; m, SA2	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
	30804–33011	43052–49251	ORFE, UL22	UL28, UL29, UL30	R, ACC78; M, SERVA; m, SA2	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	99353–101053	104765–107587	UL8	UL5, UL4, UL3.5	R, CL10; M, SERVA; m, A20	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	112731–113586	114079–114296	UL[−1]	No gene between UL[−1] and ICP4	R, V1-99; M, unknown; m, SA2	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	126079–126160	126204–126250	No gene	No gene	R, A20; M, SA2; m, SERVA	GENECONV, 3SEQ
Europe (Italy)	42226–117783	122731–126724	Entire UL region	Entire US region	R, Nobilis; M, SERVA; m, 757/11	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
United States	113909–114632	122256–124629	No gene between UL[−1] and ICP4	No gene	R, 1874c5; M, J2; m, unknown	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
	111754–112964	113276–127469	UL[−1]	ICP4, US10	R, USDA; M, 81658; m, unknown	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
	115821–119810	120055–121242	ICP4	No gene	R, BdORFC; M, 6.48.88; m, unknown	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
	117501–119320	120028–121127	ICP4	No gene	R, 81658; M, unknown; m, 6.48.88	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
	45501–49291	50588–59726	UL29, UL30	UL30, UL31, UL32, UL33, UL34, UL35, UL36	R, S2.86; M, unknown; m, USDA	RDP, GENECONV, 3SEQ
	81577–89884	90133–95942	UL19, UL13, UL15, UL17, UL14	UL13, UL12, UL11, UL10	R, 6.48.88; M, S2.816; m, J2	RDP, GENECONV, Bootscan, 3SEQ
	107225–122304	122306–128723	UL 3.5, UL2, UL1, UL0, UL[−1], ICP4	US10, Sorf4, US2, US3	R, J2; M, unknown; m, 3.26.90	GENECONV
	22886–24681	24683–26851	UL46, UL45	UL45, ORFA, ORFB	R, 6.48.88; M, unknown; m, 14.939	GENECONV, MaxChi
	14052–16151	16153–18973	UL52	UL52, UL51, UL50	R, 6.48.88; M, unknown; m, BdORFC	GENECONV
	1–26560	1–26560	UL56, ORFF, UL54, UL53, UL52, UL50, UL49.5, UL49, UL48, UL46, UL45	UL56, ORFF, UL54, UL53, UL52, UL50, UL49.5, UL49, UL48, UL46, UL45	R, USDA; M, 14.939; m, Laryngovac	RDP, MaxChi, SiScan
	42685–50586	50591–66866	UL28, UL29, UL30, UL31	UL32, UL33, UL34, UL35, UL36, UL37	R, 1874c5; M, J2; m, unknown	GENECONV, Bootscan

(Continued on next page)

TABLE 4 (Continued)

Origin of isolates	Breakpoint (in alignment), 99% CI[a]		Gene(s) at the beginning of the breakpoint location[b]	Gene(s) at the end of the breakpoint location[b]	Possible viruses involved in recombination event[c]	Method(s) by which breakpoint was detected in RDP4
	Beginning	End				
In vivo recombinants	53623–68876	120517–121888	UL35, UL36, UL37, UL38, UL39	No gene between UL [−1] and ICP4	R, sample 23; M, unknown; m, CSW-1	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	121902–124017	134435–137336	US10, SORF3	US6, US7, US8	R, sample 27; M, V1-99; m, unknown	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	71911–78480	112397–120377	UL42, UL43, UL44, UL21, UL20	ICP4	R, sample 15; M, V1-99; m, CSW-1	RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3SEQ
	121904–125713	129197–137424	US10, protein SORF3, US2, US3	US4, US5, US6, US7, US8	R, sample 15; M, V1-99; m, sample 23	GENECONV, Bootscan, 3SEQ
	74976–97499	111841–114434	UL44, UL21, UL20, UL19, UL18, UL15, UL17, UL14, UL13, UL12, UL11, UL10, UL9	No gene Between UL [-1] and ICP4	R, sample 12; M, sample 1; m, V1-99	RDP, GENECONV, Bootscan, MaxChi, Chimaera, 3SEQ
	2256–97499	106970–137424	Entire UL region	Entire US region	R, sample 12; M, unknown; m, V1-99	GENECONV, MaxChi, Chimaera
	1–32193	105020–137424	UL56, protein IF, UL54, UL53, UL52, UL51, UL50, UL49.5, UL49, UL48, UL46, UL45, protein IA-IB-IC-ID-IE, UL22	Entire US region	R, sample 12; M, unknown; m, V1-99	GENECONV, MaxChi, Chimaera, 3SEQ
	1–1909	5870–17769	No gene	UL56, protein IF, UL54, UL53, UL52, UL51, UL50	R, sample 23; M, sample 1; m, sample 12	SiScan
	1–53621	107559–120122	UL56, protein IF, UL54, UL53, UL52, UL51, UL50, UL49.5, UL49, UL48, UL46, UL45, protein IA-IB-IC-ID-IE, UL22, UL24, UL25, UL26, UL26.5, UL27, UL28, UL29, UL30, UL31, UL32, UL33, UL34	Entire US region	R, V1-99; M, unknown; m, sample 8	GENECONV, MaxChi, SiScan, 3SEQ
	1–137424	1–137424	Entire UL region	Entire UL region	R, sample 12; M, CSW-1; m, sample 27	GENECONV, Bootscan, MaxChi, 3SEQ
	1–113123	1–113123	Entire UL region	Entire UL region	R, sample 27; M, V1-99; m, sample 23	RDP, GENECONV, MaxChi, Chimaera
	1–17769	19248–137424	UL56, protein IF, UL54, UL53, UL52, UL51, UL50	UL48, UL46, UL45, protein IA-IB-IC-ID-IE, UL22, UL24, UL25, UL26, UL26.5, UL27, UL28, UL29, UL30, UL31, UL32, UL33, UL34	R, CSW-1; M, sample 15; m, sample 27	MaxChi, Chimaera, 3SEQ
	1–137424	1–137424	Entire UL and US regions	Entire UL and US regions	R, sample 8; M, sample 15; m, unknown	GENECONV, MaxChi, 3SEQ
	11775–17921	17923–137424	UL53, UL52, UL51, UL50, UL49.5	UL49, UL48, UL46, UL45, protein IA-IB-IC-ID-IE, UL22, UL24, UL25, UL26, UL26.5, UL27, UL28, UL29, UL30, UL31, UL32, UL33, UL34	R, sample 27; M, sample 8; m, unknown	MaxChi, 3SEQ
	1–137424	1–137424	Entire UL and US regions	Entire UL and US regions	R, sample 15; M, sample 1; m, unknown	3SEQ

[a]CI, confidence interval.
[b]UL, unique long; US, unique short.
[c]R, recombinant; M, major parent; m, minor parent.

needed in order to fully determine which genes within this region are more likely to be involved in recombination events and the effects on viral phenotypes.

The different numbers of isolates used in our analyses may have affected the detection of recombination hot spots, with more viruses included in the analysis of recombination in U.S. and Australian isolates. Even with the unequal numbers of isolates from the different geographical regions, higher rates of recombination were detected within Australian isolates. Vaccine strains, including the Serva, A20, and SA2 strains, were frequently involved in recombination events among the Australian isolates (2, 34). In contrast, vaccine strains did not typically feature in the recombination events detected in the U.S. strains, although the Laryngovac strain was identified as a minor parent in one recombination event and some field strains, such as 1874c5 and J2, were involved in recombination events (Table 4). In general, the recombination events were detected with more certainty in the Australian, Asian, and European isolates, with most events being detected by five or more of the RDP4 breakpoint detection methods. Some of the recombination events in the U.S. strains, and in the progeny viruses from the *in vivo* study, were detected by fewer than three of the methods within RDP4 (Table 4). Further work is needed to fully understand the significance of the recombination events described here and to better understand the role of ILTV recombination in the United States.

The precise nature of the relationship between recombination, viral diversity, and evolution of virulence remains to be determined. The results of this study suggest that there may be some correlation between recombination, increased viral diversity, and increased virulence, as the coinfected groups had significantly higher mortality rates than groups that received the same dose of only ILTV V1-99. However, the coinoculated groups did receive two times the total dose of virus compared to the birds that received a single dose of virus, and the larger dose may have resulted in more severe clinical disease. In addition, higher viral genome copy numbers were detected in many of the coinoculated birds than in birds that received just V1-99 ILTV. Future studies to characterize the phenotype of dominant recombinants would be helpful to better understand this relationship. In addition, coinoculation studies that utilize naive, in-contact birds would be useful for understanding how recombinants that arise in individual coinfected chickens may be transmitted to become established in populations of birds. Of great practical importance is an understanding of how all these processes and relationships are affected by vaccines, which could affect recombination events, and therefore have an impact on disease control. This is a complex area of study, but it should be prioritized in order to enhance the control of infectious laryngotracheitis.

MATERIALS AND METHODS

Viruses and cell culture. The V1-99 and CSW-1 ILTV wild-type strains were used as the parental viruses for *in vivo* coinoculation. These strains belong to genotype classes 2 and 4, respectively, following the Australian ILTV genotype classification system (35). The V1-99 field strain was isolated from a hen in a commercial layer flock in 1999 and is typical of the predominant field isolates detected during disease outbreaks in Australia up until 2008 (30). The CSW-1 wild type is currently used as a standard laboratory strain in Australia (36, 37) and was originally isolated in 1970 from layer birds (38). The growth characteristics of these viruses have been described previously and compared *in vitro* (18) and *in vivo* (39). Both viruses have undergone at least three rounds of plaque purification. For this study, the viruses were propagated and titrated in chicken hepatocellular carcinoma (LMH) cells (40) using a plaque assay, as previously described (41). Virus isolation and purification from clinical material collected during the *in vivo* experiment were performed in LMH cell monolayers cultured in growth medium (GM) consisting of Dulbecco's minimal essential medium (DMEM) supplemented with amphotericin B (0.005 mg/ml), gentamicin (0.05 mg/ml), and co-trimoxazole (0.01 mg of sulfamethoxazole/ml and 0.002 mg of trimethoprim/ml), 10% (vol/vol) fetal bovine serum (FBS), and 10 mM HEPES (pH 7.7).

***In vivo* coinoculation experiment.** Five-week-old SPF chickens were obtained from Australian SPF Services Pty. Ltd. and used for the infection experiments. The *in vivo* experiment extended for 8 days. The birds were maintained in separate groups in isolator units and were managed, monitored, and euthanized following protocols approved by the Animal Ethics Committee (AEC) of the University of Melbourne (AEC number 1413401.1). This committee follows the Australian Code for the Care and Use of Animals for Scientific Purposes (42). Two groups of 10 SPF chickens were coinoculated by the intratracheal route with 300 μl of a 1:1 mixture of the CSW-1 and V1-99 ILTV strains containing either 1×10^3 (low-dose-coinoculated group) or 1×10^4 (high-dose-coinoculated group) PFU of each strain. Four groups containing five SPF birds each were inoculated by the intratracheal route with 300 μl of an

inoculum containing 1×10^3 or 1×10^4 PFU of only CSW-1 or only V1-99. The birds were monitored for 8 days. At 2, 4, 6, and 8 days after inoculation, tracheal swabs were collected using standard techniques, placed into 1 ml of viral transport medium (DMEM, 3% [vol/vol] FBS, and 100 μg of ampicillin/ml), transported on ice, and then stored at −80°C until processed for virus isolation and purification. The swabbing technique was not objectively measured, and some apparent variability in viral replication may be attributable to variations in swabbing technique. Prior to storage at −80°C, 200-μl aliquots of tracheal swab suspension were collected and stored separately at −20°C for DNA extraction using a Qiagen DNA robot extractor following the manufacturer's recommendations (QIAxtractor; Qiagen) and quantification of ILTV genome copy number using a qPCR described previously (43).

Virus isolation and purification. Progeny viruses were isolated and purified as previously described (18). Material from each tracheal swab was serially diluted (10-fold) in GM in order to identify the appropriate dilution for plaque purification. Dilutions were used to inoculate LMH cell monolayers in 6-well plates. After 1 h of incubation at 37°C, the cell monolayer was covered with semisolid methylcellulose overlay medium containing 10% FBS and incubated at 37°C in a humidified atmosphere of 5% (vol/vol) CO_2. After incubation for 24 to 48 h, up to 20 plaques were picked from each sample from the coinfected chickens and up to 10 were picked from the samples from the single inoculated birds at day 4 with a micropipette, and then each plaque was propagated individually by the inoculation of LMH cell monolayers in 12-well plates. Three rounds of plaque purification were performed, with one freeze-thaw cycle between each round.

SNP genotyping assay and examination of viral diversity. To characterize the viral progeny and identify recombinants, DNA from each plaque-purified virus was extracted and used as the template in a TaqMan SNP genotyping assay, as previously described (18). Any samples that were found to contain a mixed population of viruses (i.e., both SNPs present at any of the six locations) were not included in further analyses. To measure diversity, we first identified each recombinant using a unique genotype pattern code. Afterwards, these genotype pattern codes were analyzed in RStudio 0.99.902 using the VeganR (44) and BiodiversityR (27) packages. VeganR calculates the diversity indices used to perform ecological-diversity measurements in communities (45). BiodiversityR was used to generate Renyi profiles. This package provides a graphical user interface (GUI) via R-Commander incorporating functions used by VeganR to analyze measures of diversity such as richness (x axis value = 0), Hill (x axis values = 0.25, 0.5, 4, 8, 16, 32, and 64), Shannon-Weaver (x axis value = 1), 1/Simpson (x axis value = 2), and 1/Berger Parker (x axis value = Inf) values (27).

Whole-genome sequencing, genome assembly, and recombination analysis. Six progeny viruses that were identified as recombinants using the SNP genotyping assay were selected for whole-genome sequencing (genotype pattern codes/GenBank accession numbers [9A/MF156847, 6/MF156848, 2/MF156849, 9B/MF156850, 4/MF156851, and 40/MF156852]). These viruses included those with genotype patterns that were frequently detected (codes 2, 4, 6, and 9), as well as an infrequently detected genotype pattern (code 40). Two virus isolates with the same genotype pattern (code 9) were included to enable comparisons within a genotype pattern. Each virus was first amplified in LMH cells, and then viral DNA was extracted from purified nucleocapsids as previously described (20) and sequenced using the Illumina MiSeq platform at the Medical Genomic Facility in the Monash Health Translation Precinct (MHTP), Hudson Institute of Medical Research. The genome was assembled by mapping to CSW-1 (GenBank accession number JX646899) as a reference sequence using the Geneious mapper method. Settings of medium sensitivity and up to five iterations were used, as recommended by the software. To generate the consensus sequences, SNPs were called within and outside coding regions using the Find variation/SNPs tool with default settings. CSW-1 and V1-99 genomes (GenBank accession numbers JX646899 and JX646898, respectively) were used to annotate the new genome sequences. Mapping to the reference, SNP calling, and annotations were all performed using Geneious V8.1.9 (46).

In order to expand our analysis beyond the six genome sequences of recombinant viruses generated in this study, we also analyzed 35 full-genome sequences available in the NCBI database (see Table S6 in the supplemental material). The sequences were from ILTVs isolated in different geographical regions, including Asia, Australia, Europe, and the United States. Separate recombination analyses were performed for the viruses originated from different geographical regions and for the recombinant viruses generated during this study. To enable analysis for recombination events the sequences were first aligned and curated as previously described (20, 47). Recombination analyses were then performed using reticulate network analysis and the phi test within SplitsTree4 V 4.14.4 (48), as well as seven methods for detection of recombination breakpoints (RDP, GENECONV, 3Seq, SiScan, Chimarea, MaxChi, and Bootscan) within RDP4 V 4.83 (49). As several recombination breakpoints were detected along the full-genome sequences, the same alignments were used to visualize breakpoint distribution plots in order to determine patterns and/or recombination hot spots. The CSW-1, CL9, 1874c5, and WG genome sequences (GenBank accession numbers are provided in Table S6 in the supplemental material) were used as reference strains for the recombination analysis of our *in vivo* recombinants and Australian, U.S., and Asian strains, respectively. Breakpoint distribution plots were used as a matrix, with 800 permutations and a window size of 200 nucleotides. The recombination breakpoint and the recombination hot spot location analyses were performed as recommended in the RDP4 V 4.83 manual (49).

Accession number(s). In the present study, the follow genotype pattern codes (and GenBank accession numbers) were determined: 9A (MF156847), 6 (MF156848), 2 (MF156849), 9B (MF156850), 4 (MF156851), and 40 (MF156852).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01532-17.

SUPPLEMENTAL FILE 1, PDF file, 2.5 MB.

ACKNOWLEDGMENTS

We gratefully acknowledge Mahathi Nadimpalli, Nino Ficorilli, and Mesula Korsa for their help with cell culture and Jose Quinteros, June Daly, Jenece Wheeler, and Cheryl Colson for their help with the *in vivo* studies.

This work was supported by the Australian Research Council (FT140101287). Carlos A. Loncoman is supported by Becas Chile, CONICYT, Gobierno de Chile.

The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

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