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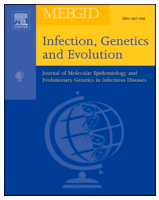

Comparative full genome sequence analysis of wild-type and chicken embryo origin vaccine-like infectious laryngotracheitis virus field isolates from Canada

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ABSTRACT

Infectious laryngotracheitis (ILT), caused by infectious laryngotracheitis virus (ILTV), occurs sporadically in poultry flocks in Canada. Live attenuated chicken embryo origin (CEO) vaccines are being used routinely to prevent and control ILTV infections. However, ILT outbreaks still occur since vaccine strains could revert to virulence in the field. In this study, 7 Canadian ILTV isolates linked to ILT outbreaks across different time in Eastern Canada (Ontario; ON and Quebec; QC) were whole genome sequenced. Phylogenetic analysis confirmed the close relationship between the ON isolates and the CEO vaccines, whereas the QC isolates clustered with strains previously known as CEO revertant and wild-type ILTVs. Recombination network analysis of ILTV sequences revealed clear evidence of historical recombination between ILTV strains circulating in Canada and other geographical regions. The comparison of ON CEO clustered and QC CEO revertant clustered isolates with the LT Blen® CEO vaccine reference sequence showed amino acid differences in 5 and 12 open reading frames (ORFs), respectively. Similar analysis revealed amino acid differences in 32 ORFs in QC wild-type isolates. Compared to all CEO vaccine strains in the public domain, the QC wild-type isolates showed 15 unique mutational sites leading to amino acid changes in 13 ORFs. Our outcomes add to the knowledge of the molecular mechanisms behind ILTV genetic variance and provide genetic markers between wild-type and vaccine strains.

1. Introduction

Gallid herpesvirus-1 (GaHV-1), traditionally known as infectious laryngotracheitis virus (ILTV), causes an upper respiratory tract disease in chickens, namely infectious laryngotracheitis (ILT). ILT is characterized by mild or severe respiratory manifestations accompanied by egg production losses (García et al., 2013a). GaHV-1 belongs to the genus *Iltovirus* in the subfamily *Alphaherpesvirinae* and the family *Herpesviridae* (Davison, 2010). Herpesviruses are known for establishing lifelong infections in their infected hosts through latency. Sites of establishment of latent infection include trachea and trigeminal ganglion (Bagust, 1986; Williams et al., 1992). Virus reactivation occurs intermittently particularly when birds are subjected to stress such as onset of laying or transportation (Hughes et al., 1989).

ILTV possess a linear double-stranded DNA genome of about 150 to 155 kbp length that is contained in an icosahedral capsid encircled with an envelope. The genome encodes 80 open reading frames (ORFs), where 65 ORFs are located within the unique long region (UL), 9 ORFs in the unique short region (US), and 6 ORFs in the inverted repeats (IR) and terminal repeats (TR) (Fuchs et al., 2007; Lee et al., 2011a). Like other DNA viruses, ILTV has a complex viral DNA replication machinery that includes a highly efficient proofreading DNA polymerase, resulting in very low rates of spontaneous mutation (Crute and Lehman, 1989; Drake and Hwang, 2005). However, the significance of natural recombination

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in the evolution of ILTV and other alphaherpesviruses is becoming increasingly recognized (Lee et al., 2012; Perez Contreras et al., 2020; Loncoman et al., 2017).

ILT is generally controlled through biosecurity and vaccination (García and Zavala, 2019). Currently, two types of vaccines are available to control ILT: live attenuated vaccines and recombinant viral vector vaccines. Depending on the choice of the attenuation of the virulent ILTV strain, there are two main categories: chicken embryo origin (CEO) or tissue culture origin (TCO) live attenuated ILT vaccines. The other type of the commercially available vaccines involves using fowlpox virus (FPV) or turkey herpesvirus (HVT) as viral vectors that express immunogenic ILTV proteins (García and Zavala, 2019). Although the live attenuated vaccines are the most effective means of ILT control, residual virulence, reversion to virulence, and recombination with field strains remain major concerns in the face of their application (Lee et al., 2012; Shehata et al., 2013; Guy et al., 1991). Several studies have shown that strains identical to or closely related to CEO vaccines have been implicated in ILT outbreaks around the world (Ojkic et al., 2006; Neff et al., 2008; Oldoni et al., 2008).

The major limitation of the currently used ILTV genotyping techniques such as restriction fragment length polymorphism (RFLP) (Kirkpatrick et al., 2006) and high-resolution melt (HRM) (Fakhri et al., 2019) is that they depend mainly on a small region of the viral genome (Sabir et al., 2020). Therefore, they may potentially be unsuccessful to differentiate all strains as variations may occur outside the areas targeted for analysis (Agnew-Crumpton et al., 2016). Additionally, the absence of difference between two strains in such a relatively small genomic region cannot necessarily be taken as evidence for identical strains. Recently, the availability of whole-genome sequences has enabled comparative analysis of gene diversity within different strains of ILTV (Loncoman et al., 2017). Since the first complete genome was assembled from partial genomic sequences of various strains in 2006 (Thureen and Keeler Jr., 2006), several molecular epidemiology studies have clustered ILTV vaccines and field strains into various clades using the full genome sequences data (Perez Contreras et al., 2020; García et al., 2013b; Zhao et al., 2015). However, the genetic alterations that cause CEO- and TCO-related isolates to revert to virulence and attenuate are generally unknown.

In Canada, while ILT is endemic in backyard flocks, outbreaks occur sporadically in commercial poultry industry in many provinces of the country (Perez Contreras et al., 2020; Ojkic et al., 2006; Sary et al., 2017). In recent years, several ILT outbreaks in Canada have predominantly involved CEO related and wild-type strains (Perez Contreras et al., 2020; Barboza-Solis et al., 2020). There are two commercially available live attenuated vaccines in Canada: LT Blen® CEO vaccine (Merial, Inc., Athens, GA, USA) and LT-IVAX® TCO vaccine (Intervet Inc., Omaha, NE, USA). Few studies have been focused in determining the difference between wild-type and vaccine-related ILTV strains targeting fragments of the ILTV genome in the Canadian chicken flocks (Ojkic et al., 2006; Barboza-Solis et al., 2020). However, due to the deficiency in the genetic information on the Canadian field strains of ILTV, progress in molecular epidemiology for ILTV has been slow in Canada. To this date, only one study was done on the comparative full genome analysis of 14 Canadian isolates from different provinces in Western Canada (Perez Contreras et al., 2020). The objective of this study was to genetically characterize the ILTV isolates obtained from ILT clinical cases in Eastern Canada (Ontario; ON and Quebec; QC) through whole-genome sequencing (WGS) and to identify genetic variation among wild-type and vaccine strains.

2. Materials and methods

2.1. Virus

Seven archived samples were received from Animal Health Laboratory, University of Guelph, Ontario and Swine and Poultry Infectious Diseases Research Center, University of Montreal, Montreal. The relevant history of the samples is summarized in Table 1.

2.2. DNA extraction, quantitative PCR (qPCR) and whole-genome sequencing (WGS)

DNA extraction from filtered homogenized tracheas was performed using QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) based on manufacturer's instructions. The extracted DNA was quantified using the Nanodrop 1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) with absorbance at 260 nm. In order to determine the amount of ILTV genome in the DNA extracted from tracheal tissue homogenates, a qPCR method was employed using a CFX96-c1000 Thermocycler (Bio-Rad laboratories, Mississauga, ON, Canada) targeting the protein kinase (PK) gene as previously described (Thapa et al., 2015). The reaction was set up as has been described previously (Perez Contreras et al., 2020; Barboza-Solis et al., 2021). The employed thermocycler conditions included 95 °C for 20 s initial denaturation, 40 cycles of denaturation at 95 °C for 3 s, annealing at 60 °C for 30 s and elongation at 95 °C for 10 s. The 7 samples had high ILTV genomic content and did not require to be propagated.

Samples were submitted for WGS at the Faculty of Veterinary Medicine, University of Montreal, QC, Canada. The libraries were started with 0.3 ng/ml of dsDNA using the Nextera XT DNA Library Preparation Kit (Illumina Corp, San Diego, CA, USA). Fragmentation and tagmentation were performed according to the manufacturer's instructions, except 14 PCR cycles were done instead of 12. Libraries were then purified using AxyPrep Mag™ PCR Clean-up Kits (Axygen®, Corning, NY, USA) as described in the manufacture's protocol. The quality of the libraries was assessed using Agilent High Sensitivity DNA Kit in a Bioanalyzer (Agilent, Santa Clara, CA, USA). LNB1 beads were used to normalize libraries (Nextera XT protocol). Libraries were sequenced using a MiSeq platform, with PhiX at about 1% as a control, for the sequencing runs in a v3 600-cycle cartridge (Illumina Corp, San Diego, CA, USA). Genome reconstruction was done as described previously (Perez Contreras et al., 2020).

2.3. Phylogenetic analysis

The whole genome sequences of our 7 ILTV isolates and 44 ILTV reference strains retrieved from GenBank (Supplementary Table 1) were aligned using Multiple Alignment with Fast Fourier Transformation (MAFFT) (Katoh and Standley, 2013). Phylogenetic trees for the full genome, UL, IR, and US alignments were generated using maximum likelihood method with 500 bootstrap replicates for branch support using MEGA X software (v.10.2.0) (Stecher et al., 2020).

2.4. Detection of network relationships

The multiple sequence alignments mentioned in the previous section were used to detect the historical recombination within our 7 ILTV sequences using reticulate network analysis and the phi test within SplitsTree4 (ver. 4.14.6) (Huson and Bryant, 2006).

3. Results

3.1. Whole-genome sequences

The genomes sizes of the 7 Canadian ILTV isolates ranged from 152,673 bp in the CAN/QC-2301711 isolate to 153,691 bp in the CAN/QC-2551439 isolate. The GC content was 48.1%, and a total of 79 predicted ORFs were identified. The accession numbers of the 7 Canadian ILTV sequences that were deposited in the GenBank are given in Table 2.

Table 1
History of the 7 ILTV isolates used in this study.

Sample ID	Province	Age (weeks)	Breed	Type	Flock size	ILT vaccine used	Mortality (# of birds)	Year
#2551439	Quebec	5	Ross	Broiler	40,500	No	650	2021
#2470159	Quebec	52	Orpington	Backyard	40	No	3	2021
#2301711	Quebec	NA	Sussex	Backyard	4	No	2	2020
#2307414	Quebec	NA	NA	Backyard	50	No	2	2020
#2236832	Quebec	3	NA	Backyard	250	NA	30	2019
#2462241	Ontario	9	NA	Backyard	NA	CEO	NA	2005
#2462242	Ontario	NA	NA	Backyard	NA	CEO	NA	1987

NA = Not available.

3.2. Phylogenetic analysis

The whole genome sequences of our 7 Canadian ILTV isolates were aligned with 44 ILTV strains, which included wild-type and TCO and CEO vaccine strains, representing various geographical areas. A phylogenetic analysis was done using the multiple sequence alignment of the 51 full genome sequences, including our 7 study sequences (Fig. 1). Two Canadian isolates recovered from ON, CAN/ON-2462241 and CAN/ON-2462242, were assigned to the CEO vaccine cluster with a nucleotide identity of 99.99% between each other and 99.97% to the USA CEO vaccine, LT Blen® (JQ083493). Of the 5 QC isolates, 1 isolate, CAN/QC-2307414, clustered with group V (CEO revertant), while the remaining 4 isolates that shared a 99.89% - 99.97% nt identity between each other, CAN/QC-2236832; CAN/QC-2301711; CAN/QC-2551439; and CAN/QC-2470159, were clustered with the wild-type group. The phylogenetic trees constructed using the UL, IR, and US regions showed similar topologies for our isolates compared to the tree constructed using the whole genome (Supplementary Fig. S1).

3.3. Recombination analysis

Analysis for the evidence of historical recombination on alignments of the whole genome, and UL, IR, and US regions was performed on 31 ILTV strains, which were selected as representative strains that showed close clustering with our 7 Canadian isolates (Fig. 2).

The phi test did find statistically significant evidence for recombination ($p < 0.05$) in the complete genome, as well as the UL, IR, and US regions, in all ILTV genome sequences used in the recombination analysis (Table 3). Recombination network topologies created by SplitsTree4 throughout the complete genome and different regions of all the ILTV sequences were consistent with the relationships observed in the phylogenetic tree.

3.4. Comparative analysis

Our 7 Canadian isolates were compared to LT Blen® CEO vaccine reference sequence. The highest nucleotide sequence identity of 99.97% and the lowest value of 99.66% were detected for the ON CEO clustered isolates and QC wild-type clustered isolate CAN/QC-2301711, respectively. The CEO revertant clustered isolate, CAN/QC-2307414, showed a 99.74% identity. All non-synonymous mutations in the coding regions of our 7 ILTV isolates compared to LT Blen® CEO vaccine reference sequence are presented in Table 4. Amino acid differences were located in a total of 35 ORFs that were unevenly distributed among our 7 differently clustered ILTV isolates.

Comparing the ON CEO clustered isolates with the LT Blen® CEO vaccine reference sequence, nucleotide changes in protein coding regions that resulted in amino acid differences were identified in UL54, UL36, ICP4, and US8 within both isolates. An additional amino acid change in UL53 was identified in CAN/ON-2462241 that was isolated two decades later than CAN/ON-2462242. Similar comparison with CEO revertant clustered isolate, CAN/QC-2307414, showed amino acid differences in ORFF, UL54, ORFC, ORFE, UL27, UL36, UL10, UL5, UL1, ICP4, US5 and US8. The highest number of amino acid changes was detected in QC wild-type clustered isolates that involved 32 ORFs (Table 4). Among a total of 62 positions of amino acid differences, 3 amino acid changes in UL54 (Met479Ile), ICP4 (Ala1152Thr), and US8 (Lys210Arg) were detected in all 7 isolates. Five changes in ORFC (Asp66AspAsp), UL27 (Ile644Thr), UL36 (Arg1347His), UL10 (Thr42Ala), and UL5 (Lys343Glu) were shared between the 5 QC isolates. QC wild-type clustered isolates demonstrated 45 amino acid differences in 28 ORFs that were not reported in the other two clusters.

To further identify the amino acid differences that are unique to our QC wild-type isolates, the 4 QC wild-type isolates, CAN/QC-2236832; CAN/QC-2301711; CAN/QC-2551439; and CAN/QC-2470159, were compared to all CEO vaccine strains available in Genbank including LT Blen® (JQ083493), Laryngo Vac® (JQ083494), CEO TRVX (JN580313), CEO low passage (JN580317), Nobilis Laringovac® (KP677881), Poulvac ILT® (KP677882), strain O (KU128407), isolate K317 (JX458824), Serva (HQ630064), A20 (JN596963), and SA2 (JN596962). Fourteen non-synonymous SNPs and 1 substitution in 13 ORFs were found unique to our 4 QC wild-type isolates compared to eleven CEO vaccine strains used. Additionally, 4 unique non-synonymous SNPs were found only in the CAN/QC-2470159 isolate. The Unique amino acid differences are summarized in Table 5. Similar analysis was conducted on phylogenetically related reference strains including J2, VFAR043, CAN/QC-

Table 2
The full genome sequences of our 7 Canadian ILTV isolates.

Isolate	Accession #	Genome length (bp)	Total reads	Mapped Reads	UL (bp)	IR (bp)	US (bp)	TR (bp)	G + C content
#2551439	ON598586	153,691	5,933,248	118,849	112,909	13,839	13,125	13,818	48.1%
#2470159	ON598585	153,607	4,009,470	65,213	112,909	13,816	13,125	13,757	48.1%
#2236832	OL354140	152,686	3,994,748	50,757	113,923	12,828	13,125	12,811	48.1%
#2301711	OK646550	152,673	3,253,586	22,832	113,911	12,828	13,125	12,810	48.1%
#2307414	OL661344	153,634	3,585,094	28,241	112,917	13,810	13,094	13,813	48.1%
#2462241	OK573459	153,589	1,071,012	531,823	113,926	12,798	13,094	12,808	48.1%
#2462242	OK624781	153,565	2,210,740	240,305	113,926	12,800	13,094	12,803	48.1%

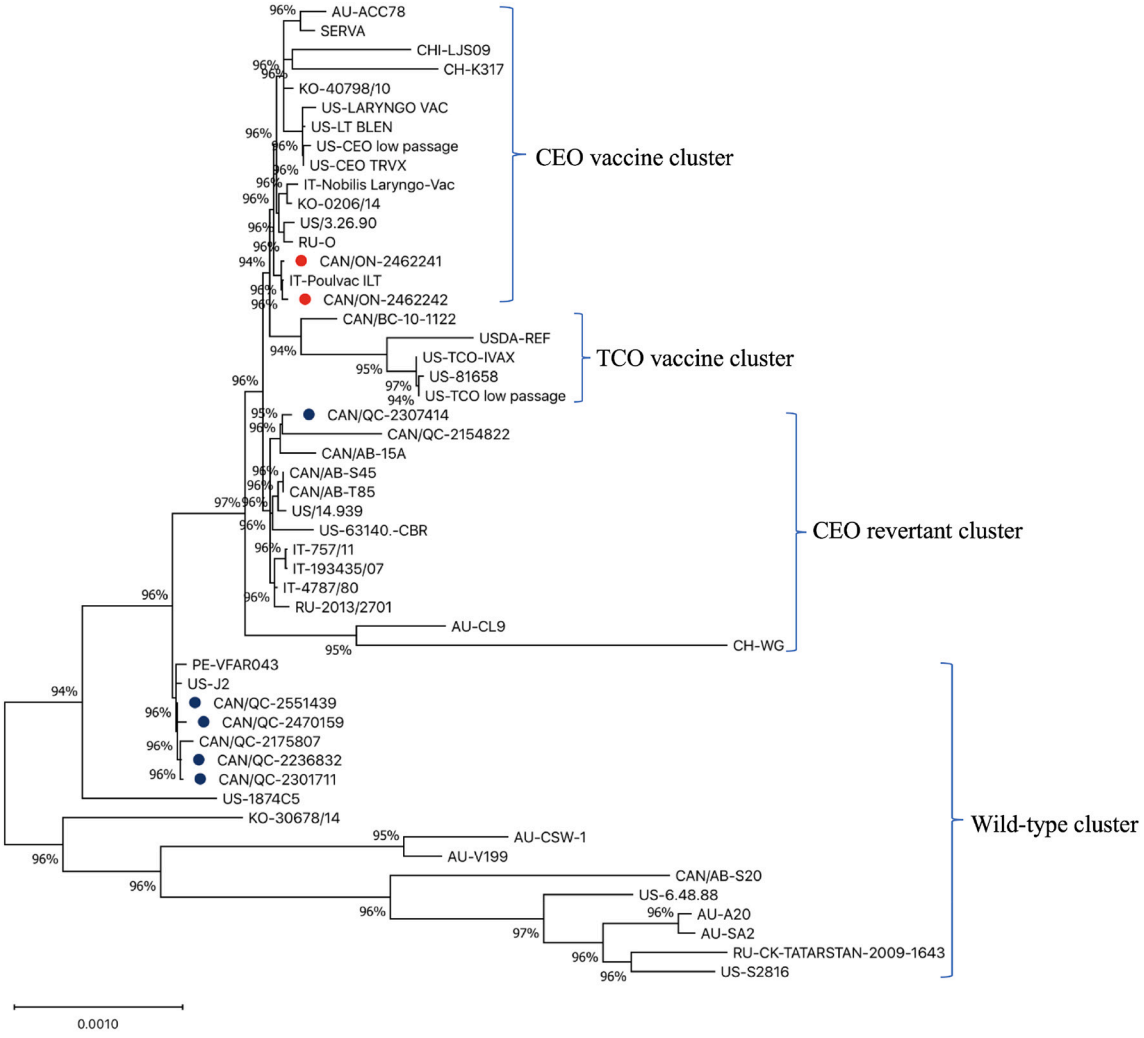


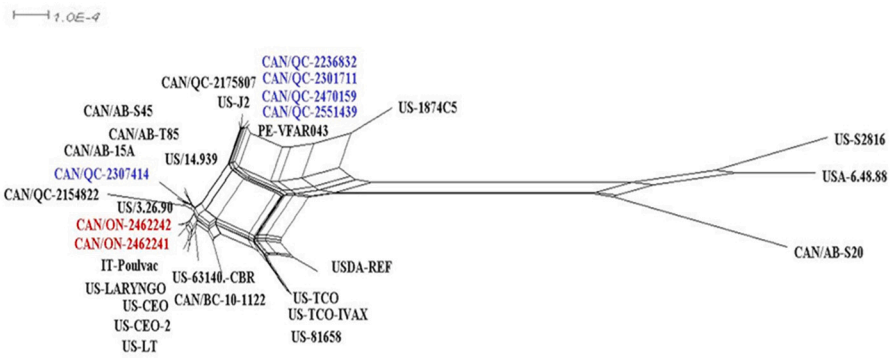
Fig. 1. Phylogenetic tree analysis for Our 7 ILTV isolates and 44 ILTV reference strains using alignment of the complete genome. Multiple genome alignment was performed using MAFFT. Maximum likelihood phylogenetic tree was constructed using the Tamura-Nei model with 500 bootstrap replicates for branch support in MEGA X software (v.10.2.0). ILTV genomes sequenced in this study are marked with red circle (ON isolates) and blue circle (QC isolates), and the clusters of the representative ILTV strains are indicated on the right side. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2175807, and 1874C5 (Fig. 1). The 15 unique amino acid changes detected in our 4 QC wild-type isolates were also identified in the reference strains.

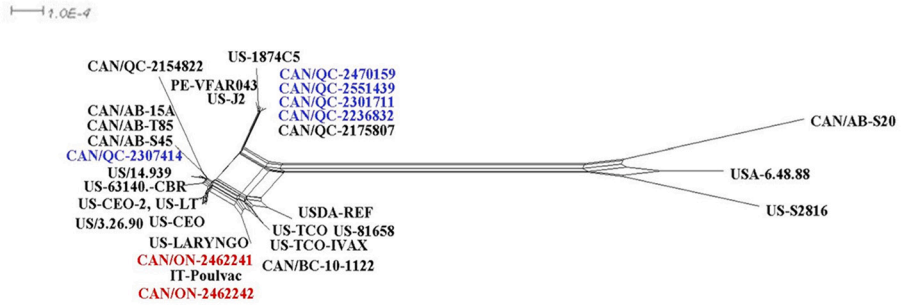
4. Discussion

Although the live attenuated ILT CEO vaccine has been routinely used to prevent and control ILTV infections over the world, the vaccine strain itself can revert to virulence following bird-to-bird transmissions, potentially resulting in outbreaks (Dufour-Zavala, 2008). Therefore, it became crucial to study the molecular epidemiology of ILTV and to distinguish between wild-type and vaccine strains. In this study, 7 Canadian ILTV isolates were genetically characterized using WGS. The isolates were phylogenetically assigned into CEO vaccine, CEO revertant, and wild-type clusters. Evidence of historical recombination in the ILTV strains circulating in Canada and other geographical regions was demonstrated. The comparison with the LT Blen® CEO vaccine genome showed that 35 ORFs differed across the 7 genomes. Fifteen unique amino acid changes in 13 ORFs were identified as genetic markers to differentiate between wild-type and CEO vaccine strains.

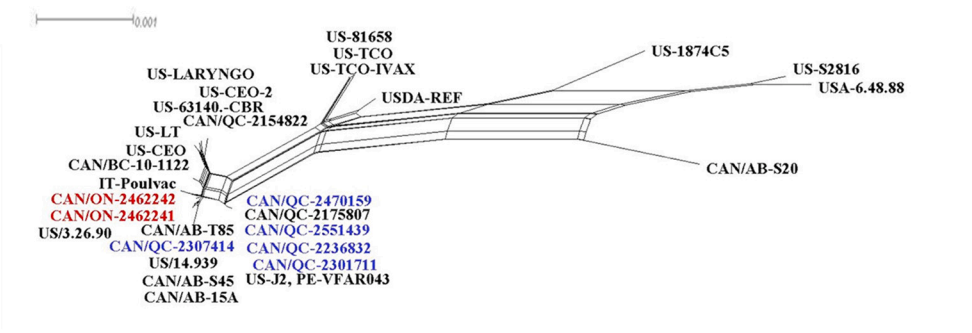
The genome lengths of CAN/QC-2236832 and CAN/QC-2301711 isolates were to some extent smaller (152,686 bp and 152,673 bp, respectively) than the other 5 isolates. In order to characterize these differences in the genome lengths, multiple sequence alignment with other reference strains was generated. Large deletion was found at the 3′ end of TR region (1041 bp for CAN/QC-2236832 and 1043 bp for CAN/QC-2301711). Interestingly, the lack of sequence fragment at the same position was also noticed (1016 bp) in the genomes of the Canadian field strain (CAN/AB-S20), European CEO vaccine (Serva), Australian CEO vaccines (SA2 and A20) and virulent strains (ACC78 and CL9), the USA 1874C5 field strain and Peruvian VFR043 field strains. This finding was in agreement with previous studies which reported significant discrepancies in the ILTV genome size ranging from 150,118 to 155,465 bp due to large insertions and deletions (INDELs) (Lee et al., 2011a; Perez Contreras et al., 2020; García et al., 2013b; Lee et al., 2013; Lee et al., 2011b; Wu et al., 2022). A deletion of 3300 bp at the 5′ ends of the USA



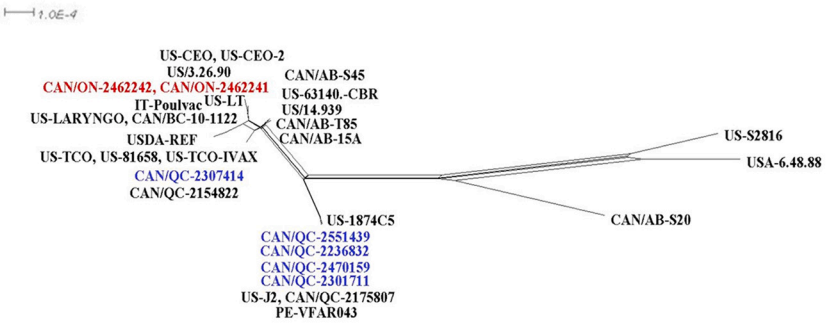
(**A**)



(**B**)



(**C**)



(**D**)

Fig. 2. Recombination network analysis of 31 ILTV sequences including our 7 ILTV sequences using the alignment of the complete genome sequence (**A**), UL region (**B**), IR region (**C**), and US region (**D**). The bar indicates sequence substitution per site. The phi test implemented in SplitsTree4 was performed in all networks and showed statistical significance ($p < 0.05$) for recombination using the complete genome and UL, IR, and US regions. The QC isolates are colored with blue, while the ON isolates are colored with red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of recombination analysis by the pair-wise homoplasy index (PHI) test implemented in SplitsTree4.

Genomic regions	Informative sites	*P*-value
Complete genome	826	0.0
UL region	446	$4.38E^{-9}$
IR region	129	$7.849E^{-9}$
US region	84	0.02273

TCO vaccines and related field strains have also been reported (García et al., 2013b; Spatz et al., 2012). It worse to mention that an error in the assembly of the Serva reference genome, which assembled based on the ILTV composite sequence, was detected. A sequence fragment with length of 1016-bp was wrongly annotated to be at the 3′ end of the UL of the Serva genome rather than the end of the TR region resulting an increase in the size of the Serva genome from 152,630 to 153,645 bp (Spatz et al., 2012).

Phylogenetic tree analyses were performed to elucidate the relationship between our 7 ILTV isolates and other ILTV reference sequences from different geographical areas. Four phylogenetic trees were generated using multiple sequence alignments of the whole genome and UL, IR, and US regions. In all cases, the 2 ON isolates were always close to the USA and European CEO vaccines. The CEO cluster included the CEO vaccine strains from USA, Europe (Italy and Russia), Chinese K317, and Serva. Only the Australian CEO vaccine strains, SA2 and A20, clustered separately. Previous studies utilizing either single genes or a smaller number of strains than our study have also indicated the distant grouping of SA2 and A20 vaccine strains from other CEO vaccine strains (García et al., 2013b; Piccirillo et al., 2016). The CAN/QC-2307414 isolate clustered with the USA 63140 strain, Italian strains (757/11, 193,435/07 and 4787/80), and Canadian strains (AB-S45 and AB-T85) that were previously identified as CEO revertant ILTV strains (Perez Contreras et al., 2020; García et al., 2013b; Piccirillo et al., 2016). The remaining 4 QC isolates were grouped with the wild-type cluster that mostly comprised virulent strains from different geographical regions except for the Australian CEO vaccine strains, SA2 and A20. An additional cluster was formed by the USA TCO vaccine strains and other related North American field strains.

The phylogenetic analysis does not take into account the recombination events while supposing phylogenetic relationships between ILTV isolates (Sabir et al., 2020; Martin et al., 2015). A split network for 31 ILTV genomes, including our 7 ILTV sequences, was built to get a wider illustration of the relationships that exist among the highly similar ILTV genomes, including the presence of recombinants. In agreement with a study done in Peru, the evidence for historical recombination in the whole genome and UL, IR and US regions was found to be statistically significant (Morales Ruiz et al., 2018). In contrast, previous studies using a smaller number of ILTV sequences than our study have shown evidence of recombination in ILTV genomes outside the US region (Zhao et al., 2015; Lee et al., 2013; La et al., 2019). Interestingly, in comparison to the UL and IR regions, the observed recombination networks in the US region were limited in the current study. This finding could indicate that recombination events are uncommon in US region (Lee et al., 2013; Morales Ruiz et al., 2018). The reticulate networks also confirmed the close relationship between the Canadian ON field isolates and the CEO vaccines. The ON isolates, CAN/ON-2462241 and CAN/ON-2462242, grouped together in the same split with the IT-Poulvac-ILT® CEO vaccine. The Poulvac ILT® CEO vaccine is a European vaccine that contains the Salisbury strain; however, it was previously shown to be closely related to the USA CEO vaccines (Piccirillo et al., 2016). These findings suggest that CEO clustered field and vaccine strains from North America and Poulvac ILT® CEO vaccine strain shared a common ancestor. The QC wild-type isolates were found in the same split with Peruvian VFAR-043 and USA-J2 field strain. The close relation between VFAR-043 and USA-J2 was determined previously in a recent study, which suggested that VFAR-043 may have been introduced into Peru illegally from unvaccinated game chickens from the USA (Morales Ruiz et al., 2018). Therefore, it is also reasonable to expect the USA origin of our QC wild-type isolates.

In this study, comparative genome analysis of 7 Canadian field isolates with the CEO vaccine licensed in Canada, LT Blen®, was performed. The CEO clustered ON isolates showed a high nucleotide sequence identity of 99.97% to the commercial LT Blen® vaccine. Only 5 amino acid differences in 4 ORFs were identified. Even though the 2 ON isolates separated by a long spanning time, only 1 additional amino acid change in UL53 was identified in CAN/ON-2462241 isolate. These findings coincide with the low mutation rate and genetic stability for the members of the *Alphaherpesvirinae* subfamily including ILTV (Piccirillo et al., 2016; Thiry et al., 2005). The CEO revertant clustered isolate, CAN/QC-2307414, showed 15 amino acid change in 12 ORFs. In a previous study, mutations in 12 genes were exclusively identified in the genome of the USA CEO revertant strain, 63140, compared to six CEO vaccine genomes (García et al., 2013b). Out of these 12 genes, mutations in 7 genes (ORFF, ORFC, ORFE, UL27, UL36, UL10, and US5) were also identified in our CEO revertant clustered isolate, CAN/QC-2307414. Additionally, 4 identical amino acid changes in ORFC (Asp66AspAsp), ORFE (Gly133Ala), UL27 (Ile644Thr), and Ul10 (Thr42Ala) were identified in both of our CEO revertant-clustered isolate and the USA 63140 strain. These results support the likelihood of that genes are being encoding for potential virulence factors (García et al., 2013b).

In order to identify genetic markers that discriminate between wild-type and vaccine strains, the genomes of our wild-type isolates were compared to all CEO vaccine genomes available in GenBank. Fifteen unique amino acid changes distributed in 13 ORFs were identified. The same 15 amino acid changes were also confirmed in other wild-types strains isolated in USA, Canada, and Peru. A single unique amino acid change was detected in each of glycoproteins gM, gG, and gJ and 2 amino acid changes in gB. These proteins play an important role in host range and pathogenicity. Glycoprotein M encoded by UL10 is an envelope glycoprotein that has roles in the entry, assembly, and release of alphaherpesviruses (Li et al., 2021). Mutants with deletions of gG (US4 gene) showed decreased virulence for chickens (Devlin et al., 2006). Glycoprotein J (US5 gene) was shown to be essential for lytic replication of ILTV (Mundt et al., 2011). Glycoprotein B (UL27) has an essential role in the herpesvirus fusion and entry (Avitabile et al., 2009). The thymidine kinase (TK) gene (UL23), a major gene related to virulence of ILTV, showed a single unique amino acid change (Asn212Asp). A TK-gene-deleted recombinant ILTV showed considerable low virulence and high protective efficacy in chickens (Han et al., 2002). Unique amino acid changes were also detected in genes encoding for structural proteins including major tegument proteins (UL36 and UL37) and capsid proteins (UL38 and UL6). In herpesviruses, these proteins have been shown to be necessary for virion assembly (Desai, 2000; Desai et al., 2001; Fan et al., 2015; Newcomb et al., 2001). Hence, the effectiveness of viral assembly and the ability to create infective viral progeny can be affected by mutations in these proteins. Unique mutations were also found in UL30 and UL9 genes. These genes encode for proteins that control the efficiency of viral DNA synthesis: DNA polymerase holoenzyme (UL30) and DNA replication origin binding protein (UL9) (Boehmer and Lehman, 1997). The US8A gene, which is predicted to encode a type II membrane protein, had a unique amino acid change. The product of this gene was recently identified as a virulence factor for herpes simplex virus 1 (HSV-1) (Kato et al., 2016).

Table 4
Summary of non-synonymous mutations in the coding regions of our 7 ILTV isolates compared to LT Blen® CEO vaccine reference sequence.

Gene		CDS		CEO vaccine		CEO revertant	Wild type			
Name	Product	Amino Acid Position	Nucleotide Change	ON-2462241	ON-2462242	QC-2307414	QC-2236832	QC-2301711	QC-2551439	QC- 2470159
ORFF	protein IF	628	C -> A	–	–	S –> Y	–	–	–	–
		633	CT –> TA	–	–	GS -> GT	–	–	–	–
UL54	multifunctional expression regulator	479	C -> T	M -> I	M -> I	M -> I	M -> I	M -> I	-> I	M -> I
		241	T -> G	E -> D	E -> D	–	–	–		
UL 53	envelop glycoprotein K	255	G –> A	H –> Y	–	–	–	–	–	–
UL50	deoxyuridine triphosphatase	320	A -> G	–	–	–	F -> L	F -> L	F -> L	F -> L
ORFA	protein IA	126	C -> T	–	–	–	P -> S	P -> S	P -> S	P -> S
ORFB	protein IB	83	T -> C	–	–	–	C -> R	C -> R	C -> R	C -> R
		129	G -> T	–	–	–	E -> D	E -> D	E -> D	E -> D
ORFC	protein IC	66	-> ATC	–	–	D -> DD	D -> DD	D -> DD	D -> DD	D -> DD
ORFE	protein IE	245	T -> C	–	–	–	Q -> R	Q -> R	Q -> R	Q -> R
		133	C -> G	–	–	G -> A	–	–	–	–
UL22	envelope glycoprotein H	568	A -> G	–	–	–	L -> S	L -> S	L -> S	L -> S
UL23	thymidine kinase	212	C -> T	–	–	–	D -> N	D -> N	D -> N	D -> N
UL26	capsid maturation protease	210	T -> G	–	–	–	I -> M	I -> M	I -> M	I -> M
UL27	envelope glycoprotein B	827	T -> G	–	–	K -> N	–	–	–	–
		805	T -> C	–	–	–	K -> R	K -> R	K -> R	K -> R
		799	G -> A	–	–	–	P -> S	P -> S	P -> S	P -> S
		644	A -> G	–	–	I -> T	I -> T	I -> T	I -> T	I -> T
UL28	DNA packaging terminase subunit 2	509	A -> G	–	–	–	I -> T	I -> T	I -> T	I -> T
UL30	DNA polymerase catalytic subunit	91	A -> G	–	–	–	R -> G	R -> G	R -> G	R -> G
		312	C -> T	–	–	–	A -> V	A -> V	A -> V	A -> V
		578	C -> T	–	–	–	A -> V	A -> V	A -> V	A -> V
UL36	large tegument protein	2179	G -> A	–	–	–	P -> L	P -> L	P -> L	P -> L
		1347	C -> T	–	–	R -> H	R -> H	R -> H	R -> H	R -> H
		817	G -> A	–	–	R -> C	–	–	–	–
		275	G -> A	A -> V	A -> V	–	–	–	–	–
UL37	tegument protein UL37	880	T -> C	–	–	–	T -> A	T -> A	T -> A	T -> A
UL38	capsid triplex subunit 1	444	C -> T	–	–	–	A -> V	A -> V	A -> V	A -> V
UL42	DNA polymerase processivity subunit	14	A -> G	–	–	–	N -> D	N -> D	N -> D	N -> D
UL17	DNA packaging tegument protein UL17	348	T -> C	–	–	–	Y -> H	Y -> H	Y -> H	Y -> H
		683	A -> G	–	–	–	H -> R	H -> R	H -> R	H -> R
UL15	DNA packaging terminase subunit 1	56	C -> A	–	–	–	–	–	–	M -> I

(*continued on next page*)

Table 4 (*continued*)

Gene		CDS		CEO vaccine		CEO revertant	Wild type			
Name	Product	Amino Acid Position	Nucleotide Change	ON-2462241	ON-2462242	QC-2307414	QC-2236832	QC-2301711	QC-2551439	QC- 2470159
UL10	envelope glycoprotein M	248	G -> A	–	–	–	P -> S	P -> S	P -> S	P -> S
		42	T -> C	–	–	T -> A	T -> A	T -> A	T -> A	T -> A
UL9	DNA replication origin-binding helicase	10	T -> C	–	–	–	V -> A	V -> A	V -> A	V -> A
		776	G -> A	–	–	–	–	–	–	R -> H
UL7	Tegument protein UL7	42	C -> T	–	–	–	–	–	–	A -> T
UL6	capsid portal protein	693	C -> T	–	–	–	E -> K	E -> K	E -> K	E -> K
		258	C -> T	–	–	–	A -> T	A -> T	A -> T	A -> T
		681	C -> T	–	–	–	–	–	–	G -> D
UL5	helicase-primase helicase subunit	343	A -> G	–	–	K -> E	K -> E	K -> E	K -> E	K -> E
UL1	envelop glycoprotein L	128	C -> T	–	–	D -> N	–	–	–	–
UL0	protein UL0	341	A -> G	–	–	–	L -> P	L -> P	L -> P	L -> P
		273	G -> A	–	–	–	S -> F	S -> F	S -> F	S -> F
ICP4	transcriptional regulator ICP4	1152	C -> T	A -> T	A -> T	A -> T	A -> T	A -> T	A -> T	A -> T
		1131	A -> G	–	–	–	V -> A	V -> A	V -> A	V -> A
		857	=+ GCGGTTGTTGCGGTTCTT	–	–	–	-> EEPQQPQ	-> EEPQQPQ	-> EEPQQPQ	-> EEPQQPQ
		88	=+CCGCGTCTTGGG	–	–	–	A -> AQDAA	A -> AQDAA	A -> AQDAA	A -> AQDAA
US3	serine/threonine protein kinase US3	291	A -> C	–	–	–	K -> T	K -> T	K -> T	K -> T
US4	envelope glycoprotein G	98	C -> A	–	–	–	H -> N	H -> N	H -> N	H -> N
		115	T -> G	–	–	–	V -> G	V -> G	V -> G	V -> G
		220	GG -> CT	–	–	–	G -> L	G -> L	G -> L	G -> L
US5	envelope glycoprotein J	684	G -> A	–	–	–	A -> T	A -> T	A -> T	A -> T
		655	=+AAATTACTCAGACTCCGAGTACGGTACCGG	–	–	–	E -> EITQTPSTVPE	E -> EITQTPSTVPE	E -> EITQTPSTVPE	E -> EITQTPSTVPE
		476	C -> T	–	–	T -> I	–	–		
US7	envelope glycoprotein I	178	C –> A	–	–	–	A -> D	A -> D	A -> D	A -> D
US8	envelope glycoprotein E	210	A -> G	K -> R	K -> R	K -> R	K -> R	K -> R	K -> R	K -> R
US8A	membrane protein US8A	91	C -> T	–	–	–	T -> I	T -> I	T -> I	T -> I
		115	G -> A	–	–	–	G -> D	G -> D	G -> D	G -> D
ICP4	transcriptional regulator ICP4	87	=+GCGGCCCAAGAC	–	–	–	-> AAQD	-> AAQD	-> AAQD	-> AAQD
		857	=+GCGGCCCAAGAC	–	–	–	Q -> QQPQEPQ	Q -> QQPQEPQ	Q -> QQPQEPQ	Q -> QQPQEPQ
		1131	T -> C	–	–	–	V -> A	V -> A	V -> A	V -> A

Table 5
Summary of non-synonymous mutations in the coding regions of our QC wild-type clustered isolates, CAN/QC-2236832; CAN/QC-2301711; CAN/QC-2551439; and CAN/QC-2470159, compared to eleven CEO vaccine reference sequences published in GenBank.

Gene	CDS Position	Nucleotide change	Amino acid change	Wild-type isolate
ORFA	126	T -> C	S -> P	*
UL23	212	T -> C	N -> D	*
UL27	805	C -> T	R -> K	*
	799	A -> G	S -> P	*
UL30	578	T -> C	V -> A	*
UL36	2179	A -> G	L -> P	*
UL37	880	C -> T	A -> T	*
UL38	444	T -> C	V -> A	*
UL15	56	A -> C	I -> M	QC-2470159
UL10	248	A -> G	S -> P	*
UL9	10	C -> T	A -> V	*
	776	A -> G	H -> R	QC-2470159
UL7	42	T -> C	T -> A	QC-2470159
UL6	693	T -> C	K -> E	*
	681	T -> C	D -> G	QC-2470159
	258	T -> C	T -> A	*
US4	220	CT -> GG	L -> G	*
US5	684	A -> G	T -> A	*
US8A	91	T -> C	I -> T	*

Asterisks (*) represent all 4 QC wild-type isolates.

Overall, our study provides valuable data about genomic variation of divergent ILTV strains. Our findings offer distinct genetic markers that can be used to differentiate between wild-type and vaccine strains.

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Informed consent statement

Not applicable.

Data availability statement

The data presented in this study are available on request from the corresponding author.

CRediT authorship contribution statement

Esraa A. Elshafiee: Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Mohamed S.H. Hassan**: Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Chantale Provost**: Formal analysis, Investigation, Writing – review & editing. **Carl A. Gagnon**: Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition. **Davor Ojkic**: Conceptualization, Resources, Writing – review & editing, Funding acquisition. **Mohamed Faizal Abdul-Careem**: Conceptualization, Methodology, Resources, Supervision, Project administration, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2022.105350.

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