Brazilian Journal of Microbiology (2022) 53:2223–2232
https://doi.org/10.1007/s42770-022-00833-y

VETERINARY MICROBIOLOGY - RESEARCH PAPER

Detection and molecular characterization of infectious laryngotracheitis virus (ILTV) in chicken with respiratory signs in Brazil during 2015 and 2016

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Received: 13 June 2022 / Accepted: 20 September 2022 / Published online: 21 October 2022

Abstract

Avian infectious laryngotracheitis (ILT) is a respiratory disease that causes severe economic losses in the poultry industry, mainly due to high morbidity and mortality and reduced egg production. Molecular characterization was performed on samples collected from flocks in the Brazilian States of São Paulo, Pernambuco, and Minas Gerais during 2015 and 2016 that presented clinical signs of respiratory disease. End-point PCR was used for viral detection, and DNA sequencing was used for differentiation of vaccine and field strains. Molecular analysis based on the infected cell protein (ICP4) gene separated four of the nine samples together with previous Brazilian isolates (São Paulo and Minas Gerais), one sample was grouped on the same branch as Minas Gerais strains (along with another related sample), one sample was separately branched but still related to the tissue culture origin (TCO) vaccine strain, and two samples were grouped on the same branch as the TCO vaccine strain. Molecular analysis of the thymidine kinase (TK) gene showed the existence of strains of both high and low virulence. The characterization of two fragments of the ICP4 gene and a fragment of the TK gene in this study suggested that the virus circulating in Guatapará, as well as those in Barretos and Itanhandu, that is causing respiratory problems in birds is a highly virulent field strain. The clinical signs point to a TCO vaccine strain that most likely underwent some reversal event and is a latent reactivated infection.

Keywords Chicken · ILTV · Detection · PCR · Molecular characterization

Introduction

ILT is a highly contagious respiratory disease of chickens and pheasants that affects the respiratory tract of birds. ILT is on the list of compulsory notifiable diseases of the World Organization for Animal Health (https://www.oie.int/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3). The causative agent is Gallid herpesvirus type 1 (GaHV-1) commonly known as Avian infectious laryngotracheitis virus (ILTV), a member of the family Herpesviridae and the genus Iltovirus [1]. It causes economic losses due to weight loss, decreased egg production, high morbidity and mortality, and susceptibility to other respiratory pathogens [2]. Infections vary according to the virulence of viral strains, presenting sub-clinically with low mortality or in a severe form in which there is high mortality [3]. ILT was the first avian disease for which effective vaccines were developed from attenuated viruses [2], specifically the chicken embryo origin (CEO)

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vaccine [4, 5] and the TCO vaccine [6, 7]. However, with these types of vaccines, there is a risk of reversion to virulence [8, 9]. Therefore, a new generation of vaccines has been developed in the last few years using vectors for the expression of GaHV-1 genes [10, 11]. These vaccines, along with good biosecurity practices, can be used as part of programs to control the disease [12, 13]. Several molecular-based techniques have been used to detect and genotype the virus, such as PCR, RFLP, qPCR, and DNA sequencing [14, 15]. These techniques were used to determine the origin of the virus (field or vaccine), monitor the animals, carry out epidemiological studies, and identify possible recombination events [16, 17]. The ILTV genome consists of a linear double-stranded chain of approximately 155 Kb [18]. The infected cell protein 4 (ICP4) gene is expressed prior to the replication of viral genomes in the infected cell and is responsible for the regulation of early and late genes during infection [19]. It has also been widely used in epidemiological studies to determine the origin of the virus involved in outbreaks of the disease [16, 19, 20]. In addition, single nucleotide polymorphisms in the thymidine kinase (TK) gene, located in the unique long region of the viral genome, has been associated with isolates of high or low virulence [21]. In Brazil, ILT was first described in 1974 [22] and since that time has been reported often in commercial poultry. In 2002, there was an outbreak in the Bastos region [16, 23], and in 2010, another outbreak occurred in the region of Minas Gerais [24]. The virus responsible for the outbreak in Minas Gerais was characterized as a non-vaccine, low-virulence strain [20].

The aim of this study was to detect and characterize ILTV in birds with respiratory signs using molecular techniques. The samples were collected between 2015 and 2016 and sent to the Laboratory of Avian Diseases from the School of Veterinary Medicine at the University of São Paulo.

Material and methods

Viral samples

In the present study, forty-two samples, obtained in the period of 2015 and 2016, from birds with respiratory signs, showing facial edema, swollen and stretched heads, and sneezing, were collected and sent to the laboratory for diagnosis. Among the samples studied, nine samples were positive for ILTV and are described below: USP 657–3 (trachea), USP 657–4 (trigeminal ganglion), USP 657–4 (eyelid), USP 657–5 (caecal tonsil), USP 657–7 (trachea), USP 695–1 (trachea), USP 695–2 (eyelid), USP 695–3 (trigeminal ganglion), and USP 729 (trachea).

Extraction of viral DNA from samples

Viral DNA from samples and from the positive control (vaccine strain) was extracted according to the protocol previously described [25]. A suspension with the samples was carried out with sufficient 0.1 M phosphate-buffered saline (PBS; 0.1 M, pH 7.4) to yield a 10% (*w/v*) solution. This solution was clarified at 12,000 × *g* for 20 min at 4 °C and 200 µL of supernatant was separated and incubated for 5 min at 37 °C with 1000 µL of phenol/guanidine thiocyanate solution. Chloroform (200 µL) was added to the solution, the mixture was centrifuged (12,000 × *g* for 15 min at 4 °C), 750 µL of propanol was added, and the whole was stored at – 20 °C for 12 h. The solution was centrifuged (12,000 × *g* for 15 min at 4 °C), the supernatant was discarded, and the DNA pellet was rinsed with 70% ethanol twice in each rinse was centrifuged (12,000 × *g* for 15 min at 4 °C). The total DNA sample was dissolved in 30 µL of TE buffer. PBS 0.1 M pH 7.4 was used as a negative control. The DNA was dissolved in 30 µL of Tris–EDTA (TE) buffer and stored at – 20 °C until the molecular tests and DNA sequencing were performed.

Procedures for detection and molecular characterization of ILTV isolates

All the samples were screened by PCR using the method and protocol previously described [26]. This protocol amplifies a fragment of the gene coding for glycoprotein E (gE). Samples that were positive by the gE PCR (657–5, 657-4 T, 657-4E, 657–5, 657–7, 695–1, 695–2, 695–5, and 729) were further subjected to PCR to amplify two fragments of the ICP4 gene using the method described [16]. The PCR reaction consisted of 0.5 µM of each of the primers (ICP4-1F and ICP4-1R or ICP4-2F and ICP4-2R) (Table 1), 2.5 µL of 10 × buffer, 4.0 µL of 1.25 mM dNTPs, 37.5 mM of $MgCl_2$, 1.25 U of Platinum Taq DNA polymerase (Invitrogen by Life Technologies, Carlsbad, CA, USA), and 2.5 µL of DNA. Ultrapure water free of DNAse was added to the reaction to bring the volume up to 25 µL. The amplification reaction occurred under the following conditions: 3 min for the initial denaturation at 94 °C; 35 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. The TK gene was amplified using a nested PCR protocol described [21]. The PCR reaction contained 0.5 µM aliquots of each of

Table 1 Primers used in the present study

Virus	Gene	Primer	Nucleotide sequences (5′–3′)	Amplicon	Assay	Reference
ILTV	gE	GE1S	CGT ATA CCA TCC TAC AGA CGG CA	540 bp	PCR	Chacón and Ferreira 2008
		GE2AS	CGT ACA ATG GTT CGG TCT TGG A			
		GE3S	AGT CCT CTT ATA GCC ATC CCC A	219 bp	*NESTED*	
		GE4AS	CAC CCC CGC GAC GAC GAA GT			
	ICP4	ICP4-1F	ACT GAT AGC TTT TCG TAC AGC ACG	687 bp	PCR	Chacón and Ferreira 2008
		ICP4-1R	CAT CGG GAC ATT CTC CAG GTA GCA			
		ICP4-2F	CTT CAG ACT CCA GCT CAT CTG	631 bp	*NESTED*	
		ICP4-2R	AGT CAT GCG TCT ATG GCG TTG AC			
	TK	TKOPS	ATC GTA TAG GCC AGC CTT	1296 bp	PCR	Han and Kim 2001
		TKOPAS	CCA CGC TCT CTC GAG TAA			
		TKIPS	CTT AGC GGA ACC TAT GCA AG	648 bp	*NESTED*	
		TKIPAS	TAG CGT CTG GTC GAT TGA AG			

ILTV, infectious laryngotracheitis virus; *gE*, glycoprotein E; *ICP4*, infected cell protein; *TK*, thymidine kinase; *bp*, base pair

the primers (TKOPS and TKOPAS) (Table 1), 2.5 µL of 10 × buffer, 4.0 µL of 1.25 mM dNTPs, 37.5 mM of $MgCl_2$, 1.25 U of Platinum Taq DNA polymerase (Invitrogen by Life Technologies, Carlsbad, CA, USA), and 2.5 µL of DNA. Ultrapure water free of DNAse was added to the reaction to bring the volume up to 25 µL. The nested reaction was performed under the same conditions except for the use of different primers (TKIPS and TKIPAS) and 2.5 µL of the amplified product from the PCR reaction. The amplification conditions were as follows: 95 °C for 3 min; 35 cycles of 94 °C for 1 min, 58 °C for 1.5 min, and 72 °C for 2 min, and a final extension at 72 °C for 10 min. Reactions were performed in a Mastercycler Nexus X1 Eppendorf thermal cycler (Eppendorf AG, Hamburg, Germany). Finally, 10 µL aliquots of the amplified product from the PCR (ICP4 gene) and nested PCR (TK gene) reactions were subjected to 1.5% agarose gel electrophoresis. A 100 bp molecular weight

Table 2 Alignment of the nucleotide sequences of the ICP4 gene analysed in the present study

Sample	Position in the gene																																					
	430	438	456	539	598	606	623	685	736	737	738	739	740	741	742	743	744	796	812	3835	3837	3838	3906	3958	3982	3993	4013	4014	4017	4048	4102	4112	4224	4299	4312	4340	4378	4388
USP 657-3	G	G	G	G	G	C	C	A	*	*	*	*	*	*	*	*	*	G	G	G	A	C	C	C	C	G	A	G	A	G	T	G	A	C	A	T	C	G
USP 657-4T	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
USP 657-4E	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
USP 657-5	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
USP 657-7	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	-	-	-	-	-	-	T	T	-	G	-	-	-	-	-	-	-	-	A	A	-
USP 695-1	-	-	-	T	-	-	T	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	T	T	-	G	-	-	-	-	-	-	-	-	A	A	-
USP 695-2	-	-	-	T	-	-	T	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
USP 695-3	-	-	-	-	-	-	T	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
USP 729	-	-	-	T	-	-	T	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-
EU104900.1 CEO VAC	-	A	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	T	-	-	-	-	-	A	-	-	-	-	-	-	A	-
EU104908.1 TCO VAC	-	-	A	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	T	-	G	-	-	-	-	-	-	-	-	A	A	-
JN542534.1 USDA	-	-	A	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	T	T	-	G	-	G	-	-	-	-	-	-	A	A	-
EU104911 12/D/02/BCK	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	A	A	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	-	-	-
EU104899 25/H/88/BCK	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	A	A	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	-	-	-
EU104910 24/H/91/BCK	-	-	-	T	-	-	T	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EU104907 13/E/03/BBR	-	-	A	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	T	T	-	G	-	-	-	-	-	-	-	-	A	A	-
EU104906 14/E/03/BBR	-	-	A	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	T	T	-	G	-	-	-	-	-	-	-	-	A	A	-
EU104912 2/A/04/BR	-	A	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
JN542536 63140/C/08/BR	-	A	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	A	-
JN542535 81658	-	-	A	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	T	T	-	G	-	-	-	-	-	-	-	-	A	A	-
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FJ477353.1 USP-02	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FJ477356 USP-06	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GQ499345 USP-07	-	A	A	-	A	-	-	G	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	A	-
FJ477357 USP-09	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GQ499346 USP-11	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GQ499350.1 USP-17	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GQ499351 USP-18	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FJ477365 USP-27	-	A	A	-	A	-	-	G	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	A	-
GQ499352 USP-32	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GQ499353 USP-45	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
GQ499355 USP-68	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FJ794467 USP-74	A	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	C	C	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C
FJ794469 USP-81	-	-	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	A	-	A	-	-	A	A	-	-	-	-	-	-
KJ028227 USP-87	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KF786292/KF786297 MG	-	-	-	T	-	T	-	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KF786293/KF786298 MG	-	-	-	T	-	T	-	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KF786294/KF786299 MG	-	-	-	T	-	T	-	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KF786295/KF786300 MG	-	-	-	T	-	T	-	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
KF786296/KF786301 MG	-	-	-	T	-	T	-	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
FJ477350 CEO Laryngo-Vac	-	A	A	-	A	-	-	G	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	A	-
FJ477351CEO Nobilis-ILT	-	A	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	-	-	G	-	A	-
FJ477349 TCO VAC LTI-IVAX	-	-	A	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	-	T	T	-	G	-	-	A	-	-	-	-	-	A	A	-
JN204827.1 AUS	-	A	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	-	G	-	A	-
JX458822.1 CHINA	-	A	A	-	A	-	-	-	C	T	C	T	T	C	C	T	C	-	-	-	-	-	T	-	-	-	-	-	-	A	-	-	G	-	-	-	A	-
DQ995291.1 CHINA	-	-	-	-	-	-	-	-	C	T	C	T	T	C	C	T	C	-	A	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	T	-	-	-
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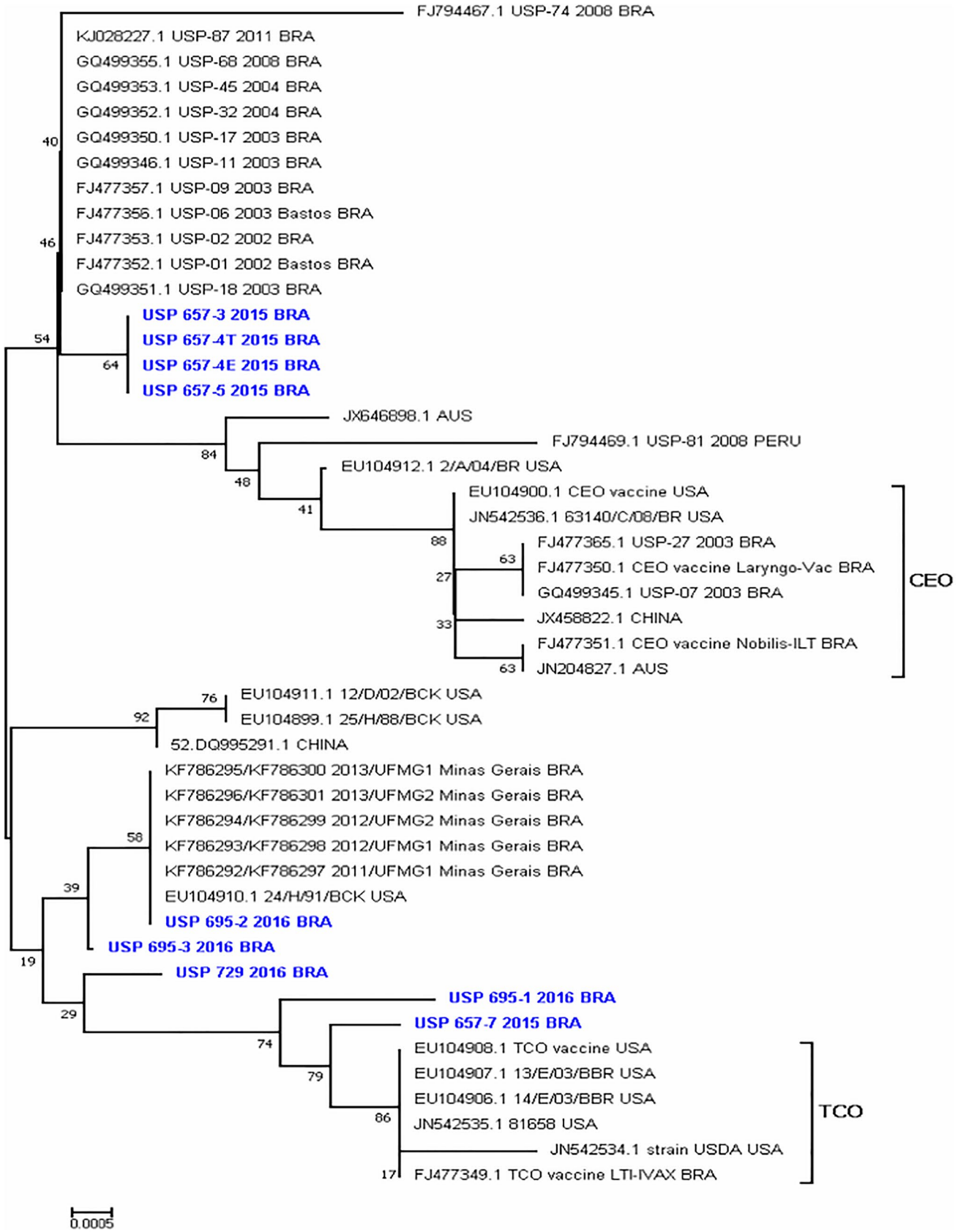
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GQ499346.1 USP-11 2003 BRA
FJ477357.1 USP-09 2003 BRA
FJ477356.1 USP-06 2003 Bastos BRA
FJ477353.1 USP-02 2002 BRA
FJ477352.1 USP-01 2002 Bastos BRA
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USP 657-3 2015 BRA
USP 657-4T 2015 BRA
USP 657-4E 2015 BRA
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JX646898.1 AUS
FJ794469.1 USP-81 2008 PERU
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EU104900.1 CEO vaccine USA
JN542536.1 63140/C/08/BR USA
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USP 695-2 2016 BRA
USP 695-3 2016 BRA
USP 729 2016 BRA
USP 695-1 2016 BRA
USP 657-7 2015 BRA
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FJ477349.1 TCO vaccine LTI-IVAX BRA
TCO
40
46
54
64
84
48
41
88
63
27
33
63
76
92
58
39
19
29
74
79
86
17
0.0005

◂**Fig. 1** Phylogenetic tree generated using the sequences of the two ICP4 gene segments from the studied strains, vaccine strains, and field strains from other countries. The phylogenetic tree was constructed using the maximum likelihood statistical method and the Tamura 3-parameter model with gamma-distributed and 1000 bootstrap replication. The scale bar represents the number of substitutions per site. The sequences of the strains studied in the present work are marked with the symbol ▼ and shown in blue

marker (Invitrogen) was used and samples were stained with Sybr Safe (Invitrogen). The results were analyzed in a transilluminator and photo-documented by an Alpha Imager Mini Analysis System (Alpha Innotech Corp., San Leandro, CA).

DNA sequencing

The ICP4 and TK amplified fragments were purified using GFX™ PCR kit DNA and Gel Band Purification (GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's recommendations. The sense and antisense strands of each purified product were sequenced using the Big Dye Terminator Version 3.1 Cycle Sequencing Kit (Applied Biosystems by Life Technologies, Carlsbad, CA, USA). Sequencing reactions were performed on an ABI 3730 DNA Analyser (Applied Biosystems by Life Technologies, Carlsbad, CA, USA).

Sequence analysis

Obtained sequences were edited and aligned using CLC Main Workbench 7.0.2 software (Qiagen Bioinformatics, Redwood City, CA, USA) and BioEdit version 7.1.3 biological sequence alignment editor (The University of Leicester, Leicester, UK). The sequences generated were analyzed using the BLAST tool to determine the similarity of the sequences to those deposited in GeneBank. The nucleotide sequences obtained were aligned and compared to other sequences of the same virus from other parts of the world using the CLUSTAL W method available in ClustalX 2.0.11 Package software (European Bioinformatics Institute, Saffron Walden, CB10 1SD, UK). Phylogenetic trees were generated using different statistical methods and phylogeny tests with 1000 replicate bootstraps integrated into the MEGA 7 program (Center for Evolutionary Functional Genomics—The Biodesign Institute, USA—Free Use) [27].

Recombination analyses

The sequences of the ICP4 region were analyzed, and perfect and imperfect repeat regions were identified and removed prior to recombination analysis using the Phobos plugin in Genious V8.1.9 with default settings and score constraints for satellites. Any repeated sequence detected was deleted in all sequences in the alignment. Recombination analysis was performed using two software: SplitsTree4 [28] and RDP4 [29]. In SplitsTree4, split network trees were generated with an uncorrected P-character transformation model, ignoring constant sites. Statistical analyses of the recombination networks were performed using the Phi test (Bruen et al. 2006) as implemented by SplitsTree4. In RDP4, six different methods were used to assess the sequences for recombination breakpoints: RDP, GENECONV, 3Seq, SiScan, MaxChi, and BootScan. Default RDP4 settings were used throughout.

GenBank accession numbers

The nucleotide sequences obtained in the present study were deposited in GenBank under the accession numbers as follow: partial ICP4 gene -USP 657–3 (MF678664; MF678673), USP 657-4 T (MF678665; MF678674), USP 657-4E (MF678666; MF678675), USP 657–5 (MF678667; MF678676), USP 657–7 (MF678668; MF678677), USP 695–1 (MF678669; MF678678), USP 695–2 (MF678670; MF678679), USP 695–3 (MF678671; MF678680), and USP 729 (MF678672; MF678681); partial TK gene: USP 657–3 (MF678655), USP 657-4 T (MF678656), USP 657-4E (MF678657), USP 657–5 (MF678658), USP 657–7 (MF678659), USP 695–1 (MF678660), USP 695–2 (MF678661), USP 695–3, (MF678662), and USP 729 (MF678663).

Results

ILTV detection based on gE, ICP4, and TK genes

Of a total of 42 samples, nine (09) samples were positive by the gE PCR, which amplifies a 219 bp long fragment. Amplification of the two ICP4 gene fragments resulted in 687 and 631 base pair products, and the amplification of the TK gene resulted in a product of approximately 694 bp.

DNA sequencing of the ICP4 gene

DNA sequencing of the two ICP4 gene fragments identified 7 samples (657–3,657-4 T, 657-4E, 657–5, 695–2, 695–3, and 729) as field strains and two samples (695–1 and 657–7) as closely related to the TCO vaccine strains; stretches of C-T-C-T-C–C-C-T-C at positions 736–744 (Table 2) were detected in the seven field strain samples. Phylogenetic analysis based on ICP4 gene sequences showed that samples 657–3, 657-4 T, 657-4E, and 657–5

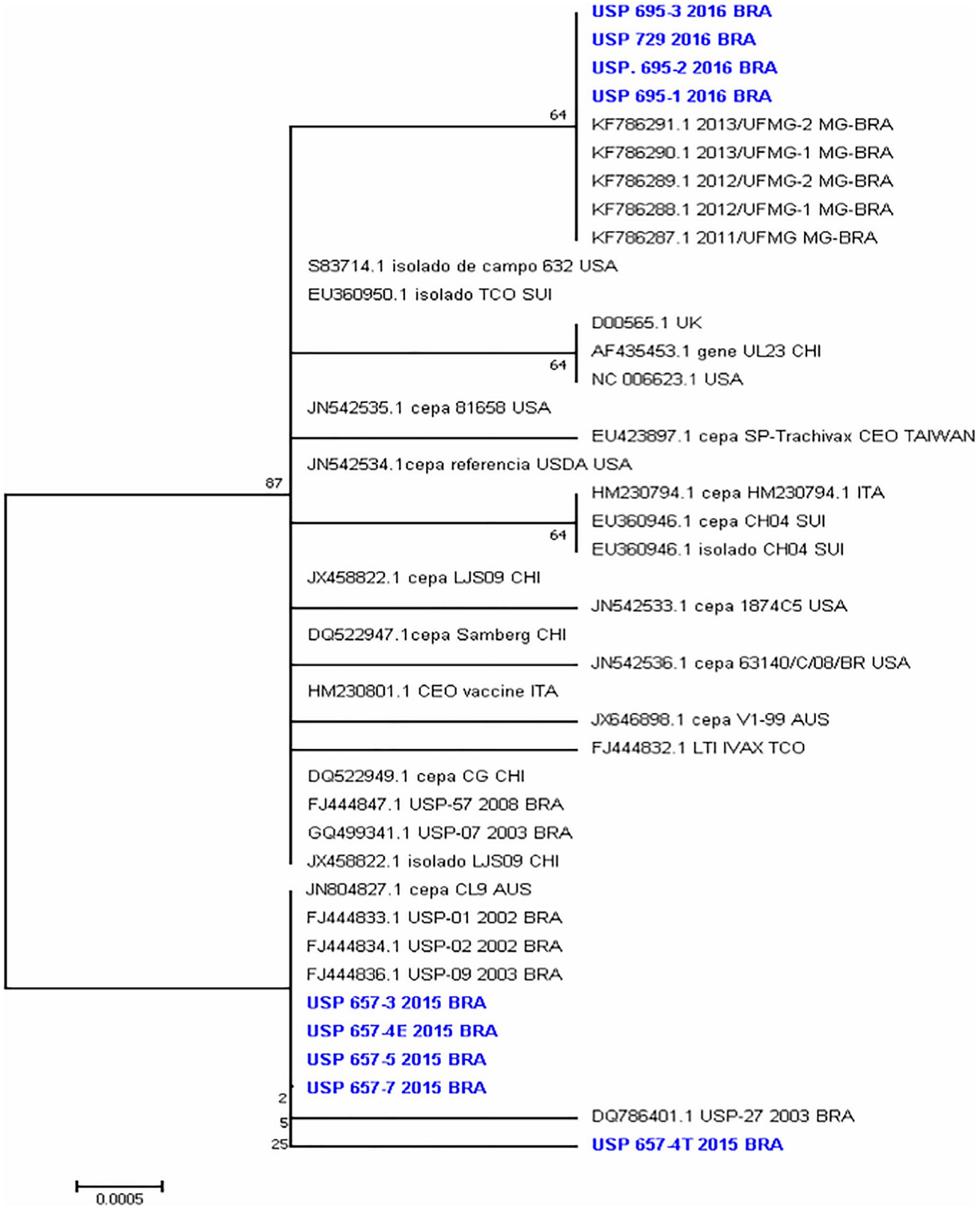

Fig. 2 The presence of methionine (M) at position 252 of the TK protein signifies high virulence, whereas the presence of threonine (T) indicates low virulence= Similarity of amino acids

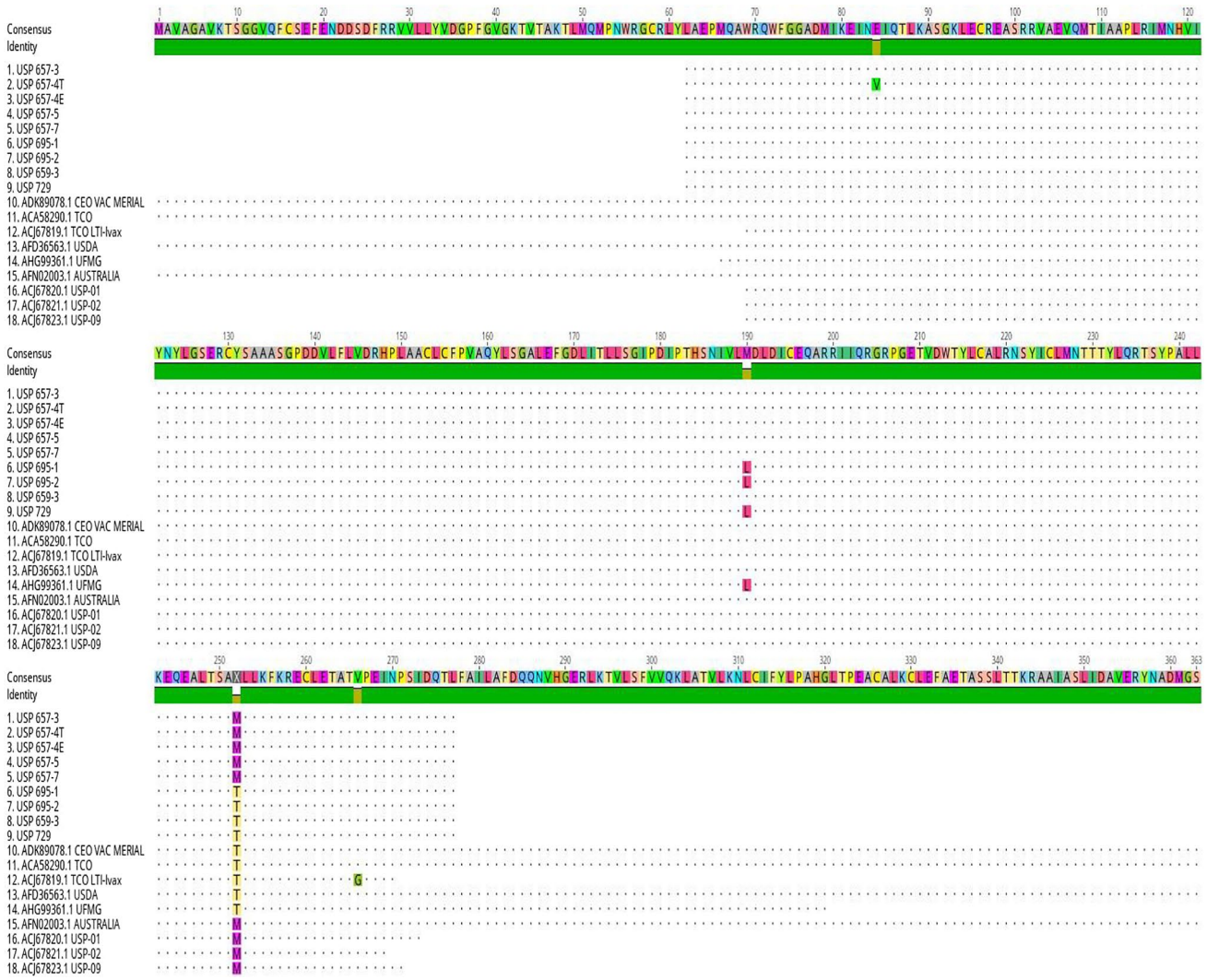

Fig. 3 Phylogenetic tree generated from a fragment of the ILTV TK gene from the studied strains, vaccine strains, and field strains from other countries. The phylogenetic tree was constructed using the maximum likelihood statistical method and the Jukes-Cantor model, with 1000 bootstrap replication. The scale bar represents the number of substitutions per site. The sequences of the strains studied in the present work are marked with the symbol ● and shown in blue

were grouped on a separate branch from the other Brazilian isolates (Bastos-SP and Minas Gerais-MG). While the ICP4 sequence obtained from samples 695–2, 695–3, and 729 collected in 2016 were closely related to the ICP4 sequence from samples collected between 2011 and 2013 from Minas Gerais, Brazil. Finally, the samples 695–1 and 657–7 are closely related to the TCO vaccine strain (Fig. 1).

TK gene sequencing

The amino acid sequence analysis of the TK gene sequenced in the present work showed that the 2015 samples (657–3, 657-4 T, 657-4E, 657–5, and 657–7) contained a methionine (M) at position 252, while the 2016 samples (695–2, 695–3, and 729) contained a threonine (T) at this same position. The presence of methionine in the former samples characterizes them as highly virulent, while the threonine in the latter samples signifies low virulence (Fig. 2), as was previously described [21]. Phylogenetic analysis of the sequences of the TK gene samples revealed that samples 657–3, 657-4 T, 657-4E, 657–5, and 657–7 were grouped on the same branch as samples from Brazil collected between 2002 and 2003 characterized as highly virulent based on the presence of a methionine at position 252 of the TK gene, while samples 695–1, 695–2, 695–3, and 729 were grouped with 2011 to 2013 samples from Minas Gerais, Brazil,

which were previously assumed to be of low virulence based on the presence of a threonine at position 252 of the TK gene (Fig. 3).

Recombination analyses

All the positive samples when analyzed with Phobos plugin in Geneious V8.1.9, SplitsTree4, RDP4 (six different methods were used to assess the sequences for recombination breakpoints: RDP, GENECONV, 3Seq, SiScan, MaxChi, and BootScan) did not present any event of recombination.

Discussion

Molecular studies were performed to determine whether the viruses that caused outbreaks of ILT originated from vaccine strains or field strains [14, 31]. The molecular structure of the ICP4 gene of the virus can often be used to differentiate between vaccine strains and field strains [16, 20, 32]. In this study, analysis of the ICP4 gene was performed to determine if viruses were related with current outbreaks in Brazil were related to vaccine strains, to previous strains that caused outbreaks in the regions of Bastos and Minas Gerais regions [20, 33], or if current outbreaks are related to the introduction of new field strains.

In Brazil, outbreaks of the disease in the Bastos region during the years 2001 to 2002 were controlled through vaccination with the TCO and CEO vaccines, which were later replaced in 2012 using a recombinant vaccine [14, 33]. In the outbreaks of Minas Gerais state that occurred from 2012 to 2014, the Governmental Agricultural Agency only authorized the use of vectorized recombinant vaccines to control the disease [20]. Genetic analysis of outbreak viruses from an outbreak in Guatapará (São Paulo state) in late 2009 and early 2010, showed that the causative ILTV strain was different from the CEO and TCO live attenuated vaccines and the virus was also different from previous viruses detected during other outbreaks of the disease in Brazil [34]. The DNA sequences obtained in the present study showed that the strains circulating in most birds are a non-vaccine field strains, different from the strains that produced the outbreaks in Bastos and Minas Gerais. However, TCO vaccine-related strains were also found.

Characterization of the two fragments of the ICP4 gene and a fragment of the TK gene in the present work suggests that the virus that is circulating in commercial bird lots and causing respiratory problems is a field strain (sample 657). Characterization of samples related to the low-virulence TCO vaccine (samples 695 and 729) was also performed, with the possibility that the clinical signs presented may result from a reactivated latent infection or virulence reversion of the vaccine as previously reported [9, 11]. Thus, it is important to constantly monitor the affected areas to avoid the appearance of new outbreaks that would cause great economic losses to the poultry industry. These results corroborate the efficacy of the use of the ICP4 gene for typing ILTV isolates [33, 35, 36]. We also suggest that this new strain (sample 657), which is related to the Bastos strain, may be the cause of the disease since some of these samples (657–3, 657-4e, 657–5, and 657–7) have a methionine instead of a threonine in position 252 of the TK gene, which suggests that this virus is virulent and is responsible for the disease, as previously indicated in the genetic characterization.

In the present study, field strains (isolated from the trachea, trigeminal ganglia, eyelid, and caecal tonsils) and vaccine strains (isolated from the trachea) were found in birds with respiratory issues, which agrees with previous studies that indicated that ILTV can actively replicate in the conjunctival trachea and establish latent trigeminal infections [12, 37]. As was reported herein, several studies have reported the presence of the virus in tissues other than those of the respiratory system [38, 39], and ILTV infection usually occurs through the reactivation of latent infections [12, 40] or through infection with new strains, which may exist due to a reversal of the virulence of vaccine strains or passage from bird to bird [41], as could it possibly have occurred in the strains studied here. However, few outbreaks have been reported because of the reversion of TCO vaccine virulence; reversion of CEO vaccine virulence is more likely [31]. A recent epidemiological study has shown that the use of CEO and TCO vaccines in a quarantine area could not eliminate the circulation of the field strain in this region and that the CEO vaccine strain and wild-type strain may spread to unvaccinated regions, despite the implementation of biosecurity measures [33].

Here, the analysis of the samples indicates that there are no existing events of recombination in the ICP4 region studied. However, other studies indicate that circulating viruses that are producing new outbreaks experimented with some type of recombination [42]. In another investigation, the authors indicate that the *Gallid herpesvirus* type 1 have been shown to display a high rate of recombination in vivo and in vitro, and in ILTV, the recombination event in natural conditions is widespread [43]. Additionally, a recent study in Canada shows that there was an event of recombination and ILTV wild-type strains can recombine with vaccinal strains [44]. Thus, La et al. indicate that the frequent detection of multiple virulent recombinants of ILTV throughout the world might suggest that natural recombination could be a common evolutionary strategy employed by ILTVs to facilitate their survival in host populations, and natural recombination should be considered one of the major risks for

the generation of revertant strains of ILTV in the field condition [45].

Currently, there are new methods and bioinformatics tools that help to determine if there are recombination events in the viral genome, in addition to helping to sequence the complete viral genome in clinical samples using NGS or Sanger Technologies. However, in our study, despite being a relatively small fragment of the ICP4 gene, it is a part of the gene that has been shown in previous studies to be used for these research purposes [16, 45].

Conclusions

The characterization of two fragments of the ICP4 gene and a fragment of the TK gene in the present study suggests that the virus that was circulating in commercial bird flocks, causing respiratory problems, is a highly virulent field strain (sample 657). There was also the characterization of samples related to low virulence TCO vaccine (samples 695 and 729), with the possibility that the clinical signs presented are the result of a reactivated latent infection or a reversal of vaccine virulence. Thus, it is important to constantly monitor the affected areas to avoid the appearance of new outbreaks that would produce great economic losses to the poultry industry.

Acknowledgements The authors would like to thank the poultry companies in Brazil that generously donated the samples for the development of this study.

Author contribution SHS-P performed the detection ILTV in the clinical samples; LFNN prepared the draft the manuscript; MRB assessed and acquired samples of ILT from chickens; CSA-F revised the manuscript; CAL contributed with molecular analysis; AJPF conceived the study and revised the manuscript. All authors contributed to the analysis and interpretation of the data and revised the manuscript.

Funding This research was supported by the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) grants number 140744/2014–2 and 306380/2014–5. A. J. Piantino Ferreira is the recipient of a CNPq fellowship grant number 301084/2019–0.

Declarations

Conflict of Interest The authors declare no competing interests.

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