



Molecular Characterization of Infectious Laryngotracheitis Virus Isolate Obtained from Backyard Poultry in Tamil Nadu

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ABSTRACT

Background: Infectious Laryngotracheitis (ILT) is a re-emerging viral disease affecting poultry in India. Currently, there are no licensed vaccines for ILTV available in India and the use of imported live vaccines poses the risk of introducing vaccine-derived ILTV infection. These imported vaccines may lead to recombination events with local field strains, complicating outbreak management. The Infected Cell Polypeptide 4 (ICP4) gene, essential for viral replication, plays a crucial role in differentiating between field isolates and vaccine strains of ILTV. Molecular characterization targeting the ICP4 gene provides valuable insights into the genetic diversity of ILTV, which is vital for monitoring viral evolution, understanding recombination events and guiding the development of region-specific vaccines in India.

Methods: A tracheal tissue sample was collected from backyard poultry suspected of ILT infection in Tamil Nadu. The virus was isolated in the chorio-allantoic membrane of 9-day-old embryonated chicken eggs. DNA extraction followed by PCR amplification targeting the ICP4 gene was performed using specific primers. The amplified product was sequenced and nucleotide as well as amino acid variations were analysed. Phylogenetic analysis was conducted using the Maximum-likelihood method with 68 representative ICP4 gene sequences from vaccine strains and field isolates obtained from GenBank. Nucleotide and amino acid variations were studied using MEGA alignment software.

Result: Isolation on the chorio-allantoic membrane resulted in thickening of the membrane along with opaque pock-like lesions. The ICP4 gene was successfully amplified at 688 bp. BLAST analysis revealed 99% similarity with other ILTV sequences reported across the world. Phylogenetic analysis grouped the isolate with field strains from India, Japan, China, Brazil and Turkey. Nucleotide analysis of ICP4 gene 688-bp fragment revealed 13 deletions and 9 variations. Amino acid analysis revealed 4 deletions and 8 variations.

Key words: Backyard poultry, Chorio-allantoic membrane, Icp4 gene, Infectious laryngotracheitis virus, Phylogenetic analysis.

INTRODUCTION

Avian Infectious Laryngotracheitis (ILT) is a highly contagious respiratory viral disease of poultry caused by Gallid alphaherpesvirus - 1 (GaHV-1) or ILT virus (ILTV), belonging to *Iltovirus* genus grouped under *Alphaherpesvirinae* subfamily of *Herpesviridae* family (Davison *et al.*, 2009). The genome of ILTV is a 150-155 kb linear, double stranded DNA and is typical of alphaherpesviruses, with a unique long (UL) region and a unique short region (US) that is flanked between Inverted Repeat (IR) sequences (Ruiz *et al.*, 2018). The UL, US and IR region consists of 65, 9 and 5 open reading frames (ORFs) respectively totaling to 80 ORFs in the entire genome (Lee *et al.*, 2011).

In India, ILT was first reported in a poultry farm in Mathura, Uttar Pradesh in the year 1964 (Mishra *et al.*, 2020) after which there were no reports until the last decade (Menendez *et al.*, 2014). In the last ten years, there has been a resurgence of ILT in various parts of India including West Bengal, Andhra Pradesh, Telangana, Karnataka and Tamil Nadu (Mishra *et al.*, 2020; Sivaseelan *et al.*, 2014; Gowthaman *et al.*, 2014; Puvarajan *et al.*, 2014; Baksi *et al.*, 2016; Priya *et al.*, 2021; Jaisree *et al.*, 2021). Hence, ILT is currently regarded as an emerging and re-emerging disease in India (Menendez *et al.*, 2014) that affects chickens of all ages and breeds, but the severity of the disease may vary. The various forms of ILT include peracute, acute, chronic or mild. Younger birds (0-16 weeks) are more susceptible,

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while older birds (over 16 weeks) may experience milder symptoms. Importantly, ILT can remain latent and can later be reactivated due to immunosuppression or stress (OIE, 2021).

Reports on ILT have been documented through various methods including isolation, molecular detection and phylogenetic analysis (Mishra *et al.*, 2020). Molecular detection involves amplification of certain genes including infected cell polypeptide – 4 (ICP4), thymidine kinase (TK), glycoprotein G (gG), glycoprotein E (gE) and UL47 (OIE,

2021) by conventional polymerase chain reaction (PCR). Among these genes, ICP4 gene located in the UL region is a conserved gene. In this conserved gene, two fragments, 635-bp and 688-bp, serve as key markers for molecular characterization (OIE, 2021). These fragments aid in understanding the origin of ILTV, identify possible recombination events, understand the genetic makeup of variants in circulation and helps in discriminating between vaccine strains and field isolates of ILTV (Lee *et al.*, 2012; Priya *et al.*, 2021). Previously, Restriction Fragment Length Polymorphism (RFLP) was used for this differentiation, however, it was reported that phylogenetic analysis demonstrated a better approach than RFLP (Chacon and Ferreira, 2009).

There is a growing need for molecular characterization of ILTV in India, as it is speculated that the virus may have entered the country through illegal trading of infected birds. Subsequent use of unauthorized imported modified live ILTV vaccines in Indian poultry may have led to recombination events and might have contributed to the re-emergence of ILTV (Mishra *et al.*, 2020). Therefore, it is essential to understand the molecular characteristics of ILTV in circulation in India. Hence, this study was construed with the objective to characterize the 688-bp ICP4 fragment of an ILTV isolate obtained from backyard poultry in Tamil Nadu using phylogenetic analysis, with the goal of contributing valuable data on ILTV in India.

MATERIALS AND METHODS

Sample processing and virus isolation

Three tracheal tissue samples (L1, L2 and L3) were collected from backyard poultry in Tamil Nadu suspected of having ILTV infection. The tissue samples were aseptically homogenized using a mortar and pestle and were re-suspended in 1:20 Phosphate Buffered Saline solution containing 50 IU/ml Penicillin and 50 µg/ml Streptomycin. After centrifugation at 3000 rpm for 10 minutes the supernatant was inoculated onto the chorio-allantoic membrane (CAM) of 9-day old embryonated chicken egg (ECE) and was incubated at 37°C for 5 days following established protocols (OIE, 2021). The work was carried out in Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai during 2018 to 2019.

DNA extraction

DNA was extracted from L1, L2 and L3 samples using QIAamp DNA mini kit (Qiagen) following manufacturer's instructions. The eluted DNA was observed for concentration and purity in spectrophotometer (NanoDrop, 2000, Thermo Scientific) and was stored at -80°C until further use.

Amplification of ICP4 gene by PCR

The DNA samples (L1, L2 and L3) were amplified by PCR using primers described by OIE, 2021 for ICP4 gene: ICP4 forward primer: 5'-ACT-GAT-AGC-TTT-TCG-TAC-AGC-ACG-3' and ICP4 reverse primer: 5'-CAT-CGG-GAC-ATT-CTC-CAG-

GTA-GCA-3'. The PCR reaction was set up using PrimeStar master mix following manufacturer's instructions at an annealing temperature of 57°C. The PCR products were separated in 1.2% agarose gel electrophoresis and were visualized under UV light using ethidium bromide staining. PCR products were gel purified with Nucleospin Gel and PCR cleanup kit (Machery Nagel, Cat#740609, Germany).

Construction of phylogenetic tree

ICP4 gene of L3 was sequenced and the same was analysed by BLAST in NCBI. Further, phylogenetic analysis was done with a total of 67 representative ICP4 gene sequences of vaccine strains and field isolates of ILTV available on GenBank using maximum-likelihood method and Tamura-Nei model with a bootstrap value of 1000 using MEGA 11 software (Tamura *et al.*, 2021).

Protein sequence analysis and structural prediction

The amino acid sequence of L3 was compared to the reference protein (YP_182397.1). Online analysis tool 310 Copilot was used for structure prediction, generating secondary and tertiary structure models with default parameters. Structural similarity was assessed using pLDDT, Pident, AAComp, length and TM-score. The results were analyzed to determine deviations in sequence and conformation between L3 and reference sequence.

RESULTS AND DISCUSSION

Virus isolation and PCR amplification

The CAM of ECE inoculated with field isolates under this study showed thickening of CAM along with opaque pock-like lesions only for L3. PCR for ICP4 gene was also positive only for L3, yielding an amplified product at an expected product size of 688 bp.

BLAST analysis and phylogenetic tree

BLAST analysis of ICP4 partial gene sequence of L3 (GenBank accession no. MH365473) showed 99% similarity with ILTV isolates of China, Russia, USA, Tunisia, South Korea and Bangladesh. The phylogenetic analysis revealed that the ILTV isolates were grouped into two major clades (Fig 1). Clade 1 comprised diverse field and vaccine strains from India, China, Japan, Turkey and Egypt. L3 isolate was branched within Clade 1 and clustered closely with other global field strains. Clade 2 predominantly contained vaccine and reference strains from the USA, Canada, Australia and Germany, indicating standardized vaccine lineages. Clade 2 was branched into two sub-clades, wherein the Tissue Culture Origin (TCO) vaccines grouped together under one sub-clade and the Chicken Embryo Origin (CEO) vaccines were grouped under another sub-clade.

Nucleotide and amino acid variations in ICP4 gene

The ICP4 gene 688-bp fragment was analysed for nucleotide and amino acid variations. L3 showed a total of 22 nucleotide (Table 1) and 13 amino acid variations (Table 2)

at various positions. At the nucleotide level, positions 214, 438, 456 and 795 showed variations with no resultant changes in amino acids. At nucleotide positions 219, 597 and 811, there were variations from C→T, G→A, A→G respectively. Additionally, deletions were observed at

nucleotide positions 259-270 and 611, while position 593-594 showed an insertion of C. On the amino acid level, L3 displayed specific substitutions. Position 73 changed serine (S) to phenylalanine (F) and positions 87-90 presented a deletion from the original sequence AAQD.

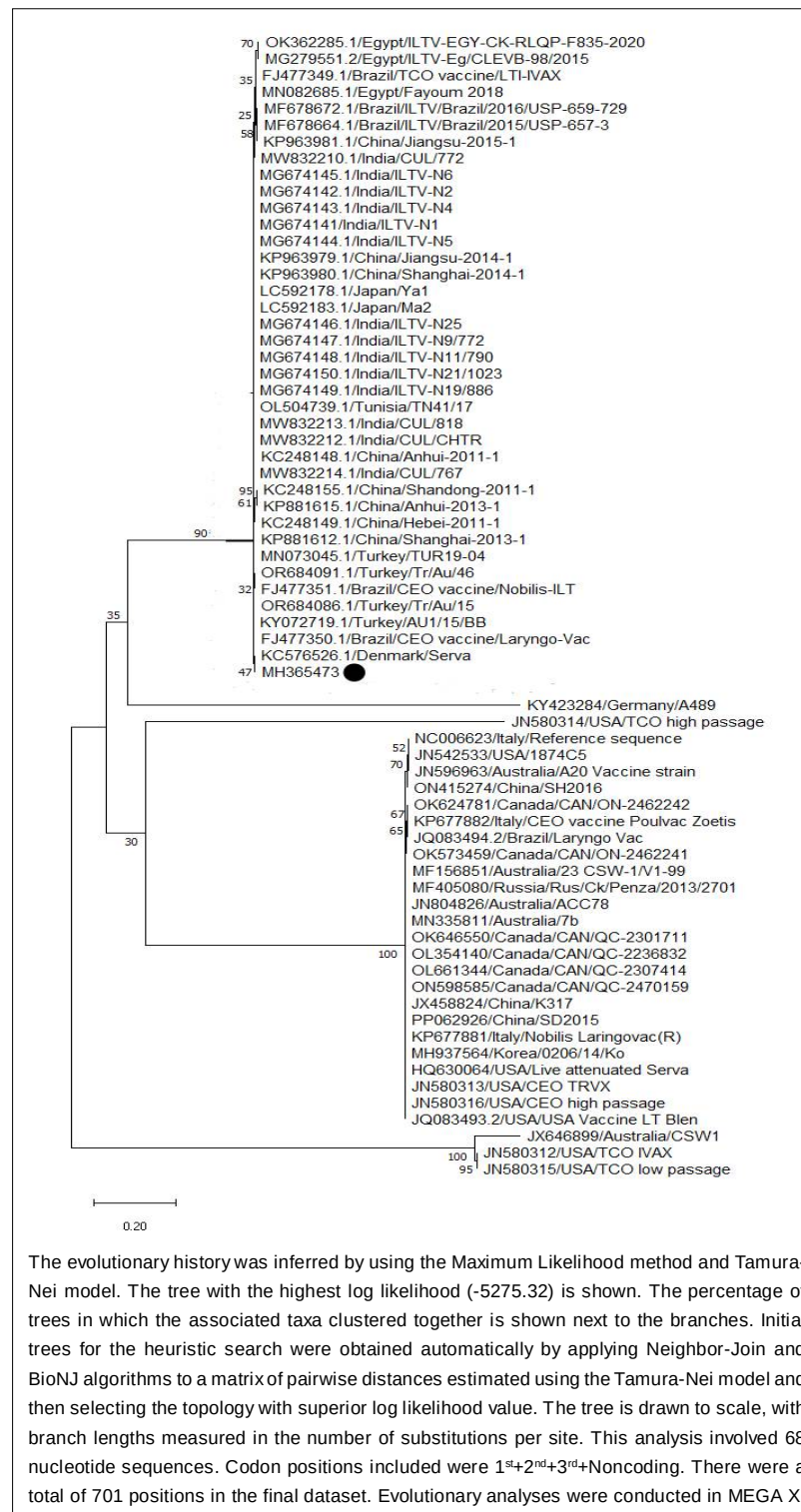


Fig 1: Phylogenetic tree of partial ICP4 (688bp) gene using maximum-likelihood method.

Table 1: Nucleotide variations in ICP4 688-bp fragment.

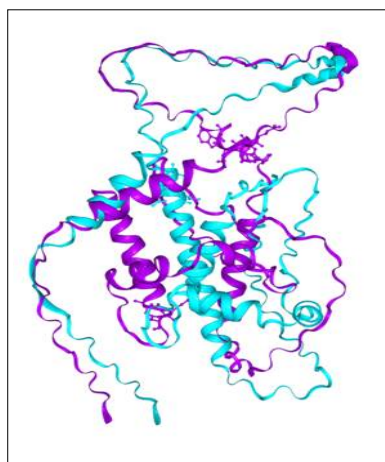
Details of the strain / isolate	214	219	259-270	438	456	593 - 594	597	611	795	811
NC_006623.1 / Reference genome	T	C	GCGGCCCAAGAC	G	G	GG	G	G	A	A
JN596963/Australia/A20 Vaccine strain	G	C	GCGGCCCAAGAC	C	G	TGGATTTT	T	C	G	C
KP677882/Italy/CEO/Poulvac Zoetis	G	C	*	C	G	CGGATTTT	T	C	G	C
FJ477350.1/Brazil/CEO/Laryngo-Vac	T	C	*	A	A	GCG	A	G	G	G
FJ477351.1/Brazil/CEO/Nobilis-ILT	T	C	*	A	A	GCG	A	G	G	G
JQ083494.2/Brazil/Laryngo-Vac	G	C	*	C	G	CGGATTTT	T	C	G	C
JN580313/USA/CEO TRVX	G	C	*	C	G	CGGATTTT	T	C	G	C
JN580316/USA/CEO high passage	C	C	*	C	G	CGGATTTT	T	C	G	C
JN580314/USA/TCO high passage	T	A	GCGGCCCAAGAC	C	G	CT	G	A	C	A
JN580315/USA/TCO low passage	G	T	GCGGCCCAAGAC	C	G	AGATAAC	A	T	A	T
FJ477349.1/TCO vaccine/LTI-IVAX	T	C	GCGGCCCAAGAC	G	A	GCG	G	G	G	G
HQ630064/USA/Live attenuated Serva	G	C	*	C	G	CGGATTTT	T	C	G	C
JQ083493.2/USA/USA Vaccine LT Blen	G	C	*	C	G	CGGATTTT	T	C	G	C
JN804826/Australia/ACC78	G	C	*	C	G	CGGATTTT	T	C	G	C
JX646899/Australia/CSW 1	C	G	GCGGCCCAAGAC	C	G	AGATAAC	A	T	A	T
MF678664.1/Brazil/2015/USP-657-3	T	C	GCGGCCCAAGAC	G	G	GCG	G	G	G	G
OK573459/Canada/CAN/ON-2462241	G	C	*	C	G	CGGATTTT	T	C	G	C
ON415274/China/SH2016	G	C	GCGGCCCAAGAC	C	G	TGGATTTT	T	C	G	C
KP881615.1/China/Anhui-2013-1	T	C	*	A	A	GCG	A	G	G	G
KC248149.1/China/Hebei-2011-1	T	C	*	A	A	GCG	A	T	G	G
KC576526.1/Denmark/Serva	T	C	*	A	A	GCG	A	G	G	G
OK362285.1/Egypt/F835-2020	T	C	GCGGCCCAAGAC	G	A	GCG	G	G	G	G
KY423284/Germany/A489	T	G	GCGGCCCAAGAC	A	A	AGTC	G	T	C	C
MG674142.1/India/LTV-N2	T	C	*	A	A	GCG	A	G	G	G
LC592183.1/Japan/Ma2	T	C	*	A	A	GCG	A	G	G	G
MH937564/Korea/0206/14/Ko	G	C	*	C	G	CGGATTTT	T	C	G	C
MF405080/Russia/Penza/2013/2701	G	C	*	C	G	CGGATTTT	T	C	G	C
OL504739.1/Tunisia/TN41/17	T	C	*	A	A	GCG	A	G	G	G
JN542533/USA/1874C5	G	C	GCGGCCCAAGAC	C	G	TGGATTTT	T	C	G	C
MH365473	G#	T	*	A#	A#	GCG	A	*	G#	G

* Nucleotide deletions. #Variation of nucleotide did not cause amino acid changes.

Table 2 : Amino acid variations in ICP4 688-bp fragment.

Details of the strain / isolate	Protein ID	Amino acid position with reference to NC_006623.1			
		73	87-90	199-204	271
NC_006623.1 / Reference genome	YP_182397.1	S	AAQD	PWRDLW	T
JN596963/Australia/A20 Vaccine strain	AER28186.1	S	AAQD	AVARSL	T
KP677882/Italy/CEO/Poultvac Zoetis	AJR27615.1	S	AAQD	AMARSL	A
FJ477350.1/Brazil/CEO/Laryngo-Vac	ACM89316.1	S	*	AMARSL	A
FJ477351.1/Brzil/CEO/Nobilis-ILT	ACM89318.1	S	*	AMARSL	A
JQ083494.2/Brazil/Laryngo-Vac	AEW67891.1	S	*	AMARSL	A
JN580313/USA/CEO TRVX	AFM36352.1	S	*	AMARSL	A
JN580316/USA/CEO high passage	AFM36587.1	S	*	AMARSL	A
JN580314/USA/TCO high passage	AFM36430.1	S	AAQD	AVARSL	A
JN580315/USA/TCO low passage	AFM36508.1	S	AAQD	AVARSL	A
FJ477349.1/TCO vaccine/LTI-IVAX	ACM89313.1	S	AAQD	AVARSL	A
HQ630064/USA/Live attenuated Serva	AEB97360.1	S	*	AMARSL	A
JQ083493.2/USA/USA Vaccine LT Blen	AEW67812.1	S	*	AMARSL	A
JN804826/Australia/ACC78	AFN01970.1	S	*	AMARSL	A
JX646899/Australia/CSW1	AGC23187.1	S	AAQD	AMMARSL-	A
MF678664.1/Brazil/2015/USP-657-3	AXA98625.1	S	AAQD	AVARSL	A
OK573459/Canada/CAN/ON-2462241	UPW36744.1	S	*	AMARSL	A
ON415274/China/SH2016	WOK15002.1	S	AAQD	AVARSL	T
KP881615.1/China/Anhui-2013-1	ALA63882.1	S	AAQD	AMARSL	A
KC248149.1/China/Hebei-2011-1	AGS36514.1	S	*	AMARSL	A
KC576526.1/Denmark/Serva	AGJ51146.1	S	*	AMARSL	A
OK362285.1/Egypt/F835-2020	UJO27537.1	S	AAQD	AVARSL	A
KY423284/Germany/A489	ASR80986.1	S	AAQD	AVARSL	A
MG674142.1/India/ILTV-N2	AYM26229.1	S	*	AMARSL	A
LC592183.1/Japan/Ma2	BCM78301.1	S	*	AMARSL	A
MH937564/Korea/0206/14/Ko	QAA96051.1	S	*	AMARSL	A
MF405080/Russia/Penza/2013/2701	AWL83383.1	S	*	AMARSL	A
OL504739.1/Tunisia/TN41/17	UXO30725.1	S	*	AMARSL	A
JN542533/USA/1874C5	AFD36530.1	S	AAQD	PWRDLW	T
MH365473	QBB89769.1	F	*	AMARSL	A

* Amino acid deletions.

**Fig 2:** Superimposed 3D structure of L3 (Aqua blue colour) and reference protein (Purple colour) showing deletions and variations of amino acid sequences at 87 - 90th position and 199 - 204th position.

The sequence from amino acids 199-204 was altered from PWRDLW to AMARSL and position 271 showed a change from threonine (T) to alanine (A).

Protein sequence analysis and structural prediction

Sequence identity was 96%, but reference protein had four additional residues, leading to structural deviations. The superimposed 3D structure of L3 and reference protein, generated using 310 Copilot, is shown in Fig 2.

The Indian poultry industry encompasses both commercial and backyard poultry, with the latter accounting for approximately 37.2% of the total poultry population in India as per 20th Livestock Census of Department of Animal Husbandry and Dairying, Ministry of Fisheries, Animal Husbandry and Dairying. Backyard poultry play a crucial role in supplementing the income and nutritional requirements of rural families. Outbreaks of various viral diseases have been reported in backyard poultry flocks, of which ILT (Garcia and Zavala, 2019) is currently gaining attention due to its re-emergence almost after four decades

of disappearance (Ponnusamy *et al.*, 2022). Due to the recent resurgence, molecular characterisation of ILTV would help us understand the spread, origin and variations in the strains of ILTV in circulation. This in turn can guide the development of region-specific ILT vaccines for the Indian poultry (Chacon and Ferreira, 2009). In this study, therefore, we report the molecular characterisation of L3, an ILTV isolate obtained from backyard poultry in the state of Tamil Nadu, India.

The L3 isolate was obtained from a tracheal tissue sample collected from a 32-week-old backyard chicken displaying symptoms of the disease, consistent with the fact that ILT can affect chickens of all ages (Gowthaman *et al.*, 2014). Isolation of L3 on the CAM of ECE resulted in thickening of the CAM and the formation of opaque pock lesions characteristic of ILT (OIE, 2021). PCR was done targeting the 688-bp fragment of ICP4 gene, which is a major immediate-early regulatory gene of ILTV and a differential marker between field and vaccine strains (Chacon and Ferreira, 2009). Sequencing and BLAST analysis of L3 ICP4 gene showed high sequence similarity (99%) with strains circulating in China, Russia, USA, Tunisia, South Korea and Bangladesh. This implies that strains of ILTV may circulate globally, with the potential to recombine and adapt to local environments and host populations (Bayoumi *et al.*, 2020). Phylogenetic analysis revealed that L3 isolate clustered within Clade 1 alongside other ILTV strains reported from commercial poultry in India (Mishra *et al.*, 2020; Jaisree *et al.*, 2021), suggesting a shared origin between the strains circulating in backyard and commercial poultry populations. Furthermore, Clade 1 included isolates reported from China, Brazil, Turkey, Denmark and Egypt. This reinforces the notion of genetic diversity and possible recombination events within ILTV strains across geographic regions (Bayoumi *et al.*, 2020). Clade 2 revealed two distinct sub-clades, with the TCO vaccine strains clustered together in one sub-clade, while the standardized vaccine strains were grouped in another. The separation between Clade 1 and Clade 2 underscores the genetic variability between field and vaccine strains.

Sequence analysis of L3 ICP4 688-bp fragment showed a deletion of four amino acids (AAQD) at position 87-90, which is a characteristic feature of vaccine strains (Can-Sahna *et al.*, 2020). The presence of this deletion, along with the clustering of L3 in Clade 1 in phylogenetic analysis, is consistent with previous studies (La *et al.*, 2019). This suggests that ILTV may have entered the country through illegal bird trade or unauthorized use of imported live vaccines. It also raises concerns about vaccine-virus recombination or the reversion of vaccine strains to virulence, especially in backyard flocks, which could make disease control more challenging (Lee *et al.*, 2012; Yang *et al.*, 2020). Therefore, molecular characterization of ILTV isolates from backyard poultry is highly essential to monitor the evolution of the virus for the benefit of both commercial and rural poultry production systems in India.

CONCLUSION

In the past few decades, the annual growth rate of Indian poultry industry has increased to 8-10%. This remarkable growth underscores the crucial role of poultry industry in driving economic growth and enhancing food security. However, viral diseases continue to pose significant challenges, impacting both commercial and backyard poultry production. Among these, ILT has re-emerged in India after decades, raising concerns about its spread, evolution and potential economic losses. This study underscores the re-emergence of ILTV in India and highlights the need for molecular surveillance to monitor both backyard and commercial poultry, in order to develop region-specific vaccines and better control ILT across poultry systems.

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Conflict of Interest

The authors report no conflict of interest.

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