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Molecular detection and sequence analysis of envelope glycoproteins (gC, gD) and tegument protein (UL47) of infectious laryngotracheitis virus isolated from commercial poultry flocks in Tamil Nadu, India

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Abstract
Infectious laryngotracheitis is a respiratory tract infection of chickens. There is re-emergence of ILT especially in intensive poultry production region in Tamil Nadu causing severe economic loss. Vaccination is the best way to control ILT. The rise in ILTV infections have been correlated with use of live attenuated vaccines. This study has been attempted to understand the genetic variation in ILTV genes coding for immune dominant glycoproteins and tegument protein as candidates for next generation safer vaccines. The full-length amplification and sequencing of gC, gD and UL47 genes of an Indian isolate of ILTV has been recorded for the first time in India and accessioned in Gen Bank. This study revealed more than 99% identity matrix with published isolates from different countries, proving their conserved nature and possibility to use as effective vaccine candidate. The phylogenetic tree analysis depicts that the Indian ILTV sequences clustered with major reference sequences of the world.

Keywords: Infectious laryngotracheitis, Molecular characterization, Sequence analysis, Phylogenetic analysis, gC, gD, UL47

1. Introduction
The infectious laryngotracheitis (ILT) is a highly contagious, acute upper respiratory tract disease that primarily affect the chickens of all age groups [1]. ILT is caused by Gallid alphaherpes virus 1 under genus Iltovirus and family Herpesviridae. The ILTV genome is linear measuring 155kb double stranded DNA consisting of Unique long (UL), Unique short (US) and two inverted repeat regions [Internal repeat (IR) and Terminal repeat (TR)] flanking on either side of US region. This virus causes huge economic loss in poultry industries worldwide including India and is a major concern for poultry farmers.
After the first report of ILT during 1963 at Uttar Pradesh in India [2] and two more reports in late 1960s, no reports of ILT were recorded in the country till last decade. In the recent past re-emergence of ILT were being reported especially from state of Tamil Nadu [3-9]. It has been reported that Infectious laryngotracheitis virus (ILTV) is one of the re- emerging diseases across the world [10].
The clinical cases of ILT in field conditions may be observed either as severe acute form with prominent clinical signs like conjunctivitis, nasal discharge, dyspnoea, coughing and expectoration of bloody mucus or as very mild form with no major clinical signs. The acute cases often result in establishment of latent infection in trigeminal ganglion of birds and this may lead to reactivation and subsequent infection of naïve birds [11]. Nationwide epidemiological study is warranted to assess the magnitude of ILTV infections particularly in the intensive poultry production state like Tamil Nadu.
The envelope glycoproteins and tegument proteins of ILTV were particularly targeted for development of new generation vaccines and for development of diagnostic tools. They were found to be the important protective immunogens, playing an important role in entry and replication of virus into the cell [12].

Glycoprotein C (gC) encoded by *UL44* mediates the initial attachment of virus by interacting with cellular Heparan Sulfate determinants [13] and also found to be the target for cytotoxic T cell cellular immune response [14]. Glycoprotein D (gD) encoded by *US6* were identified as abundant virion proteins in ILTV and is essential for receptor binding and virus entry [15]. The tegument protein UL47 plays a vital role in particle assembly in the nucleus and virion maturation in the cytoplasm [16].

Sequence analysis of individual genes or several genes has been used by a number of researchers for reliable molecular characterization of ILTV isolates [17,18]. Molecular detection and related sequence analysis using the envelope glycoproteins and tegument protein of ILT virus has not been attempted in India. This study describes for the first time in India; the molecular confirmation of ILT virus from natural outbreaks in Tamil Nadu using gC (*UL44*), gD (*US6*), UL47 genes; their sequence analysis and phylogenetic relationship with reference isolates in the database.

2. Materials and Methods

2.1 Sample collection

A total of 11 commercial poultry farms (32 flocks, n=10) in Namakkal region of Tamil Nadu wherein ILT outbreaks were reported during March 2023 to June 2023 were covered under this study. The farms in this study includes both layer and broiler farms with different commercial strains like Cobb, Bovans, Babcock, Hubbard, Hyline, Lohman and Ross. Tracheal swabs were collected from birds showing mild to severe signs of nasal discharge, dyspnoea and coughing. The whole trachea was collected aseptically from dead birds for further investigation in the laboratory.

2.2 Virus isolation

Five samples from each flock were pooled and a total of 32 pooled samples were processed for virus isolation. The tissue samples were minced using sterile mortar and pestle to prepare 10% homogenous suspension with sterile Phosphate buffered saline solution. The tissue suspension was then centrifuged at 4000 rpm for 10 mins at 4 °C. The clear supernatant was transferred to a sterile Eppendorf tube and treated with 10× concentration of antibiotic and antimycotic solution (Himedia cat #A002A) for about 30 mins at 37˚C. The supernatant was filtered through 0.45µm syringe filter (Sartorius) and used for inoculation into 9-11day old SPF chicken eggs through Chorio allantoic membrane (CAM) route by artificial air sac method. 200µl of sample was inoculated into each egg, incubated at 37˚C and examined daily for dead embryos for up to 120 hours. Then the infected CAM was harvested and used for further passages.

2.3 Total DNA extraction

The infected CAM were triturated using sterile Phosphate buffer saline to get 10% tissue homogenate and subjected to three cycles of freezing/thawing at -20℃ to facilitate DNA extraction. Total DNA was extracted using the EZ-10 Genomic DNA extraction kit, Bio-basic Canada Inc as per the manufacturer's protocol. The purity and concentration of DNA was checked using Nanodrop 2000, Thermo scientific. The DNA eluted in TE Buffer (pH 8.0) was stored at -20˚C until use.

2.4 Polymerase chain reaction targeting gC, gD and UL47 genes of ILTV

The DNA was initially subjected to diagnostic PCR for amplification of partial ICP4 gene to confirm ILT virus. After confirmation, the genomic DNA was subjected to PCR amplification of full length gC, gD and UL47 genes. Oligonucleotide primers were designed in-house and synthesized at Eurofins Genomics India Pvt Ltd, Bangalore. The position of forward and reverse primers was designed in such a way to include extra nucleotides before start codon and after stop codon of each gene so as to get full length data in sequencing reaction. The details of primers used and the cycle parameters standardized after gradient PCR reaction were listed in the Table.1. The PCR was carried out in 20µl volume reaction using 2× red dye master mix (Ampliquon®, Denmark) in C1000 Biorad® thermal cycler. After amplification, 5µl of PCR products were analysed by running on 1.5% agarose gel stained with ethidium bromide and image was recorded using gel documentation system (Syngene®). Bio-Helix® 100bp DNA ladder was used to determine the size of double stranded DNA on agarose gel.

Table 1: List of Primers used for amplification of gC, gD and UL47 genes in this study

Target Gene	Primer Name	Primer sequence (5'-3')	PCR Cycle conditions
gC	gC-FP	CGGACTGTGTCCATAGTAGC	98 °C 2 min 30s; 35 × (98 °C 20s, 62 °C 45s, 72 °C 1 min 30s); 72 °C 10 mins
	gC-FP	GCTTGAGACTACCTGCCAG	
gD	gD-FP	CTGGCCAAAGTACAGTGTGG	98 °C 2 min 30s; 35 × (98 °C 20s, 62 °C 45s, 72 °C 1 min 40s); 72 °C 10 mins
	gD-FP	CCATGCTTTTCGAACGTCC	
UL47	UL47-FP	GACGGAGATAGAGAACTGAAGC	98 °C 3 min; 35 × (98 °C 20s, 62.5 °C 25s, 72 °C 2 min); 72 °C 10 mins
	UL47-RP	GGATGCGTAGCTCAGTTACG	
ICP4	ICP4 OIE-FP	CTTCAGACTCCAGCTCATCTG	94 °C 3 min; 35 × (94 °C 1 min, 62 °C 1 min, 72 °C 1 min 30s); 72 °C 10 mins
	ICP4 OIE-RP	AGTCATGCGTCTATGGCGTTGAC	

2.5 Nucleotide sequencing

The PCR products of each gene was confirmed by analysing on agarose gel electrophoresis. Then the bulk PCR products were purified using QIAquick® PCR purification kit (Qiagen, Germany). The purified PCR products were sequenced at Eurofins Genomics India Pvt Ltd, Bangalore by Sanger dideoxy method using their respective forward and reverse primers. The sequence data of forward and reverse strands were assembled to get complete coding sequence (CDS) of the genes using LaserGene software (DNAStar). The sequence data were then subjected to BLASTn analysis in NCBI database. The confirmed sequences of gC, gD and UL47 genes were deposited in GenBank database and accession numbers were received.

2.6 Phylogenetic analysis

The sequences obtained in this study were subjected to phylogenetic analysis. Reference homologous gene sequences showing high similarity were retrieved from GenBank database and aligned using ClustalW for sequence comparisons. A phylogenetic tree was constructed by maximum likelihood method using 1000 bootstrap

replications in MEGA XI software to infer the evolutionary association.

3. Results

3.1 Epidemiology of ILT outbreaks in poultry farms

All the poultry farms under this study showed typical clinical signs in multiple age group of birds. Although the incidence of ILT was being reported round the year in Namakkal, more than 75% of total cases were recorded during March to June month. The details of ILT positive flocks screened in this study were presented in Table 2. Both layer and broiler farms were found to be susceptible for ILTV infections. Among layer birds the incidence was found to be higher among 10 to 20 weeks age group. The mortality percentage in this study varied from 1.5% to 6.2% in various farms with morbidity range of about 30 to 70% in a flock. The average mortality was found to be 3.21% among layer flocks and 4.98% among broiler flocks. The major clinical signs observed in this study includes drop in egg production, conjunctivitis, blood mixed nasal discharge, Coughing and pump handle type of respiration. On Post-mortem examination of dead birds, typical haemorrhagic tracheitis with caseous exudate could be found in many birds (Figure 1).

3.2 Cultivation of ILT virus in embryonated eggs

Four isolates from different farms in this study were successfully passaged in the embryonated SPF chicken eggs. The infected CAM was harvested after 120 hrs. of incubation and were confirmed for ILT virus by diagnostic PCR for partial ICP4 gene with a specific amplicon of size 635bp as shown in Figure 2. The positive samples produced highly haemorrhagic and more thickened CAM which could be well evidenced on comparison with healthy negative control eggs. A few pock lesions could be seen in the infected CAM of eggs.

Table 2: Details of Poultry flocks from where samples are collected for this study

Sl.no	Region/state	Farm size	Type of farm	Month/year	Age of birds	Mortality %	ILT Vaccination
1	Namakkal, TN	2,50,000	Layer	March, 2023	35 weeks	1.5	Not done
2	Namakkal, TN	1,50,000	Layer	March, 2023	12 weeks	3.2	Not done
3	Namakkal, TN	50,000	Broiler	March, 2023	3 to 4 weeks	5.0	Not done
4	Namakkal, TN	35,000	Broiler	April, 2023	3 to 4 weeks	4.2	Not done
5	Namakkal, TN	1,65,000	Layer	April, 2023	8 to 20 weeks	3.6	Not done
6	Namakkal, TN	1,50,000	Layer	April, 2023	15 weeks	4.0	Not done
7	Namakkal, TN	80,000	Layer	April, 2023	60 weeks	1.8	Vaccinated
8	Namakkal, TN	1,00,000	Layer	May, 2023	20 weeks	4.4	Not done
9	Namakkal, TN	1,20,000	Layer	May, 2023	18 weeks	4.0	Not done
10	Namakkal, TN	10,000	Broiler	May, 2023	4 weeks	6.2	Not done
11	Namakkal, TN	25,000	Broiler	June, 2023	5 weeks	4.5	Not done

Fig 1: Haemorrhagic tracheitis with caseous exudate

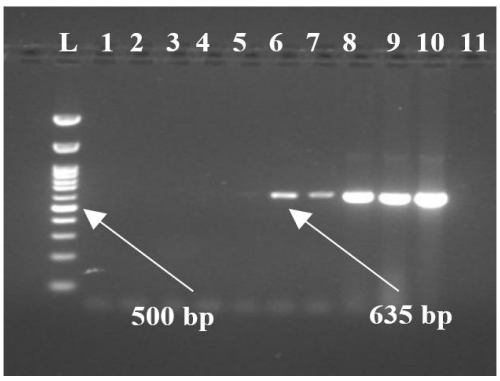

Fig 2: PCR amplification of partial ICP4 gene of ILTV

Figure 2: L-100bp ladder, Lane1-5: Negative samples, Lane 6-9 Positive samples, 10- Positive control, 11- Negative control.

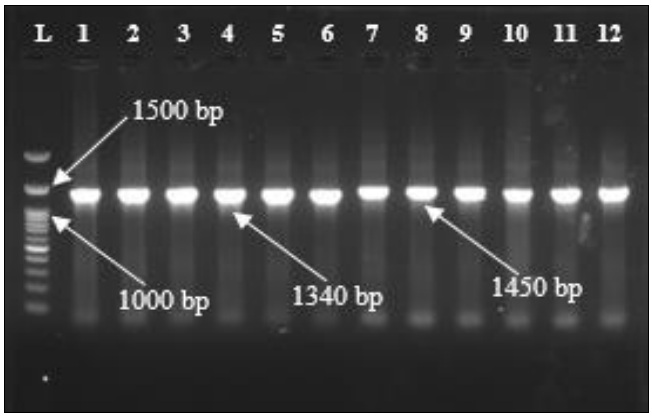

Fig 3: L-100bp ladder, Lane1-6: gC gene, Lane 7-12: gD gene

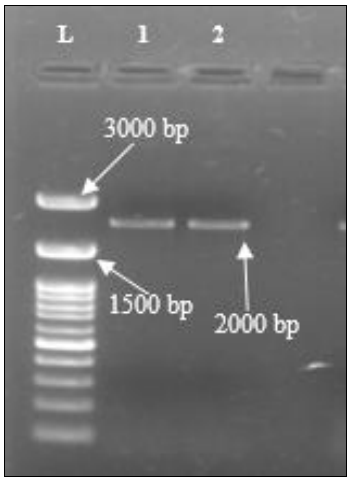

Fig 4: L-100bp ladder, Lane 1 and 2: UL47 gene

3.3 PCR amplification of gC, gD and UL47 genes

All the four isolates of ILTV isolated in this study were subjected to full length amplification of gC, gD and UL47 genes. Single, clear bands of specific size were obtained for all the three genes which were confirmed and documented on agarose gel electrophoresis as shown in Figure 3 and 4. This yielded expected product size of about 1340bp, 1450bp and 2000bp for gC, gD and UL47 genes respectively.

3.4 Nucleotide sequencing gC, gD and UL47 genes

On sequencing, the products of gC, gD and UL47 genes yielded good results with clear chromatogram peaks. The chromatogram data were also checked manually for forward and reverse reads. The FASTA sequences for three genes were trimmed from start to stop codon after assembling both strands of read to get complete CDS and were submitted in GenBank with accession numbers: gC gene: PP133317.1, PP133318.1, PP133323.1; gD gene: PP133319.1, PP133320.1, PP133321.1, PP133322.1; UL47 gene: PP133324.1 and PP133325.1. BLASTn analysis with reference sequences in GenBank produced percent identity matrix of 99.52 to 99.84% for gC, 99.62 to 99.92% for gD and 99.36 to 99.41% for UL47 genes. The gC, gD and UL47 sequences of different isolates obtained in this study showed 99% nucleotide identity with a few single nucleotide polymorphic sites but without any gaps.

3.5 Phylogenetic analysis: The full-length sequences of gC, gD and UL47 genes submitted in this study were the only Indian sequences available in GenBank. They were used for phylogenetic analysis in comparison with sequences available in GenBank from different countries for their evolutionary relationship which are shown in the Figure 5a, 5b, and 5c. The phylogenetic analysis based on full-length sequence of three isolates of gC gene revealed that two of them clustered a one branch and one isolate as a separate branch from sequences of various countries. The phylogenetic tree constructed based on full-length sequence of four isolates of gD gene revealed that two sequences clustered with the sequences of other countries and two forms a separate branch from that cluster. The two isolates of UL47 genes bifurcate as a separate branch from that of main cluster containing sequences of various countries.

Fig 5a: Phylogenetic tree based on full length sequences of gC gene in this study.

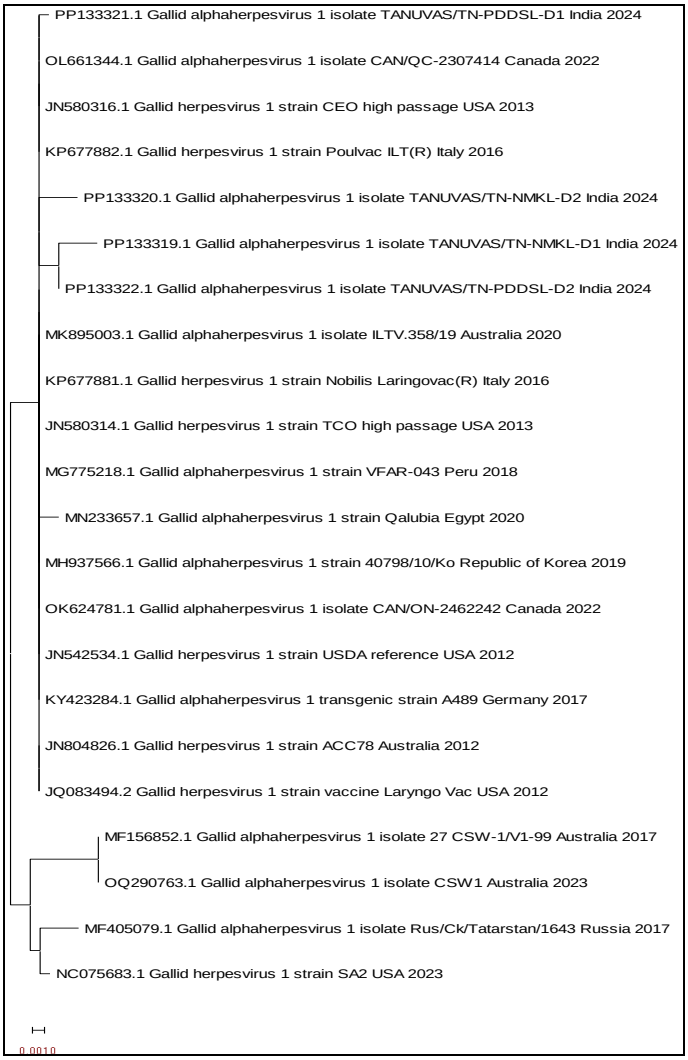

Fig 5b: Phylogenetic tree based on full length sequences of gD gene in this study.

4. Discussion

ILT is a highly contagious acute respiratory disease affecting chickens of all age groups. The incidence of ILT has geared up highly during the last decade especially in state of Tamil Nadu which poses major challenge to intense poultry production regions like Namakkal. The re-emergence of ILT in many countries necessitate detailed molecular epidemiological study to assess the genetic diversity in the ILTV.

The cases of ILT outbreaks reported during the month of March to June 2023 in various commercial poultry farms of Namakkal region were included in this study. More than 3/4th

of total cases of ILT in a year were found to occur during summer months. Although ILT is infectious to all age groups of birds, 12-20 weeks of birds were found to be highly susceptible. The average mortality percentage of 3.2% (layers) and 4.98% (broilers) recorded in this study suggests that the outbreaks are due to mild form of ILTV in accordance to reports of many researchers [1]. The ILT virus was successfully passaged by CAM route of inoculation in 9-11 day old embryonated chicken eggs. Apart from thickening and highly haemorrhagic lesions, the intensity of pock lesions on infected CAM was found to increase on further higher number of passages in the eggs. Vaccination combined with biosecurity is the best way to control ILT. The recent rise in outbreaks of the disease have been correlated to the use of live attenuated vaccine strains especially Chicken Embryo Origin vaccine that have regained virulence after bird-to-bird passage [19] and has become the source of long-lasting outbreaks. This clearly defines the need for development of safer vaccines against ILT.

The in-house designed PCR primers in this study, covering the extra portions before and after complete CDS of the gene has positively amplified the full-length which were confirmed on sequencing data. The amplification and sequencing of gC, gD and UL47 gene of an Indian isolate of ILTV has been recorded for the first time in India.

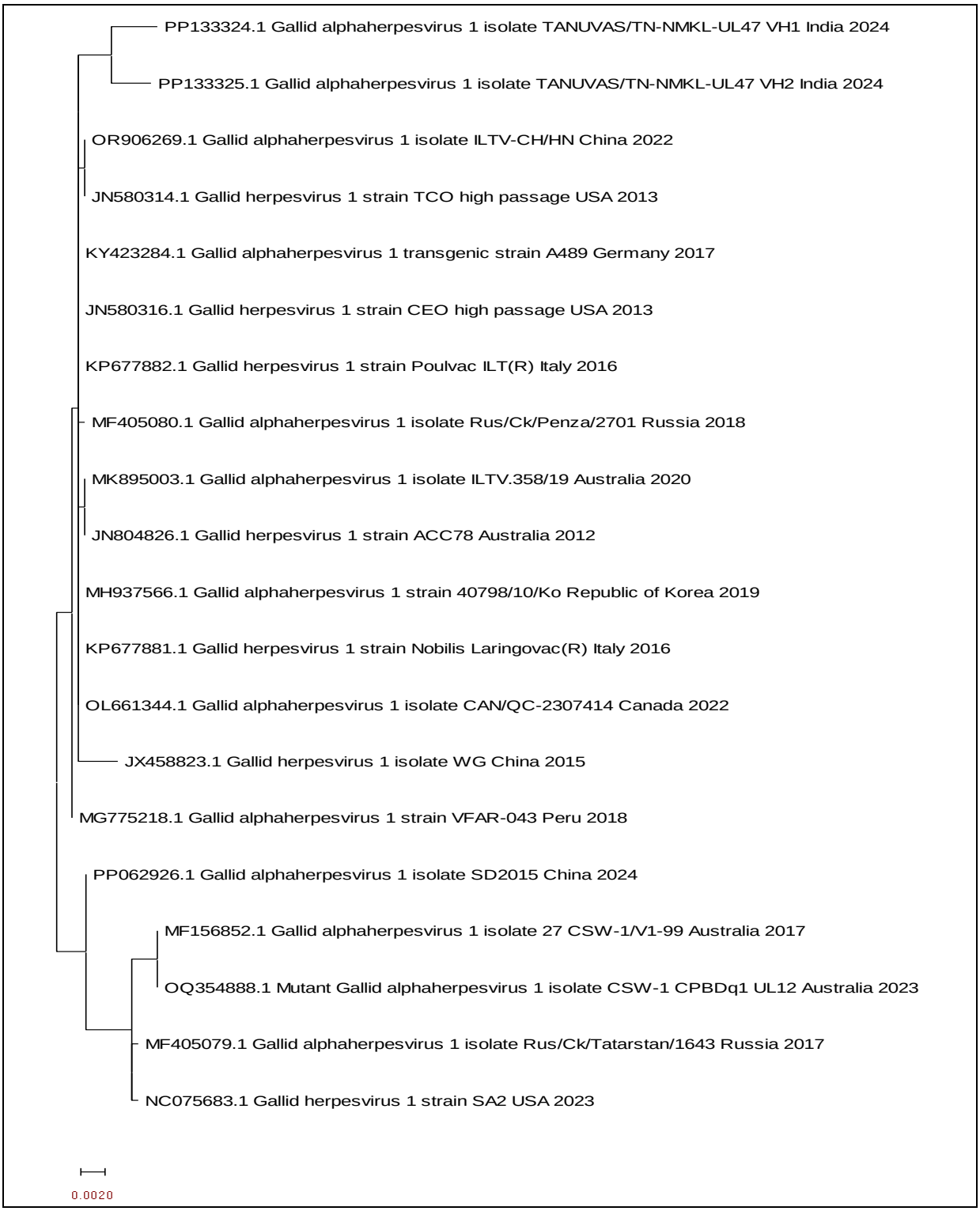

Fig 5c: Phylogenetic tree based on full length sequence of UL47 gene in this study.

A recombinant vaccine expressing the protective immunogen (s) of the ILTV would be safer vaccine. With the objective of targeting the most appropriate genes for development of next generation vaccines for ILT, three genes gC, gD and UL47 were selected for molecular characterization and sequence analysis in this study. These three genes are important candidates for future vaccines as reported by researchers [18, 20]. The sequence analysis of UL47 gene of ILTV will serve as useful tool for investigations of ILT viruses [21]. The sequence data of UL44, US6 and UL47 of an Indian isolate of ILTV has been elucidated for the first time in the country. They revealed that these sequences share more than 99% percent identity matrix with reference isolates of the globe, proving their conserved nature and possibility of using them as effective vaccine candidate. The phylogenetic tree constructed with these sequences clearly depict that they all clustered with sequences published from various countries in the world.

5. Conclusion

The present vaccination strategies against ILTV are not ideal and detailed investigations of the genetic diversity of protective immunogens against the disease need to be focussed. In this context, this preliminary study was attempted for molecular epidemiological analysis of two major envelope glycoproteins (gC, gD) and one tegument protein (UL47). The genes for gC, gD and UL47 were found conserved and are suitable candidate antigens for development of safer next generation vaccines.

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