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Studies on molecular diagnosis and respiractory tract pathology of infectious laryngo-tracheitis disease in chickens in Karnataka

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Abstract
The present study mainly focussed on respiratory tract pathology and molecular diagnosis of field occurrences of ILT in 783 chickens from 50 chicken farms in Karnataka during May 2017 to May 2018. Among 200 pooled tracheal samples from 50 farms, 24 samples belonging to six layer farms were positive for ILT by PCR results. Clinical signs included nasal discharge, infraorbital swelling, dyspnoea, expectoration of bloody mucus with depression. Post mortem lesions revealed respiratory tract inflammatory changes, laryngo-tracheal hemorrhages with mucous plug and diptheretic membrane formation, congestion and edema of lungs with patchy hemorrhages, pleuritis, polyserositis and airsacculitis. Histopathologically, degenerative and hyperplastic changes in laryngo-tracheal region consisting of syncytial epithelial giant cell containing eosinophilic inclusion bodies were mainly recorded. Analysis of sequencing by phylogenetic tree construction revealed 100% similarity between field and few CEO vaccine strains as far as the 688bp ICP4 gene segment was concerned.

Keywords: ILT, PCR, ICP4 gene, laryngo-tracheitis, inclusion bodies, Karnataka

1. Introduction
Infectious laryngotracheitis (ILT) is an acute viral disease involving upper respiratory tract infection of chickens, and, rarely, pheasants and peafowl characterized by marked dyspnoea, coughing, gasping, and expectoration of bloody exudate. It is caused by a member of the genus Iltovirus, subfamily Alphaherpesvirinae of the Herpesviridae family within the order Herpesvirales (Crawshaw and Boycott, 1982; Davison, 2010; Garcia and Spatz, 2020) [1, 2, 3]. The GaHV-1 virion has a nucleocapsid of icosahedral symmetry surrounded by a protein tegument layer, encapsulated by an outer envelope with incorporated virus encoded glycoproteins. The GaHV-1 genome, contained within the nucleocapsid, is a linear double-stranded DNA molecule composed of a unique long (UL) and unique short (US) region flanked by inverted repeats (Mc Geoch *et al*., 2000; Ruiz *et al*., 2018) [4, 5].
The denser areas of poultry industry in India and various ILT endemic continents of world (United States, North America, South America, Europe and Asia) experience multimillion dollar losses each year as a consequence of GaHV-1-induced mortality, decreased egg production, cost of vaccination, biosecurity measures and therapy to counteract secondary infection by other avian pathogens and performance penalty due to vaccination reactions particularly in broilers (Jones, 2010; Garcia and Spatz, 2020) [6, 3]. Aiming at the present scenario of occurrence of ILT, the study was done using diagnostic techniques like histopathology and molecular methods, using PCR and gene sequencing to diagnose, to study the pathomorphology, to ascertain the usefulness of particular primers for ILT disease diagnosis in Karnataka and to know the degree of homology of the genetic material of the above viruses by sequencing and comparing with already known strains.

2. Materials and Methods
2.1 Source of samples
The present study was carried out in the Department of Veterinary Pathology, Veterinary College, Hebbala, Bengaluru, during May 2017 to May 2018. A total of 783 birds showing respiratory signs were examined from 50 poultry farms in southern Karnataka including adjacent districts of Hyderabad and Namakkal. Among 783 birds, 200 birds (four birds from each farm) were considered as a source material for histopathological and molecular studies in the present study.

Detailed postmortem examination was carried out on all the birds showing respiratory tract infection. Representative samples from organs such as nasal passage, larynx, trachea, lungs, air sacs and any other organ showing gross changes were collected for histopathological studies in 10 per cent neutral buffered formalin. They were suitably processed by routine standard paraffin embedding technique and 5µ thick sections were cut and stained by hematoxylin and eosin (H and E) (Luna, 1968) [7]. Pooled samples of larynx, trachea and lungs from dead birds and laryngotracheal swab contents in 50 per cent glycerol-PBS from live birds were collected aseptically in 50mL sterile polycarbonate/2mL cryo-vials and carried on cold carrier for immediate storage at -20 ºC. From field, where immediate freezing was not possible, the samples were collected in 50 per cent glycerol-PBS (Cumming, 1969 and Mc Martin, 1993) [8, 9].

2.2 Polymerase chain reaction and sequencing

Triturated pooled sample (in sterile 200µl PBS) or scraping from laryngo-tracheal region (≤25 mg) or swab dipped PBS (200µl) was used for DNA isolation by DNA extraction kit (QIAmpr DNA Mini Kit 50, Cat. No 51304) as per standard kit protocol. DNA was quantified by NanoDrop® Spectophotometer (ND-1000). The A260/A280 ratio approximately 1.8 for DNA was considered pure. Amplification ICP4 gene was done by using gene specific primers *viz*., 5'ACTGATAGCTTTTCGTACAGCACG3' and 5'CATCGGGACATTCTCCAGGTAGCA3' with a product size of 688 bp for ILTV (Chacon and Ferreira, 2009) [10]. Primers were synthesized commercially from M/s Bio Serve Biotechnologies (India) Pvt. Ltd, Hyderabad. These primers were reconstituted in 1x TE buffer (TE) to obtain the required concentration of 200 picomol/µL. the PCR reaction was carried out using Amplicon Taq 2x Master mix with initial denaturation at 94 ºC for 3 minutes followed by denaturation at 94 °C for 1 minute, annealing at 62 °C for 1 minute, extension at 72 °C, for 1.5 minute, final extension at 72 °C for 10 minutes and finally held at 4 ºC, as per the conditions in OIE manual. About 2 µl of amplified products were subjected to agarose gel electrophoresis with 100 kb marker and DNA bands were visualized and the images were captured by Gel Doc. The PCR product (about 50µl) of field samples of ILT were sequenced at Eurofins Genomics India Pvt. Ltd. Both forward and reverse sequence were analysed using NCBI database and phylogenetic tree construction was done.

3. Results and Discussions

3.1 Occurrence of ILT and age group involvement

ILT was confirmed by PCR assay using primers for ICP4 gene (Fig 1). PCR band was amplified at 688bp, which was read corresponding to 100bp ladder. The forward and reverse primers were located at positions 181–204 and 846–869 of the ILTV ICP4 gene sequence. Among 200 representative samples from 50 farms with respiratory disorder, ILT was recorded in six layer farms (24 birds, 12%) with a population of 151 birds. The ILT positive birds were aged between 10 to 70 weeks. In the age group of 10-20 weeks, 20-30 weeks, 40-50 weeks, 50-60 weeks, one outbreak each was recorded whereas there were two outbreaks in the age group of 60-70 weeks. These were in tune with the observations of Boulianne (2012) [11] and Dufour-Zavala (2008) [12] who reported that most outbreaks occurred in chickens aged more than 3-4 weeks or in matured or nearly matured chickens and stated that all age groups were susceptible for ILT. In the present study ILT infections occurred in mild enzootic form in two farms and in moderately severe form in four farms.

3.2 Clinical signs in ILT positive layer birds

Clinical signs in ILT positive birds in the present study included persistant nasal discharge, conjunctivitis, swelling of eyelids with lacrimation and unthriftiness which was followed by rhinitis leading to sneezing and sinusitis with swollen infraorbital sinuses along with dyspnea, tracheal rales and gasping sounds which could be due to exudate secretion caused by tracheitis during viral multiplication phase. These clinical signs were typical to milder form of ILT and were also recorded by Linares (1994) [13], Timurkaan (2003) [14], Sellers *et al.* (2004) [15], Gowthaman *et al*. (2014) [16], Reddy *et al.* (2017) [17] and Garcia and Spatz (2020) [3]. Virus replication cause severe epithelial damage and hemorrhages of the larynx and the tracheal mucosa (Reddy *et al.,* 2017) [17]. Coughing is usually noticed when the birds try to expel the clotted blood and debris from the obstructed trachea (Garcia and Spatz, 2020; Nazir *et al.,* 2020) [3, 18]. In the field outbreaks of ILT clinical signs were seen between 6 to 14 days post exposure (Garcia and Spatz, 2020) [3] with detection of virus replication in trachea as early as 2 to 7 dpi (Oldoni *et al.,* 2009) [19]. Waidner *et al*. (2011) [20] reported that clinical signs such as conjunctivitis with mucous or serous nasal discharge, cough, dyspnoea, gasping, decreases egg production and death in severe cases occur during lytic infection of ILT.

Other associated general clinical signs like anorexia, depression/listlessness/lethargy/ apathy, ruffled feathers and droopy wings, sudden death, cyanosis of comb and huddling together recorded in the present study were also described by earlier workers (Garcia and Spatz, 2020 and Nazir *et al.,* 2020) [3, 18] who indicated multisystemic involvement.

3.3 Gross Pathology of ILT positive birds

Nasal passage in all groups of chicken in the present study showed gross pathological changes like swollen infraorbitals, mucus exudate, fibrinous exudate, caseous/ mucopurulent exudate, hemorrhages and infraorbital swelling. Nasal discharge is a common observation in all respiratory tract affections in response to either irritation or injury to respiratory nasal mucosa by causative factors and has been reported by Koare *et al.* (2018) [21] with multiple etiologies in chicken along with conjunctivitis and swollen sinuses with purulent exudate.

The gross lesions observed in the laryngotracheal region were, congestion, haemorrhage/ blood clots, mucous plug and plaques white debris on laryngeal opening mucous/ catarrh, fibrinous exudate, thickening, caseous plug/ crumbly cast and diphtheritic/ pseudomembrane (Fig 2 and Fig 3). Mucoid inflammation of the tracheal mucosa occurs during early infection, while degeneration, necrosis, and hemorrhage of the tracheal mucosa at later stages of ILT disease (Reddy *et al.,* 2017) [17]. Diphtheritic changes are common in the trachea mucosa and may be seen as mucoid casts that can extend its entire length. Garcia and Spatz (2020) [3] reported that severe hemorrhage into the tracheal lumen may result in blood casts and blood may be found in mucus and necrotic tissue. Similar lesions were also recorded by Boulianne (2012) [11] in more pathogenic strains of ILT virus with tracheal casts resulting in tracheal occlusion and death from suffocation.

Lungs showed congestion, areas of gelatinous edema, areas of hemorrhages and pus oozing from cut surfaces. These

findings were in tune with Purcell and McFerran (1969) [22] who also observed that posterior ventral angle of lungs was more involved and was clearly demarcated as areas of gelatinous edema in the early stages and as dark areas of consolidation in later stages. The inflammation due to ILT may extend into the primary bronchi and airsacs (Boulianne, 2012) [11]. The concurrent secondary bacterial and viral infections are uncommon in conjunction with ILT but 3-4 weeks age broilers with an outbreak of ILT and staying in field for additional 3 to 4 weeks prior to processing showed a severe *Escherichia coli* associated airsacculitis. Pus in lungs of ILT affected birds could be thus explained as per the observations of Boulianne (2012) [11] and Picoux *et al.* (2015) [23].

Gross changes observed in airsacs in the present study included clear to whitish exudate, increased vascularity, cloudiness, cheesy material and fibrinous covering/ thickened air sac (Fig 4) which were in tune with the observations of Boulianne (2012) [11] and Picoux *et al.* (2015) [23] but might occur in other associated secondary bacterial and mycoplasmal complications and more occurrence may be due gas flow pathway in avian lung and the mechanisms present in the parabronchi (Tully, 1995; Fedde, 1998) [24, 25].

3.4 Histopathology of nasal passage, larynx, trachea, lungs and airsacs in ILT

The microscopical changes in the respiratory tract of ILT positive birds included congestion/ hyperemia and edema in mucosal and submucosal blood vessels of nasal passage, larynx, trachea, lung parenchyma and air sac interstitial blood vessels. Mild to severe focal to multifocal hemorrhages were recorded in laryngo-tracheal mucosa and submucosal region, inter alveolar spaces of lungs and air sac membrane.

Inflammatory changes included mononuclear cell infiltration (lymphocytes and plasma cells), heterophils and macrophages in mucosal and submucosal regions of the respiratory tract. Yavuz *et al*. (2018) [26] attributed various degrees of mucosal thickening to oedema, mononuclear cell infiltrates (lymphocytes, plasma cells), hyperemia, and heterophil granulocyte accumulation in the mucosa of the trachea and larynx. Milder inflammation of nasal mucosa with mucous secretion was noticed in nasal passage while laryngotracheal region showed much more severe inflammatory changes with serous to mucous exudate in lumen containing fibrin, blood and necrotic epithelium along with inflammatory cells (Fig 5,6 and 7). Abdel-Aziz, (2016) [27] described that marked heterophilic infiltrates and intraluminal fibrinoheterophilic exudates are common in the early acute phases of ILT infection with non-necrotic epithelium showing loss of cilia and increased mucous production. They recorded lymphoid cell infiltrates in the lamina propria of the tracheal mucosa as common response if affected chickens survive several days.

Degenerative changes consisted of segmental epithelial desquamation in nasal passage, ciliary loss in milder form and epithelial erosions to mucosal desquamation in laryngo-trachea region in moderately severe form of disease resulting in cast formation in lumen. Ciliary loss followed by epithelial erosions which lead to exposure of blood vessels leading to hemorrhages.

Erosions were repaired by regeneration/ stratification of non-ciliated epithelium and adjacent goblet cell hyperplasia leading to thickened mucosa, lined by eosinophlilic exudate, in the birds that survive the initial acute phase of infection. Degenerative changes in lungs included serofibrinous exudate and casts of sloughed off epithelial cells in the bronchial lumen and acute alveolar emphysema whereas in air sacs, inflammatory exudate in interstitium with fibrin deposition were recorded.

The thickening and opaqueness of air sacs recorded in the present sudy was also previously reasoned out by Yavuz *et al*. (2018) [26] who explained that inflammatory changes including inflammatory cellular infiltration in the interstititum, proliferation of fibrous connective tissue and formation of inflammatory exudate on the surface cause thickening and opaqueness of air sacs.

Hyperplastic changes resulted in glandular hyperplasia (goblet cells), mucosal thickening and syncytial epithelial giant cell formation (by cytoplasmic fusion of adjacent cells) in nasal passage (Fig 8) and laryngo-tracheal region (Fig 9). Laryngeal mucosa of some ILT affected birds was lined by eosinophilic exudate containing inclusion bodies in syncytial cells (Fig 10, 11 and 12). In lungs hyperplastic changes included smooth muscle hypertrophy and obliteration of atria and infundibulum.

Vareille *et al.* (2011) [28] and Coppo *et al.* (2013) [29] attributed the cascade of events that occur in ILT infection to chemokines and cytokines that are released by the destruction of respiratory epithelium. They discovered that ILTV infection significantly increased the expression of interleukins (6 and 8) and CXC K60, with much smaller increases in the expression of CCL17, CCL20, CCL4, chemokine ah221, CXCL14 and interleukin-15.

These mediators are responsible for directing both the innate inflammatory and the adaptive immune responses. They also stated that the extensive hyperplasia of respiratory epithelium observed in trachea was consistent with the up regulation of genes related to cell growth and proliferation, while up regulation of cytokine and chemokine genes was consistent with the infiltration of inflammatory cells.

During ILTV infection, ILTV encodes glycoprotein G (gG), a viral chemokine binding protein (vCKBP) that is secreted and capable of inhibiting chemokine function (A) and this inhibitory effect results in reduced migration of heterophils to the site of infection and adaptive immune responses to infection skewed towards the production of non-protective antibody (Devlin *et al*., 2010 and Vareille *et al.,* 2011) [30,28]. Coppo *et al.,* (2013) [29] reported that in spite of mucous containing many innate immune mediators such as interferons, collectins, IgA, defensins and lactoferrin (Vareille *et al.,* 2011) [28], the relevance of these molecules during ILTV infection of the trachea is still not known.

3.5 Sequencing results

The PCR product (about 50µl) of field sample of ILTV were sent to Eurofins Genomics India Pvt. Ltd for sequencing. Both forward and reverse sequencing was done by Sanger sequencing.

Analysis of sequencing by phylogenetic tree construction revealed 100% similarity between field strain and CEO vaccine strains, as far as the 688bp ICP4 gene segment is concerned (Fig 13).

The present scenario was elaborated by Gowthaman, (2014) [16] who reported that the percent identity matrix calculated for Indian ILT viruses shared 99.8 % homology with chicken embryo origin (CEO) vaccine strains of Italy, USA and China, and 98.4 % with Brazilian strain and concluded that Indian ILTV sequences were clustered with CEO vaccine strains of Italy, USA, China and Brazil.

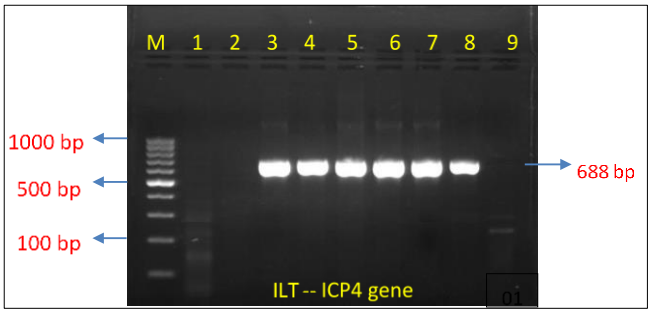

Fig 1: PCR Gel doc photograph of agarose gel electrophoresis; screening of tissue samples for ILT (ICP4 gene at 688bp). Lane M: 100bp DNA ladder; Lane 1 and 2: Negative control and NTC control; Lanes 3: Positive control (ILT vaccine); Lanes 4-8: Positive bands from field samples

Gross changes in trachea and airsacs

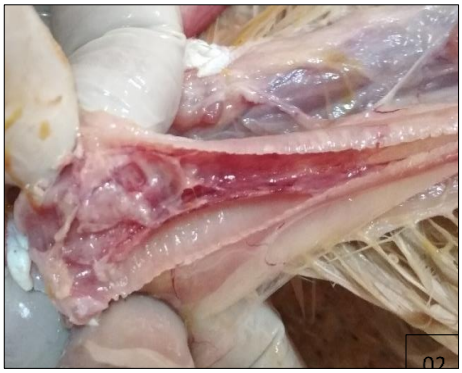

Fig 2: Gross picture sloughing off of diphtheritic/ degeneration and necrosis of mucous membrane of trachea and larynx, along with presence of fibrinopurulent material, in an ILT positive broiler bird

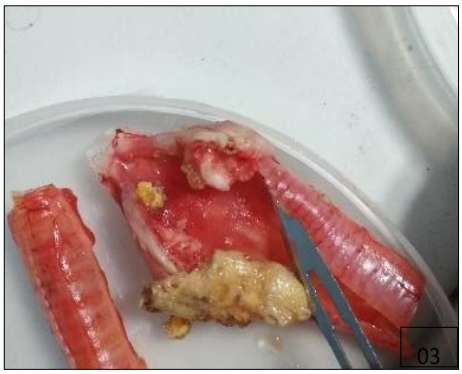

Fig 3: Gross picture severely congested trachea and laryngeal mucous membrane along with presence of fibrinopurulent plug in laryngo-tracheal region of ILT positive broiler bird

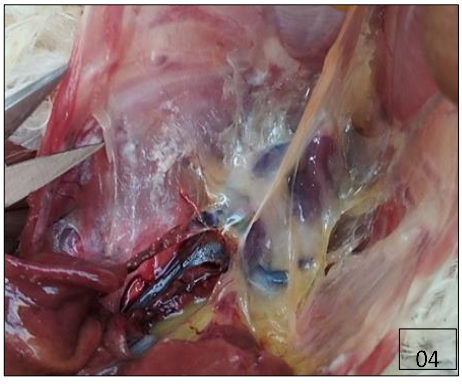

Fig 4: Gross picture cloudy air sacs in an ILT positive broiler bird

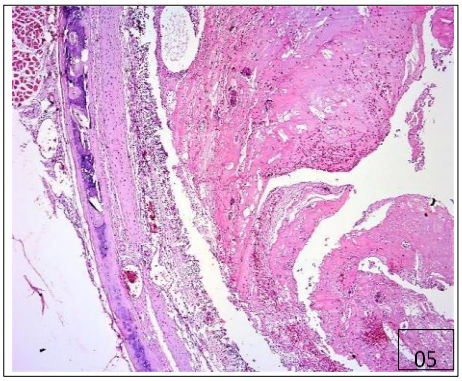

Fig 5: Section of trachea from ILT positive bird showing presence of thick sero-fibrinous exudate in the lumen. Note necrotic mucosal layer. H&E X20

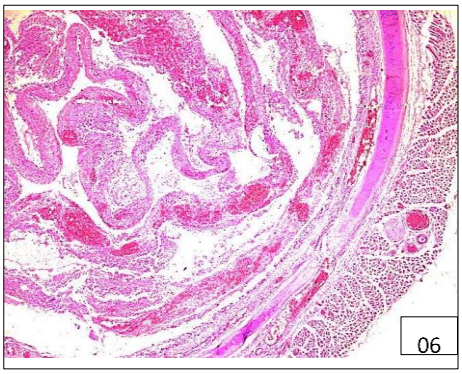

Fig 6: Section of trachea showing from ILT positive bird showing tracheal lumen filled with fibrino-hemorrhagic exudate mixed with desquamated mucosa. H&E X20

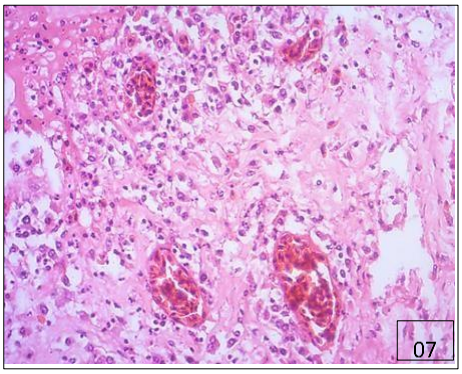

Fig 7: Section of laryngo-tracheal region from ILT positive bird showing fibrinous exudate with presence of syncytial cells with inclusion bodies. H&E X1000

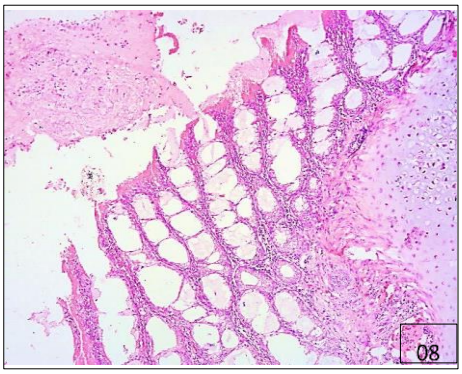

Fig 8: Section of nasal passage from ILT positive bird showing thickened mucosal layer with numerous dilated mucous glands and mucous exudate in caudal concha. H&E X50

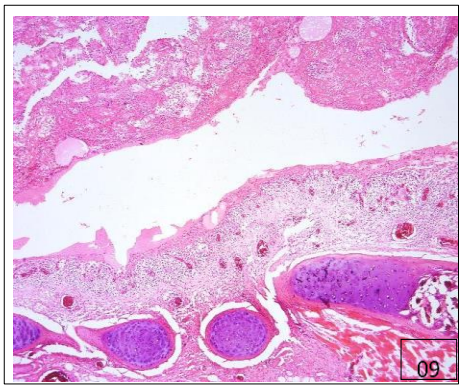

Fig 9: Section of laryngo-tracheal region from ILT positive bird showing thickened mucosa lined by thick eosinophilic exudate. Also note presence of serofibrinous exudate in the lumen. H&E X50

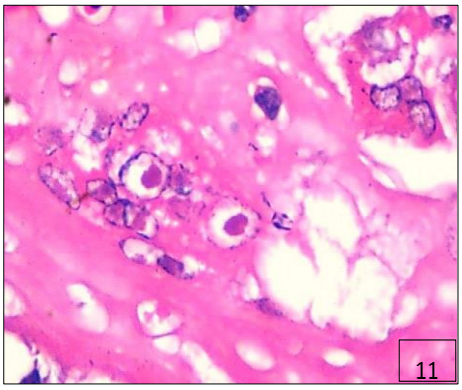

Fig 11: Syncytial cells showing intranuclear amphophilic Cowdry type A inclusions with a halo around and margination of chromatin at the periphery of nucleus. H&E X100

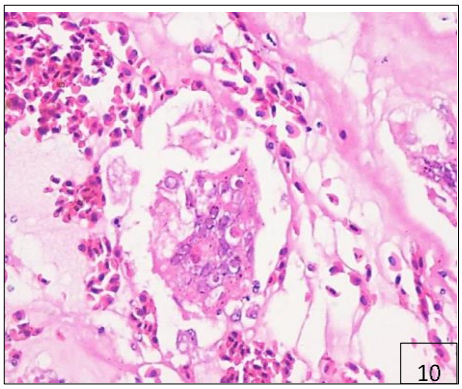

Fig 10: Tracheal serofibrinous exudate from ILT positive bird showing syncytia formations consisting of numerous cells with inclusions. H&E X40

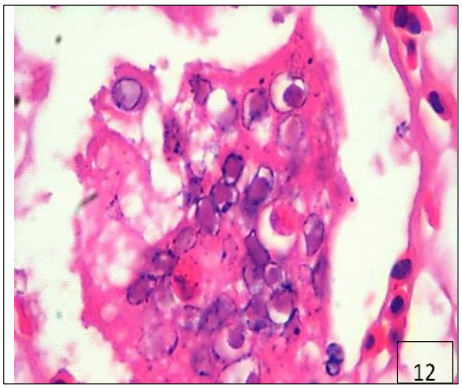

Fig 12: Syncytial cells showing intranuclear amphophilic Cowdry type A inclusions with a halo around and margination of chromatin at the periphery of nucleus. H&E X100

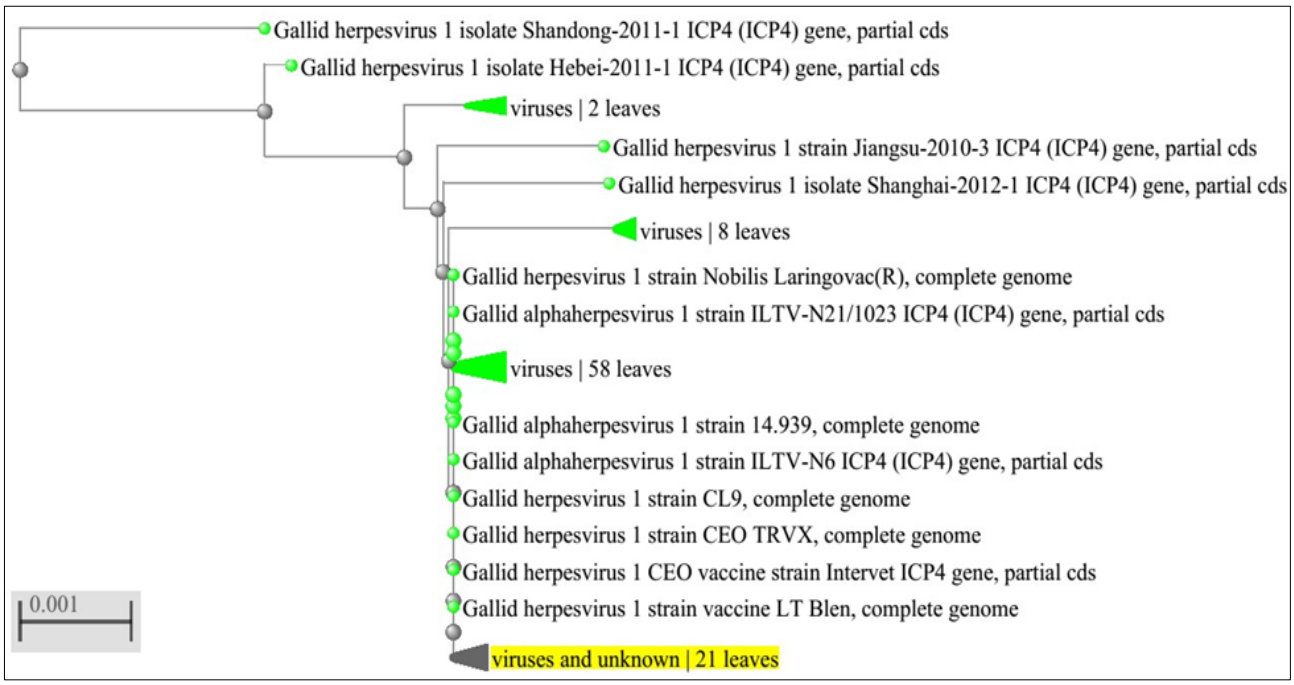

Fig 13: Phylogenetic tree for ILTV field sample

4. Conclusions

The present study findings indicate that the vaccine or field strain is already establishing in Karnataka and warrants for further detailed investigations on screening for ILT virus occurrence, its strain identification and necessary vaccination programme or strict biosecurity control measures.

5. Acknowledgments

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