Pathology and Molecular Detection of Infectious Laryngotracheitis (ILT) In Commercial Layer Chicken

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Abstract

Poultry sector is one of the fastest growing agricultural sectors in India. Poultry industry has always been confronted with challenges in the form of various diseases. Among these, diseases caused by infectious agents are more common causing severe mortality and economic loss. Infectious laryngotracheitis (ILT) is a respiratory disease of chicken often noticed in intensive poultry rearing regions of the world. The present study was aimed to investigate the pathology and molecular confirmation of Infectious laryngotracheitis in commercial layer chicken at Namakkal, Tamil Nadu and Puducherry, India. Out of 62 farms investigated, 40 farms (64.5%) were reported to be affected by ILT. The clinical signs varied from mild respiratory distress to difficulty in breathing, gasping, pump handle-type of respiration, oculo-nasal discharge, swelling of the infraorbital sinuses and closed eyes. Grossly, the tracheal mucosa revealed congestion, hemorrhage, catarrhal, fibrinous and diphtheritic inflammatory changes. Histopathology of the trachea included congestion, hemorrhage, inflammatory and necrotic changes. Occasionally, syncytial formation with intranuclear inclusion bodies, cystic dilatation of submucosal glands and inflammatory changes in trigeminal ganglion were also observed. Inoculation of tracheal samples from ILT suspected cases into 9-11day-old embryonated chicken eggs revealed numerous pinpoint white pock lesions in the chorioallantois membrane (CAM) characteristic to ILT virus. Further, DNA extracted from the trachea, CAM and trigeminal ganglia subjected for PCR gave amplification of 237 bp target of ICP4 gene and confirmed that the trigeminal ganglia was the site of latency of ILT virus.

Keywords: Commercial Layers; ILT; Gross; Histopathology; Molecular confirmation

Abbreviations: ILT: Infectious Laryngotracheitis; CAM: Chorioallantois Membrane; NRSF: Neuronal Restrictive Silencing Factor; REST: Repressor Element Silencing Transcription; NBF: Neutral Buffered Formalin; H&E: Hematoxylin and Eosin; ICP: Infected-Cells Polypeptide.

Introduction

Respiratory disease complex has emerged as a great challenge to poultry industry. The superior germplasm of intensely reared birds intended for increased production coupled with environment and other managemental practices have made them highly vulnerable to respiratory disease complex, which is one of the most common and complex syndromes, surfacing too often in the field [1]. The complex comprises a group of diseases viz Avian pneumovirus infection, Avian influenza, Infectious bronchitis, Newcastle disease, Infectious laryngotracheitis, Infectious coryza, Ornithobacteriosis, Mycoplasmas and Colibacillosis [2]. These diseases consistently involve the respiratory system and produce closely resembling symptoms in poultry. As a result, diagnosis of each disease, its differentiation from the others, their treatment, control and prevention have become extremely complicated.

Avian infectious laryngotracheitis (ILT) is one of the important contagious viral respiratory diseases that cause significant economic losses in the poultry industry due to increased mortality, decreased growth rate and lower egg production [3]. The disease mostly affects the upper respiratory tract of chickens and is caused by infectious laryngotracheitis virus (Gallidherpesvirus-1), a member of the sub-family Alphaherpesvirinae (Genus Iltovirus). This virus is only transmitted horizontally and primarily infects the conjunctiva and tracheal mucosa [4].

Like alpha herpesviruses in other species, Infectious laryngotracheitis virus can establish a carrier state in recovered birds. The latent infection in the trigeminal ganglion in the central nervous system was established by both vaccine and challenge

strains in an experimental study [5]. During viral replication, The DNA released into nucleus transcribes the genes in three stages: immediate-early (IE), early (E) and late (L). In the IE stage, infected-cells polypeptide (ICP) 4 is mainly expressed and functions as a transcriptional trans activator for other virus genes by binding to viral promoters [6]. A cellular alpha trans-inducing factor (α-TIF) protein helps ICP4 to regulate the viral transcription activity in the IE stage [7]. The function of ICP4 is inhibited by neuronal restrictive silencing factor (NRSF) and human repressor element silencing transcription factor (REST) in the latent infection [8,9]. The latently infected virus can be reactivated by the ICP0 and ICP4 from the dissociation of NRSF in illness or physical stress conditions [10].

Clinical signs generally appear 6-12 days following natural exposure and include nasal discharge, moist rales, conjunctivitis and labored breathing with expectoration of blood-stained mucus in severe cases with morbidity and mortality rates of 100% and 70%, respectively [11,12]. Grossly, ILT is characterized by catarrhal, hemorrhagic and/or necrotic tracheitis. Microscopic changes may vary from epithelial degeneration, edema, hemorrhage, epithelial hyperplasia, syncytial formation with intranuclear inclusion bodies and infiltration by inflammatory cells to complete necrosis of the epithelium.

Infectious laryngotracheitis is usually diagnosed in the laboratory because other diseases such as avian influenza, bronchitis, new castle's disease, infectious coryza, and mycoplasmas cause very similar clinical signs and lesions. The diagnosis based on clinical signs is only reliable in cases of acute severe disease, with high mortality and expectoration of blood [13]. Pathological diagnosis of ILT by the detection of intranuclear inclusion bodies is highly specific, but they are no longer visible, once tissue regeneration starts after approximately six days [14]. The virus isolation is more sensitive, but the samples should be collected as soon as possible after the establishment of clinical signs because isolation attempts are successful 6-7 days after infection [15]. Comparatively, molecular methods for the detection of viral DNA by PCR are more sensitive than virus isolation or electron microscopy as it allows detection of virus in samples containing other viral agents [16].

Infectious laryngotracheitis was first described during 1925 in the USA and in India, it was first reported in Mathura, Uttar Pradesh and subsequently in other parts of India up to 1970 [17,18]. Namakkal District in Tamil Nadu, India was chosen for the present study because this area has made a remarkable progress in commercial layer farming with 50-million-layer stock with a production of 40 million eggs per day in about 550 poultry farms contributing 80% to the egg production in Tamil Nadu, India and accounting for 90% of the total egg exports from the country. There are no published data on ILT in the Namakkal district of Tamil Nadu until reported during 2012 [19]. Considering the above, the present paper describes the pathological features and molecular diagnosis of ILT in commercial layer chicken. In addition, the role of trigeminal ganglia was also evaluated.

Materials and Methods

In the present study, the samples were collected from various farms located in Namakkal region of Tamil Nadu and also from poultry carcasses referred to the Department of Veterinary Pathology, RIVER, Puducherry, India. Samples were collected from those birds which had lesions suggestive of ILT. A total of 105 samples (100 from Namakkal and 5 from Puducherry) were collected. A total of 62 commercial layer farmers were investigated. The details of the farms such as total flock size (Chick, Grower and Layer), system of rearing, affected age group, mortality, morbidity, production loss and concurrent infection were collected using a questionnaire. Wherever possible, clinical signs in affected birds were recorded. In dead birds, a detailed gross examination was carried out and samples were collected from carcasses which had lesions suggestive of ILT.

In such cases, representative tissue samples from trachea, conjunctiva, respiratory sinus and trigeminal ganglia were collected and fixed in 10% neutral buffered formalin (NBF) for routine histopathological examination. Representative tracheal samples were also collected and stored at -20°C for PCR and virus isolation. In addition, trigeminal ganglia from infected, recovered and uninfected flocks (4 samples from each) were also collected and stored at -20°C.The tissue samples fixed in 10% NBF were processed by routine paraffin-embedding technique and 4-5μm thick sections were prepared and stained by routine Hematoxylin and Eosin (H&E) stains for detailed histopathological examinations. In selected cases, histochemical and special staining techniques were carried out for PAS positive substance (Periodic Acid Schiff) and inclusion bodies (Lendrum staining and Page-Green method) respectively [20].

Tracheal samples were subjected to virus isolation using 9-11 day-old embryonated chicken eggs [21]. Thirty milligram of trachea was taken in a sterile mortar and pestle along with sterile sand and phosphate buffer saline and triturated well (10% w/v suspension). After trituration the suspension was transferred in to the sterile centrifuge tube and centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant of each sample was filtered through 0.45μm membranes (Millipore, India). The filtrate was collected into another sterile 2ml Eppendorf tube and stored at -20°C until further use.

The processed samples were inoculated into chorioallantois membrane (CAM) of 9-day old embryonated chicken eggs. The eggs were incubated at 37°C for 72 hrs. along with control. After incubation the CAM were harvested and examined. Subsequently suspension prepared from harvested CAM was filtered through 0.45 μm membranes and utilized for three blind passages in chicken embryonated eggs. After third passage, DNA was extracted from triturate of CAM by boiling lysis method and subjected to PCR.

Polymerase chain reaction: Samples from trachea, CAM and the trigeminal ganglion were subjected to PCR. DNA was extracted using Phenol-chloroform method [22] and the quality of DNA was checked by using Agarose gel electrophoresis [23]. PCR was conducted using the following set of primers [24] (Table 1).

Table 1.

Region of Amplification	Primer Sequence (5′◻3′)	Expected Size
ICP4 gene	Forward: CCTTGGTTCGGGATGAAACC	237bp
	Reverse: TTCATTACCTCCAGGCGTTCC	

The reaction was carried out in an Eppendorf thermocycler, with a primary denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 40 seconds, extension at 72°C for 40 seconds and a final extension at 72°C for 10 minutes. The amplified products were checked by agarose mixed with ethidium bromide (1.5%) gel electrophoresis at 100 volts for 45 minutes. The DNA bands (237 bp) were visualized under UV trans-illumination and documented using Bio-Rad gel documentation system.

Results and Discussion

Out of 62 commercial layer farms investigated in the present study, birds from 40 farms were reported to have signs of ILT. The study covered different regions of Namakkal. The flock size of the poultry farms ranged from 18,000 to 250,000 birds. The flocks consisted of chicks, growers and multi-aged laying hens reared in same place in majority of the farms and in few farms chicks and growers reared in one place and layers were reared in another place. All the flocks were reared under cage system following the standard managemental conditions recommended by the breeding companies. In India, ILT was first reported in Mathura, Uttar Pradesh [17]. Subsequently it was reported in Andhra Pradesh and Madhya Pradesh [25, 26]. There are no published data on ILT until it was reported in the Namakkal district of Tamil Nadu during 2012 [18]. Subsequently many authors have reported the prevalence of ILT in Namakkal [2,27,28]. This clearly indicates the re-emergence of ILTV infection in India. Namakkal, the egg town of Tami Nadu with an area of 3368.21 square kilometers concentrating 550 poultry farms and housing about 50 million layers, undoubtedly satisfied the necessary criteria for the prevalence of ILT.

The affected chicks showed varied clinical signs including dullness, reduced feed and water intake (Figure 1). Depending on the severity of the disease, the degree of respiratory signs ranged from mild respiratory distress to difficulty in breathing, gasping, coughing up blood or blood-tinged mucus and pump handle-type of respiration (Figure 2) and sharp laryngeal sounds. Other signs observed were conjunctivitis, oculo-nasal discharge, swelling of the infraorbital sinuses and closed eyes (Figure 3). Laying birds showed depression, anorexia and the egg production dropped up to 12 %.

Figure 1: Chick showing dullness and closed eyes.

Figure 2: Grower showing the characteristic pump handle-type of respiration.

Figure 3: Swelling of the infraorbital sinuses and closed eyes.

Gross lesions were recorded mainly in the upper respiratory tract, particularly in trachea. In mild cases, the laryngeal and tracheal mucosa showed discrete congestion and generalized catarrhal changes. The lumen contained whitish or yellowish exudate partially occluding the lumen. In severe form, the mucosa revealed hemorrhagic lesions (Figure 4) and necrotic tissue were observed sticking on the laryngeal and tracheal mucosa (Figure 5). Occlusion of tracheal and syrinx lumen by whitish or yellowish or reddish plaques were also observed in severely affected cases (Figure 6).

The clinical signs and the pathological changes observed in the present study concurred with the observations of earlier workers [2,27-30]. However, the pathological changes were predominant in the trachea indicating that ILTV is highly cytolytic for the trachea [31-33]. This resulted in the accumulation of either mucoid or hemorrhagic and/or fibrine necrotic exudate causing partial or complete occlusion of the lumen. The accumulation of mucus and blood varies during the course of the infection and also depend on the strain of the virus [34]. Small amounts of mucus with slight hemorrhages are seen in the early phase of infection

and progress to yellow-cheese like mucus plugs in the late stages. The mucus plugs may block the airway and can lead to death by asphyxiation.

Histopathologically, the inflammatory changes noticed in the trachea were catarrhal, fibrinous/diphtheritic and hemorrhagic in nature. Cellular reaction was generally moderate, consisting of heterophils, lymphocytes, macrophages and a few plasma cells. In some cases, trachea revealed diffuse inflammation leading to accumulation of fibrino-haemorrhagic and necrotic debris which resulting in obliteration of tracheal lumen (Figure 7). Tracheal lumen was also filled with casts of sloughed off epithelial cells. In moderate number of cases, the desquamated or the hyperplastic epithelial cells resulted in the formation of syncytia with the

presence of eosinophilic intranuclear inclusion bodies. The inclusion bodies were best visualized using Lendrum staining technique (Figure 8). Histopathological examination of the trachea for typical intranuclear inclusions is considered characteristic for ILT. However, in the present study, inclusion bodies were either absent or difficulties were experienced to appreciate the inclusion bodies. Many samples, which yielded positivity in PCR technique, did not reveal inclusion bodies under routine H&E technique, but the same samples showed inclusion bodies when subjected for Phloxine-Tartrazine staining technique. From this, it is inferable that Phloxine-Tartrazine staining technique could yield better demonstration of inclusion bodies of ILT when compared to H&E technique. These features are similar to those described by earlier workers [28].

Figure 4: Trachea showing congestion and haemorrhages.

Figure 5: Yellowish exudate completely occluding the laryngeal opening.

Although, histopathology is considered a valid and relatively rapid test for ILT [35], the probability of finding typical lesions is decreased dramatically when histopathology is performed after eight to ten days (subacute to chronic stage) of infection, due to desquamation of the epithelial cells [13,36]. The other changes observed were cystic dilatation of the submucosal glands and

focal or multifocal areas of lymphoid aggregates were seen in few cases. The conjunctiva and respiratory sinus did not present any ILT characteristic lesions, except for congestion and infiltration of inflammatory cells. Infiltration of few mononuclear cells was the only lesion seen in the trigeminal ganglion.
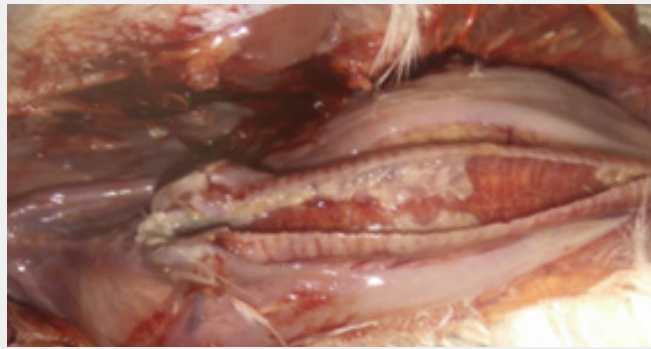
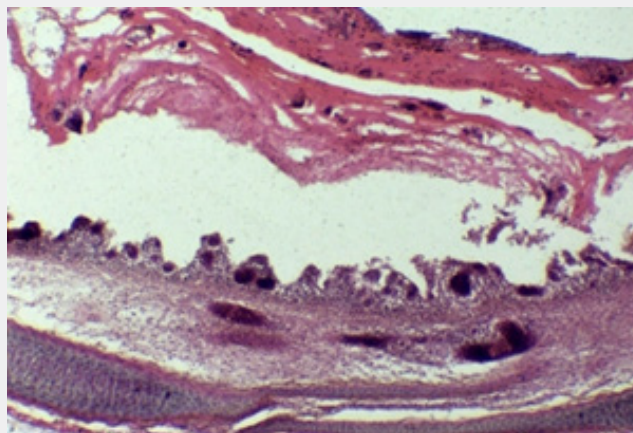**Figure 6:** Diphtheritic membrane occupying entire length of the tracheal lumen.

Figure 7: Trachea- Accumulation of fibrino-haemorrhagic and necrotic debri. H&E x100.
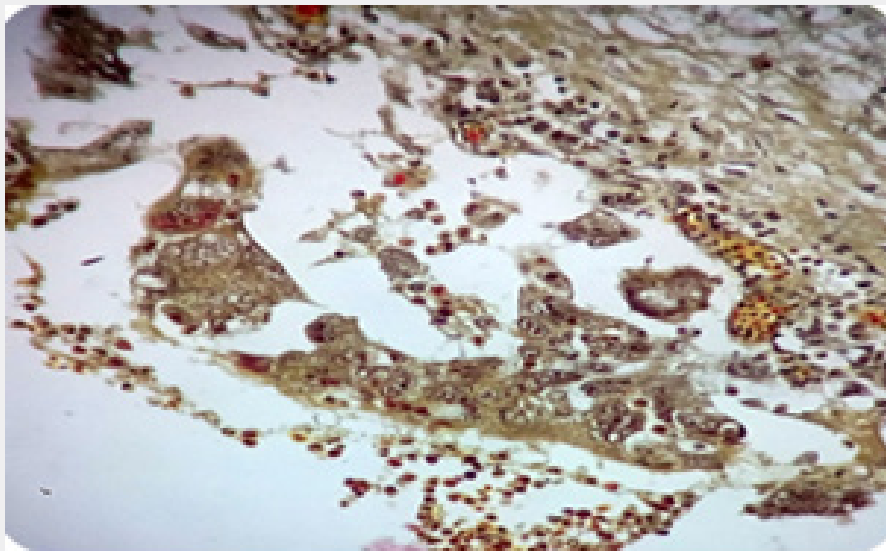

Figure 8: Trachea- Syncytia with intranuclear inclusion bodies. Lendrum staining technique x400.

The CAM harvested from the inoculated embryonated eggs revealed cloudy and thickened membrane with numerous pin point white pocks after three passages (Figure 9). In addition, the inoculated embryos revealed stunted growth and congestion (Figure 10). In the embryonated eggs, the characteristic lesions observed on the CAM in the present study corroborated with the observations of earlier workers [37,38]. From the present study and from the works of earlier author's, it is understood that the time taken/number of passages required for the development of characteristic pock lesion on CAM is highly variable. This could be attributed to the differences in the concentration of virus or the samples might have had inactivated viral particles and consequently replication in embryonated eggs did not occur. Other possibilities are low virus concentration in original sample and the need of additional passages [39]. The time of pock development can also be related to the virus ability to adapt to embryonated eggs. The harvested CAM was subjected to PCR that yielded a specific PCR product of 237 bp confirming isolation of ILTV [40].

Figure 9: Numerous pinpoint white pocks on the CAM.

Figure 10: The inoculated embryos showed stunted growth (left) and congestion when compared with the control eggs (right).

DNA from CAM (4), trachea (30) and trigeminal ganglia (12) were extracted by phenol-chloroform method. All the samples were subjected for PCR. Then the PCR products were subjected to agarose (1.5%) gel electrophoresis at 100 volts for 45 minutes along with 100bp DNA ladder. Two samples from CAM and 27 samples from trachea gave an expected product size of 237 bp (Figure 11). Out of 4 samples of trigeminal ganglia from each group, all the 4 were found positive in the infected birds, 1 in the uninfected birds and 3 in the recovered birds (Figure 12&13). In the present study, 90% tracheal samples were found to be positive for ILTV amplification of 237bp target of ICP4 gene. The ICP4 gene plays a role in the regulation of early genomic expression in infection, and it is used to differentiate between vaccine strains and field and wild strains in epidemiological studies [41,42]. The correlation between clinical signs, histopathology features and PCR results demonstrate the importance of the molecular analysis as more rapid tool for ILT diagnosis [2, 29].

Indeed, the PCR technique is also more sensitive than virus isolation and histopathology analyses [15], especially when other contaminant viruses or bacteria are present in tested samples. Another study revealed that nucleotide sequencing of TK gene revealed re-emergence of ILT due to vaccinal and virulent strains [43]. Sequencing of a portion of the ICP4 region of ILTV allowed differentiation of ILTV field, CEO, and TCO vaccine strains [44]. They concluded that the outbreaks caused in Tamil Nadu from 2019-2020 were due to CEO- vaccine like strains.
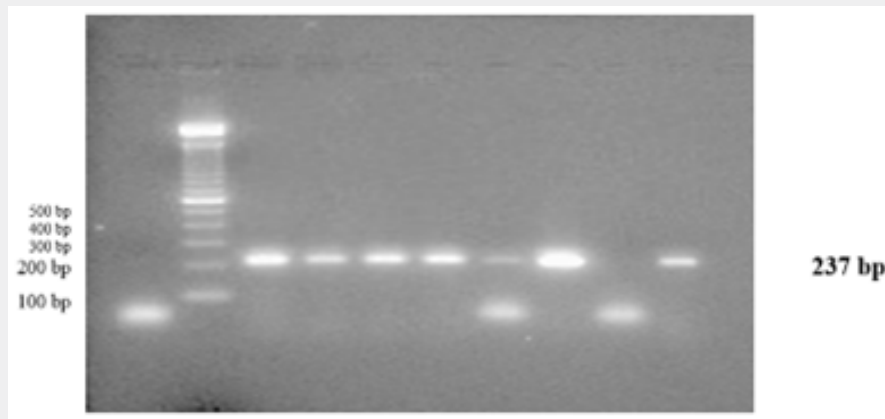
Figure 11: Agarose (1.5%) Gel Electrophoresis of PCR product (237 bp) from trachea. Lane 1: Negative control, Lane 2: 100 bp DNA ladder, Lane 3, 4, 5, 6, 7, 8, 10: Samples positive, Lane 9: Samples negative.
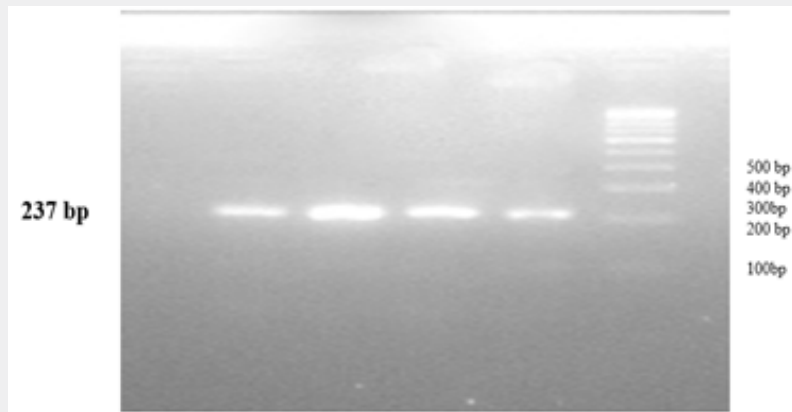

Figure 12: Agarose (1.5%) Gel Electrophoresis of PCR product (237 bp) from Trigeminal ganglia (Infected Flock) Lane 1: Negative control, Lane 2 to 5: Samples from Infected flock, Lane 6: 100 bp DNA ladder.
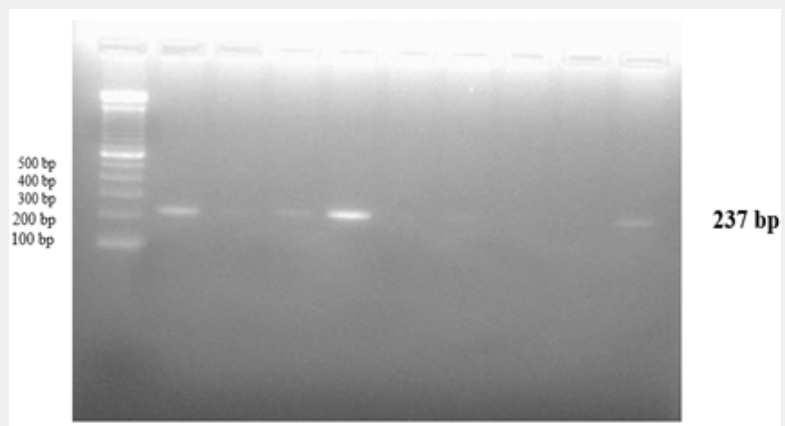

Figure 13: Agarose (1.5%) gel electrophoresis of PCR product (237 bp) from Trigeminal ganglia of recovered and un-infected flock, Lane 1: 100 bp DNA ladder, Lane 2 to 5: Samples from recovered, Lane 6: Negative control, Lane 7 to 10: Samples from Un-infected.

Invasion of the trigeminal ganglion by ILTV has been found to occur regularly from days three to six, during the acute phase of ILT infections by field and vaccine strains [31]. In the present study, out of 4 samples from each group (infected birds-4, unaffected birds-4 and recovered birds-4), all the 4 were found positive in the infected birds, 1 in the uninfected group and 3 in the recovered birds after 3 months. The positive samples gave an expected product size of 237 bp. Inapparent infection of the respiratory tract is a trait of ILTV persistence. Earlier observations by GIBBS, 1933 demonstrated a "field" carrier rate of approximately 2% for periods up to 16 months after a disease outbreak. Later, latent tracheal infections were demonstrated for similar periods of time in 50% or more of infected chickens [31,45]. Latent ILTV was detected by PCR in trigeminal ganglia of birds that recovered from the disease 61 days after infection, but it was not possible to isolate the virus [15].

During the study period, a single flock of 100 Giriraj birds at Puducherry, India that had gross lesions suggestive of ILT were however found to be negative for ILTV by molecular technique. Further survey studies in this region during reported outbreaks are required to confirm the occurrence disease [46].

Conclusion

The results of this study demonstrate that ILTV is circulating in laying flocks reared in Namakkal, Tamil Nadu. PCR can be a better diagnostic test especially in cases in which inclusion bodies could not be demonstrated. Trigeminal ganglia can be used as a potential tool for forecasting the prevalence of ILTV in endemic areas. Above all, it is important to note that management practices, vaccines (recombinants), and strict biosecurity measures can be a better tool for the control and prevention of ILT.

Acknowledgement

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