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Histopathological and molecular confirmation of infectious laryngotracheitis virus in desi chicken

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Abstract
Tracheal samples were collected from desi chickens suspected of infectious laryngotracheitis virus (ILTV) infection. The tracheal samples were triturated and inoculated into embryonated chicken eggs through the chorioallantoic membrane route for isolation of ILTV. After five days of inoculation, chorioallantoic membranes were harvested for histopathological examination. Histopathology of pock lesions on chorioallantoic membrane revealed the presence of syncytia, intranuclear inclusion bodies and infiltration of heterophils and lymphocytes. DNA was extracted from the infected chorioallantoic membrane. Further confirmation, PCR was carried out using the TKOP gene and found positive for infectious laryngotracheitis virus by PCR. This study confirmed the presence of ILT infection in chickens by histopathology and molecular method.

Keywords: infectious laryngotracheitis virus, desi chicken, histopathology, PCR and TKOP gene

1. Introduction
Infectious laryngotracheitis disease in poultry was first reported by May and Tittsler in 1925. Though there were no reports of infectious laryngotracheitis disease before the 1920s, it may have existed in chickens much earlier (May and Tittsler, 1925) [8]. Gasping with the expectoration of mucus and blood was a common symptom. The infectious laryngotracheitis disease is commonly noticed in chickens and causes significant losses to the poultry farming community due to high mortality and lowered production performances viz. drop in egg production and reduced body weight gain (García *et al*., 2013) [4]. The virus causing ILT has been classified by the International Committee on Taxonomy of Viruses (ICTV) under the family *Herpesviridae*. The species *Gallid herpesvirus* 1 has been reported to cause ILT belongs to the *Iltovirus* genus (Davison *et al*., 2009) [3].
The ILT virus is usually transmitted by inhalation, direct contact, and indirectly by fomites and people (Menendez *et al.*, 2014) [9]. The virus is also transmitted by oral routes, after that the virus must expose to nasal epithelium to produce infection (Garcia *et al.*, 2013) [4]. Diagnosis of ILT infection is carried out by conventional methods like virus isolation in embryonated chicken eggs and cell cultures. The ILT infection is also identified by histopathological examination of intranuclear inclusion bodies in infected tissues. The conventional methods are replaced by molecular methods for rapid and accurate detection of ILT infection. The present study was aimed to confirm the presence of ILTV in desi chicken by histopathological examination as well as the molecular method by PCR using amplification of the TKOP gene.

2. Materials and Methods
Around 50 village chicken aged 20 weeks were maintained as a backyard farming system in Siruvachur village, Salem district, Tamil Nadu. Mortality was observed in the native chicken unit in July'2021. Hemorrhagic tracheitis and blood mixed with mucus exudate in the tracheal lumen were noticed during necropsy conducted at avian disease laboratory, Thalaivasal. The tracheal samples were collected aseptically for detection of infectious laryngotracheitis virus. The collected tracheal samples were triturated with sterile PBS for preparation of 10 per cent virus suspension. The virus suspension (0.2 ml) was inoculated into 9-11 days old embryonated chicken eggs through dropped chorioallantoic membrane route and incubated at 37 ^{0}C for 5 days. The eggs dead within 24 hours were discarded. After 5 days of incubation, the chorioallantoic membrane was collected for further analysis.

2.1 Histopathology
The infected chorioallantoic membrane was fixed in 10% formalin, processed and embedded in paraffin wax. Hematoxylin and eosin stains were used for staining the tissue sections as previously described. Further, the histopathological lesions were analyzed using a research microscope at various magnification.

2.2 PCR targeting TKOP gene
The DNA was extracted from infected CAM using a QIAamp DNA mini kit (Qiagen, Germany) following the manufacturer's instructions. PCR was carried out using forward primer 5′-CGGGATCCATCGTATAGGCCAGCCTT-3′ and reverse primer 5′- GCTCTAGACCACGCTCTCTCGAGTAA-3′ as previously reported by Han and Kim, (2001) [5]. The primers were specifically amplifying the 1.3 kbp length of the TKOP gene of ILTV. PCR was carried out in a thermocycler (Bio-Rad) in a total reaction volume of 20 µl containing 10 µl of master mix (2x), 1 µM of forward and reverse primers each and 3 µl of extracted DNA. The thermal cycling conditions for the TKOP gene were as follows, initial denaturation of 95 ^{0}C for 3 min, then 30 cycles consisting of 1 min denaturation at 94 ^{0}C, 1.5 min annealing at 59 ^{0}C and 1.5 min extension at 72 ^{0}C, followed by a final extension step of 72 ^{0}C for 10 min (Kim *et al*., 2013) [7].

3. Results and Discussion
Infectious laryngotracheitis disease is an upper respiratory tract infection in chickens and affects the poultry farming community worldwide. The affected birds show dullness, depression, conjunctivitis and sinusitis in milder form whereas respiratory distress and mucous mixed with blood are noticed in the severe form of ILT infection. Mortality in case of the peracute form of ILT infection may exceed 50 per cent and in case of acute infection may vary from 10-30 per cent (OIE, 2014) [10].

In this study affected birds showed sudden death with exhibiting clinical signs like matting of eyelids, lacrimation and conjunctivitis during 20 weeks of age. On postmortem examination, hemorrhagic tracheitis and blood mixed with mucous were observed in the tracheal lumen. The post mortem lesions on the trachea were following earlier reports of ILTV in poultry. ILT virus was cultivated in embryonated chicken eggs through chorioallantoic membrane route produced enlargement and pock lesions. The lesions on the chorioallantoic membrane after inoculation of ILTV were similar to that of previous reports.

On histopathological examination of pock lesion on chorioallantoic membrane tissue section revealed hemorrhage, congestion and epithelial hyperplasia with lymphocytic and heterophilic infiltration. Edematous swelling with congestion and infiltration of heterophils were also noticed on histopathological examination. The syncytial cells with intranuclear inclusions were also observed in the infected chorioallantoic membrane (Figures 1-7). Our findings were in correspondence with histopathology examination of the infected chorioallantoic membrane after inoculation of ILTV in embryonated chicken eggs. (Kammon *et al*., 2020) [6].

Fig 1: Ailing birds showed matting of eyelids and lacrimation

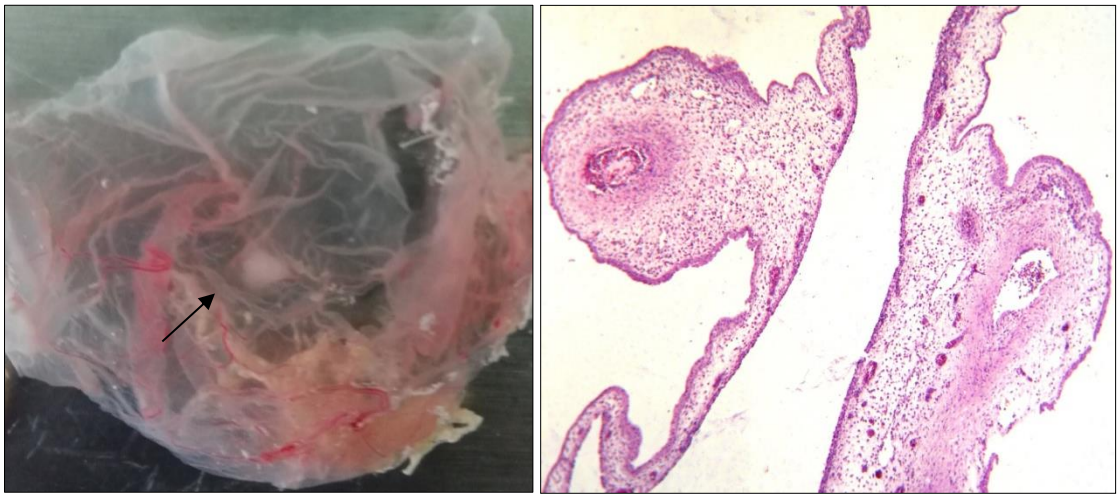

Fig 2: Pock lesion on the chorioallantoic membrane and its microscopic view (H&E 100x)

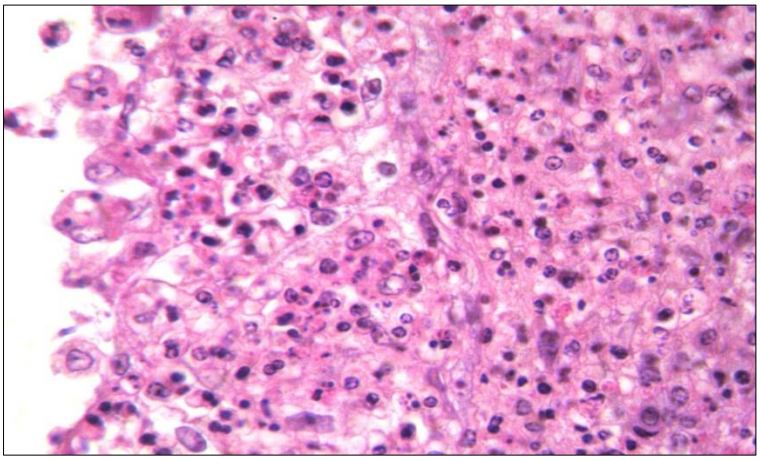

Fig 3: Epithelial hyperplasia with lymphocytic and heterophilic infiltration (H&E 400x)

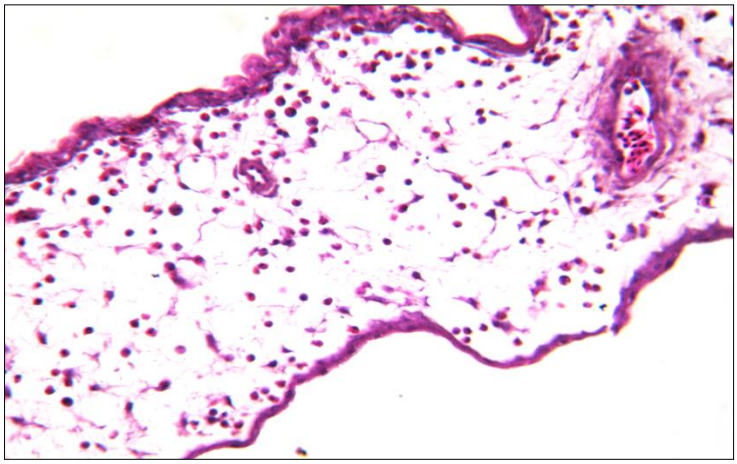

Fig 4: Edema with congestion and heterophilic infiltration (H&E 400x)

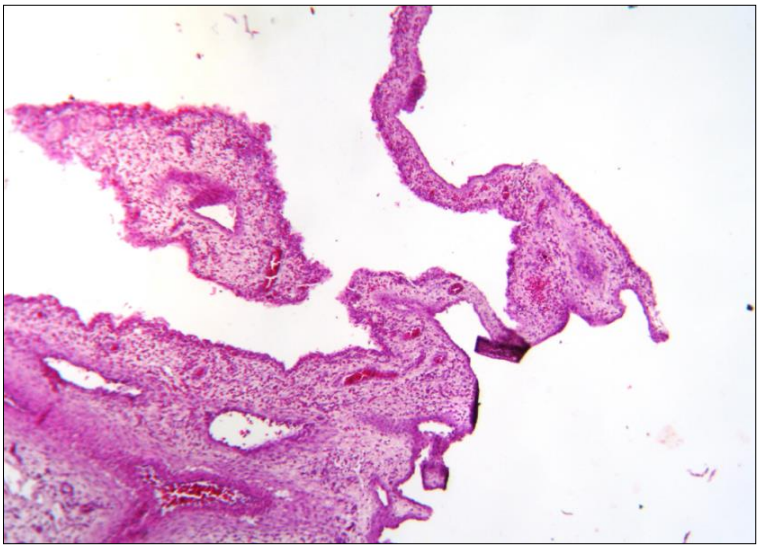

Fig 5: Congestion and hemorrhage of infected CAM (H&E 100x)

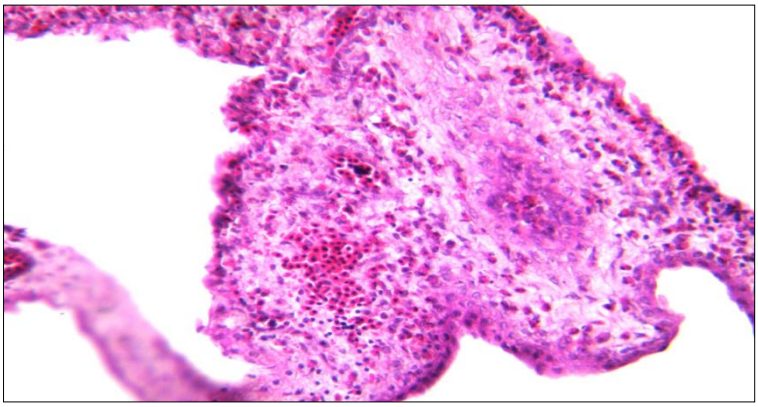

Fig 6: Congestion and hemorrhage of infected CAM (H&E 400x)

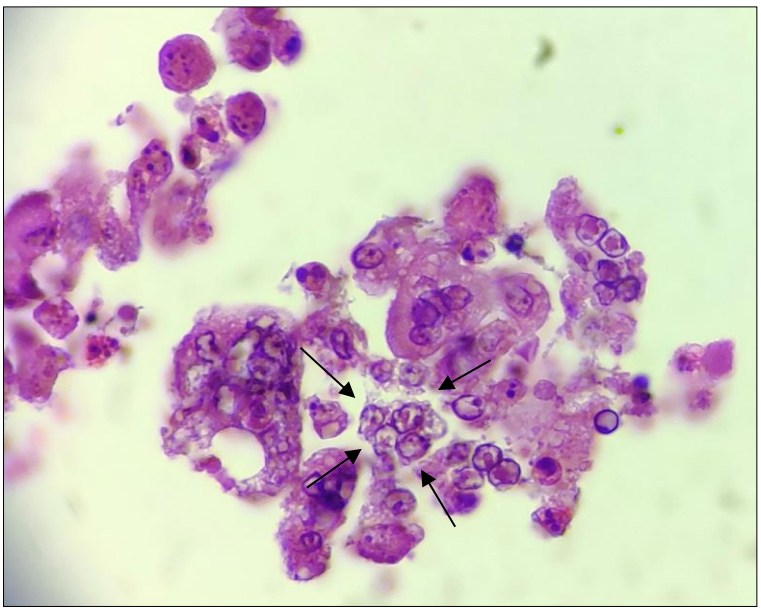

Fig 7: Syncytia showing intranuclear inclusions in the infected CAM (H&E 1000x)

For further confirmation, DNA was extracted from the infected CAM and was subjected to PCR with specific primer sets targeting the TKOP gene of ILTV and yielded approximately 1.3 kbp length of PCR product from the infected CAM (Figure 8).

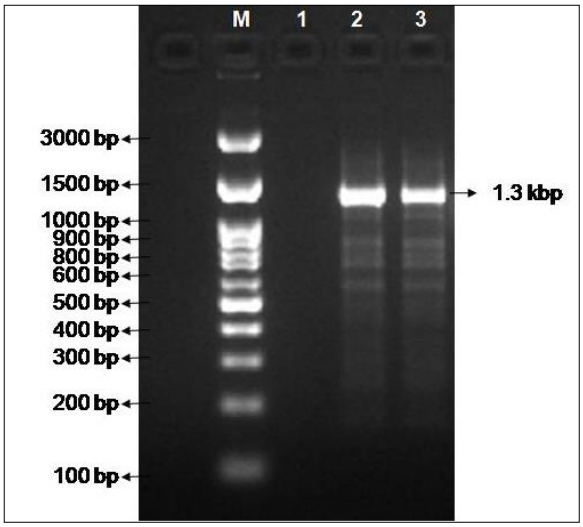

Fig 8: PCR for TKOP gene of ILTV. Lane M: DNA Marker (100-3000bp) Lane 1: No template control, Lane 2-3: Samples

The results obtained in this study were in agreement with previous reports for the detection of ILTV using the TKOP gene (Davidson *et al*., 2009) [2]. Molecular detection of ILTV outbreak in the field was routinely diagnosed by a polymerase chain reaction and it is considered to be more sensitive, rapid and accurate compared to that conventional method of virus isolation. Alexander and Nagy (1997) [1] standardized the PCR as a diagnostic tool for detection of ILTV in conjunctiva from experimentally infected chickens.

The native chickens reared under the backyard system are tolerant to diseases than commercial chickens. Poor management and biosecurity result in the occurrence of diseases like Newcastle disease, Avian influenza, Fowlpox and Marek's disease in native chickens. The occurrence of ILTV infection in Aseel chickens has been reported by Vijayalingam *et al*. 2019 [12]. The detection of circulating antibodies against ILTV infection in backyard chicken was reported in central and south Ethiopia. The seropositivity of ILT infection was higher in backyard poultry (34.4%) than commercial poultry (13.3%) production (Tesfaye *et al*., 2019) [11].

It is concluded that the present study confirms the presence of ILTV infection in desi chicken by histopathological and molecular methods. Further investigation of more samples, as well as molecular characterization, would throw better light on the prevention, control as well as source of ILT infection in desi chicken.

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