

Tissue Tropism and Pathobiology of Infectious Laryngotracheitis Virus in Natural Cases of Chickens

Sivaseelan, S.,^{1*} Rajan, T.,¹ Malmarugan, S.,² Balasubramaniam, G.A.¹ and Madheswaran, R.¹

¹ Department of Veterinary Pathology, Veterinary College and Research Institute, Namakkal – 637002, Tamil Nadu, India.

² Department of Veterinary Microbiology, Veterinary College and Research Institute, Tirunelveli – 627001, Tamil Nadu, India.

* **Corresponding Author:** Dr. S. Sivaseelan, M.V.Sc., Ph.D Associate Professor, Department of Veterinary Pathology, Veterinary College and Research Institute, Namakkal – 637002, Tamil Nadu, India. Tel: +91 9442937227, Email: pathologysiva@yahoo.co.in

ABSTRACT

Tissue tropism of Infectious Laryngotracheitis (ILT) virus in naturally occurring cases of field outbreaks of ILT in chicken was evaluated. Symptoms manifested by affected birds and the gross lesions from postmortem examination were recorded. The virus tissue tropism and histopathological changes was evaluated in tissue samples collected from respiratory sinuses, larynx, trachea (anterior one third, middle, and posterior one third), bronchi, lungs, air sacs, liver, spleen, intestine, and conjunctiva. About 60 sets of these samples were subjected to Polymerase Chain Reaction (PCR) to confirm the ILT virus. The severity of microscopic tissue damage caused by ILT virus in different tissue was assessed by hematoxylin-eosin staining technique. It was concluded that ILT virus possesses greater affinity towards the middle portion of the trachea and conjunctiva, and lesser affinity towards larynx and other portions of the trachea. Viral affinity was not detected in other tissues tested.

Keywords: Infectious Laryngotracheitis; Tissue Tropism; Pathobiology; Chicken.

INTRODUCTION

Infectious Laryngotracheitis (ILT) caused by Gallid herpesvirus-1 causes a severe respiratory tract infection of chicken, which produces considerable economic losses due to mortality of infected chicks, pullets and adult birds and/or decreased weight gain and egg production. The virus spreads horizontally by aerosol and the disease is rapidly propagated among the commercial layers being reared in intensive farms of closer vicinity. Mild forms of infection, sometimes enzootic are characterized by mucoïd tracheitis, sinusitis, unthriftiness and low mortality. However, serious disease outbreaks continue to occur periodically whenever ILT virus strains can move from persistently infected flocks to non-vaccinated birds. The incidence of ILT is on the rise in many parts of the world, which may be due to the usage of modified live vaccines that could establish latent infections similar to wild ILT virus strains (1).

Infectious laryngotracheitis virus can establish a carrier state in recovered birds. In chickens, this is characterized by periods of latency interspersed with episodes of intermittent virus shedding detectable by tracheal swabbing of live recovered birds. Virus shedding or re-excretion may occur spontaneously, but certain factors such as stress, rehousing and onset of laying have also been shown to induce virus shedding from latent carrier birds (2).

Infection of susceptible chickens results in virus replication in the epithelium of larynx and trachea, and potentially other mucus membranes such as conjunctiva, respiratory sinuses, air sacs and lungs (3).

Viral DNA replication occurs by a rolling circle mechanism with the formation of concatemers which are cleaved into monomeric units and packaged into preformed nucleocapsids within the nucleus (1). In the nucleus, capsids are formed and filled with viral DNA. Then, the nucleo-

capsids are transported into the cytoplasm by subsequent envelopment and de-envelopment at the inner and outer leaflets of the nuclear membrane. The cytoplasmic capsids associate with electron dense tegument and are then re-enveloped by a second budding event in the trans-Golgi region followed by release of mature particles by exocytosis (4).

Diagnosis of ILT can be performed by DNA detection and virus (5) isolation and identification of intranuclear inclusion bodies (6). This study has been conducted in chicken to evaluate the organ/tissue preference of ILT virus based on the pathobiology.

MATERIALS AND METHODS

Sample collection

A total of 60 poultry farms (population varied from 10,000 to 100,000 birds) with history and symptoms of ILT were investigated. Necropsy was carried out on recently dead chicken carcasses and ailing birds. Tissue samples including respiratory sinuses, larynx, trachea (anterior one third, middle, and posterior one third), bronchi, lungs, air sacs, liver, spleen, intestine, and conjunctiva were collected during necropsy.

Preparation of tissue homogenate

Individual tissue samples were finely minced and 20% suspension was prepared with phosphate buffer saline containing Penicillin (2000 IU / ml) and Streptomycin (2 mg / ml). The samples were kept at room temperature for 1 hour and then centrifuged at 2000 rpm for 15 minutes. The supernatant was collected, filtered through Millipore membrane filter, treated with antibiotics and stored at -10°C. This material was used for egg inoculation.

Egg inoculation technique

About 0.2 mL of supernatant fluid was inoculated onto the dropped chorioallantoic membrane (CAM) of 10 to 12 days old embryonated chicken eggs. The eggs were incubated at 37°C for 4-6 days. The CAMs were harvested and examined for the formation of pock lesions (7). The CAM with pock lesions were utilized for histopathological examination and the presence of ILTV was confirmed by PCR technique.

Polymerase Chain Reaction

DNA was extracted by a phenol:chloroform:isoamyl alcohol method (7) and PCR was conducted in a thermal cycler (Eppendorff) as per the procedure of Villarreal *et al.* (8). The following forward and reverse primers were used for the amplification of target sequence of p32 gene of ILTV that produced a 588-bp fragment.

Forward primer

5' - CTACGTGCTGGGCTCTAATCC - 3'

Reverse primer

5' - AAACCTCTCGGGTGGCTACTGC - 3'

The reaction was carried out in a Eppendorff thermocycler, with a initial denaturation at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1min, 55°C for 1.5 min, 72°C for 2 min, and a final extension at 72°C for 10min. Ten microliters of the PCR product were analyzed by electrophoresis in 2 per cent agarose gel, stained with 0.5 mg/mL ethidium bromide and observed under UV light for visualization of the 588-bp fragment.

Pathobiology – gross lesions and histopathology

Detailed necropsy was conducted on randomly selected birds and the gross lesions were recorded. A transverse section of tissue approximately 0.5 cm in thickness was taken from the selected organs. The CAMs with pock lesions were preserved as such. Tissue pieces were fixed in 10 percent buffered neutral formalin and trimmed to a thickness of about 3 mm. The tissues were dehydrated, cleared and embedded in paraffin in a routine manual processing. Tissues for histopathological examinations were cut at 3 to 5 µm thicknesses, mounted on glass slides and subjected to Haematoxylin-Eosin (H&E) staining technique, Phloxine-Tartrazine staining technique, and Gordon and Sweet's method for reticulin staining technique for (9).

Cytology

Scraping of an intact sheet of epithelium, including as little blood and exudate as possible, from affected trachea was taken with a round blade scalpel. The scraping was deposited near the mid-point of a clean, oil free slide. With a second clean slide, the tissue was pressed onto the first slide with a gentle rotary pressure until it was flattened out in a thin layer. The two slides were drawn apart parallel to one another. The smears were stained with haematoxylin and eosin.



Figure 1: ILT affected bird; pump hand like respiration with wide open beak.



Figure 2: Trachea from ILT affected chicken; haemorrhagic lesion.

RESULTS

Clinical signs and gross pathological findings

The chickens affected with ILT showed nasal discharge and moist rales followed by coughing, gasping, sneezing, depression, labored breathing, expectoration of blood-stained mucus and pump hand respiration (Figure 1). Respiratory sound was audible from few affected birds. Affected birds showed conjunctivitis, swelling of the infraorbital sinuses and closed eyes. In severe cases, eyelids were adhered with dry, crusty, ocular discharge which stuck to the eyelids and skin around the eye. Some birds coughed up bloody mucus, which was found on and around the beak and in the oral cavity. In severely affected flock, spots of dried bloody exudates were found on the sides and bottom of cages. In some farms, dried blood spots were noticed on the wall of feeder and at watering points. Laying birds showed depression, anorexia and decreased egg production. In broilers, young chicks stretched out their neck due to dyspnea. Mortality rate progressively increased daily and within 3 to 5 days about 30 to 40 per cent of the flock died.

At post mortem the laryngeal and tracheal mucosa showed discrete congestion and generalized catarrhal changes in the less severe form of the disease. The lumen contained whitish or yellowish exudate partially occluding the lumen. In severe form, the mucosa revealed hemorrhagic lesions varying from discrete to diffuse foci (Figure 2). Tiny blood clots were observed sticking on to the laryngeal and tracheal mucosa in some cases.

Occlusion of tracheal and laryngeal lumen by whitish or yellowish or reddish plaques were observed in severely affected cases. Pneumonic changes of congestion, consolidation and red hepatisation were noticed in both the lungs of the severely affected birds.

Histopathology and tissue tropism

In the ILT affected flocks, histopathological evaluation of respiratory organs was carried out sequentially from birds affected with manifestations of respiratory distress.

Mid-portions of trachea of chickens in the earlier stages of the disease showed congestion of mucosal and submucosal blood vessels, followed by discontinuation of the intact mucosa, deciliation and appearance of eosinophilic intranuclear inclusions in the surface epithelial cells. Formation of multinucleated giant cells (syncytia) with the presence of intranuclear inclusions was observed in the next stage. It was followed by appearance of heterophils in the mucosal areas of the trachea in addition to the presence of inclusions. Disassociation of tracheal epithelium and infiltration of lymphocytes were observed subsequently. The size of the inclusions was increased and almost all epithelial cells of trachea showed inclusion bodies under Phloxine-Tartrazine staining technique in the following stage (Figure 3). Disruption of blood vessel walls with escape of RBCs was noticed in severely affected birds.

Gordon and Sweet's method for reticulin staining technique revealed the rupture of reticulin fibres, disassociation of cells and disruption of blood vessels in the tracheal epithe-

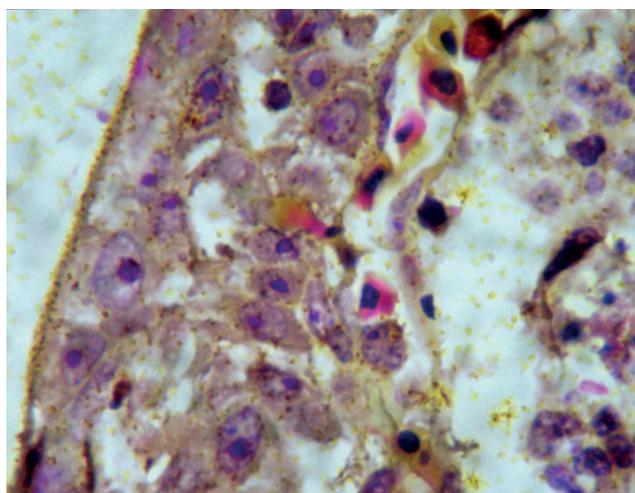


Figure 3: Trachea from ILT affected chicken; epithelial cells with eosinophilic intranuclear inclusion bodies. Phloxine-Tartrazine; 1500x.

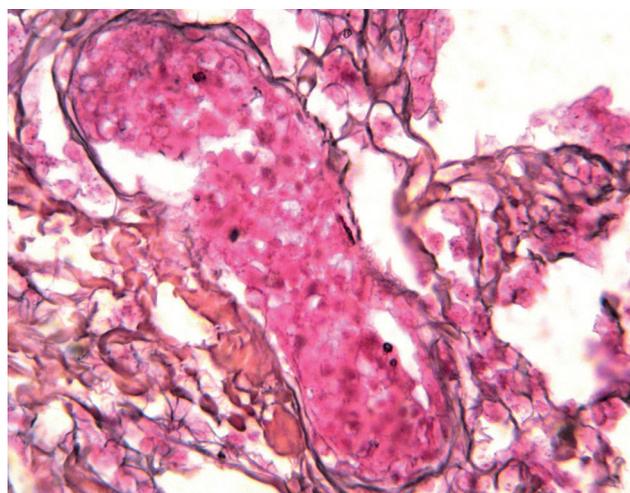


Figure 4: Trachea from ILT affected chicken; disruption of blood vessel by rupture of reticulin fibres. Silver nitrate-Gold chloride; 1000x.

lium leading to the haemorrhagic tracheitis (Figure 4). After this stage, the disrupted and necrosed epithelial cells started sloughing and some of these cells also contained the intranuclear inclusion bodies. Cytological examination of scraping from middle portion of trachea showed the intranuclear inclusion bodies. Middle portions of trachea showed inclusion bodies in 30 farms. In 12 farms (Farm No. 30 - 41), in addition to the middle portion of trachea even the larynx and anterior portion of the trachea also revealed inclusion bodies.

Conjunctiva showed severe congestion and infiltration of inflammatory cells in between and under the epithelium. Eosinophilic intranuclear inclusion bodies were observed in several epithelial cells under Phloxine -Tartrazine staining

technique in 30 farms in which the middle portion of the trachea also revealed inclusion bodies.

Microscopic observation of lesions from the larynx and other portions of trachea revealed degenerative changes of epithelium, vascular congestion and sporadic development of intranuclear inclusion bodies even in the severely affected birds.

Tissue samples of respiratory sinus, bronchi, air sacs, liver, spleen and intestine did not reveal either any characteristic microscopic lesions or inclusion bodies.

In severely affected cases, the lung showed hemorrhages and infiltration of heterophils, mononuclear cells and plasma cells into the interalveolar spaces. Blood vessel congestion



Figure 5: Chorioallantoic membrane; chicken embryo, opaque plaques induced by ILTV.

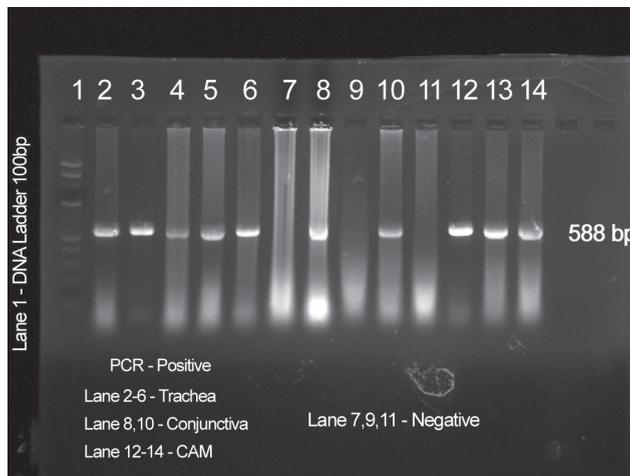


Figure 6: 588 bp PCR product on agarose gel electrophoresis.

Table 1: Farm wise PCR Result and Inclusion body observation of ILT in natural cases chicken

Organs tested	Farm Numbers															
	1-12	13	14-26	27-29	30-41	42-46	47-57	58-60	1-12	13	14-26	27-29	30-41	42-46	47-57	58-60
	PCR Result								Inclusion body							
Sinus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Larynx	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
Trachea Anterior	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-
Middle	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-
Posterior	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bronchi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lung	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Airsac	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Liver	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Spleen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Intestine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Conjunctiva	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-

- Negative; + Positive (Sample size: Five to ten sets of samples per farm)

was noted. Acute alveolar emphysema was observed in some birds. Mild edema was observed within the air spaces. Some birds also revealed hemorrhage in the intra- and inter- alveolar spaces and terminal bronchioles.

Diagnosis

The inoculation of different portions of trachea, larynx and conjunctiva from 42 farms out of 60 suspected for ILT caused the formation of opaque plaques on the CAM (Figure 5) resulting from necrosis and proliferative tissue reactions induced by the ILTV. Plaques were observed as early as 2 days post inoculation and embryo deaths occurred 2-8 days later. All the 42 isolates produced band at 588-bp by PCR (Figure 6). All these PCR results coincided with the inclusion body observation in different tissue samples.

Farm details, number of samples per farm, tissue samples details, PCR result and inclusion body observation are summarized in the table. Tissue samples of respiratory sinus, bronchi, lung, air sacs, liver, spleen and intestine from the same birds were negative by PCR.

DISCUSSION

In embryonated chicken eggs, ILT virus causes formation of opaque plaques on the CAM which serves as a preliminary screening for ILTV (7). Observation of opaque plaque on the CAM and formation of a band at 588 bp by PCR confirmed the ILTV in different portions of trachea, larynx and conjunctiva. Tissue samples of respiratory sinus, bronchi, lung,

air sacs, liver, spleen and intestine from the same birds proved negative by PCR indicating ILT virus does not replicate in these tissues.

Conjunctivitis, swelling of the infraorbital sinuses and closed eyes noticed in this study correlated well with the earlier report of Guy and Bagust (10). These lesions probably were induced by the proliferation of ILTV in conjunctiva, which has been supported by the detection of ILTV in conjunctiva by PCR technique and demonstration of inclusion bodies by histopathology. The gross and histopathological lesions observed in this study correlated well with the earlier reports of Hidalgo (1) and Tahseen (11).

Sloughing of tracheal epithelial cells and release of RBCs and inflammatory cells due to the rupture of blood vessels might be the reason for the formation of plaques noticed grossly. Plaques formed by the mixture of blood, desquamated epithelial cells, and exudate and fibrin from trachea have caused total occlusion of trachea, especially at larynx region causing suffocation and death of severely affected birds. Partial occlusion of airways was probably the reason for the emphysema in less severe cases. Pneumonic changes observed in the lung might be due to the extension of inflammation to the bronchi (11), which is also supported by negativity for ILTV by PCR. Eye and conjunctival lesions may have been caused by the proliferation of ILTV in these regions.

Guy *et al.* (12) reported that diagnosis of ILT could be based on the demonstration of the inclusion bodies in tissues but his experience infers that its sensitivity is poor and

also requires pathological expertise. In this study, detection of inclusion body of ILTV was considered as an evaluation tool to assess the tissue preference based on the level of inclusion bodies formed and the degree of damage induced by the virus. Though, PCR could only designate either positivity or negativity of the sample tested. Poor sensitivity to detect inclusion bodies by the routine H&E technique was improved by the utilization of Phloxine – Tartrazine technique, which yielded better appreciation of ILTV intranuclear inclusions.

Demonstration of inclusion bodies in the middle portion of the trachea and conjunctiva indicates the greater affinity of ILT virus towards these tissues. Sporadic development of intranuclear inclusion bodies in the larynx and other portions of the trachea even in the severely affected birds reveal lesser affinity to these tissues. Inclusion bodies could not be detected in tissue samples of respiratory sinuses, bronchi, air sacs, liver, spleen and intestine, indicating that ILT virus has no affinity to these tissues.

Based on the PCR results and histopathological evidence, it is concluded that ILT virus has greater affinity towards the middle portion of the trachea and conjunctiva, lesser affinity towards the larynx and other portions of the trachea and no affinity to all other tissues. These findings are supported by the recent report of Wang *et al.* (13).

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