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Immunohistopathological studies on infectious laryngotracheitis disease in poultry birds (Immunohistopathological studies on infectious laryngotracheitis disease)

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Abstract
The present study was conducted to diagnose infectious laryngotracheitis disease (ILT) using gross, histopathological, and immunohistochemical approaches. A total of 25 samples were collected from the four different poultry farms from Ludhiana and the nearby districts. Upon gross analysis of the necropsied birds, the relevant tissue samples such as trachea, larynx, lungs and air sacs were collected in 10% Neutral buffered formalin and were then processed for histopathological and immunohistochemical studies. Grossly haemorrhages in the trachea and larynx were observed. The accumulation of small amount of mucus in the lumen of nares, larynx and trachea was also observed. Pneumonic changes of congestion, consolidation and red hepatisation were noticed in both the lungs. Histopathologic picture revealed loss of cilia, presence of mucus, congestion, increase number of goblet cells and lymphocytic infiltration. Formation of multinucleated giant cells (syncytia) with the presence of intranuclear inclusions was also observed. In two representative cases (2/25, 8%) of one outbreak, ILT antigen were mainly detected in the nucleus of tracheal epithelial cells as well as in cilia of trachea. Further, reliability of various techniques employed was considered and positive correlation (r=0.958833) was emerged out with conventional pathological scoring. It was concluded that the trachea acts as an organ of choice for demonstrating ILTV antigen for specific diagnosis of disease using immunohistochemistry.

Keywords: Histopathology, immunohistochemistry, infectious laryngotracheitis disease, poultry

Introduction
Infectious laryngotracheitis (ILT) is an acute, highly contagious upper-respiratory infectious disease of chicken, responsible for serious production losses in the poultry industry [1]. It was first described in the USA in 1925 [2, 3] but some reports suggest that it may have existed earlier [4]. Chickens are the primary host [5] but it may affect pheasants. Starlings, sparrows, crows, pigeons and ducks seem to be resistant to the virus [1]. The causative agent is a pneumotropic virus of the family *Herpesviridae*, genus *Iltovirus*. Taxonomically, this virus is classified as a *Gallid herpes virus* 1 [6]. Chickens of all ages are susceptible to the infection by the laryngotracheitis virus, but birds older than three weeks are more sensitive [7]. The main transmission routes are ocular and respiratory. Because of the biological proprieties of Infectious Laryngotracheitis Virus (ILTV) and the environmental conditions, the virus persists in the affected flocks and regions in its endemic form, resulting in sporadic and mild outbreaks [1]. Clinical signs associated with the severe form of the disease include gasping, depression, nasal discharge, conjunctivitis, and expectoration of bloody mucus. Upon gross examination of the trachea, characteristic severe hemorrhages and mucus plugs are observed [8, 9]. The clinical signs associated with less severe forms of the disease include conjunctivitis, swelling of the infraorbital sinuses, closed eyes, persistent nasal discharge and mild tracheitis [10]. Gross lesions may be found in the conjunctiva and throughout the respiratory tract of ILTV infected chickens, but they are most consistently observed in larynx and trachea [11].
Disease can be diagnosed through clinical signs, gross lesions, histopathology, PCR, virus isolation [12], immunohistochemistry, fluorescent antibody technique [13], conventional enzyme linked immunosorbent assay (ELISA) [14] and agar gel immunodiffusion [15]. In our study we diagnosed ILT through gross examination, histopathology and immunohistochemisytry. Immunohistochemistry is more reliable diagnostic test than other conventional methods.

Materials and Methods

Material

Collection of samples

A total of four poultry farms were visited in and around the areas of Ludhiana and the other districts of Punjab, as shown in Table 1. Out of all birds collected, total 25 birds suspected for Infectious Laryngotracheitis disease were necropsied.

Processing of samples

Birds suspected for disease were necropsied and gross lesions were noted. The relevant representative tissue samples such as trachea, larynx, lungs and air sacs were subsequently collected in 10% neutral buffered formalin.

Methods

Histopathology

The tissues were processed and the 4 µ thick tissue sections were cut out of the paraffin embedded tissue blocks and stained with hematoxylin and eosin staining for routine histopathology.

Triple shorr staining

The slides were stained with Harris Hematoxylin Solution for 13-15 minutes after subsequent clearing and rehydration. The slides were then differentiated in acid alcohol and were given a dip in ammonia water solution. The slides were then stained with triple shorr stain for ten minutes. After rinsing in 95% alcohol, the slides were dehydrated in absolute alcohol and clear in xylene and mounted using DPX mount.

Immunohistochemistry

Immunohistochemistry (IHC) was performed as per previous studies [16, 17]. Briefly, tissue section(s) were taken on poly-L-Lysine coated slide and then subjected to clearing and then rehydration. The antigen retrieval was carried out in citrate buffer in microwave at 99 °C for 3 minutes and 70 °C for 7 minutes. These slides were then washed in phosphate buffer saline for 5 to 10 minutes. Serum blocking was done using normal goat serum and subsequently non-specific binding and endogenous peroxidase blocking was followed by overnight incubation at 4 °C with chicken polyclonal to infectious laryngotracheitis virus (1:500 dilution) (Abcam, United Kingdom). It was then followed by 30 min incubation with horseradish peroxidase-conjugated goat polyclonal secondary antibody to chicken IgY-Fc (Abcam, UK) at room temperature. Color was developed with substrate diaminobenzidine (DAB, Vector) and counterstained with Mayer's hematoxylin stain. For negative control addition of primary antibody was omitted. The immunohistopathological scoring was done using the scoring scale of 0-5, where, five sites in each tissue section were observed under the microscope and scored [18].

Statistical analysis

The correlation between the histopathological changes in the trachea from affected birds and the immunohistopathological scoring of the histopathologically positive samples were studied using the Graphpad Prism software.

Results and Discussion

A total of 25 samples from four different farms were collected and gross, histopathological and immunohistochemical observations were recorded.

Result

Gross: The majority of gross lesions observed in the birds necropsied for suspected Infectious Laryngotracheitis disease included haemorrhages in the trachea and larynx in all the outbreaks. In most of the cases lumen of the trachea was blocked by mucoid blood clots and sometimes with yellow caseous exudate/cheesy plug. The accumulation of small amount of mucus in the lumen of nares, larynx and trachea was also observed. In both the lungs of the severely affected birds, there were changes associated to pneumonia characterized by congestion, consolidation and red hepatisation.

Histopathology

Histopathological evaluation and scoring the lesions of respiratory organs was carried out sequentially from birds affected with manifestations of respiratory distress (Table 2). In one of the outbreak histopathologic picture revealed loss of cilia, presence of mucus, congestion, increase number of goblet cells and lymphocytic infiltration (Figure 1). Formation of multinucleated giant cells (syncytia) with the presence of intranuclear inclusions was also observed. In some of the cases marked necrosis, severe hemorrhage and desquamation of respiratory epithelium was also observed [10, 19, 20].

Intranuclear inclusion bodies were also observed in the trachea by Triple shorr staining as shown in (Figure 2). Similar inflammatory lesions, but in lesser intensity were observed in the mucosa of distal trachea and lungs. In the lungs, there was heterophilic and histiocytic inflammatory infiltrate in the parenchyma and also in the lamina propria of the bronchi [21]. In severely affected cases, the lung showed hemorrhages and infiltration of heterophils, mononuclear cells and plasma cells in the interalveolar spaces [19].

As per the analysis of the tracheal lesions in these four different outbreaks, the epithelial thickening was seen the highest in the third outbreak (2.2±0.44), followed by first (2.12±0.64) and fourth (1.8±0.44) outbreak, while it was least in the second outbreak (1.75±0.5). Similarly cellular infiltration in the third outbreak (2.6±0.54) was seen highest followed by fourth outbreak (1.8±0.44) while in first outbreak it was seen the least (1.75±0.46). In the similar way, the extent of hemorrhage in the trachea was also highest in the third outbreak (3±0.70), but here unlike cellular infiltration it was followed by first (2.5±0.53) and then fourth outbreak (2.2±0.83). It was again least seen in second outbreak (2±0)). The syncitial cell formation was again highest in the third outbreak (1.2±0.83) followed by first (0.375±0.51) and then fourth outbreak (0.2±0.44). On statistical analyses of epithelial thickening, extent of hemorrhage, cellular infiltration, and syncitial cell formation in the outbreak third, it is proposed that these four histopathological changes follow a parallel trend in the pathogenesis of the disease.

Immunohistopathology

In IHC, trachea and lung showed positive signal and scoring of immunohistopathological lesions are shown in table 3. In two representative cases (2/25, 8%) of one outbreak, ILT antigen were mainly detected in the nucleus of tracheal epithelial cells as well as in cilia of trachea (Figure 3). Further in one bird, epithelial cells lining the parabronchi showed moderate positive reaction indicating the presence of ILT antigen. The specific reaction was seen in both the nucleus and cytoplasm of epithelial cells. Intense staining of epithelial cells was observed with involvement of large, focal areas of

tracheal mucosa.

For a statistical comparison to be drawn, the mean and the standard deviation of the respective histopathological changes was calculated, as shown in the table, this mean represents the overall damage to the histo-architecture which was compared with the immunohistochemical scoring of the respective cases using the Graphpad Prism software. The correlation coefficient between the mean histopathological scoring and the immunohistochemical scoring was also found to be positive, 0.958833 for the respective cases, which proves the reliability on IHC for the positive cases. Further it was also represented graphically, which also follows similar trend.

Discussion

Sivaseelan *et al.* [19] have also reported similar changes grossly i.e., haemorrhages in the trachea and larynx along with pneumonic changes in both the lungs of affected birds. Pries *et al.* [20] reported in 2014, alike histopathologic picture revealing loss of cilia, presence of mucus, congestion, increase number of goblet cells and lymphocytic infiltration in the trachea of affected birds. They also reported syncytial cell formation and presence of intra nuclear inclusion bodies in some of the lungs which goes correct with the present study.

Diallo *et al.* [23] has also reported Gallid Herpes virus-1 lesions in the trachea characterized by multinucleated cells with intra nuclear inclusion bodies. Kaboudi *et al.* [22] has also reported the similar changes like epithelial necrosis, hemorrhagic exudates, syncytium cell formation, intranuclear inclusion bodies and heterophil infiltration in larynx and trachea.

Preis *et al.* [20] also confirmed the presence of antigen in the trachea through immunohistochemistry. But they also confirmed the presence of antigen in conjunctiva, turbinates, paranasal sinuses and lungs. Guy *et al.* [24] have also identified antigens of ILT virus in tracheal sections of infected chickens by immuno peroxidase staining. Timurkaan *et al.* [10] have also confirmed the presence of antigen in the tracheal epithelial cells and also in larynx which was in accordance with our study.

Tadese *et al.* [25] diagnosed ILT virus in infected tissue as well as individual cells using IHC and multiplex polymerase chain reaction (PCR) but he demonstrated ILTV using monoclonal antibodies through double IHC techniques.

Table 1: Various farms visited for the sample collection for the suspected ILT outbreaks

S. No.	District of Punjab	Number of samples collected	Total Birds	Age group of affected group (weeks)
Farm I Organized Poultry Farm	Ludhiana	8	25000	10
Farm II Ghola Poultry farm,Gahaur	Ludhiana	6	16000	8
Farm III Ghumman poultry farm, Batala	Gurdaspur	6	100	Adult
Farm IV Local Laboratory	Ludhiana	5	Not intimated	6
	Total	25		

Table 2: Scoring of the tracheal lesions in four outbreaks for inter-outbreak analysis of ILT

Case no.	Epithelium thickening	Presence of mucous glands	Thickening of mucosa	Cell infiltration	Congestion/ Hemorrhage	Syncytia cell formation
Outbreak 1						
J02A	2	1	0	1	2	1
J02B	2	2	1	2	3	0
J02C	2	1	0	2	3	0
J04A	1	1	0	2	2	0
J04B	3	2	2	2	3	1
J04C	2	2	1	2	2	0
J06A	2	2	1	1	2	0
J06B	3	2	2	2	3	1
Mean ± Std Deviation	2.12±0.64	1.625±0.51	0.875±0.83	1.75±0.46	2.5±0.53	0.375±0.51
Outbreak 2						
J39A	2	1	1	2	2	0
J39B	2	2	1	2	2	0
J40A	1	0	0	1	2	0
J40B	2	1	0	2	2	0
Mean ± Std Deviation	1.75±0.5	1±0.81	0.5±0.57	1.75±0.5	2±0	0±0
Outbreak 3						
J64A	3	2	2	2	3	2
J64B	2	1	2	2	2	0
J64C	2	2	2	3	3	1
J65A	2	1	1	3	3	1
J65B	2	1	2	3	4	2
Mean ± Std Deviation	2.2±0.44	1.4±0.54	1.8±0.44	2.6±0.54	3±0.70	1.2±0.83
Outbreak 4						
J70A	2	2	1	2	2	0
J70B	2	2	0	1	1	0
J75A	1	1	1	2	2	0
J75B	2	0	1	2	3	0
J75C	2	0	0	2	3	1
Mean ± Std Deviation	1.8±0.44	1±1	0.6±0.54	1.8±0.44	2.2±0.83	0.2±0.44

Table 3: Overall Histopathological scoring and the immunohistochemical scoring of ILT

Case no.	Epithelium thickening	Presence of Mucous glands	Thickening of mucosa	Cell infiltration	Congestion/ Hemorrhage	Syncytial cell formation	Mean	SD	IHC score
Outbreak 1									
J02A	2	1	0	1	2	1	1.16	0.75	0
J02B	2	2	1	2	3	0	1.66	1.03	0
J02C	2	1	0	2	3	0	1.33	1.21	0
J04A	1	1	0	2	2	0	1	0.89	0
J04B	3	2	2	2	3	1	2.16	0.75	0
J04C	2	2	1	2	2	0	1.5	0.83	0
J06A	2	2	1	1	2	0	1.33	0.81	0
J06B	3	2	2	2	3	1	2.16	0.75	0
Outbreak 2									
J39A	2	1	1	2	2	0	1.33	0.81	0
J39B	2	2	1	2	2	0	1.5	0.83	0
J40A	1	0	0	1	2	0	0.66	0.81	0
J40B	2	1	0	2	2	0	1.16	0.98	0
Outbreak 3									
J64A	3	2	2	2	3	2	2.33	0.51	1.9
J64B	2	1	2	2	2	0	1.5	0.83	0.98
J64C	2	2	2	3	3	1	2.16	0.75	1.6
J65A	2	1	1	3	3	1	1.83	0.98	1.2
J65B	2	1	2	3	4	2	2.33	1.03	2.2
Outbreak 4									
J70A	2	2	1	2	2	0	1.5	0.83	0
J70B	2	2	0	1	1	0	1	0.89	0
J75A	1	1	1	2	2	0	1.16	0.75	0
J75B	2	0	1	2	3	0	1.33	1.21	0
J75C	2	0	0	2	3	1	1.33	1.21	0

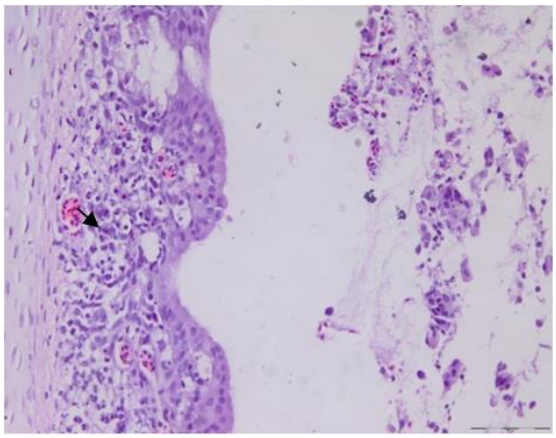


Fig 1: Trachea: Photomicrograph showing lymphocytic cellular infiltration in the tracheal mucosa. Presence of mucus exudate with syncytial cells (arrow) were observed in the lumen of the trachea. H&E, Bar= 100µm

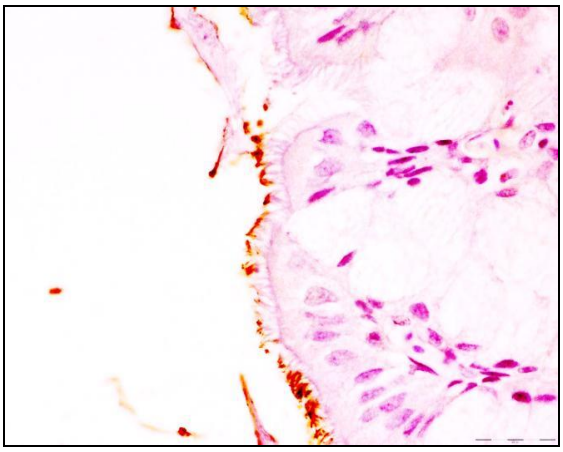


Fig 3: Trachea: Photomicrograph of immunohistochemistry for ILT antigen in nucleus of tracheal epithelial cells. Highly brown stained ILT antigens were observed in the cilia of the tracheal epithelial cells. IHC, Bar=20µm

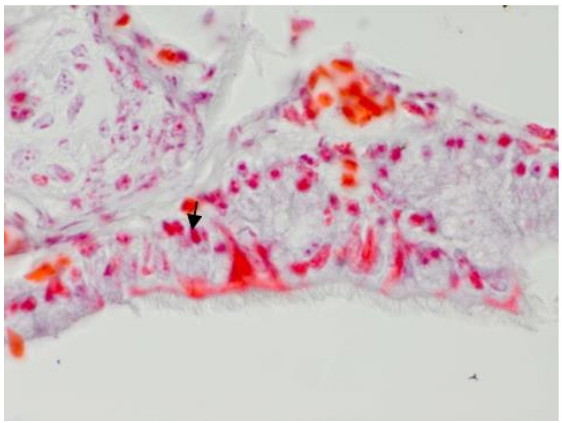
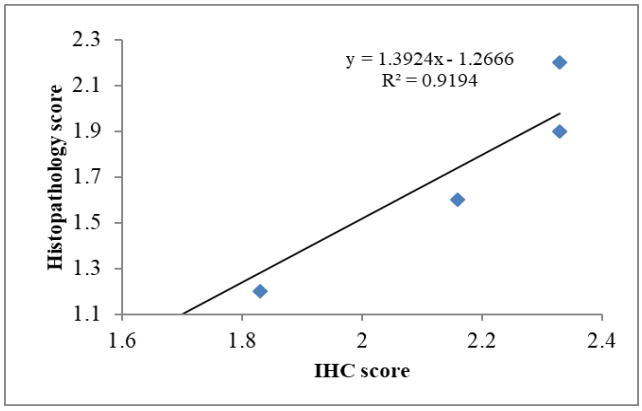


Fig 2: Trachea: Photomicrograph showing intranuclear inclusion bodies (arrow) observed in the tracheal epithelial cells, Triple shorr. X100



Graph: Correlation between Histopathology (HP) score and Immunohisto-chemistry (IHC) score for ILT (Pearson's correlation coefficient, r=0.958833).

Conclusion

Thus, the present study showed the prevalance of ILT affecting the poultry flocks in Punjab. Immunohistochemistry helps in confirmatory diagnosis of the disease. Further, the trachea acts as an organ of choice for demonstrating ILTV antigen for specific diagnosis of disease using immunohistochemistry. Large scale studies are required in future using molecular biological tools for further confirmation of the disease.

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