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Differential tissue expression of infected cell protein-4 gene in avian infectious laryngotracheitis by PCR

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Abstract
In this study, a molecular based survey on the overall occurrence of natural cases of Infectious laryngotracheitis in commercial poultry flocks from Tamil Nadu, India for the period from 2019 to 2021 were studied. A total of 420 clinical samples from 70 commercial chicken flocks were investigated. The PCR technique was used to explore the variation in detection of ILTV-ICP4 gene in different clinical samples from the field outbreaks like oro-pharyngeal swabs, trachea, paranasal sinuses, caecal tonsils and harderian gland of birds. Out of 420 samples, 180 (42.86%) samples were found positive for ILT virus-specific ICP4 gene. Significantly higher tissue expression was recorded in the trachea and harderian glands of layer and broiler birds of all age groups, when compared to lower detection in oro-pharyngeal swabs. To our knowledge, this is the first documented report on ILTV outbreak in the state attributed to distribution pattern and detection efficiency of viral ICP4 gene in various types of flocks and different age groups. The findings of the study suggests that variation in viral antigen localization in trachea, paranasal sinuses, caecal tonsils and harderian gland will serve as an useful tool to assess the severity and route of transmission of the disease among different flocks and age group of poultry and will have an impact on sampling methods in disease diagnosis.

Keywords: Poultry, Infectious laryngotracheitis, Tissue expression, Harderian gland, ICP4 gene, PCR

1. Introduction
Infectious laryngotracheitis (ILT) is an acute, highly contagious upper-respiratory infectious disease of chickens, which was first described in the USA in 1925 [1]. The disease is globally distributed and results in economic losses; due to a drop in egg production, reduced weight gain, and mortality [2]. The symptoms of ILT are nasal discharge, conjunctivitis, reduced egg production, gasping, coughing, expectoration of bloody mucus, and marked dyspnea that may lead to suffocation. ILT is now appearing as one of the major re-emerging disease affecting poultry in India [3, 4] and Tamil Nadu [5, 6, 7, 8]. Infectious laryngotracheitis is caused by *Gallidherpesvirus I* (GaHV1), a double stranded DNA virus of 150 kb in size belongs to the genus *Iltovirus*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, according to the Ninth International Committee on the Taxonomy of Viruses (ICTV) [9, 10].
Several molecular techniques have been used to characterize the virus strains with regard to the origin (vaccine or field) of the virus. Although virus isolation has been used to detect ILTV, it is time consuming. Serological tests, including fluorescent antibody technique (FAT) [11], indirect immunofluorescence (IIF) [12], conventional enzyme linked immunosorbent assay (ELISA) [13], serum neutralization (SN) [14] and agar gel immunodiffusion (AGID), have also been used, but they are generally of low sensitivity and laborious.
DNA detection by conventional polymerase chain reaction (PCR) or real-time PCR has become a preferred method of viral diagnosis [10, 15, 16, 17, 18] due to its improved rapidity, sensitivity and reproducibility. The transcriptional regulatory protein, also known as infected-cell protein 4 (ICP4), is produced before the initiation of the replication cycle. The ICP4 gene is responsible for regulating the expression of early and late genes of herpesviruses during infection [19].
Earlier studies demonstrated that detection of long-term tracheal carriers (approximately 2%) among convalescent birds recovered from acute ILT infection play a major role in the establishment of latency [20]. Recent experimental studies revealed sustained detection of ILTV genome in the Harderian gland, trachea, lung and kidney up to 28 days post-infection [21].

In this study, a rapid and sensitive ICP4 gene specific PCR assay for detection of ILTV was evaluated in field outbreaks. By using this technique, the detection efficacy of ICP4 gene in the various tissues of the ILTV affected chickens at different age groups and in the clinical cases from different types of flocks was determined.

2. Materials and Methods
2.1 Sample Collection
The disease investigation was carried out in a total of 420 suspected tissue samples from 70 ILT suspected poultry flocks of Tamil Nadu. The flocks consisted of multi-aged layers and broiler breeders and reared under cage and deep litter system, respectively. The flock size of the poultry farms ranged between 200 and 1.5 lakh (0.15 million) birds. The age of the flocks under investigation ranged between 5 and 80 weeks. Necropsy examination was carried out. Oro-pharyngeal swabs and tissue samples like trachea, paranasal sinus, harderian gland and caecal tonsil were collected aseptically for the virus identification and disease diagnosis by PCR. The tissue samples collected were homogenized in Phosphate Buffered Saline (PBS). The suspension was centrifuged at 5000 rpm for 10 minutes and the supernatant was collected in 1.5 ml eppendorf tubes and stored at-70ºC until use.

2.2 Polymerase Chain Reaction
DNA was extracted using DNeasy Blood and Tissue Kit (M/s Qiagen, Germany) as per manufacturer's instructions. The oligonucleotide primers targeting the Infected Cell Protein-4 gene (ICP4 gene) of GaHV-1 *viz.* ICP4-2F 5'-TTCAGACTCCAGCTCATCTG-3'and ICP4-2R 5'-AGTCATGCGTCTATGGCGTTGAC-3' were used in the PCR. The reaction was carried out using 2X Emerald Amp GT PCR Master mix (M/s. Takara, USA), with an initial denaturation at 94^0 C for 3 min, followed by 35 cycles at 94^0 C for 1 min, 62^0 C for 30 sec, 72^0 C for 90 sec, and a final extension at 72^0 C for 7 min [10, 22]. Ten micro litres of the PCR product were analyzed by electrophoresis in 1.5% agarose gel.

2.3 Statistical analysis
The data obtained were analysed using Analysis of variance (ANOVA) test using SPSS software version 20.0 (IBM®SPSS®Ver20.0 for Windows®) [23] to identify the difference in the expression efficiency of ICP4 gene of ILTV in various tissues.

3. Results and Discussion
3.1 Clinical Findings
The ailing birds revealed varied clinical signs including dullness, difficulty in breathing, gasping, purulent conjunctivitis watery to tenacious oculo-nasal discharge and decrease in egg production. Grossly, caseous plugs in the larynx, catarrhal or haemorrhagic exudates or combinations of these were noticed in the tracheal lumen [10, 24, 25]. Recent work has confirmed considerable variation among ILTV strains in their tropism, capacity to induce mortality, clinical signs and lesions in different tissues [2, 26].

3.2 Polymerase Chain Reaction
The PCR detection of ILTV highly correlated with the characteristic clinical signs and pathology of ILT observed in birds [27, 28, 29]. The targeted ICP4 gene was successfully amplified in various tissue samples at a molecular size of 635 bp in agarose gel electrophoresis (Figure 1). This study demonstrated that there is significant detection variability of ICP4 gene in various tissues between the age groups and types of flocks. Similarly, an experimental study [30] have detected ILTV by real-time PCR assay in the heart, liver, spleen, lung, kidney, larynx, tongue, thymus, glandular stomach, duodenum, pancreatic gland, small intestine, large intestine, cecum, cecal tonsil, bursa of Fabricius, and brain of experimentally infected chickens and concluded that larynx and trachea revealed highest detection level [31, 32].

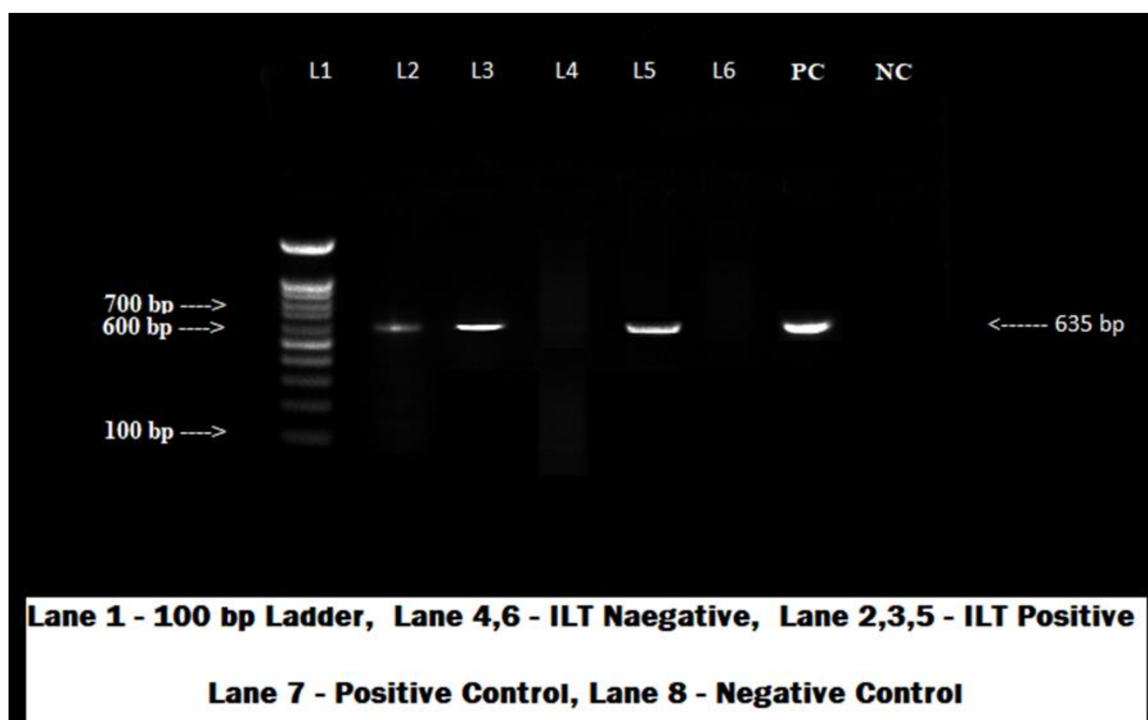

Fig 1: PCR amplification of ICP4 gene from ILTV suspected samples

3.3 Detection of ILTV-ICP4 gene by PCR in different flocks and age groups

Out of 420 clinical samples tested, 180 samples were found positive for ILT from various chicken flocks. Among 180 positive cases, highest detection of ICP4 gene was observed in pooled tracheal tissues (81.66%) followed by Harderian gland (58.88%), Paranasal sinus (50.00%) and Oro-pharyngeal swabs (41.66%). Lowest percent detection of ICP4 gene of ILTV was observed in caecal tonsils (17.77%) in both layer and broiler flocks (Table 1). Among layers and broiler flocks, pooled tracheal tissue revealed highest expression of ICP4 gene as 82.57% and 79.16%, respectively, while the ceacal tonsils revealed lowest detection (24.24%) in layer birds and absent in the broiler birds.

Table 1: Flock wise Incidence and Percent detection of ILTV- ICP4 gene in various tissues based on PCR

Type of Flock	No. of Flocks positive	No. of Positive Samples (n=6)					Total no. of birds
		Oro-pharyngeal swab	Paranasal Sinus	Trachea	Harderian Gland	Caecal tonsil	
Layer	22	60(45.45)	72(54.54)	109 (82.57)	84(63.63)	32(24.24)	132(100)
Broiler breeder	8	15(31.25)	18(37.5)	38 (79.16)	22(45.83)	0(0.00)	48(100)
Total	30	75(41.66)	90(50.00)	147 (81.66)	106 (58.88)	32(17.77)	180(100)

Figures in parenthesis are percentage to the Column Total

During the study period, the disease was found to affect all age groups of birds, as described earlier [8, 33, 34]. According to the age of the chicken in both commercial layer and broiler flock positive for ILTV, the highest prevalence (Table 2) was found in the age group between 20-30 weeks which was 30.00% (9/30) followed by 23.33% (7/30), 16.66% (5/30), 10.00% (3/30) in 10-20 weeks, 30-40 weeks and 60-70 weeks of aged birds, respectively. Lowest prevalence (6.66%) of ILTV was found in birds aged 0-10 weeks, 40-50 weeks and 50-60 weeks old. Our study revealed high prevalence of Infectious laryngotracheitis (62.85%) in layer flock. The findings agreed with the previous reports in India. ILT was first reported in India in 1964 [35]. The re-emergence of ILT was reported many authors in their reports from South India [4, 5, 6, 7, 8] and the incidences gradually increased and became a predominant disease affecting layers causing substantiate level of economic loss to the poultry farmers. Earlier it was believed that adult birds are highly susceptible to ILTV than young birds [36]. Later, it was reported that all age group of birds, breeds and strains are equally susceptible to the infection [34].

Similarly, Age wise percent detection of ILTV-ICP4 gene revealed overall highest expression in tracheal samples (81.11%) in all age groups, followed by harderian gland (67.77%), paranasal sinus (42.77%), oro-paryngeal swab (36.66%) and caecal tonsil (22.22%), respectively. These findings suggests that, the ILTV virus is significantly localised in the trachea, harderian gland and para-nasal sinuses of the affected birds during clinical course and thus elucidating the concept of transmission of virus through discharges from conjunctival route and nasal route [10].

Table 2: Age wise Incidence and Percent detection of ILTV- ICP4 gene in various tissues based on PCR

Age in Weeks	No. of Flocks positive	No. of samples positive (n=6)					Total No. of birds
		Oro-pharyngeal swab	Paranasal Sinus	Trachea	Harderian Gland	Caecal tonsil	
0-10	2(6.66)	3(25.00)	4(33.33)	8(66.66)	7(58.33)	2 (16.66)	12(100)
10-20	7(23.33)	16(38.09)	19(45.24)	35(83.33)	28(66.66)	7 (16.66)	42(100)
20-30	9(30.00)	25(46.29)	32(59.26)	45(83.33)	42(77.77)	20(37.04)	54(100)
30-40	5(16.66)	11(36.66)	12(40.00)	26 (86.66)	23(76.66)	7(23.33)	30(100)
40-50	2(6.66)	4(33.33)	5(41.66)	9(75.00)	6(50.00)	3(25.00)	12(100)
50-60	2(6.66)	4(33.33)	3(25.00)	8(66.66)	7(58.33)	1(8.33)	12(100)
60-70	3(10)	3(16.66)	2(11.11)	15(83.33)	9(50.00)	0(0.00)	18(100)
Total	30	66(36.66)	77(42.77)	146(81.11)	122(67.77)	40 (22.22)	180 (100)

Figures in parenthesis are percentage to the Column Total

3.4 Tissue expression of ILTV-ICP4 gene by PCR

The present study was validated for its ability to detect ICP4 gene of ILTV from various samples like oro-pharyngeal swab, paranasal sinus, trachea, harderian gland and caecal tonsil collected from layer and broiler flocks suspected for ILTV infection by conventional PCR. Our results revealed that among the layer flocks (Table 3), the tracheal samples had highest detection efficiency for ICP4 gene (18.38 ± 0.43). There was no significant difference between the detection efficiency of ILTV in harderian gland (16.46 ± 0.46) and paranasal sinuses (15.31±0.38) while caecal tonsil and oro-pharyngeal swab revealed detection efficiency of 13.92±0.56 and 13.85 ± 0.38, respectively, which were comparatively lesser than the detection level in trachea, harderian gland and paranasal sinuses. Among the broiler flocks (Table 4), highest detection efficiency of ILTV-ICP4 gene was observed in Trachea (5.50 ±0.86) and paranasal sinus (3.50 ±0.38), and ICP4 gene expression was significantly lesser in oro-pharyngeal swab (1.00 ± 0.57), harderian gland (1.50 ±0.65) and caecal tonsil (0.75 ± 0.75). Analysis of variance (ANOVA) test of significance revealed a highly significant difference in the detection efficiency of ILTV-ICP4 gene among various samples in layer flocks (17.80**) and broiler flocks (9.49**).

In a study, PCR method was applied in fresh larynx, trachea, paranasal sinus, and lung from ILT suspected chickens [37] and they found positive reactions in 63.2% of larynx/ trachea, 56% in sinus and 57.6% in the lung. The findings of highest detection efficiency of ILTV-ICP4 gene in trachea of affected layer and broiler flocks from this study well correlated with the previous reports. In the pathogenesis of the disease [38, 39], the virus has high cytolytic activity and primary affinity to tracheal and laryngeal epithelium and it was also emphasized that the virus replicates in other sites such as conjunctiva, sinus, air sacs and lungs [36, 40].

Table 3: Analysis of Variance among Layers flocks for Detection of ILTV- ICP4 gene in various tissues based on PCR

S. No.	PCR Detection	Total Number of Flocks	Mean±SE	F value
1.	Oro-pharyngeal swab	22	13.92 ^{c}±0.56	17.80**
2.	Paranasal Sinus	22	15.31 ^{b}±0.38	
3.	Trachea	22	18.38 ^{a}±0.43	
4.	Harderian Gland	22	16.46 ^{b}±0.46	
5.	Caecal tonsil	22	13.85 ^{c}±0.38	

**: Highly significant at 99% confidence interval
a, b, c: Groups with same superscript are homogenous and groups with different superscript are significantly different

Table 4: Analysis of Variance among Broilers flocks for Detection of ILTV- ICP4 gene in various tissues based on PCR

S. No.	PCR Detection	Total Number of Flocks	Mean±SE	F value
1.	Oro-pharyngeal swab	8	1.00 ^{c}±0.57	9.49**
2.	Paranasal Sinus	8	3.50 ^{b}±0.38	
3.	Trachea	8	5.50 ^{a}±0.86	
4.	Harderian Gland	8	1.50 ^{c}±0.65	
5.	Caecal tonsil	8	0.75 ^{c}±0.75	

**: Highly significant at 99% confidence interval
a, b, c: Groups with same superscript are homogenous and groups with different superscript are significantly different

4. Conclusion

A rapid and sensitive conventional PCR assay for detection of ILTV was used and evaluated for localization of virus specific ICP4 gene in various tissues. The results emphasised on the detection efficiency of the virus in various tissues during disease outbreak. Highest detection of ICP4 gene expression was apparent in tracheal tissues followed by harderian gland and paranasal sinus when compared to the oro-pharyngeal swabs. This study concludes that ILTV virus has differential distribution pattern among age groups and types of flocks and were significantly localised in the trachea, harderian gland and para-nasal sinuses during clinical course, elucidating the concept of disease transmission of virus through discharges from conjunctival and nasal route, hence recent control and biosecurity approaches for ILTV needs to be reassessed to avoid further outbreaks and spreading.

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