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Technical Note

Occurrence of Infectious Laryngotracheitis Virus (Iltv) in 2009-2013 in the State of São Paulo – Brazil

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ABSTRACT

Infectious laryngotracheitis is a very important respiratory disease because it causes significant economic losses in the poultry industry. The target of ILTV infections is the respiratory system, and the main organ in which the virus remains latent is the trigeminal ganglia. However, the virus has demonstrated tropism for other organs as well. The present study was conducted to determine the presence of Infectious Laryngotracheitis Virus (ILTV) in the state of São Paulo. Samples submitted to LABOR- USP during the last four years (2009-2013) analyzed by a nested/PCR technique. Out of the 682 samples from layers tested for LTIV, 12.46 % were positive, and derived from in both traditional (trachea and trigeminal ganglion) and untraditional (cecal tonsils, digestive tract and kidneys) organs utilized for ILTV diagnosis. The present work showed that ILTV is circulating in commercial layer flocks in São Paulo State, and that the LTIV is present in other organs in addition to the respiratory tract and trigeminal ganglion; however, it was not determined if the circulating virus is a vaccinal or field strain.

INTRODUCTION

Infectious laryngotracheitis (ILT) of birds is a highly contagious disease that primarily affects chickens, pheasants, and partridges, with hens as the primary host. Starlings, sparrows, crows, pigeons, and ducks seem to be resistant to the virus (Guy & Garcıa, 2008). The causative agent is a pneumotropic virus of the family *Herpesviridae*, genus *Iltovirus.* Taxonomically, this virus is classified as a *Gallid herpesvirus* 1 (Zhao *et al.*, 2013). This disease is included in the list of mandatory notification of terrestrial and aquatic animal diseases of the OIE. Its notification to the Brazilian Official Service is also mandatory (http://www.oie.int/en/animal-health-in-the-world/oie-listed-diseases-2014/).

Infectious laryngotracheitis was first described in 1925 (May & Tittsler, 1925), and since then it has been reported in many countries, where it is endemic, especially in regions with intensive poultry production with large concentrations of birds and multi-age farms, such as in North America, China, Europe (especially in Poland), Australia, Africa, southwest Asia, New Zealand, and South America (Chacón & Ferreira, 2009; Hidalgo, 2003). Viral transmission occurs via horizontal transfer, and the primary replication sites are in the tracheal mucosa and conjunctiva, where it can causes inflammation, mucoid or serous discharge, cough, and dyspnea. Poor egg production and weight gain are also observed (Coppo *et al.*, 2013b).

The virus invades the trigeminal nerve during the lytic phase of infection, resulting in a latent infection that may be present during the entire life of the animal. Some stressors, such as the onset of lay and placement with other birds, may reactivate virus replication and

shedding (Coppo *et al.*, 2013a; Hughes *et al.*, 1991; Hughes *et al.*, 1989; Williams *et al.*, 1992). Recent experimental studies indicate that the virus can also be detected in other organs, such as the heart, liver, spleen, lung, kidney, tongue, thymus, proventriculus, pancreas, duodenum, small intestine, large intestine, cecum, cecal tonsils, bursa, and brain (Oldoni *et al.*, 2009; Wang *et al.*, 2013; Zhao *et al.*, 2013).

Molecular techniques, such as polymerase chain reaction (PCR), have been successfully used for the detection of ILTV in respiratory and other organs (Chacón *et al.*, 2007; Oldoni *et al.*, 2009; Rodríguez Avila *et al.*, 2007). Polymerase chain reaction presents high sensitivity and specificity for the detection of ILTV. It is able to detect viral DNA when other tests, such as histopathology and immunofluorescence, yield negative results (Crespo *et al.*, 2007). Therefore, PCR provides a rapid diagnostic test that can aid in the differentiation between field and vaccinal strains (Clavijo & Nagy, 1997).

The aim of this study was to evaluate the occurrence of the Infectious Laryngotracheitis Virus in samples submitted to the Laboratory of Avian Diseases of the School of Veterinary Medicine of the University of São Paulo between 2009-2013.

MATERIALS AND METHODS

Sampling

A total number of 682 samples from layer flocks from several cities of the state of São Paulo (Bastos, Iacri, Tupã, Guapiaçu, Rancharia, Parapuã, Ibiúna) was analyzed. Out of the total number of samples, 337 derived from the trachea, 308 from the trigeminal ganglia, 11 from the lungs, six from the cecal tonsils, seven from the digestive tract, two from the liver, two from the spleen, one from the pancreas, and eight from the kidneys (Table 2).

DNA extraction

Total DNA was extracted according to the method of described by Chomczynski (1993). Briefly, samples were homogenized in sufficient 0.01 M phosphate buffered saline (PBS; pH 7.4) to yield a 10% (w/v) suspension, and clarified at 3000 x g for 20 min at 4°C. The supernatant was separated and an aliquot (200 µL) incubated for 5 min at 37°C with 1000 µL of phenol/guanidine thiocyanate solution. Chloroform (200 µL) was then added to the solution, the mixture was centrifuged (12,000 x g for 15 min at 4°C), propanol (750 µL) was added, and the whole mixture was cooled at -20°C for at least 2 h. Precipitated DNA was collected by centrifugation (12,000 x g for 20 min at 4°C). Any DNA that remained adhered to the wall of the tube was rinsed off with 70% ethanol and the solvent was allowed to evaporate. The total DNA sample was dissolved in 30 µL of Tris-EDTA buffer.

Viral Detection

Viral detection was performed by a nested-PCR technique, which was oriented to the amplification of the gene that encodes protein E, as described by Chacón & Ferreira (2008).

In a DNAse-free fresh microtube, 1 X PCR Buffer, 1.25 mM of each dNTP, 0.5 pmol of each of the forward (GE1S) and reverse (GE2AS) primers (as described in table 1), 1.25 U of Platinum® Taq polymerase (Invitrogen by Life Technologies, Carlsbad, CA, USA), and 2.5 µL of extracted DNA were added. DNA free ultrapure water was included to bring the volume to 25 µL. Amplification was performed in a Mastercycler® Nexus X1Eppendorf (Eppendorf AG, Hamburg, Germany). Thermal cycling consisted of an initial denaturation step of 3 min at 94 °C, followed by 45 cycles of denaturing at 94 °C for 1 min, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s. The end cycle was followed by an extension step at 72 °C for 10 min. The second round of amplification (nested-PCR) was performed in a similar manner, although a second set of primers (GE3S forward and GE4AS reverse) was employed, as described in Table 1. The PCR products were visualized after separation by electrophoresis in an agarose gel (1.5%) using Blue Green Dye (LGC, Sao Paulo, Brazil) to stain the DNA. The size of the amplified product was estimated using the 100 base pair DNA Ladder molecular size marker (Invitrogen).

Table 1 – Primers, nucleotide sequences, amplified products (in bp), and references used in the nested-PCR test.

Virus	Reaction	Primer	Nucleotide Sequence (5` - 3`)	(bp)	Reference
Infectious Laryngotracheitis Virus	PCR	GE1S GE2AS	CGTATACCATCCTACAGACGGCA CGTACAATGGTTCGGTCTTGGA	540	(Chacón & Ferreira, 2008)
	NESTED	GE3S GE4AS	AGTCCTCTTATAGCCATCCCCA CACCCCCGCGACGACGAAGT	219	

Table 2 – ILTV frecuency in the analyzed organs, as detected by nested-PCR.

Organs analyzed	Positive Samples	Percentage (%) per organ analyzed	Total Samples Analyzed (n)
Trachea	56/337	8.21	337
Trigeminal ganglia	19/308	2.79	308
Lungs	7/11	1.03	11
Cecal tonsils	1/6	0.15	6
Digestive tract	1/7	0.15	7
Liver	0/2	-	2
Spleen	0/2	-	2
Pancreas	0/1	-	1
Kidneys	1/8	0.15	8
Total	85/682	12.46	682

RESULTS

Using the nested-PCR technique, an amplified product of 219 bp from analyzed samples was obtained (Figure 1). The nested-PCR technique did not amplify any product other than the expected size product. Positive results were obtained in 56/337 (16.6%) of the tracheal samples, 19/308 (6.1%) of trigeminal ganglia, 7/11 (63.6%) of the lungs, 1/6 (16.6) of the cecal tonsils, 1/7 (14.3%) of the digestive tract, and 1/8 (12.5%) of the kidneys. Negative results were found in 2/2 livers, 2/2 spleens, and 1/1 pancreas. (Table 2). Out of the 682 samples analyzed for ILTV, 85 (12.46%) samples were positive for ILTV and 597 (87.54%) samples were negative. The highest number of ILTV-positive samples were trachea samples (56), which showed the highest percentage (8.21%) of positivity for ILTV. On the other hand, liver, spleen, and pancreas samples were negative for ILTV (Table 3).

Table 3 – ILTV distribution in the 682 analyzed samples from different organs of layers in São Paulo State.

Organs analyzed	Positive Samples	Negative Samples	Total Samples Analyzed (n)
Trachea	56 (8.21%)	281 (41.20%)	337 (49.41%)
Trigeminal ganglia	19 (2.79%)	289 (42.38%)	308 (45.16%)
Lungs	7 (1.03%)	4 (0.59%)	11 (1.61%)
Cecal tonsils	1 (0.15%)	5 (0.73%)	6 (0.88%)
Digestive tract	1 (0.15%)	6 (0.88%)	7 (1.03%)
Liver	0	2 (0.29%)	2 (0.29%)
Spleen	0	2 (0.29%)	2 (0.29%)
Pancreas	0	1 (0.15%)	1 (1.15%)
Kidneys	1 (0.15%)	7 (1.03%)	8 (1.17%)
Total	85 (12.46%)	597 (87.54%)	682 (100%)

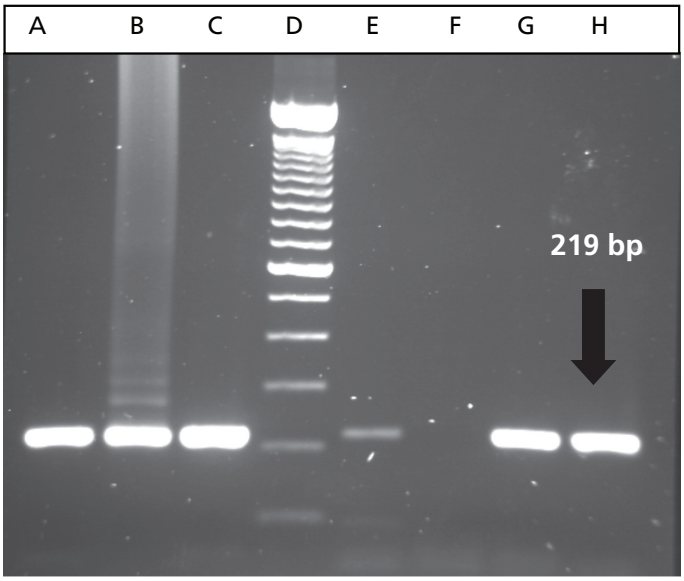

Figure 1 – Nested-PCR product (219 bp) of the partial amplification of ILTV glycoprotein E gene. Lanes A, B, E, G, H: tested samples. Lane C: positive control. Lane F: negative control, and Lane D: Molecular size marker (100 bp).

DISCUSSION AND CONCLUSION

Infectious laryngotracheitis (ILT) is a disease that causes singnificant economic losses due to increased mortality and reduced growth rates and egg production (Guy & Garcıa, 2008). The target of ILTV infections is the respiratory system, specifically the epithelium of the trachea and larynx, although the sinuses and lungs may also be infected. The target site of infection largely depends on the route of inoculation (Bagust & Johnson, 1995). In the present study, ILTV was detected in seven (63.6%) lung samples and 57 (16.6%) tracheal samples, showing a wide viral distribution and strong tropism for the respiratory organs, despite its detection in the intestines, cecal tonsils, and trigeminal ganglia.

The main organ in which ILTV is latent is the trigeminal ganglia, possible because the trigeminal nerve is the main enervator of the upper respiratory tract, tongue and eyes, and the distal part is involved in the innervation of the trachea (Bagust & Johnson, 1995; Bagust, 1986; Williams *et al.*, 1992). The presence of ILTV was detected in 19 of the evaluated trigeminal ganglia samples, showing that the virus became latent in a few chickens.

Our study indicates that viral detection was detected in higher numbers in the lungs, trachea, and trigeminal ganglia. This is consistent with previously reported results, indicating that greater viral replication was detected in the respiratory organs (Oldoni *et al.*, 2009; Rodríguez Avila *et al.*, 2007). However, other organs, such as the lungs, cecal tonsils, digestive tract,

and kidneys were also positive. These findings are in agreement with other studies (Wang *et al.*, 2013; Zhao *et al.*, 2013), in which viral DNA was detected and quantified in the heart, liver, spleen, kidneys, tongue, thymus, proventriculus, duodenum, pancreas, small intestine, large intestine, cecum, cecal tonsils, bursa, and brain. These results indicate that circulating ILTV strains may show tropism for other organs in addition to the respiratory system. Further studies, using ILTV-specific immuno-histochemical techniques should be performed in order to confirm such finding.

Five hundred ninety-seven (597) out of 682 (87.5%) samples were negative by the aforementioned molecular tests, implying partial success of the vaccination program regarding reducing viral activity. Indeed, the positive samples (12.46%) revealed viral presence in healthy chickens. In addition, viral presence could also mean that a field strain is circulating among layer flocks. This is suggested by the detection of ILTV in uncommon organs and indicates that the pathogenesis of the disease is not well understood. Several studies using molecular techniques, such as PCR for detection of ILTV (Chacón *et al.*, 2007; Chacón & Ferreira, 2008; Clavijo & Nagy, 1997; Crespo *et al.*, 2007), prove that these techniques are very sensitive and specific. In the present study, a reaction of nested-PCR, oriented to the amplification of part of the gene encoding glycoprotein E, was successfully used to investigate the presence and tissue location of ILTV in layer chickens.

The results of this study demonstrate that ILTV is circulating in laying flocks reared in São Paulo state; however, it is unknown if the circulating virus is a vaccinal or a field-derived strain. Further studies should focus on differentiating the nature of these strains. Additionally, it must be noted that, at the time of diagnosis, organs other than those of the respiratory system presented ILTV infection.

REFERENCES

Bagust TJ. Laryngotracheitis (Gallid-1) herpesvirus infection in the chicken. 4. Latency establishment by wild and vaccine strains of ILT virus. Avian Pathology 1986;15: 581–595.

Bagust TJ, Johnson MA. Avian infectious laryngotracheitis: Virus-host interactions in relation to prospects for eradication. Avian Pathology 1995;24: 373–391.

Chacón JL, Brandao PE, Villareal LY, Gama NM, Ferreira AJP. Survey of Infectious Laryngotracheitis Outbreak in Layer Hens and Differential Diagnosis with other Respiratory Pathogens. Brazilian Journal of Poultry Science 2007; 9: 61–67.

Chacón JL, Ferreira AJP. Development and validation of nested-PCR for the diagnosis of clinical and subclinical infectious laryngotracheitis. Journal of Virological Methods 2008;151: 188–193.

Chacón JL, Ferreira APJ. Differentiation of field isolates and vaccine strains of infectious laryngotracheitis virus by DNA sequencing. Vaccine 2009;27:6731–6738.

Chomczynski P. A reagent for the single-step simultaneus isolation of RNA, DNA and protein for the cell and tissues samples. Biotechniques 1993;15: 532–536.

Clavijo A, Nagy E. Differentiation of Infectious Laryngotracheitis Virus Strains by Polymerase Chain Reaction. Avian Diseases 1997;41:241–246.

Coppo MJC, Hartley CA, Devlin JM. Immune responses to infectious laryngotracheitis virus. Developmental and Comparative Immunology 2013a;41:454–462.

Coppo MJC, Noormohammadi AH, Browning GF, Devlin JM. Challenges and recent advancements in infectious laryngotracheitis virus vaccines. Avian Pathology 2013b;42:195–205.

Crespo R, Woolcock PR, Chin RP, Shivaprasad HL, Garcia, M. Comparison of Diagnostics Techniques in an Outbreak of Infectious Laryngotracheitis from Meat Chickens. Avian Diseases 2007;51:858–862.

Guy JS, Garcıa M. Laryngotraqueitis. In: Saif YM, Glisson JR, Fadly AM, Mcdougald LR, Nolan LK, Swayne DE, editors. Diseases of poultry. 12thed. Ames: Iowa State University Press; 2008. p.137–152.

Hidalgo, H. Infectious Laryngotracheitis : A Review. Brazilian Journal of Poultry Science 2003; 5:157–168.

Hughes CS, Gaskell RM, Jordan FTW, Bradbury JM, Jones RC. Effects of certain stress factors on the re-excretion of infectious laryngotracheitis virus from latently infected carrier birds. Reserch in Veterinary Science 1989; 46:274–276.

Hughes CS, Williams RM, Gaskell RM, Jordan FTW, Bradbury JM, Bennett M, Jones RC. Latency reactivation of infectious laryngotracheitis vaccine virus. Archives of Virology 1991; 121:213–218.

May HJ, Tittsler RP. Tracheo-laryngotracheitis in poultry. Journal of American Veterinarian Medical Association 1925;67:229–231.

Oldoni I, Rodríguez-Avila A, Riblet SM, Zavala G, García M. Pathogenicity and growth characteristics of selected infectious laryngotracheitis virus strains from the United States. Avian Pathology 2009;38:47–53.

Rodríguez-Avila A, Oldoni I, Riblet S, García M. Replication and Transmission of Live Attenuated Infectious Laryngotracheitis Virus (ILTV) Vaccines. Avian Diseases 2007;51:905–911.

Wang LG, Ma J, Xue CY, Wang W, Guo C, Chen F, Qin JP, Huang NH, Bi YZ, Cao YC. Dynamic distribution and tissue tropism of infectious laryngotracheitis virus in experimentally infected chickens. Archives of Virology 2013;158:659–666.

Williams RA, Bennett M, Bradbury J M, Gaskell RM, Jones RC, Jordan FTW. Demonstration of sites of latency of infectious laryngotracheitis virus using the polymerase chain reaction. Journal of General Virology 1992;73:2415–2420.

Zhao Y, Kong C, Cui X, Cui H, Shi X, Zhang X, Hu S, Hao L, Wang Y. Detection of infectious laryngotracheitis virus by real-time PCR in naturally and experimentally infected chickens. PLOS ONE 2013;8:1–10.

In the article entitled Litter Characteristics and Pododermatitis Incidence in Broilers Fed a Sorghum-Based Diet published in the Revista Brasileira de Ciência Avícolas/Brazilian Journal of Poultry Science, v16 (3): 291-295, in page 291 where it was written

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