Outbreak of infectious laryngotracheitis in large multi-age egg layer chicken flocks in Minas Gerais, Brazil[1]

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ABSTRACT.- Preis I.S., Braga J.F.V., Couto R.M., Brasil B.S.A.F., Martins N.R.S. & Ecco R. 2013. **Outbreak of infectious laryngotracheitis in large multi-age egg layer chicken flocks in Minas Gerais, Brazil.** *Pesquisa Veterinária Brasileira 33(5):591-596.* Setor de Patologia Veterinária, Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 30123-970, Brazil. E-mail: ecco@vet.ufmg.br

A recent (November 2010) outbreak of infectious laryngotracheitis (ILT) in a multi-age laying hen facility in Minas Gerais state, Brazil, is described. Previous ILT outbreak in laying hens was only notified in São Paulo state, Brazil, in 2002. In the outbreak described here, the affected population was approximately eight million hens, with flock sizes ranging from 100,000 to 2,900,000 chickens. The average mortality ranged from 1 to 6%, and morbidity was around 90% (most of the twenty seven farms of the area were positive for ILT virus). Three multi-age laying farms from one company were selected for this report. Clinical signs included prostration, dyspnea, conjunctivitis, occasional swelling of the paranasal sinuses and bloody mucous nasal discharge. Severely affected chickens presented with dyspnea, gasping and became cyanotic before death. At necropsy, these chickens had fibrinous exudate blocking the larynx and the lumen of cranial part of the trachea. In addition, conjunctivitis with intense hyperemia, edema and sinuses with caseous exudate were present. On histopathology, there were marked necrosis and desquamation of respiratory ephitelium and conjunctiva with numerous syncytial cells formation and fibrinous exudate. Moderate to marked non suppurative (especially lymphocytes and plasma cells) infiltration in the lamina propria also was observed. Sixteen out of 20 examined chickens, eosinophilic intranuclear inclusion bodies were observed in the syncytial cells. The DNA extracted from larynx and trachea produced positive PCR results for ILT virus (ILTV) DNA using formalin-fixed, paraffin embedded (FFPE) samples. Amplicons from a small region of ICP4 gene were submitted to sequencing and showed 100% identity with ILTV EU104910.1 (USA strain), 99% with ILTV JN596963.1 (Australian strain) and 91% with ILTV JN580316.1 (Gallid herpesvirus 1 CEO vaccine strain) and JN580315.1 (Gallid herpesvirus 1 TCO vaccine strain).

INDEX TERMS: Laying hen, avian infectious laryngotracheitis, Gallid herpesvirus 1, histopathology, conventional PCR, sequencing.

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RESUMO.- [**Surto de laringotraqueíte infecciosa em granjas de galinhas poedeiras de múltiplas idades em Minas Gerais, Brasil.**] Um surto recente (Novembro de 2010) de laringotraqueite infecciosa (LTI) em granjas de postura de múltiplas idades em Minas Gerais, Brasil, é descrito. Um surto de LTI em galinhas de postura havia sido previamente relatado apenas no Estado de São Paulo em 2002. No surto aqui descrito, a população afetada foi de aproximadamente oito milhões de galinhas, com lotes variando de 100.000 a 2.900.000 galinhas. A mortalidade média variou de 1 a 6% e a morbidade atingiu cerca de 90% (a

maioria das 27 granjas foram positivas para o virus da LTI). Três granjas com aves de múltiplas idades pertencentes a uma empresa foram selecionadas para o presente relato. Os sinais clinicos incluíram prostração, dispneia, conjuntivite, edema ocasional dos seios paranasais e secreção nasal mucosa e/ou sanguinolenta. As aves severamente afetadas apresentaram acentuada dispneia, aparente engasgo e tornaram-se cianóticas antes da morte. Nestas aves, exsudato fibrinoso denso obstruindo o lúmen da laringe e parte cranial da traqueia foi observado na necropsia. Havia também, conjuntivite com hiperemia intensa e edema, além de sinusite com exsudato caseoso. Na histopatologia, observaram-se necrose e descamação acentuada do epitélio respiratório e da conjuntiva com formação de numerosos sincícios e exsudato fibrinoso. Além disso, infiltrado inflamatório mononuclear (especialmente linfócitos e plasmócitos) moderado a acentuado na lâmina própria foi observado. Corpúsculos de inclusão intranucleares nas células sinciciais foram observados em 16 das 20 aves examinadas. Resultados positivos pela PCR para o virus da LTI foram obtidos de DNA extraído das laringes e traqueias utilizando amostras fixadas em formol e incluidas na parafina. O produto amplificado de uma região pequena do gen ICP4 foi submetido ao sequenciamento e quando comparado com outras sequências depositadas no *Genbank* mostrou os seguintes resultados: 100% de identidade com uma estirpe do virus de LTI dos Estados Unidos (JN596963.1), 99% de identidade com uma estirpe Australiana e 91% com a estirpe vacinal CEO (JN580316.1) e TCO (JN580315.1).

TERMOS DE INDEXAÇÃO: Galinhas de postura, laringotraqueite infecciosa das aves, herpesvirus tipo 1 dos galináceos, histopatologia, PCR convencional, sequenciamento.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a viral respiratory disease caused by a *Gallid herpesvirus* I. ILTV belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Iltovirus* (Index of Viruses 2006, McGeoch et al. 2006). Natural infections with ILTV occur mainly in chickens, and both young and adult chickens are susceptible to infection. Broilers older than 3 weeks are most susceptible to ILTV (Fahey et al. 1983). Natural ILTV infections may affect other species, including pheasants and peafowl, and also turkeys (Portz et al. 2008). ILTV of chickens is responsible for serious production losses and decreased egg production. Clinical signs can be observed 6-12 days post infection. There are two clinical forms of ILTV infections, severe and mild. Clinical signs of the severe form include marked dyspnea and expectoration of bloody mucus, watery eyes and hemorrhagic conjunctivitis (Guy & Garcia 2008). This form can cause 90%-100% morbidity with mortality ranging from 5% to 70% and average mortality being 10-20%, variable depending of the viral strain. Mild ILT forms generally results in morbidity lower than 5% and mortality ranging from 0.1% to 2% (Bagust et al. 2000). Clinical signs of the mild form include depression, reduced egg production and reduced weight gain, conjunctivitis, swelling of the paranasal sinuses and nasal discharge. Gross lesions of the severe form include necrosis with hemorrhage and fibrinous exudate in the conjunctiva, larynx, trachea and nasal mucosa. Gross lesions with the mild form include swollen palpebrae, hyperemic conjunctiva and mild to moderate tracheitis (Sellers et al. 2004). Histopathologic changes with the severe form include fibrinonecrotic and hemorrhagic laryngotracheitis and syncytial cell formation with intranuclear inclusion bodies (Guy & Garcia 2008). Mild forms exhibit mild to moderate fibrinous laryngotracheitis and conjunctivitis, with occasional intranuclear inclusion bodies, and may occur with vaccine virus strain infections (Sellers et al. 2004). Attenuated vaccines, mainly the CEO (chicken embryo origin) strains have been isolated from ILT outbreaks in different parts of the world (Kirkpatrick et al. 2006, Oldoni & Garcia 2007; Blacker et al. 2011). The attenuated virus can spread from vaccinated bird to non-vaccinated bird, and revert to the virulent form after sequential bird-to-bird passage (Guy et al. 1991). The present report describes clinico pathological findings and diagnostic workup of a recent outbreak of avian infectious laryngotracheitis in multi-age egg layer farms from a high production area in Minas Gerais State, Brazil.

MATERIALS AND METHODS

Birds and pathology. Sampling for this study included twenty chickens from three multi-age laying hen farms (one company), clinically diagnosed with respiratory disease with a high suspicion of ILT. Clinical and epidemiologic data (monthly average morbidity and mortality rate) were collected on each farm by a veterinarian of *Instituto Mineiro de Agropecuaria* (IMA). Data and tissue samples were collected monthly from December 2010 through June of 2011. Twenty layer chickens of 21-60 weeks of age from three farms were selected and examined for this report. Birds were necropsied and samples for diagnosis including conjunctiva, conchas nasais, seios paranasais, larynx, trachea and lungs were collected. Samples were only removed outside the outbreak zone after being placed in 10% buffered formalin, due legal restrictions. After 52 h in formalin, tissues were processed routinely, embedded in paraffin, sectioned at 5µm, and stained with hematoxylin and eosin. Two pools of fresh-frozen traqueal swabs were sampled from 20 chickens of an ILTV positive flock.

Molecular detection of ILT virus. Formalin-fixed, paraffin embedded (FFPE) tissue samples, including larynx and trachea from twenty chickens ILT histopathology confirmed were used for DNA extraction. DNA was extracted from FFPE tissues using QIAGEN DNA Extraction kits (QIAGEN, Valencia, California) according to the manufacturer's instructions. Tissue samples were cut at 5µm (8-10 sections), and placed in DNase-free, 2ml microtubes. Extracted DNA (70-924ng/µl) was stored at -80 C until used for DNA amplification by polymerase chain reaction (PCR). Primers were designed to amplify a 237 bp fragments from the ILTV diploid gene ICP4. The primers ICP4-1F (5'- CCTTGGTTCGGGATGAAACC-3') and ICP4-1R (5'- TTCATTACCTCCAGCGGTTCC-3'), bind at positions 117,057-117,076 and 117,255-117,275 within the internal repeat short (IRS) and at positions 149,473-149,492 and 149,274-149,294 within the terminal repeat short of the 63140/C/08/BR strain of ILTV (Accession number JN542536). Primers were designed manually. Hairpin structures, homo- and heterodimers were examined using the Oligoanalyzed program (Integrated DNA technologies). PCR oligonucleotides sense and antisense were synthetized by IDT technologies (CA, USA). PCR components included 2µl (200ng) of the extracted DNA (templa-

te), in a 25µl reaction of enzyme Taq polymerase and dNTP mix kit (Promega) and 10µmol of each primer. Cycling parameters for the amplification were 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 2 minutes, and a final extension at 72°C for 15 minutes. A positive control from FFPE tissue was kindly provided by Dr. Maricarmen García from Poultry Diagnostic and Research Center of University of Georgia, Athens, Georgia, USA. Fresh-frozen traqueal swabs sampled from chickens of ILTV positive flock were used to confirm the sequencing result observed for FFPE tissues. An aliquot of 200µl of ground and centrifuged sample was subjected to DNA extraction using sodium iodide and silica according to the methodology described previously (Boom et al. 1990). In order to confirm the PCR results, products were visualized on 1.5% agarose gel electrophoresis and three FFPE tissues and two fresh-frozen positive samples were selected for direct sequencing. Sequences were determined bi-directionally using BigDye® Terminator v3.1 cycle kit (Applied Biosystems, Inc., Foster City, California, USA) following the manufacturer's protocol on an ABI 3130 Genetic Analyzer. Sequences were analyzed using SeqScape® Software v2.5 and identified by searching the GenBank database using the BLASTn platform.

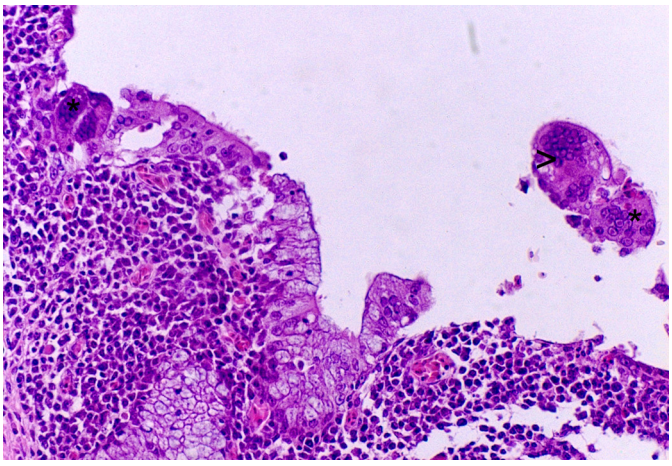
RESULTS

Case history. In November 2010, flocks from an area with multi-age egg layer production were reported to be experiencing increased mortality ranging 1% to 6%. One month later (December), mortality had increased (3-9%), and related to restrictions on removal and transportation of chickens and litter, and sanitary culling of sick birds. The affected area included a zone of very high poultry density, of approximately eight million hens, with farm flock sizes ranging from 100,000 to 2,900,000 chickens. Chickens showed clinical signs characterized by depression, open mouth breathing and gargling respiratory noises. Some birds stretched out their necks while trying to breath (Fig.1). In these birds, a necrotic caseous yellow material could be seen blocking the laryngeal opening. Also, the eyelids were often covered with a dry and crusty exudate. In some chickens, there was mild to moderate swelling of the paranasal sinuses. Respiratory signs were observed simultaneously to a sudden decline in egg production (particularly in two farms). Less severely affected birds showed swollen eyelids, reddened conjunctivae, and excessive lacrimation, without severe respiratory signs. Flocks were vaccinated against Newcastle disease, infectious bronchitis, fowlpox, infectious coryza but not ILT. Based on the clinical signs, the official communication of ILT suspicion was submitted to the official veterinary service (IMA) by November 11, 2010. After notification, the official veterinary service immediately isolated the suspected area and samples from several birds were collected. Samples were submitted to the official reference laboratory for ILTV investigation. After positive official result for ILTV, the outbreak biosecurity zone was established and all measures to contain and avoid virus spread were implemented. All samples submitted for diagnosis outside the outbreak area were placed in 10% buffered formalin for zero transportation risk.

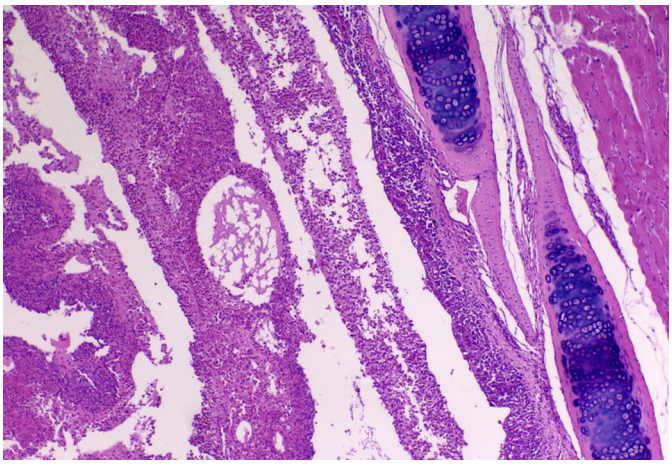
Fig. 1. Forty five week-old chicken, naturally infected by ILTV stretching its neck and opening the beak while is trying to breath.

Gross and histopathology. Gross lesions were characterized by fibrinous or caseous exsudate blocking the larynx and the lumen of cranial part of the trachea in four chickens (Fig.2). In another twelve chickens, there was hyperemia and a fibrin layer covering the mucosa of larynx and, especially the cranial and medial part of tracheal mucosa. Conjunctivitis with intense hyperemia and edema were seen in five hens (25%). Also, thickening of nasal turbinates and wall of the paranasal sinuses with fibrinous exudate in the lumen could be seen in most of chickens. On histopathology, the conjunctiva and mucosa of the nasal turbinates, sinuses (Fig.3), larynx (Fig.4 and 5) and trachea (Fig. 6) were markedly thickened due to intense infiltration of lymphocytes and plasma cells in the lamina propria. The distribution and intensity of these lesions in all examined birds were variable. In multifocal areas, the inflammatory cells replaced the mucosal glands in the trachea and nasal turbinate. In addition, necrosis of epithelial cells, hyperplasia and desquamation, along with fibrin, cellular debris and heterophils, formed a diphtheritic membrane. Also, protruded and fused epithelial cells forming syncytia of about 40 to 90 µm in diameter and containing eosinophilic intranuclear inclusion bodies were observed. The syncytial cells contained about 10 to 50 nuclei, and almost 100%

Fig. 2. Larynx and trachea, 35-week-old chicken, naturally infected by ILTV. Abundant caseous fibrinous exsudate blocking the lumen, characterizing the typical gross lesion of infectious laryngotracheitis.



3



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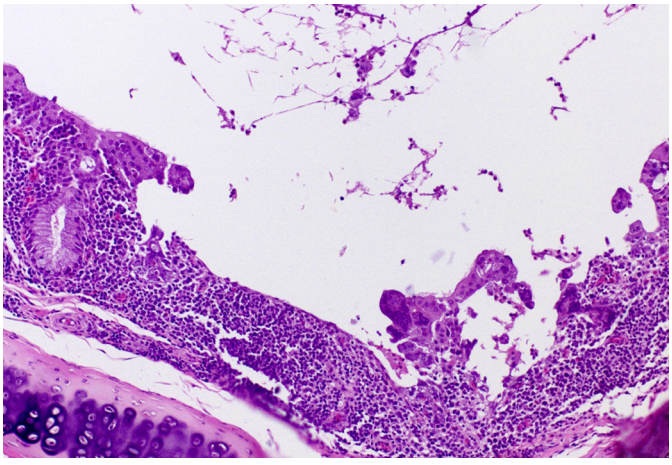

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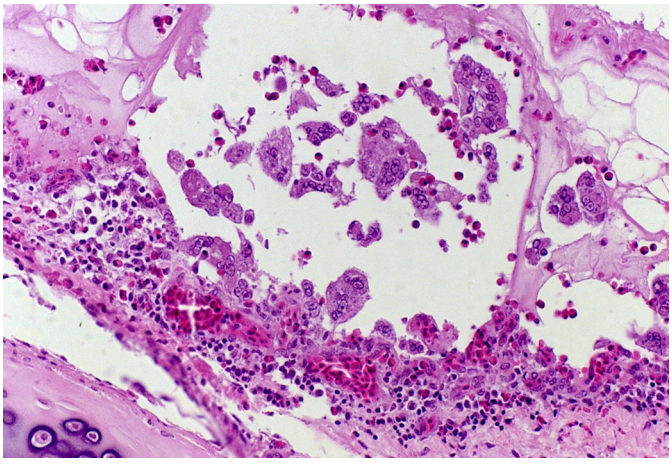

6

Fig.3. Syncytial cells containing intranuclear inclusion bodies Cowdry A (>) and B (*) detaching from the epithelium associated with marked lymphoplasmacytic sinusitis. Paranasal sinus, 30-week-old chicken, naturally infected by ILTV. HE, obj.60x.

Fig.4. The lumen of the larynx is filled with abundant fibrin and debris cells. Inflammatory cells are expanding the lamina propria and epithelial cells were loosed by desquamation. Syncytial cell are not seen, characterizing more advanced cases of ILT. Larynx, 30-week-old chicken, naturally infected by ILTV. HE, obj.10x.

Fig.5. There is desquamation of epithelial cells and syncytial cells contain several nuclei, characterizing typical lesions observed in acute cases of ILT associated with severe lymphoplasmacytic laryngitis. Larynx, 45-week-old chicken, naturally infected by ILTV. HE, obj.20x.

Fig.6. Lymphocytes and plasm cells are expanding the lamina propria, and desquamation of epithelial cells are forming syncytial cells. Fibrin, heterophils and syncytial cells with intranuclear inclusion bodies also can be observed into the lumen. Lower trachea, 25-week-old chicken, naturally infected by ILTV. HE, obj.40x.

were filled with a basophilic (Cowdry Type B intranuclear inclusions) and/or eosinophilic dense material (Cowdry Type A intranuclear inclusions) (Fig.7). These morphologic changes were coincidental with the acute phase of this disease. Similar inflammatory lesions, but in lesser intensity were observed in the mucosa of distal trachea and lungs. Syncytial cells with eosinophilic intranuclear inclusion bodies were observed in the conjunctiva (20%), nasal turbinate and sinus (25%), larynx (50%), trachea (70%) and primary and secondary pulmonary bronchi (10%) of chickens. Most chickens presented intranuclear inclusion bodies simultaneously in the larynx, trachea and nasal turbinates or paranasal sinus. In more advanced cases, areas with epithelial desquamation resulted in lacking of epithelial covering or a thin layer of cuboidal cells. Thus, in two birds, detached syncytial cells with intranuclear inclusion bodies were seen in the lumen of the primary bronchus only. In these birds, there was no syncytial cell with inclusion bodies in the upper or lower respiratory system or conjunctiva, but only necrotic and lymphoplasmacytic laryngitis, tracheitis and sinusitis.

Molecular detection of ILT virus. All twenty layer chickens showed positive PCR results for ILTV of DNA extracted from larynx and trachea. Three FFPE samples and two fresh-frozen traqueal swabs samples from three farms, representing an area containing twenty seven farms, were selected for sequence study. The products submitted to sequencing showed 100% identity with ILTV EU104910.1 (USA strain), 99% with ILTV JN596963.1 (Australian strain) and 91% with ILTV JN580316.1 (*Gallid* herpesvirus 1 CEO vaccine strain) or JN580315.1 (*Gallid* herpesvirus 1 TCO vaccine strain). The sequence ILTV Brazil/2011/UFMG from the present study was deposited in the GenBank database under the accession number KC182579. Sequence re-

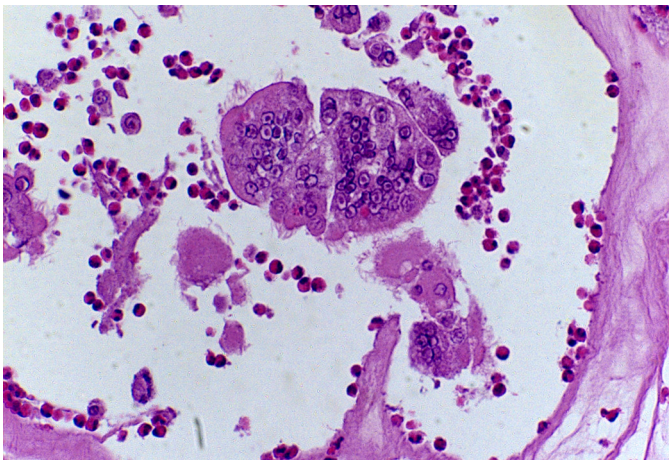

Fig.7. Syncytial cells containing many intranuclear inclusion bodies Cowdry A. Lower trachea, 25-week-old chicken, naturally infected by ILTV. HE, obj.60x.

sults from fresh-frozen traqueal swabs and FFPE samples were identical.

DISCUSSION

Clinical and pathologic findings were consistent with the severe form of infectious laryngotracheitis. This form is characterized by marked dyspnea, high mortality and marked hemorrhagic and/or diphtheric laryngotracheitis, and are attributed to wild-type ILTV (Guy et al. 1990). The development of intranuclear inclusions bodies in the respiratory and conjunctiva epithelium is considered pathognomonic for ILT (Purcell 1971) and is frequently seen in the moderate and severe form (Bagust et al. 2000). Most chickens selected for this study had syncytia with intranuclear inclusion bodies associated with the respiratory epithelium allowing confirmatory histopathology diagnosis. The herpesvirus inclusion bodies are intranuclear accumulations of assembled viral particles, proteins and genome. Initially, the basophilic intranuclear inclusion bodies are called Cowdry type B. This is an early stage which later shrinks to produce the "halo", becomes eosinophilic, and is then designated as Cowdry type A. In this stage virus is in the cytoplasm just following release and degeneration of syncytial cell (Cowdry 1934).

Most birds in the present report showed typical lesions in the sinus, larynx and the entire trachea, although the conjunctiva and lungs were less frequently affected. A study in Australia demonstrated that different strains vary in their capacity to induce mortality, clinical signs and lesions in different tissues. Some strains demonstrated high affinity for the trachea but little affinity for conjunctiva. In contrast, other strains showed high affinity for conjunctiva but lower affinity for trachea (Kirkpatrick et al. 2006). Researchers reported that the occurrence of the typical lesions was higher between three and nine days post infection in experimental conditions (Hayashi et al. 1985) and between five and nine days post infection in natural conditions (Bagust et al. 2000). The diagnosis by histopathology is considered a valid and relatively rapid test for ILT (OIE 2009), but the probability of finding typical lesions is decreased dramatically when histopathology is performed after eight to 10 days (subacute to chronic stage) of infection, due to desquamation of the epithelial cells (Hayashi et al. 1985, Bagust et al. 2000). For later ILT histopathology diagnosis, it is important to collect and examine primary and secondary bronchi, because detached syncytial cells from larynx and trachea may be found in the lumen, as observed in two chickens of the present report. Complementary tests are necessary and very important for the demonstration of the etiology of unspecific conjunctivitis and/or tracheitis cases. This condition is described in mild forms of laryngotracheitis related to ILTV vaccine strains (Sellers et al. 2004; Dufour-Zavala 2008). In cases where the typical lesions are missing, ancillary diagnostic tests are strongly recommended, such as PCR for definitive diagnosis. The PCR is considered more sensitive than virus isolation (Williams et al. 1994) and conventional PCR was applied successfully for our FFPE samples, on the definitive diagnosis of ILT in association with histopathology. Also, the primers designed to amplify a product from the ILTV ICP4 gene demonstrated good sensitivity. The detection limit of ILTV DNA from FFPE samples was as down as 0.1ng/ul (data not shown). The nucleotide sequencing confirmed the results and validated the primers delineated for this study. The tests in FFPE and fresh-frozen tissues showed identical results. This study showed that the validated primers are a good choice for diagnosis using FFPE or fresh-frozen samples and conventional PCR. In order to verify relationships among Brazilian isolates and vaccine virus strains, further sequencing of a larger ICP4 sequence and other genomic regions is under way.

The first report on the occurrence of ILTV in Brazil was published in 1974, in Rio de Janeiro (RJ) state, based on virological and serological tests (Hipólito et al. 1974). In 1981 and 1982, an outbreak in laying hens also in RJ was reported by Araujo et al. (1982). Antibody titers were demonstrated in chickens in Rio Grande do Sul (RS) state by 1995 (Vargas 1995). Later, ILTV was described in RS state by Beltrão et al. (2004). Nevertheless, severe outbreaks involving a wide and highly populated poultry area was not reported before 2002. However, at the end of 2002 and during 2003, an ILT epidemic with high mortality was observed in Bastos region of the São Paulo State, in a population of 14 million layer chickens, the largest concentration in Brazil, causing great economic losses. During the outbreak, the mortality ranged from 2-20% (Chacon et al. 2007). In the present outbreak, the monthly mortality rate was lower (1-9%), and for a shorter time. Approximately two to three months after the beginning of the outbreak, the mortality rate spontaneously decreased without the implementation of a live attenuated vaccination program, although latent infectious birds still were detected by PCR test.

The characterizations of ILTV isolates involved in the outbreak in Bastos have shown that two CEO vaccine types and a wild-type virus strains were co-circulating in the region. The origin of vaccine-type isolates is unknown, because the use of ILTV vaccines was not previously authorized for use in the country (Chacon et al. 2010).

For the outbreak here described, the highest mortality rate occurred during the first two to three months. After

this period, the average mortality decreased to below 0.5%. Nine months after the outbreak (August 2011), a vaccination program using vectored fowl pox vaccine was introduced for all new flocks entering the affected area. By early 2012, the average mortality was recorded lower than 0.3%, although latent infectious birds still were detected by PCR test. The origin of the virus remains unknown. No official live attenuated vaccine strains were employed in Minas Gerais due to legal restrictions.

Finally, the viral molecular characterization will provide better comprehension of the potential virulence of ILTV strains, and the relationship between isolates from this outbreak and isolates from other field or vaccine strains. For instance, no new outbreak besides the originally interdicted area was detected. The outbreak area remains interdicted and biosecurity measures were applied. It was apparent that the measures taken for avoiding virus spreading including biosecurity strategies, no live virus vaccination and re-routing transportation were effective.

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