Title

Case Report: Esophagitis and pharyngitis associated with avian infectious laryngotracheitis in backyard chickens: 2 cases

Running title: Atypical avian infectious laryngotracheitis in backyard chickens

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Summary

Infectious laryngotracheitis (ILT) is a contagious viral respiratory disease of great economic importance for the global poultry industry, caused by *Gallid herpesvirus 1* (GaHV-1). Lesions of the upper digestive tract caused by this virus have not been reported before. Two small flocks of backyard chickens experienced an outbreak of ILT, one in 2006 and the other in 2014. These birds had typical ILT lesions, characterized by a necrohemorrhagic laryngitis and tracheitis but were also affected by a severe erosive and necrotic esophagitis and pharyngitis. On microscopic examination of the esophagus and pharynx, numerous individual epithelial cells were degenerated or necrotic. Syncytial cells were present in the mucosa or sloughed in the overlying inflammatory crust and some of these cells contained an amphophilic intranuclear viral inclusion. GaHV-1 was detected in tissues, both from respiratory and digestive tracts, by polymerase chain reaction (PCR) and/or immunohistochemistry (IHC) diagnostic assays. This case stresses the importance for veterinarians, owners and technicians to pay attention to different or atypical clinical manifestations of ILT given its highly contagious nature.

Key words: avian infectious laryngotracheitis, *Gallid herpesvirus 1*, poultry, pharyngitis, esophagitis

Abbreviations:

ILT: avian infectious laryngotracheitis

ILTV: avian infectious laryngotracheitis virus

GaHV-1: *Gallid herpesvirus 1*

IHC: immunohistochemistry

Introduction

Infectious laryngotracheitis (ILT) was first recognized as a disease in the 1920's and was associated with clinical signs of the upper respiratory tract in chickens. The infectious laryngotracheitis virus (ILTV) is classified in the *Herpesviridae* family of the *Alphaherpesvirinae* subfamily, genus *Iltovirus* and identified as *Gallid herpesvirus* 1 (GaHV-1) (14, 18). Chicken is the natural primary host of this virus, which has also been reported in pheasant, partridge, young turkey and peafowl (7, 19). Starling, sparrow, crow, dove, duck, pigeon, guinea fowl appear to be refractory to the virus. It can affect chickens of all ages, but usually occurs in chickens older than three weeks. GaHV-1 has been identified worldwide.

GaHV-1 is a highly contagious virus and may infect nearly all birds in a flock (90 to 100%) (19). Depending on the pathogenicity of the strain, mortality could reach up to 70% but usually ranges between 10 to 20% (19). Accurate estimate of the economic impact of this disease on small backyard flocks in Quebec is difficult to assess because these flocks are not under a regular veterinary supervision. However, ILT has been regularly diagnosed in these flocks over the last ten years and they are considered a potential risk for the transmission of the virus to commercial poultry farms (3). This risk cannot be neglected as, for example, an outbreak of ILT in Quebec commercial broiler chickens in 2009 has led to a total economic loss estimated at 2M $CAN (2)

The usual route of entry of ILTV in the body host is through the upper respiratory tract and conjunctiva. Viral replication is generally known to take place in the epithelium of

the larynx, trachea and conjunctiva and less frequently in other parts of the respiratory tract with no or little signs of viremia (18, 19). Transmission occurs through acutely infected birds and latent carriers of the virus, such as vaccinated birds which may be able to excrete the live vaccinal strain (18). Clinical signs vary according to the pathogenicity of the strain (19). In the severe form, the infected birds show nasal discharge, rales, coughing, gasping, marked dyspnea and expectoration of blood-stained mucus, while in the mild form, birds may present unthriftiness, conjunctivitis, swelling of the infraorbital sinuses and mild tracheitis. Characteristic intranuclear inclusion bodies and syncytia can be observed in epithelial cells of the larynx, trachea and conjunctiva on microscopic examination (19).

The following are reports of two atypical cases of necrotic pharyngitis and esophagitis associated with ILTV. Lesions in the upper gastrointestinal tract caused by ILTV have not been documented before.

Flock history and animals

Case 1. The owner of 26 backyard chickens of various breeds (three pheasants, one peacock and an unknown number of pigeons) complained about a marked increase in morbidity and mortality in the backyard flock. Clinical signs were first observed in one of the pheasants in early July 2014. The bird had bilateral swelling of the periorbital area and was unable to open his eyes . The following week, five chickens developed similar clinical signs and four of them died. During the same week, on July 22nd 2014, a local veterinarian, after a visit to the premises, submitted a 6 month-old hen to the Laboratoire

d'épidémiosurveillance animale du Québec (LEAQ) in Saint-Hyacinthe for suspicion of a *Mycoplasma gallisepticum* infection. The birds had been bought at two different auctions of exotic fowl in the spring of 2014. They had been kept in a shed with free access to an outside pen. A local feed mill provided the owner with commercial feed.

Case 2.

A retrospective study of the diagnoses made on birds submitted to our laboratory revealed a similar case in 2006. A flock of mix breed laying hens aged 18 weeks and older was kept in a backyard setting. This flock had no epidemiological link with the 2014 case. Half of the flock experienced respiratory signs and some were found dead within 24 hours after the onset of clinical signs. Six of the laying hens were submitted to the LEAQ for diagnostic investigation and surveillance for avian influenza.

Diagnosis

Gross findings

The hen from case 1 was in a moribund state and had a low body score index. Birds from case 2 were in moderate or good body condition. All birds submitted alive (case 1 and one from case 2) were humanely euthanized by electrical stunning. A necropsy was performed on all animals and opening of the carcasses revealed similar lesions to the upper digestive tract. These consisted of numerous, coalescing, round and well-delineated, cream to white raised plaques on the buccal mucosa at the caudo-dorsal aspect of the pharynx and palate, as well as on the tongue and over the larynx opening (Figure 1). Similar plaques, ring-shaped in the case 1 bird, were also found in the proximal third

of the esophagus, as well as fine ulcers in many individuals from case 2. Dissection of the upper respiratory tract of case 1 bird revealed a very mild accumulation of white debris in the larynx with no other significant findings. In case 2, however,obvious blood clots were present in the larynx and trachea of most of the birds and a pseudomembrane was observed in the trachea of one bird. They also had mild edema of the eyelids and some mucus in the nasal cavities. Some nematodes, compatible with *Heterakis* spp., were also found in their ceca. Their sinuses, lungs and air sacs looked normal and other organs showed no gross abnormalities.

Histopathology

Tissue sections from nervous (brain), digestive (tongue, larynx, esophagus, crop, proventriculus, small and large intestines, liver), respiratory (pharynx, trachea, lungs), cardiac, haematopoietic (spleen) and skeletal (muscles) systems were collected, fixed in neutral buffered 10% formalin and routinely processed for microscopic examination.

The epithelium of the tongue, pharynx and esophagus was multifocally eroded and moderately to severely hyperplastic, mildly disorganized and infiltrated by a variable amount of macrophages, lymphocytes and heterophils. A crust of degenerated heterophils, sloughed keratinocytes and necrotic debris sometimes covered the eroded epithelium, especially in the pharynx, and was colonized, in its superficial layers, by a numerous and mixed population of bacteria (Figure 2). Individual keratinocytes were necrotic and some multinucleated syncytial cells were present, sometimes necrotic also. Rare to numerous intranuclear eosinophilic inclusion bodies were seen in keratinocytes

and syncytial cells (Figures 3 and 4). The underlying lamina propria was moderately infiltrated by lymphocytes, plasma cells, macrophages and heterophils. Esophagal glands near the lesions often showed invasion and effacement of their epithelium by numerous lymphocytes. Also, many glands presented erosions and some of them, in the birds from case 2, demonstrated marked hyperplasia and immaturity with complete loss of mucus cells (Figure 5). The larynx and trachea of all birds presented typical ILT lesions, characterized by a hyperplastic, disorganized and superficially eroded epithelium, a mixed inflammation in the lamina propria and an abundant exudate in the lumen composed of red blood cells, mucus, heterophils, necrotic debris and sloughed epithelial cells, some of which were multinucleated and/or contained intranuclear eosinophilic inclusion bodies. No significant findings were present in other organs, except for a moderate and multifocal interstitial lymphoplasmacytic inflammation in the proventriculus of the bird of case 1.

Immunohistochemistry

Both cases were analyzed by IHC using an immunoperoxidase method (EnVision^{+} System HRP Labeled Polymer Anti-Mouse, Dako, Carpinteria, CA). Four-µm-thick paraffin sections were mounted on charged slides, deparaffinized and rehydrated by routine methods, followed by peroxidase inhibition by hydrogen peroxide. Antigen retrieval was performed using a pressure cooker and a citrate buffer solution (Diva Decloaker, Biocare Medical, Concord, CA) for 10 minutes. The slides were rinsed with TBS-Tween buffer and a casein-based blocking solution (Background Punisher, Biocare Medical, Concord, CA) was applied. Tissue sections were incubated with a mouse

monoclonal antibody (1:4000) directed against the ILT virus for 30 minutes at room temperature. Following incubation with the HRP-labeled anti-mouse polymer for 30 minutes at room temperature, color was developed with 3-amino-9-ethylcarbazole (AEC Substrate Chromogen, Ready-to-Use, Dako, Carpinteria, CA) and sections were counterstained with Mayer's Hematoxylin. Many superficial keratinocytes in the esophageal epithelium of both cases exhibited a positive reaction for ILTV, as well as sloughed and necrotic keratinocytes.

Microbiological and molecular tests

Bacteriology

Case 1.

Tissues from lungs and liver as well as swabs from the esophagus and pharynx were submitted to the Bacteriology Laboratory of the LEAQ for culture and identification. Columbia blood agar and MacConkey agar plates were inoculated and incubated for 48hrs at 35 ± 2°C. The Columbia blood agar plates were incubated with 5% CO_2.

Only mild to high growth of contaminants were isolated. Selective media (RAPPAPORT-VASSILIADIS broth, brilliant green with novobiocin agar plates and xylose lysine agar Tergitol 4 plates) were used for *Salmonella* detection on swabs from esophagus and pharyngeal plaques. All were negative. Direct smear examination of the plaques showed a mixture of Gram negative and Gram positive bacillus bacteria. There was no evidence of yeasts or protozoans.

Case 2.

Liver and spleen tissues were tested for general and specific bacterial growth as in case 1. Few non-hemolytic *E. coli* (1+), *Mannheimia haemolytica* and *Staphyloccocus spp*. were cultured from spleen tissues. Sinuses culture for *Mycoplasma spp.* was positive.

Molecular biology

Case 1

Tissues were submitted to the Molecular diagnostic laboratory of the Faculté de médicine vétérinaire (Université de Montréal) for PCR testing. Briefly, viral genome was extracted from tissues homogenates (pooled lung and trachea, pharynx lesions as well as pharyngeal swabs) using the QIAamp cador Pathogen Mini Kit with the Qiacube apparatus following the manufacturer instructions. Thereafter, two molecular PCR methods were conducted to confirm the presence of GaHV-1 in tissues (lung/trachea and pharynx). The first molecular method used was a GHV-1 specific real-time PCR assay (qPCR) as previously described by Callison et al. (2007) (4). Briefly, this qPCR used the following set of primers and probe: ILTV-F: 5'- CCTTGCGTTTGAATTTTTCTGT-3'; ILTV-R: 5'-TTCGTGGGTTAGAGGTCTGT-3'; and ILTV-S: 5'-(6-FAM) CAGCTCGGTGACCCCATTCTA (BHQ1)-3'. The second molecular method used was a pan-herpesvirus nested PCR assay (nPCR), as previously described (23). The nPCR assay was designed to target the herpesviruses DNA polymerase (DPOL) gene and is able to detect a broad range of herpesvirus species. The herpesvirus PCR product was

subsequently sequenced to confirm its genomic relationship to GaHV-1 as previously described (1). An amplicon of 210 nucleotides (nt) in length was generated and submitted to GenBank Basic Local Alignment Search Tool (BLAST) for comparison. Interestingly, both PCR methods were positive for GaHV-1 on all tested samples and the sequence analysis of the nPCR amplicon indicated that the nt sequence was 94% identical to several GaHV-1 wildtype and vaccine strains such as the US strain 1874C5; Genbank accession number JN542533.

Mycoplasma gallisepticum, *M. meleagridis*, *M. synoviae*, Avian poxvirus, *Trichomonas gallinae*, type A avian influenza virus, infectious bronchitis virus and avian Paramyxovirus type 1 were tested by PCR on chosen tissues and results were negative.

Case 2

Paraffin-embedded sections of esophagus were processed using an adapted protocol from Innis et al., 1990 (15). The GaHV-1 specific qPCR was performed on this tissue and was positive. When these birds were submitted in 2006, selected swabs were processed with PCR assays to detect the presence of Type A avian influenza virus and *Mycoplasma spp.* with the same protocols as in case 1. PCR for avian influenza on a pool of cloaca and pharyngeal swabs was negative. PCR for *Mycoplasma spp*. on sinuses swabs was positive and sequencing of the DNA revealed 98% homology with *M. gallinarum*.

Virus isolation

Specific-pathogen-free 10-day-old embryonated chicken eggs were inoculated with tissue homogenates from case 1 hen via the chorioallantoic sac for virus isolation as previously described (10). Presence of the GaHV-1 within the allantoic fluid collected at the second, third and/or fourth passage was tested by electron microscopy and qPCR assays. Unfortunately, GaHV-1 isolation attempts from qPCR positive tissues were unsuccessful.

Parasitology

Wisconsin testing was performed on feces from case 1 bird and pooled feces of case 2 birds. Large numbers of coccidia were detected (semi-quantitatively evaluated as 3+) in case 1 and a lesser amount in case 2 (graded 1+). In addition, 28 eggs (case 1) and >100 eggs per 5 grams of feces (case 2) of Heterakis spp. and/or Ascaridia were observed. Zinc sulfate testing on a scraping of the pharyngeal mucosa of case 1 bird was done to try to detect *Capillaria* parasites and was negative.

Discussion

Upper gastrointestinal lesions caused by ILTV have never been described before except in one of the first papers written on this disease in 1931. The authors then reported, in a large proportion of experimentally inoculated birds, small thin white patches on the mouth mucosa, microscopically characterized by necrosis of the epithelium. However, they haven't seen inclusion bodies in these lesions and they finally concluded this lesion was not characteristic ~~non-specific~~ of ILTV because it could have been caused by other diseases and dismissed it in the lesions description (21). Rare cases of ILT with mild pharyngeal and esophageal erosions and typical intranuclear inclusions have been

sporadically observed in our laboratories but never with macroscopic lesions such as in these cases.

Tropism of ILTV for the digestive tract is questioned. This virus is known to gain entry and replicate in the conjunctiva and the epithelium of the upper respiratory tract, then establishing a lifelong latency in the trigeminal ganglions and trachea (9). An experimentally induced infection has since demonstrated that this virus can be detected by quantitative PCR in other tissues such as throat, lung, trachea, cecum, kidney, pancreas and, interestingly, esophagus (24). ILTV genome has also been detected by qPCR (Taqman®) in the conjunctiva, sinuses, trachea, trigeminal ganglia, cecal tonsils, thymus and cloaca in all chickens that were vaccinated with a live modified virus or after infection by a field strain in another study in the United-States, hence demonstrating that the virus could infect other tissues than initially thought (17). However, none of these papers reported lesions in other locations than the upper respiratory tract or had done IHC to identify the virus *in situ* in other locations. Histopathology was done along with PCR in another study on naturally infected birds, but they observed inclusion bodies and detected ILTV DNA by PCR only in the larynx, trachea and conjunctiva. Liver, spleen, intestines, air sacs, respiratory sinus, bronchi and lungs were all negative (22). This is the first time that ILTV is detected by PCR and/or by IHC in the upper digestive tract of a bird, in association with compatible microscopic lesions, thus confirming that the virus, under certain conditions, could not only infect, but also replicate in and damage other types of cells than the conjunctival and respiratory epitheliums.

Atypical lesions in the esophagus and pharynx with concomitant typical ILT lesions in the larynx of these birds could raise the hypothesis that they were co-infected by two different viruses, ILTV (as demonstrated by PCR and sequencing in case 1) and another herpesvirus that could have been undetected by the precedent methods. In fact, other *Alphaherpesvirinae*, such as the duck enteritis virus (duck plague) and pigeon herpesvirus (*Columbid herpesvirus* 1) have the potential to invade other epithelium than the respiratory one. Linear ulcers in the esophagus are typical of duck enteritis (5, 20). Pigeons severely infected by *Columbid herpesvirus* 1 show small ulcers or foci of necrosis that covers the mouth, pharynx and larynx (16). This herpesvirus not only infects pigeons but has also been demonstrated to cause lesions in falcons and owls, as well as in psittacine birds (6, 11). Most pigeons are asymptomatic carriers of this herpesvirus and reactivate it from time to time. However, the design of the pan-herpesvirus nested PCR assay would have allowed the detection of *Columbid herpesvirus* 1 but sequence analyses of PCR amplicons confirmed instead the presence of GaHV-1only.

Concomitant diseases or conditions could have favored the development of these lesions in the esophagus. History of these cases was minimal and, to our knowledge, no feed analysis was performed to analyze the vitamin content or the presence of mycotoxins in those cases. It is however difficult to dismiss a possible subclinical hypovitaminosis A. Vitamin A contributes to epithelial integrity and deficient levels leads to the development of squamous metaplasia of, among others, the submucosal glands and ducts of the pharynx and esophageal mucosa with characteristic raised, ring-shaped gross lesions around the opening of the glands (19). Such ring-shaped lesions were observed in the

upper third of the esophagus of the case 1 bird but typical microscopic lesions were not present. However, we cannot completely exclude the possibility that mild squamous metaplasia could have been obscured by the viral-induced necrosis and inflammation in some glands. In case 2, changes seen in the eosophageal glands were even more ambiguous as hyperplasia and goblet cells loss gave a pseudostratified aspect to the glands, but without any evidence of keratinization. In the absence of vitamin A dosage in tissues or feed, we cannot confirm nor invalidate this hypothesis. Another possibility to consider is the presence of mycotoxins such as T2-toxin in the feed, which was been reported not only for inducing proliferative esophagitis proventriculitis in ducks (13) but also buccal mucosa necrosis (19). Again, there was no analysis to confirm this hypothesis.

Salmonella infection has been shown to cause ulcerative lesions similar to those reported here in the upper alimentary tract of birds, (8, 12). *Salmonella* was not detected in the case 1 bird, neither by routine MacConkey agar nor by specific enriched broth and was not tested in case 2, as the tissues were not available anymore at the time of the study. We could speculate on whether this is a truthful absence of *Salmonella* in case 1 or that its presence is hidden by the bacterial contamination of the esophagus. In fact, the normal pharyngeal bacterial flora could certainly have contributed to worsen pre-existing mild viral lesions, as it was concluded in the first article to report these lesions in ILTV-infected chickens (21).

We could not exclude the possibility that immunosuppression might also have contributed to some extent in this unusual manifestation of the disease. In fact, the case 1 bird had a low body score index and was moderately infected with coccidia. Its bursa of Fabricius and thymus were physiologically atrophied due to age thus its immunoresponsiveness was difficult to assess. However, birds from case 2 were all in good body condition and their lymphoid tissues appeared histologically normal, suggesting there was no relationship between the occurrence of these atypical lesions and previous immunosuppression

This report presents two, non-related cases of backyard chickens with typical ILT laryngitis as well as unusual necrotic and proliferative lesions associated with ILTV in the esophagus and pharynx. Veterinarians should be aware that this unusual manifestation of ILT might be confused with other upper digestive system diseases such as salmonellosis, hypovitaminosis A, parasites such as *Trichomonas*, *Capillaria* or *Ascaridia* and mycotoxins (T2-toxin) intoxication, thus highlighting the importance of using molecular diagnosis tools along with histopathology to confirm such cases. This was particularly true for the bird from case 1, in which the pharyngeal and esophageal lesions were far more obvious than the laryngeal lesions, which were mild and non specific and could have easily been missed by an unacquainted observer. ILTV can propagate rapidly among fowls in auctions or in commercial barns and these atypical lesions could be misdiagnosed, delaying biosecurity and sanitary measures to confine the outbreak with potential severe consequences for the local poultry industry.

Short abstract

Atypical upper gastrointestinal lesions can be observed with infectious laryngotracheitis. Given the contagious nature of this disease, diagnosis or delayed diagnosis might have important financial implications for the poultry industry.

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