



A five-year surveillance study of vaccination schedules using viral-vectored vaccines against infectious laryngotracheitis in a high-density layer region¹

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ABSTRACT.- Santos W.H.M., de Oliveira L.B., Leão P.A., Hergot I.G., Wenceslau R.R., da Rocha C.M.B.M., Ferreira H.L., Resende M., Martins N.R.S., Spatz S.J. & Ecco R. 2022. **A five-year surveillance study of vaccination schedules using viral-vectored vaccines against infectious laryngotracheitis in a high-density layer region.** *Pesquisa Veterinária Brasileira* 42:e07037, 2022. Departamento de Clínica e Cirurgia, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG 31270-901, Brazil. E-mail: ecco@vet.ufmg.br

The effectiveness of vectored recombinant vaccines to control infectious laryngotracheitis (ILT) in chickens from a region (State of Minas Gerais, Brazil) with ~10 million layers was evaluated under field conditions from 2014-2018. During this period, only recombinant turkey herpesvirus (rHVT) or fowl poxvirus (rFPV) vaccines that express antigens of infectious laryngotracheitis virus (*Gallid herpesvirus-1*; GaHV-1) were used. Layer chickens (n=1,283), from eight different egg-producing companies, were individually sampled and examined (active surveillance), and in instances when government poultry health veterinarians were notified due to respiratory disease (passive surveillance). Clinical, macroscopic, and histopathology examinations were performed to diagnose ILT as well as molecular techniques for the detection and characterization of the GaHV-1 DNA from the trachea and trigeminal ganglia (TG). The layer hens sampled and examined belonged to flocks and farms that used different vaccination protocols (non-vaccinated, single dose vaccination, and prime/boost vaccination). This is the first long-term field study of the effectiveness of ILT vectored vaccines in a high-density multiple age layer hen region. Using various diagnostic methods, the occurrence of GaHV-1 infection and ILT clinical disease in layer hens vaccinated with vectored recombinant vaccines in one quarantined region of Brazil were investigated. The number of ILTV positive chickens by PCR and ILT clinical disease cases was lower in farms when all chickens were vaccinated with at least one vaccine. However, the difference in the

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detection rates of GaHV-1 infection was significant only when compared farms with prime/boost and farms using single dose of HTV-LT.

INDEX TERMS: Vaccination, vectored vaccine, laryngotracheitis, layer chickens, *Gallid herpesvirus 1*, histopathology, PCR, sequencing.

RESUMO.- [Cinco anos de monitoramento de esquemas vacinais com vacinas virais vetorizadas contra laringotraqueíte infecciosa em uma região altamente povoada por galinhas poedeiras.] A efetividade das vacinas recombinantes vetorizadas para o controle da laringotraqueíte infecciosa (LTI) nas aves de uma região (Minas Gerais, Brasil) com aproximadamente 10 milhões de poedeiras foi avaliada em condições de campo, no período de 2014 a 2018. Durante este período, somente as vacinas recombinantes “turkey herpesvirus” (rHVT) ou “fowl poxvirus” (rFPV), que expressam antígenos do vírus da laringotraqueíte (*Gallid herpesvirus-1*; GaHV-1) foram utilizadas. Galinhas poedeiras (n=1.283), de oito diferentes granjas produtoras de ovos, foram individualmente amostradas e examinadas por monitoramento ativo e, na ocorrência de notificação de doença respiratória aos veterinários do serviço oficial, por monitoramento passivo. Exames clínicos, macroscópicos e histopatológicos foram realizados para o diagnóstico de LTI, bem como técnicas moleculares para a detecção e caracterização do DNA de GaHV-1 da traqueia e gânglio trigêmeo. As galinhas poedeiras pertenciam a lotes e granjas que usavam diferentes protocolos de vacinação (não vacinadas, uma dose ou tipo de vacina e duas doses ou tipos de vacina). Este é o primeiro longo estudo a campo sobre a efetividade das vacinas vetorizadas em uma região com população elevada de poedeiras de múltiplas idades. Utilizando vários métodos de diagnóstico, a ocorrência da infecção por GaHV-1 e a LTI clínica em poedeiras de uma região interdita do Brasil foi investigada. O número de galinhas positivas para o vírus GaHV-1 e para casos clínicos de LTI nas granjas foi menor quando todas as aves estavam vacinadas com, pelo menos, um tipo ou dose de vacina. Entretanto, a diferença na taxa de detecção da infecção por GaHV-1 foi significativa somente quando a comparação foi realizada entre granjas com aves vacinadas com duas doses e aves de granjas vacinadas com uma única dose de HVT-LT.

TERMOS DE INDEXAÇÃO: Vacinação, vacinas vetorizadas, laringotraqueíte, galinhas poedeiras, *Gallid herpesvirus 1*, histopatologia, PCR, sequenciamento.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a highly contagious viral respiratory disease, which affects layer and broiler chickens of all ages (Guy & García 2008). However, chickens older than three-week-old are more susceptible (Fahey et al. 1983). The disease has also been reported in other avian species such as peafowl, pheasants (Guy & García 2008), and turkeys (Portz et al. 2008). ILT is caused by *Gallid herpesvirus - 1* (GaHV-1), belonging to the subfamily Alphaherpesvirinae of family Herpesviridae (ICTV 2011). This disease is associated with significant economic losses because of high morbidity, increased mortality, decreased growth rates, reduced egg production, and secondary respiratory infections (Guy & García 2008). The major control measures for this disease include the

combination of biosecurity measures and vaccination (Chin et al. 2009). Both virulent field and live attenuated vaccine strains of GaHV-1 can establish latent infections within the trigeminal ganglia of chickens (Bagust 1986, Hughes et al. 1991, Williams et al. 1992, Thilakarathne et al. 2019). Latency is a distinct characteristic of herpesviruses, which makes ILT difficult to control in layers and breeder type chickens (Bagust 1986, Guy & García 2008). Two types of live GaHV-1 vaccines have been used in different countries to control ILT. Live vaccines have been attenuated by sequential passages in embryonated eggs (chicken embryo origin - CEO) or tissue culture (tissue culture origin - TCO) (Guy & García 2008). A mild form of ILT (so-called vaccinal ILT) with low morbidity and low mortality (0.1 to 2.0%) has been reported and associated with live-attenuated vaccines (Sellers et al. 2004). Various studies have demonstrated that live-attenuated vaccine strains can become more virulent as a result of simple bird-to-bird passage (Guy et al. 1991). Compounding this is the fact that live ILTV vaccine strains can also establish a life long latent infection (Hughes et al. 1991). Both virulent field and live attenuated vaccine strains of GaHV-1 can establish latent infections within the trigeminal ganglia of chickens (Bagust 1986, Hughes et al. 1991, Williams et al. 1992, Thilakarathne et al. 2019). Consequently, the latent vaccine-derived virus can reactivate so that a flock can become a source of infection with continuous outbreaks of disease. Because of this and the use of high-density poultry housing with infrequent environmental cleaning, there is a continuous virus reservoir (both vaccine and field strains) in the flocks capable of recombination and evolving to newer highly virulent strains (Loncoman et al. 2017). Recombination events between different live attenuated vaccine strains have also been reported (Lee et al. 2012). Because of these shortcomings, viral-vectored recombinant vaccines expressing infectious laryngotracheitis virus (ILTV) proteins were developed and used in the field. Three viral vectors have been used to construct recombinants, fowl poxvirus (FPV) (Vagnozzi et al. 2012), turkey herpesvirus (HVT) (Saif 1994) and lentogenic Newcastle disease virus (Sun et al. 2008, Zhao et al. 2014).

GaHV-1 was first detected in Brazil in 1970, on layer farms from the State of Rio de Janeiro (Hipólito et al. 1974). Outbreaks of the disease remained unreported for several years. Then in 2002, an outbreak was reported on 182-layer chicken farms in the state of São Paulo, resulting in significant economic losses (Chacón et al. 2007). The viruses were molecularly characterized as both field and vaccine strains (Chacón & Ferreira 2009). From 2002 to 2005, periodic outbreaks occurred on farms in South Brazil (Beltrão et al. 2004) and other regions in the São Paulo State. Noteworthy, GaHV-1 was also detected in turkeys on farms in the southern Brazilian states (Portz et al. 2008). In November 2010, an outbreak was reported in the state of Minas Gerais (region of this study), affecting 27 layer-farms populated with approximately ten million chickens (Preis et al. 2013). This warranted mandatory outbreak management and quarantine measures. In August

2011, two recombinant vaccines (HVT-LT and FPV-LT) were licensed and used to immunized layers in this affected region, and no live-attenuated vaccines were authorized for use in the State of Minas Gerais (Couto et al. 2015).

ILT continues to be a problem in Brazil and according to an OIE (Office International des Epizooties - World Organization for Animal Health) report, ILT, as of 2018, was present in more than one region. Large field studies on the effectiveness of viral-vectored recombinant vaccines in controlling ILT outbreaks have not been previously reported. Here, a surveillance study on the effectiveness of viral-vectored recombinant vaccines against ILT in high-density (eight million) multiple-age layer hens from a single quarantined region of Brazil was performed. During this long-term monitoring study from 2014 to 2018, various methods were used to investigate the occurrence of GaHV-1 infection and ILT clinical disease in layer hens vaccinated with either FPV or HVT-based recombinant vaccines using different immunization protocols.

MATERIALS AND METHODS

Geographic location and characterization of the farms and chickens. In November 2010 an outbreak of ILT was diagnosed in layer-type chickens in a region of Minas Gerais, Brazil. Following this outbreak, the area was placed in quarantine by the veterinary service of the “Instituto Mineiro Agropecuário” from the state of Minas Gerais (Agricultural and Livestock Institute - IMA). Depopulation was not conducted on these farms. This large region with 24 commercial layer-type farms was classified as a single epidemiologic unit with viral circulation, due to the proximity of the farms and the

epidemiological temporal linkage of the recurring outbreaks. The immunization using the recombinant vaccines started in 2011 for vaccinated farms, and by the end of 2013 all layers were vaccinated. The vaccines were administered at hatchery and at four to five week-age (subcutaneously), explaining the long time needed to have all layers vaccinated in these farms.

Poultry farms information were collected during the technical visits by local poultry association in the quarantine region. Distances between farms and their geographic location were determined using Google Maps® (Fig.1). The quarantine region comprised 12 egg-producing companies within four neighboring municipalities. Several of these companies have more than one poultry operating centers (totaling 27 at the beginning of the field study and reduced to 24 at the end). The chicken populations ranged from 40,000 to 3,586,000 layer-type chickens on each farm. The smallest farm had three sheds and the largest had 34 sheds. Layers were housed in battery cages with manual egg collection or with automatic egg collection system, which varied from simple two tiers layer cages to 4-6 automatized-ventilated tiers layer cages.

The biosecurity measures implemented at the start of the outbreak (Hergot et al. 2021) varied among the farms, as did the immunization protocols using either HVT or FPV based ILTV recombinant vaccines. Chickens were initially vaccinated in 2011 and sampled for diagnostic purposes thereafter (Fig.2).

Three farms had natural barriers (i.e. trees and shrubs) around the perimeter of the sheds, three had partial hedges around the perimeter of the sheds, and two did not have any fences. The distances between farms ranged from 0.03-1 kilometer. Production sheds were less than 0.45km from a highway or urban area (Table 1). The biosecurity measures implemented by each farm were evaluated in all technical visits. These measures included high secluded of farms, carcasses disposal, traffic and visits, pest control and access of other animals to the sheds, poultry density, cleaning and disinfection of the sheds, compliance with the prohibition of forced molting, and vaccination practices.

Sampling of active surveillance. Selection of the farms and sample size were defined based on the geographical location of production units. Farms were considered a productive unit (poultry operation unit) when they had the same workers and managers and similar chicken management procedures. The farm or the production unit

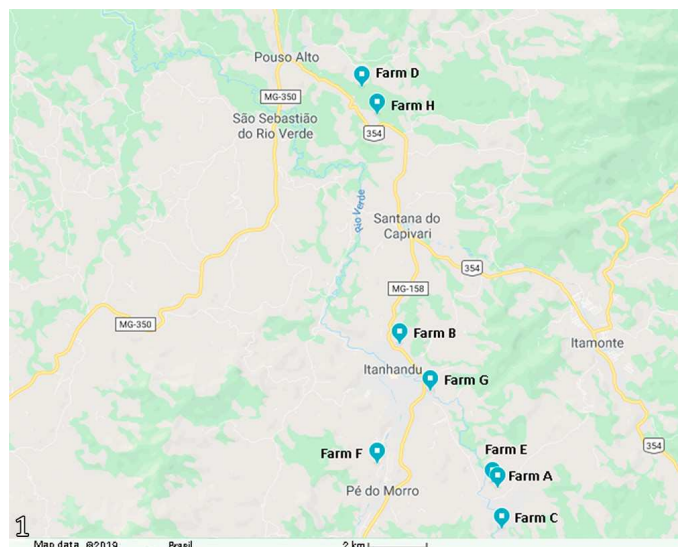


Fig.1. Geographical location of farms: adapted from Google Maps®. Scale bar = 2km. Farms: A = non-vaccinated, B = vaccinated twice with both turkey herpesvirus (HVT-LT) and fowl poxvirus (FPV-LT), C = FPV-LT, D = FPV-LT, E = vaccinated twice, F = HVT-LT, G = HVT-LT, H = non-vaccinated. The central municipality (Itanhandu) is located at -22.301794 South, -44.932864 West, and the region is characterized by hills, with peaks of 1,300m to 2,357m in altitude.¹⁰

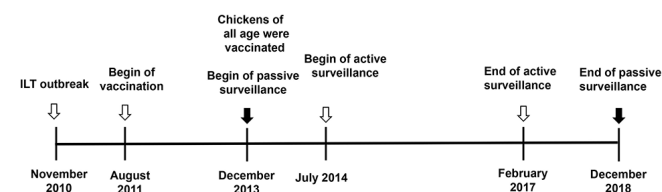


Fig.2. Timeline of study scenario. ILT = infectious laryngotracheitis. Outbreak = ILT outbreak of State of Minas Gerais, Brazil. Vectored vaccination beginning: turkey herpesvirus (HVT-LT) and/or fowl poxvirus (FPV-LT) vaccines. Farms with different vaccination programs (no vaccination, a single dose of FPV-LT, a single dose of HVT-LT, or prime/boost vaccination - HVT-LT at hatchery and FPV-LT at 21-35 days of age) were sampled by active surveillance (programmed visits for clinical examination and sample collection) and passive surveillance (notification of respiratory disease and veterinarian from Official Veterinary Services (SVO) collected samples from animals and submitted them for analysis).

¹⁰ Available at <https://en.wikipedia.org/wiki/Mantiqueira_Mountains> Accessed on Sep. 18, 2019.

was defined as a sampling unit. The production unit with the highest sanitary risk in terms of age, origin/breed diversity, and biosecurity was selected when farms had more than one unit.

From June 2014 to February 2017, 962 chickens were sampled from eight different farms (Fig.2). Technical visits and sampling occurred twice per year, during the Summer and Winter months. The week-long sampling was performed according to the mandatory active surveillance, accompanied by employees from the Official Veterinary Services (SVO) of the IMA.

The sampling and criteria for sampling was design as follows: the type of vaccination, location of farms, density in house-population, and season. The immunization criterion for the sampling of chickens varied according to whether they remained non-vaccinated or vaccinated: two farms without vaccination; two farms with single dose of HVT-LT vaccination (subcutaneously at hatchery); two farms with single dose of FPV-LT (membrane wing at 21-35 days of age); and two farms with two doses of vaccination (HVT-LT at hatchery and FPV-LT at 21-35 days of age). Therefore, the two farms that did not vaccinate were considered the negative control farms. The vectored vaccines used in these farms were: 1. HVT-LT, a recombinant turkey herpesvirus (HVT) that expresses glycoproteins D and I (gD and gI) of GaHV-1 (Gimeno et al. 2011), and 2. FPV-LT, a recombinant fowlpox virus (FPV) that expresses glycoprotein B (gB) and UL32 gene product of GaHV-1 (Davison et al. 2006, Coppo et al. 2013).

The number of chickens per farm was scrutinized with respect to the proximity of the farms and their sheds, and the high density of chickens. Eight farms were selected and designated A-H. Concerning the epidemiological parameters, ethical and economical limitations were also taken into account and the standard for official surveillance of “Ministério da Agricultura, Pecuária e Abastecimento” (Ministry of Agriculture and Livestock and Supply - MAPA) was followed. At each farm, flocks were selected according to their production phase. Nine chickens were collected per phase. Samples were collected from each chosen flock/shed during laying peak (25-60 weeks) and at laying end (>60 weeks of age), comprising a total of 18 chickens per simultaneous sampling (Farms A-D and F-H). In the farm with the highest density (Farm E), nine chickens were collected at the beginning, middle, and end of production, totaling 27 chickens (Table 2). Flocks presenting respiratory clinical signs were preferentially tested. Random selection of chickens was performed in the absence of clinical signs. Two additional sample sets (flocks) were sampled from younger flocks because clinical signs of respiratory disease were observed. These samples were from two largest farms

and consisted of six immatures growing layers (<18 weeks of age) and nine chickens before the laying peak (<24 weeks).

The layers were firstly examined for respiratory clinical signs of disease, euthanized by cervical dislocation, then necropsied for macroscopic examination, and sample collection. During these times, number of chickens in the sampled flocks, their age, date of the onset of clinical disease, and if any, mortality rate and type of observed clinical signs were recorded. Information about management and biosecurity measures were also collected.

Gross and histopathology. The macroscopic evaluation for lesions on organs was performed at necropsy. The larynx, proximal and distal part of the trachea, lungs, conjunctivae, and nasal concha (turbinates) were fixed in 10% neutral buffered formalin for 48 to 52 hours. Subsequently, the tissues were cleaved and processed in an increasing series of alcohol, xylene, paraffin embedded, sectioned at 4µm thickness, and stained with hematoxylin and eosin (HE). Tissues were examined under light microscopy for the presence or absence of lesions. A detailed description of the histological lesions was performed for all tissues by a trained pathologist to characterize the lesions and associated them with the etiology. Lesions in the respiratory mucosae and conjunctivae consistent with ILT were characterized as previously described (Hayashi et al. 1985, Guy et al. 1990, Preis et al. 2013). Morphologic diagnoses including distribution and intensity of the lesions was performed to each altered tissue. For the diagnosis of other respiratory infectious diseases, chickens were examined for mycoplasmosis, infectious bronchitis, infectious coryza, respiratory colibacillosis, and diphtheritic fowlpox. Immunohistochemistry was used to identify samples for mycoplasmosis (Casagrande et al. 2014). Good pasture special stain (Gram histologic stain) was also used to detect intralosomal bacteria, and in the absence of ILT typical lesions, the histologic characterization indicated for the further investigation of the etiology, as routinely performed in a pathology laboratory for avian diagnosis.

Sampling for passive surveillance. From June of 2014 to December 2018, passive surveillance of the quarantine region was also carried out by the SVO from IMA. The SVO were notified, if caretakers observed any respiratory signs, and performed visits to the farms for necropsy and sample collections. Farms that had respiratory diseases and notified SVO were Farms B, E and F. Six to ten sick chickens were sampled (total=321 chickens) from each flock with respiratory distress. Relevant data based on a questionnaire (see above) were also recorded. Samples of the conjunctivae, nasal

Table 1. Sampled farms characteristics

Farm	A	B	C	D	E	F	G	H
Vaccination protocol	Non-vaccinated	Prime/boost (HVT-LT and FPV-LT)	FPV-LT	FPV-LT	Prime/boost (HVT-LT and FPV-LT)	HVT-LT	HVT-LT	Non-vaccinated*
Housing capacity	40,000	558,000	200,000	199,000	3,586,000	684,000	370,000	150,000
Number of hens per production unit	1	12	9	5	34	13	17	2
Number of chickens per houses	6	13	16	10	34	24	17	3
Vegetation barrier	Absent	Present	Absent	Present	Partial	Partial	Partial	Present
Distance to the nearest farm	30 m	440 m	500 m	1000 m	30 m	420 m	850 m	1000 m
Distance to the nearest road or urban area	131 m	195 m	100 m	332 m	170 m	435 m	300 m	175 m

* One of these farms was deactivated after the second sampling, thereafter remaining only one farm without vaccination.

conchae, larynx, trachea, lungs, and air sacs were fixed in 10% neutral buffered formalin and sent for histopathological evaluation in the Veterinary Pathology Sector of the “Escola de Veterinária” (Veterinary School) at “Universidade Federal de Minas Gerais” (UFMG).

Detection of GaHV-1 in tracheal and TG samples by polymerase chain reaction (PCR). Tracheal (medial portion) and TG samples (Fig.3-4) were individually collected from all chickens, stored in sterile microtubes, and frozen at -20°C for subsequent detection of GaHV-1 DNA using the polymerase chain reaction (PCR). As an endogenous DNA extraction quality control, all samples that were negative for the ICP4 gene of GaHV-1 were also tested for the β -actin gene (Li et al. 2005).

For DNA extraction, the tracheal samples were individually opened in individual petri dishes, the mucosa was scraped using a sterile surgical steel blade, and transferred into DNA/RNase-free microtubes. The TG samples, after transferred into sterile DNA/RNase free microtubes, were ground with an individual mortar and pestle (Couto et al. 2015). Thereafter, mucosal scraping of the trachea or macerated TG were mixed with three volumes of 6M sodium iodide for

cell lysis and release of the total DNA and adsorption to silica particles (Vogelstein & Gillespie 1979, Couto et al. 2015). The oligonucleotides that were used ICP4-1F (5'-CCTTGGTTCCGGATGAAACC-3') and ICP4-1R (5'-TTCATTACCTCCAGCGGTTC-3') were based on a report by Preis et al. (2013). The amplification reactions were done in a volume of 25µl containing 12.5µl of master mix (PCR Master Mix, Promega, Madison/WI, USA), 1.5µl of each primer and 2 microliters of total DNA (approx. 200ng). The positive control was obtained from pooled field tracheal samples that were previously confirmed by PCR-sequencing to contain GaHV-1 DNA (Couto et al. 2015).

Molecular characterization. GaHV-1 was characterized in five biological samples using three amplicons, representing two regions in the ICP4 genes and one in the thymidine kinase (TK) gene of GaHV-1, and sequenced as previously described (Couto et al. 2015). Sequences were evaluated for nucleotide insertion and deletions (INDELs); and single nucleotide polymorphisms. Samples were selected for sequencing based on the criteria of vaccination (two doses or single dose vaccination), passive or active surveillance,

Table 2. Sample size of active surveillance per farms, year, age and vaccination type

Farm	June 2014		February 2015		August 2015		February 2016		August 2016		February 2017		Total
	N	Age/VT*	N	Age/VT	N	Age/VT	N	Age/VT	N	Age/VT	N	Age/VT	
A	9	36/NV	9	47/NV	-	-	-	-	-	-	-	-	45
	9	56/ NV	9	107/NV	-	-	-	-	-	-	-	-	
	9	116/ NV											
B	9	32/HVT-LT	9	25/Both vaccines	9	96/Both vaccines	9	70/Both vaccines	9	38/HVT-LT	9	33/HVT-LT	152
	9	18/Both vaccines	9	68/Both vaccines	9	43/Both vaccines	9	27/HVT-LT	10	54/HVT-LT	9	79/HVT-LT	
									15	35/HVT-LT	8	9/Both vaccines	
									12	15/HVT-LT	8	13/Both vaccines	
C	9	38/FPV-LT	9	35/FPV-LT	9	38/FPV-LT	9	55/FPV-LT	9	30/FPV-LT	9	20/FPV-LT	108
	9	71/FPV-LT	9	83/FPV-LT	9	82/FPV-LT	9	65/FPV-LT	9	88/FPV-LT	9	73/FPV-LT	
D	9	36/FPV-LT	9	29/FPV-LT	9	36/FPV-LT	9	46/FPV-LT	9	40/FPV-LT	9	31/FPV-LT	110
	9	58/FPV-LT	9	93/FPV-LT	9	85/FPV-LT	9	96/FPV-LT	9	89/FPV-LT	9	97/FPV-LT	
									2	72/FPV-LT			
E	18	33/Both vaccines	9	35/Both vaccines	9	45/HVT-LT	9	34/HVT-LT	9	26/HVT-LT	9	38/HVT-LT	176
	18	60/Both vaccines	9	65/Both vaccines	9	65/Both vaccines	9	43/HVT-LT	12	34/HVT-LT	9	25/HVT-LT	
			9	89/Both vaccines	9	30/HVT-LT	9	81/HVT-LT	9	84/HVT-LT	9	86/HVT-LT	
									2	13/HVT-LT			
F	9	41/HVT-LT	9	30/FPV-LT	6	19/HVT-LT	9	28/HVT-LT	9	43/HVT-LT	9	39/HVT-LT	126
	9	88/HVT-LT	9	90/FPV-LT	9	34/HVT-LT	9	88/HVT-LT	9	88/HVT-LT	8	97/HVT-LT	
					9	87/HVT-LT	3	21/HVT-LT	6	36/HVT-LT	4	19/Both vaccines	
G	9	38/HVT-LT	9	34/HVT-LT	9	34/HVT-LT	9	37/HVT-LT	9	34/HVT-LT	9	38/HVT-LT	108
	9	85/HVT-LT	9	73/HVT-LT	9	87/HVT-LT	9	81/HVT-LT	9	79/FPV-LT	9	80/HVT-LT	
H	9	50/NV	9	44/NV	9	40/NV	9	75/NV	13	88/NV	10	109/NV	137
	9	67/NV	9	98/NV	9	70/NV	9	95/NV	14	98/NV	10	20/NV	
					9	49/NV	9	66/NV					
TOTAL	171		153		150		147		185		156		962

N = number of chickens sampled, VT = vaccine type, HVT-LT = turkey herpesvirus, FPV-LT = fowl poxvirus, Both vaccines = HVT-LT and FPV-LT, NV = non-vaccinated; * The ages of chickens are in months.

season, year, and DNA quality based on 280/280nm ratios. Three samples from active surveillance were collected from Farms E, F, and F during Summers in 2016, 2016, and 2017, respectively. Two additional samples from passive surveillance were selected from Farms F and B collected in Winter 2016 and 2018, respectively.

The obtained sequences were evaluated for quality using the Sequence Scanner™ Software 2 (Applied Biosystems, Foster City) and edited by MEGA7 (Kumar et al. 2016). Afterward, the obtained sequences and sequences available in GenBank were aligned using MAFFT (Katoh et al. 2005), Clustal W (Thompson et al. 1994) and Bioedit Sequence Alignment software version 7.2.5 (Hall 1999). Phylogenetic analysis using the concatenated sequences of a region of the TK gene and regions 1 and 2 of the ICP4 gene and tree construction were also performed using MAFFT and Geneious using the Maximum Likelihood method with a total of 1000 replications on the bootstrap. The sequence of strain 63140/C/08/BR (JN542536) was used as a reference for nucleotide numbering.

Laboratory tests were carried out at the Molecular Pathology Laboratory at the “Escola de Veterinária” (Veterinary School) of the “Universidade Federal de Minas Gerais” (EV-UFMG).

The nucleotide sequences of the PCR products were deposited in GenBank under the following accession numbers: MN643590-643594 for the TK gene, MN689091-689095 for the ICP4 gene (region 1) and MN689801-689805 for the ICP4 gene (region 2).

ELISA (enzyme-linked immunosorbent assay). At active surveillance, blood samples were collected from 158 and 153 chickens during the Winter of 2016 and the Summer of 2017, respectively, for ELISA testing. In the total, sera from 311 chickens were collected for serological examination using an ELISA kit. Those chickens were also examined by histopathology and by PCR and the results were correlated. Sera samples were analyzed using an indirect ELISA commercial test (CK124 ILT Biocheck®, The Netherlands) according to the manufacturer's instructions. Results were read on a Thermo Plate® TP-READER Microplate Reader at 405nm and analyzed using Biocheck II Diagnostic software®. Sera were considered positive if titers were higher than 1.071 at 1:500 dilution.

Statistical analysis of results. Absolute and relative frequencies of clinical signs, histological lesions, as well as, GaHV-1 DNA detection by PCR from trachea and TG tissues were described. Proportions of GaHV-1 DNA detection by PCR from the trachea and TG tissues were

tested between year, sampling periods, genetic lineages, age categories, farms and vaccine protocols by Chi-square or Fisher exact test when appropriate. A mixed logistic regression was applied to predict the probability of GaHV-1 DNA detection (PCR positivity by trachea or trigeminal ganglia). In the final model, sampling periods and vaccine protocols were modeled as fixed effects, but the farm was considered as a random effect (random intercept) to adjust repeated observations along time. The final model was chosen by AIC and study design. Standard errors, confidence intervals and odds ratios for coefficient estimates were obtained by bootstrap. The same model was applied to study vaccinated vs non-vaccinated for GaHV-1 DNA detection (PCR positivity). ELISA titer was compared among vaccination protocols by using Tukey test after adjustment of a linear mixed model considering titer as a function of farm and vaccine protocol. ELISA titer was transformed with logarithmic function for ANOVA but results were presented in the original scale. In this model, the farm was modeled as a random effect to account for repeated measures in the same local. Cohen's Kappa coefficient was made to measure the reliability between PCR positive chickens using TG and trachea samples. For all analyzes, a significant level equal to 5% was considered. The software R 4.0.2 (R Core Team 2020) was used in the analyzes.

Weather data. The average minimum and maximum temperatures during the periods of scheduled collections and at times of unscheduled collections were obtained from the website of the “Instituto Nacional de Meteorologia do Brasil” (National Institute of Meteorology - INMET) of the MAPA¹¹. These records were collected from the meteorological station of the municipality of São Lourenço/MG, the closest to the monitored region.

Ethics in the use of animals. The project was approved by the Committee on Ethics in the Use of Animals (CEUA) of the UFMG, protocol number 78/2014, along with the terms of consent of the owners or managers of the farms.

RESULTS

Sampling

During the study, some sampling conditions were changed. One of the non-vaccinated (negative control) farms was closed after the second sampling (Table 2). The two largest farms

¹¹ Available at <<http://www.inmet.gov.br>> Accessed on Jan. 20, 2019.

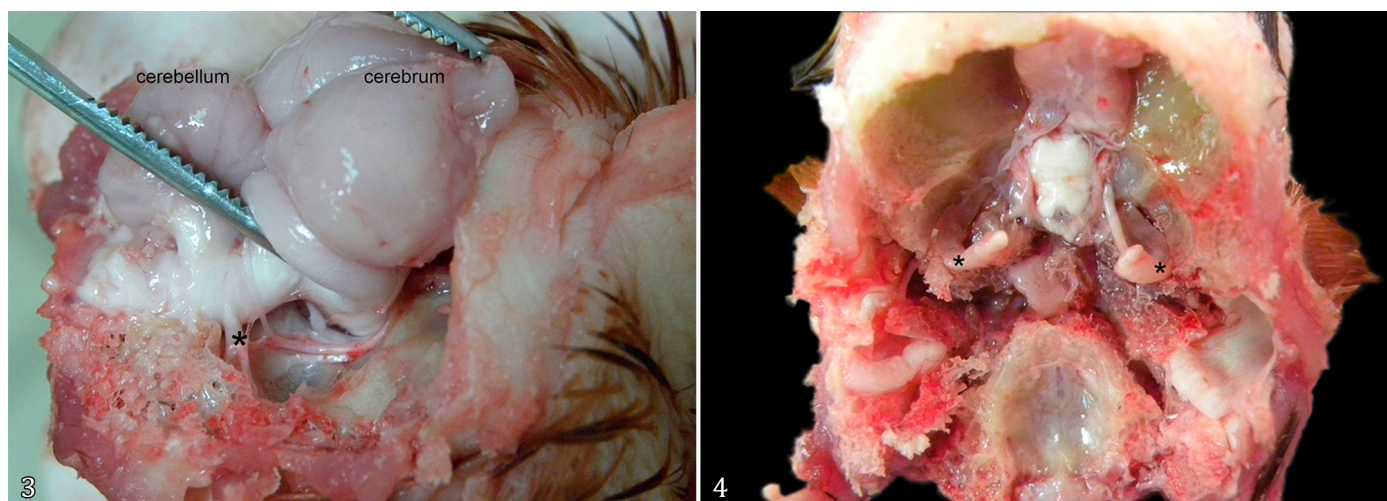


Fig.3-4. (3) Anatomic location of the trigeminal nerve (*). (4) Anatomic location of the trigeminal ganglia (*). The trigeminal ganglia were sampled from all chickens examined by active surveillance for DNA extraction to detect infectious laryngotracheitis virus (ILT) by PCR.

modified the vaccination protocol, and instead of the prime/boost vaccination (HVT-LT and FPV-LT) previously used, from the third sampling and onwards, adopted a single type vaccine, and only (HVT-LT) was maintained. As a consequence, at the sampling in the Summer and Winter of 2016, the available number of sampled chickens that received prime/boost vaccination decreased to nine and zero, respectively. These changes were unexpected and the surveillance was extended for three more seasons, to compare chickens that received two and single dose vaccination in two largest farms. During the five years of the study, a total of 1,283 chickens, collected from active (n=962) and passive (n=321) surveillance, were examined for signs of respiratory distress caused by GaHV-1 infection and/or for lesions consistent with ILT.

Active surveillance

Clinical, gross and histopathology results. Clinical signs and/or gross lesions were found in 118 (12.2%) chickens out of the 962 sampled chickens during active surveillance. Of these 118 chickens, only 64.4% had macroscopic lesions in organs of the respiratory tract. The majority of these changes represented different respiratory diseases. Chickens, especially pullets, from two additional farms presented respiratory clinical signs with mortality increase during the Winter of 2016. In these pullets, although the clinical signs were suggestive of ILT, the lesions of airsacculitis and necrotic bronchopneumonia were diagnosed as colibacillosis. Mortality rate ranged from low to medium for flocks sampled until Summer of 2016. After that, mortality rate in flocks with respiratory clinical signs varied from mild increase (0.8%) to moderate increase (1.5%-2.0%).

Respiratory tissue sections from 902 chickens obtained during the active surveillance program, were examined using histopathology (Table 3). Lesions consistent of ILT were found in only 0.6% of chickens sampled during the active surveillance. Mycoplasmosis was far more common and diagnosed in 19% of the chickens. Other diseases diagnosed in these chickens were coryza and colibacillosis characterized by pneumonia and airsacculitis. Nonspecific moderate infiltration of lymphocytes and plasma cells in the mucosa of trachea (probably related to the regular immunization for multiple respiratory agents) were found in chickens without macroscopic lesions and clinical signs of respiratory disease (69.9%). No lesions were found in the respiratory tissues of 8.5% of the chickens (77 out of 902).

GaHV-1 detection in the tracheal and TG samples. Primers specific for the ICP4 genes of GaHV-1 were used in

amplification reactions with DNA isolated from trachea and trigeminal ganglia of 962 chickens. The database for scheduled collections and all PCR results by year, season, farm and age are presented in Table 4. Overall, GaHV-1 DNA was detected in 28.8% of chickens using either trachea or TG DNA samples; specifically, 184 (19.1%) and 127 (13.4%) trachea and TG samples, respectively (Table 5). The proportions of positive chickens in TG and trachea were different. The Cohen's Kappa coefficient was 0.06 ($P=0.04$), a weak agreement when comparing PCR detection in trachea and TG samples, suggesting no correlation between these tissues. Also, GaHV-1 DNA detection in TG decreased gradually from 2015 to 2017 (Fig.5).

GaHV-1 detection according to collection period. All PCR results after the implementation of vaccination (August of 2011), from first sampling (Winter of 2014, June), to last sampling (Summer of 2017), can be seen in the Figure 5 and 6, and Table 4. When evaluating the results per year, from 2014 through to 2017, the proportion of positive and negative chickens were different comparing the years ($P<0.0001$). The highest detection rates using trachea and TG samples were obtained in Winter of 2014, with 56.4% of positives chickens. While the lowest detection rate of positive chickens was obtained in the Summer of 2016 (19.0%). This was the period when all chickens were vaccinated. Hence, a decrease in detection rates was observed when all chickens were vaccinated. However, a slight increase in detection of GaHV-1 was observed in the Winter of 2016, when only one dose of the viral-vectored vaccine was used on the farms instead of the using the two recombinants. This probably reflected in increasing of positive chickens observed in the results of trachea from the Summer of 2017.

GaHV-1 detection in chickens according to farm. There was a significant difference in the presence of GaHV-1 DNA among farms in the field study ($P=0.001$). This was not surprising since proximity to other farms, biosecurity, shed design, vaccine type, and vaccine regimen differed. Farm B and D had a lower proportion of positive animals than Farms C, E, F, G, and H (Table 4). The lowest GaHV-1 DNA detection rate was 15.4% and 18.4% in samples from Farms D and B, respectively. Farm D is furthest away from the nearest farm (Farm H; Fig.1), and maintains Bovans chickens, having the third-smallest population among the analyzed farms. This farm has a population of 199,000 chickens in 10 sheds, with the greatest isolation among the farms and natural barriers around the perimeter of the property's perimeter. Farm B, the second-lowest GaHV-1 detection rate (18.4%) was the

Table 3. Results of histopathological examination according to sampling period, diagnoses and number of chickens (active surveillance)

Samples period	Summer 2013 Number	Winter 2014 Number	Summer 2015 Number	Winter 2015 Number	Summer 2016 Number	Winter 2016 Number	Summer 2017 Number	Total
Histopathologic diagnosis								
Infectious laringotracheitis	1	0	0	1	0	3	1	6
Mycoplasmosis	9	38	12	40	22	29	24	174
Nonspecific (vaccinal) inflammatory infiltrate	38	112	124	91	77	95	93	630
No lesions	0	11	8	15	7	16	20	77
Other respiratory diseases	0	2	2	0	0	10	1	15
TOTAL	48	163	146	147	106	153	139	902

Table 4. Database for scheduled collections and all PCR results from trachea and trigeminal ganglia of all 962 chickens examined during active surveillance

Farm	Winter 2014				Summer 2015				Winter 2015				Summer 2016				Winter 2016				Summer 2017				Total of chickens per farm /PCR positive
	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	
A	9/4 ^{TC}	36/NV	-	9/1 ^T	47/NV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45/13 (28.9%) ^{ab}
	9/2 ^T	56/NV	-	9/3 ^C	107/NV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
	9/3 ^{TC}	116/NV	-																						
B	9/6 ^{TC}	32/HVT-LT	I	9/1 ^G	25/Both vaccines	II	9/2 ^G	96/Both vaccines	I	9/0	70/Both vaccines	I	9/2 ^G	38/HVT-LT	VI	9/1 ^T	33/HVT-LT	II							
	9/9 ^C	18/Both vaccines	II	9/0	68/Both vaccines	I	9/1 ^T	43/Both vaccines	I	9/0	27/HVT-LT	I	10/4 ^T	54/HVT-LT	II	9/0	79/HVT-LT	II							152/28 (18.4%) ^a
C	9/7 ^{TC}	38/FPV-LT	I	9/0	35/FPV-LT	IV	9/1 [*]	38/FPV-LT	IV	9/7 ^T	55/FPV-LT	IV	9/2 ^{TC}	30/FPV-LT	IV	9/0	20/FPV-LT	V							108/32 (29.6%) ^b
	9/6 ^{TC}	71/FPV-LT	I	9/1 ^T	83/FPV-LT	IV	9/1 [*]	82/FPV-LT	IV	9/0	65/FPV-LT	IV	9/4 ^C	88/FPV-LT	IV	9/3 ^T	73/FPV-LT	IV							
D	9/2 ^G	36/FPV-LT	III	9/4 ^C	29/FPV-LT	III	9/1 ^G	36/FPV-LT	III	9/2 ^G	46/FPV-LT	III	9/0	40/FPV-LT	III	9/1 ^C	31/FPV-LT	VII							
	9/3 ^C	58/FPV-LT	III	9/1 [*]	93/FPV-LT	III	9/2 ^T	85/FPV-LT	III	9/0	96/FPV-LT	III	9/0	89/FPV-LT	III	9/1 ^T	97/FPV-LT	III							110/17 (15.4%) ^a
E	18/12 ^T	33/Both vaccines	I	9/5 ^C	35/Both vaccines	I	9/5 ^{TC}	45/HVT-LT	I	9/6 ^C	34/HVT-LT	I	9/2 ^T	26/HVT-LT	I	9/4 ^T	38/HVT-LT	I							
	18/3 ^T	60/Both vaccines	I	9/5 ^{TC}	65/Both vaccines	I	9/1 ^T	65/Both vaccines	I	9/2 [*]	43/HVT-LT	I	12/1 ^T	34/HVT-LT	I	9/1 ^T	25/HVT-LT	I							177/61 (34.4%) ^b

Farm	Winter 2014			Summer 2015			Winter 2015			Summer 2016			Winter 2016			Summer 2017			Total of chickens per farm /PCR positive
	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	Number of chickens/PCR positive	Age (weeks)/Vaccine	Breed	
F	9/7 ^{TC}	41/ HVT-LT	II	9/4 ^T	30/FPV-LT	I	6/3 ^{TC}	19/ HVT-LT	IV	9/0	81/HVT-LT	I	9/3 ^T	84/HVT-LT	I	9/3 ^T	86/HVT-LT	I	126/44 (34.9%) ^b
	9/8 ^{TC}	88/ HVT-LT	II	9/1 ^G	90/FPV-LT	I	9/2 ^{TC}	34/ HVT-LT	I	9/0	88/HVT-LT	II	9/2 ^G	13/HVT-LT	I	8/1 ^T	97/HVT-LT	IV	
							9/2 ^{TC}	87/ HVT-LT	I	3/0	21/HVT-LT	II	6/3 ^{TC}	36/HVT-LT	II	4/1 ^T	19/Both vaccines	II	
	9/4 ^G	38/ HVT-LT	I	9/4 ^T	34/ HVT-LT	I	9/1 ^T	34/ HVT-LT	II	9/3 ^T	37/HVT-LT	I	9/3 ^T	34/HVT-LT	II	9/3 ^T	38/HVT-LT	III	
G	9/3 ^{TC}	85/ HVT-LT	IV	9/1 ^G	73/ HVT-LT	I	9/0	87/ HVT-LT	IV	9/6 ^T	81/HVT-LT	I	9/3 ^T	79/FPV-LT	III	9/7 ^T	80/HVT-LT	I	108/38 (35.2%) ^b
	9/9 ^{TC}	50/NV	I	9/3 ^{TC}	44/NV	I	9/1 ^T	40/NV	I	9/1 ^G	75/NV	I	13/3 ^T	88/NV	I	10/3 ^T	109/NV	I	137/45 (32.8%) ^b
H	9/9 ^{TC}	67/NV	I	9/5 ^T	98/NV	I	9/4 ^T	70/NV	I	9/0	95/NV	I	14/2 ^T	98/NV	I	10/2 ^T	20/HVT-LT	VII	
							9/2 ^T	49/NV	I	9/1 ^G	66/NV	I							
Total of chickens per sampling/PCR positive	171/97 (56.4%) ^b			153/44 (28.7%) ^a			150/32 (21.3%) ^a			147/28 (19%) ^a			185/38 (20.5%) ^a			156/39 (25%) ^a			962/278 (28.8%)

NV = non vaccinated; ^T Positive chickens only in the trachea, ^G positive chickens only in trigeminal ganglia (TG), ^{*} positive chickens in the trachea and trigeminal ganglia; Breed: I = Hyline White, II = Hisex White, III = Bovans White, IV = Dekalb White, V = Loman White, VI = Hisex Brown, VII = Isa Brown; ^{ab} Distinct letters in superscript state difference in frequencies for column (farms) or line (periods) by Chi-square test ($P < 0.05$).

third-biggest farm and had one period with the protocol for both vaccines. On the other hand, Farm G had 35.2% of positive samples. This farm is located next to a highway that services other farms of the region. This farm has 370,000 chickens housed in 17 sheds, located 850 meters from the nearest farm and 300 meters from a highway. The farm is partially surrounded by vegetation barriers and had the highest percentage of GaHV-1 infected chickens- 35.2% (38/108).

GaHV-1 detection in chickens according to season. The region under surveillance in this field study is prone to dry and rainy seasons. In the dry season (April to September), including the Winter period (May to August), there is little to no rain. The average temperatures during the sampling periods ranged from 15°C and 20°C in Winter. The highest temperatures were close to 30°C and the lowest close to 2°C.

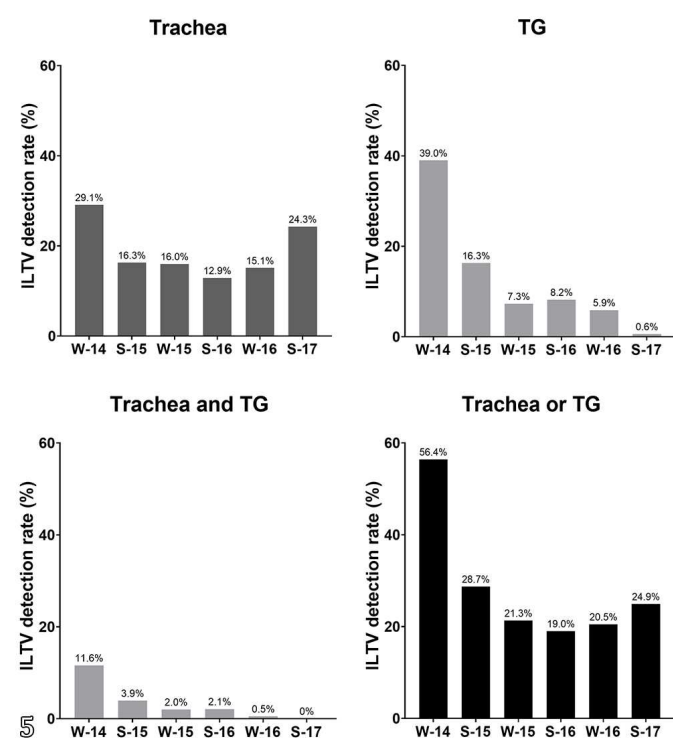


Fig.5. Detection (in percentage) of the GaHV-1 by PCR in the trachea, trigeminal ganglia or both from chickens sampled by active surveillance and examined by seasons from 2014 to 2017. S = Summer, W = Winter.

In Summer, average temperatures ranged between 20°C-25°C with highest around 35°C and the lowest around 15°C. No high reduction in temperature occurred during 2013 to May 2016. The incidence of GaHV-1 varied based on the season of the year, Winter or Summer, ($P=0.04$). A higher percentage of infected chickens were obtained in the Winter, especially in the Winter of 2014 (Fig.5 and 6). Of the 506 chickens sampled in the Winter, 167 (33.0%) were infected with GaHV-1 using both trachea and TG samples. Of the 456 chickens sampled during the Summer months, 111 (24.3%) were infected (Table 4). An important meteorological change occurred in June 2016, when the average temperatures were lower (average between 10-15°C) and the lowest temperature reached negative degree Celsius. Coincidentally, during this period, outbreaks of ILT and other respiratory diseases resurged.

GaHV-1 detection, according to the type of vaccine and vaccination program. A total of 182 non-vaccinated chickens from two farms were initially sampled. However, one of these farms was deactivated after the second sampling, thereafter only chickens on Farm H served as non-vaccinated controls (Table 4). This farm had three houses that were more secluded. Of the non-vaccinated chickens on this non-vaccinated farm, GaHV-1 was detected in 58 (31.8%) layer-type chicken. The number of chickens tested PCR-positive for GaHV-1 DNA based on the type of vaccines used on the farms per season is presented in Figure 5 and 6. Differences in the number of positive chickens were observed only in the Summer of 2017 when comparing chickens receiving prime/boost vaccination and chickens with one dose of HVT-LT vaccine ($P=0.04$). Differences between these two types of vaccination protocols were also observed when evaluating all collection periods and all farm data in conjunction (Fig.6 and Table 6). According to mixed logistic regression analysis, the chance of a chicken to be positive for GaHV-1 when using a one-shot of HVT-LT vaccine is 1.78 times higher than if a prime/boost vaccination is done. Also, no significant differences in the total proportion of chickens positive for GaHV-1 DNA were observed regardless of whether layers were vaccinated with a single vaccine or non-vaccinated.

GaHV-1 detection, according to chicken breed and age. The proportion of samples positive for GaHV-1 DNA varied significantly among the breeder of chickens ($P=0.0049$). Six distinct breeds were identified, Isa Brown ($n=19$) with three (15.8%) positive hens, Bovans ($n=119$) with 22 (18.5%) positive hens, Hisex ($n=161$) with 40 (24.8%) positive hens, Dekalb ($n=122$) with 34 (27.9%) positive hens, and Loman ($n=67$)

Table 5. Absolute and relative GaHV-1 positive chickens from trachea and trigeminal ganglia (TG) employing different diagnostic techniques and vaccination protocols

Vaccination protocol	PCR		ELISA		Histopathology
	Positive (tested samples) Percentage of positives		Positive (tested samples) Percentage of positives		Positive (tested samples) Percentage of positives
	Trachea	TG			
Non-vaccinated	(38/182) 20.8% ^a	26(168) 15.4% ^a	14(37) 37.8% ^{bc}		1(158) 0.6% ^a
HVT-LT	84(389) 21.6% ^a	42(387) 10.8% ^a	134(175) 76.5% ^a		4(374) 1.0% ^a
FPV-LT	36(245) 14.7% ^a	32(245) 13.0% ^a	16(79) 20.2% ^c		0(224) 0% ^a
Both vaccines (2 doses)	26(146) 17.8% ^a	27(146) 18.4% ^a	9(20) 45% ^b		1(146) 0.6% ^a
TOTAL	184(962) 19.1% ^a	127(949) 13.4% ^a	173(311) 55.6%		6(902) 0.6%

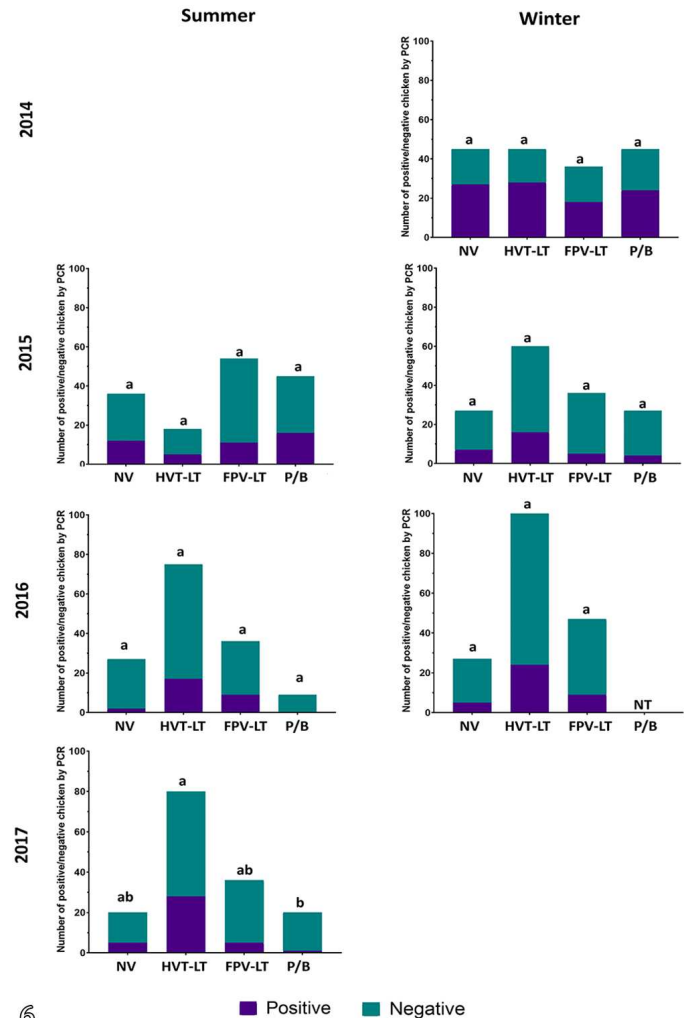
HVT-LT = herpesvirus of turkey - Laryngotracheitis recombinant virus vaccine subcutaneously at hatching, FPV-LT = Fowl poxvirus - Laryngotracheitis recombinant virus vaccine via wing web at 21-35 days of age, Both vaccines = HVT-LT and FPV-LT; ^{a,b,c} Distinct letters show difference in positive proportions between vaccination protocols (column) by chi-squared test or Fisher exact test ($P<0.05$).

with 22 (32.8%) positive hens. The Hyline breed ($n=453$) had the highest percentage of GaHV-1 positive with 167 (33.7%) positive hens. Chickens from Hyline genetic lineage had 1.69 times greater chance than others to be positive for GaHV-1. The age of the chickens (rearing, laying peak, and end of laying) was not a determinant factor in the susceptibility of layers to GaHV-1 ($P=0.20$).

Antibodies against GaHV-1. In total, 173 (55.6%) of sera collected from 311 chickens in the two last samplings (2016 and 2017) were positive for GaHV-1 specific antibodies by ELISA. Among those, 79 (50%) out of 158 sera were positive for samples collected during the Winter of 2016. The highest number of chickens, 61% (93/153), tested positive during the Summer of 2017. Out of 37 non-vaccinated chickens on farms that did not vaccinate, 14 (37.8%) were serologically positive. These serological results, according to type of vaccination, are presented in Table 4. There were no statistical serological differences among the age groups of chickens.

GaHV-1 detection, according to diagnostic methodology and vaccination protocol. The highest number of GaHV-1 positive chickens was detected serologically (55.6%), followed by the PCR-based method using trachea (19.1%) and TG DNA samples (13.4%). The ILT was diagnosed by histopathology in 0.6% of chickens collected by active surveillance (Table 5 and 6).

Comparison between detection of GaHV-1 in tissue samples, antibodies, and histological lesions. Out of the 1,283 examined chickens tested, 962 chickens were tested by PCR for the detection of GaHV-1 DNA during active surveillance (2016-2017). Only 278 (28.9%) chickens tested positive for the 237 bp amplicon representing the ICP4 gene. Amplicons were generated using DNA isolated from either trachea or TG samples, or both. In these same chickens, histopathology of the respiratory and conjunctival tissues was performed. In six (6/902) (0.6%) of these chickens only, lesions consistent with ILT (syncytial cells and intranuclear inclusion bodies) were found in the last year of active-surveillance sampling (Table 7). The presence of antibodies against GaHV-1 in chickens from the last two active surveillance samplings were compared to PCR and histopathological results. Out of 311 chickens tested by ELISA, PCR and histopathology, one, 41 and 185 chickens were positive in the three tests (histopathology, PCR and ELISA), two tests (PCR and ELISA) and one test (ELISA or PCR), respectively. In two of the farms whose chickens were positive by ELISA and PCR with the absence of lesions, outbreaks of ILT have not been diagnosed since 2012. Eighty-four chickens were negative in all tests.



⑥

Fig.6. Detection (in percentage) of the GaHV-1 by PCR in the trachea, trigeminal ganglia or both from chickens sampled by active surveillance and examined by seasons from 2014 to 2017. NV = non-vaccinated, HVT-LT = turkey herpesvirus - Laryngotracheitis recombinant virus vaccine subcutaneously at hatching, FPV-LT = fowl poxvirus - Laryngotracheitis recombinant virus vaccine via wing web at 21-35 days of age, LT and FPV-LT; P/B = Prime/boost (both vaccines - HVT-LT and FPV-LT), NT = not tested. Distinct letters show difference in positive proportions between vaccination protocols within period by Fisher exact test ($P<0.05$). Chi-squared statistics for general vaccination protocols comparison has P -value equal to 0.16.

Table 6. Absolute and relative GaHV-1 positive chickens according to vaccination protocols, season and years

Vaccination protocol	Chicken number/PCR positive						Total (n%)
	Winter 2014	Summer 2015	Winter 2015	Summer 2016	Winter 2016	Summer 2017	
Non-vaccinated	45/27 ^a	36/12 ^a	27/7 ^a	27/2 ^a	27/5 ^a	20/5 ^{ab}	182/58 (31.8%) ^a
HVT-LT	45/28 ^a	18/5 ^a	60/16 ^a	75/17 ^a	111/24 ^a	80/28 ^a	389/118 (30.3%) ^a
FPV-LT	36/18 ^a	54/11 ^a	36/5 ^a	36/9 ^a	47/9 ^a	36/5 ^{ab}	245/57 (23.2%) ^a
Both vaccines	45/24 ^a	45/16 ^a	27/4 ^a	9/0 ^a	0/0 ^a	20/1 ^b	146/45 (30.8%) ^a
TOTAL (n/%)	171/97	153/44	150/32	147/28	185/38	156/39	962

HVT-LT = Herpesvirus of turkey - laryngotracheitis recombinant virus vaccine subcutaneously at hatching, FPV-LT = fowl poxvirus - laryngotracheitis recombinant virus vaccine via wing web at 21-35 days of age, Both vaccines = HVT-LT and FPV-LT; Number of tested/number of positive; ^{a,b} Distinct letters show difference in positive proportions between vaccination protocols within period by Fisher exact test ($P<0.05$); Chi-squared statistics for general vaccination protocols comparison has P value equal to 0.16.

Passive surveillance

ILT diagnosis and differential diagnosis at passive surveillance. Respiratory tissues and conjunctivae from a total of 321 chickens were submitted by veterinarians from IMA (December of 2013 to December of 2018) to the Veterinary pathology sector at UFMG for histopathological diagnosis. For these samples, chickens representing one or more flocks presented clinical signs of acute respiratory diseases. Of these, 124 (38.6%) of these chickens (Table 8) were diagnosed with ILT based on the pathognomonic lesions. Other respiratory diseases (diphtheritic avian pox, infectious bronchitis, infectious coryza and mycoplasmosis) were also diagnosed. Immunohistochemistry confirmed the mycoplasmosis cases. Chickens with nonspecific changes characterized by lymphocytic and plasma cells infiltration in the respiratory mucosa (probably related to an immune inflammatory response to several vaccines used against respiratory diseases in these chickens) or with chronic and regenerative lesions in larynx and trachea without definitive etiologic diagnostic were also found. Mortality rate in flocks with respiratory clinical signs reached 1.4% to 14.4%, independent if outbreaks was by ILT or for other respiratory diseases. It was interesting to note that in the region (Fig.1) ILT remained undiagnosed, as monitored by both active and passive surveillance for three years before the resurgence of new outbreaks.

Phylogenetic analysis of GaHV-1 strains based on the thymidine kinase and ICP4 genes. All sequences were classified as virulent Brazilian ILTV strains based on phylogenetic analysis of concatenated sequences from selected regions of the ILTV genome. One region contained sequences from the TK gene, and the other two regions contained sequences from the large ICP4 gene (Fig.7). Phylogenetic analysis clustered the four samples (Brazil virulent MG/FarmE-P871/Summer/2016, Brazil virulent -MG/FarmG-P982/Summer/2016, Brazil virulent -MG/FarmF-P1253/Winter/2016, and Brazil virulent -MG/FarmF-P1369/Summer/2017) within the strain characterized previously in the same area that was detected in 2011 (KF786297.1, KF786292.1, and KF786287.1). The sequence Brazil virulent -MG/FarmB-H1096/Winter/2018 was slightly different with three single nucleotide polymorphism (SNP) at positions 3,854 (119,133), 3,937 (119,216), and 4,020 (119,299) in the ICP4 gene (complete genome). Sequences

from 2016 and 2017 had bases G, A, and C, while the sequence from 2018 had A, C, and T at those positions described above. When comparing the field virus from the area and Brazilian virulent strain from the state of São Paulo detected in 2004 (FJ477366 and FJ477367), the latter viruses also had the bases A, and C at positions 3,854 and 3,937 as described above, similar to the ILTV/Brazil-MG/FarmB-H1096/Winter/2018. However, the FJ477366 and FJ477367 sequences also have C at the residue 4,020 (119,299) as the other ICP4 sequences. The comparison of sequence from Minas Gerais strains and São Paulo strains revealed no close genetic relatedness.

DISCUSSION

The data obtained in this study indicated that the percentage of GaHV-1 infected chickens did not statistically differ among the various farms that used the single dose recombinant vectored vaccines and non-vaccinated farms. However, when the comparative evaluation was carried out among chickens from a subset group vaccinated with two doses and from a group vaccinated with a single dose (rHVT-LT), chickens that were vaccinated with two doses had a lower positivity rate. In the Winter of 2016, two larger farms experienced a resurgence of new clinical outbreaks of ILT, probably resulted from changes in the vaccination programs along with increased densities in these farms, and low temperature. Additionally, data of the current study suggested that the virus continued to evolve after circulating in the area for a few years or a novel virus was introduced from other region.

ILT is a disease with a worldwide distribution and endemic in countries with high density poultry production in geographically concentrated commercial farms. Usually, live attenuated vaccines, along with biosecurity, are the control measures in regions where cyclic outbreaks occur (Guy & García 2008, Menendez et al. 2014). In Brazil, recombinant vaccines are used to control ILT present in three quarantined regions with large numbers of layers. On the other hand, outbreaks of ILT in broiler chickens in Brazil are rare events, possibly associated with optimally designed biosecurity measures, with flocks being depopulated following notification (Department of Animal Health, personal communication). The region of the present study currently has 24 laying farms with an estimated

Table 7. Summary of number of chickens examined, type of tests and percentage of chickens with positive diagnosis for GaHV-1 and/or ILT clinical disease

	PCR total	PCR trachea	PCR TG	ELISA	Histopathology (active surveillance)	Histopathology (passive surveillance)
Total of chickens	962	962	949	311	902	321
% of positive	28.9%	19.1%	13.4%	55.3%	0.6%	38.6%

ILT = infectious laryngotracheitis, TG = trigeminal ganglia.

Table 8. Passive surveillance results according to number of chickens and diagnosis

Year	Diphtheritic fowlpox	Infectious bronchitis	Infectious coryza	ILT	Mycoplasmosis	Response to vaccination	Chronic tracheitis/ regeneration	Total
2016	13	17	9	53	0	5	5	103
2017	2	8	0	20	45	40	0	115
2018	1	0	0	51*	39	12	0	103
TOTAL	16	25	9	124	84	57	5	321

ILT = infectious laryngotracheitis; * Seven chickens with concurrent lesions by GaHV-1 and *Mycoplasma* spp.

total population of over 10 million chickens, the third-largest eggs-producing region in the country (IBGE 2017). From 2011 to 2018, the control of ILT in these regions was authorized by the exclusive use of recombinant vectored vaccines.

The long-term study, reported here, is the first large-scale field study, evaluating the effect of the use of recombinant vectored vaccines with different vaccination protocols for the control of ILTV within the quarantined area. It started when all chickens at the initial phase of production were immunized with the vectored vaccination protocols on each farm. PCR positivity tended to decline, as residual non-vaccinated flocks were gradually substituted by vaccinated, as shown early in the second sampling (Summer 2015). This declining trend continued until the fourth sampling (Summer 2016). The

decrease in PCR positivity in some periods was greater than 50%, indicating that vaccination reduced the replication of field virus curtailing its circulation. These results are in agreement with an experimental study using vectored ILT vaccines (Vagnozzi et al. 2012).

Despite the high prevalence of GaHV-1 PCR positive chickens, the clinical disease frequency during the first three years of the study was low. Outbreaks of ILT were not noticed between December of 2013 (Couto et al. 2015) through June of 2016. From the end of June 2016 until the end of December 2018, outbreaks of respiratory diseases reemerged on the three largest farms sampled with a high frequency. The immune response to GaHV-1 may have influenced the absence of ILT outbreaks during the period when all chickens were vaccinated.

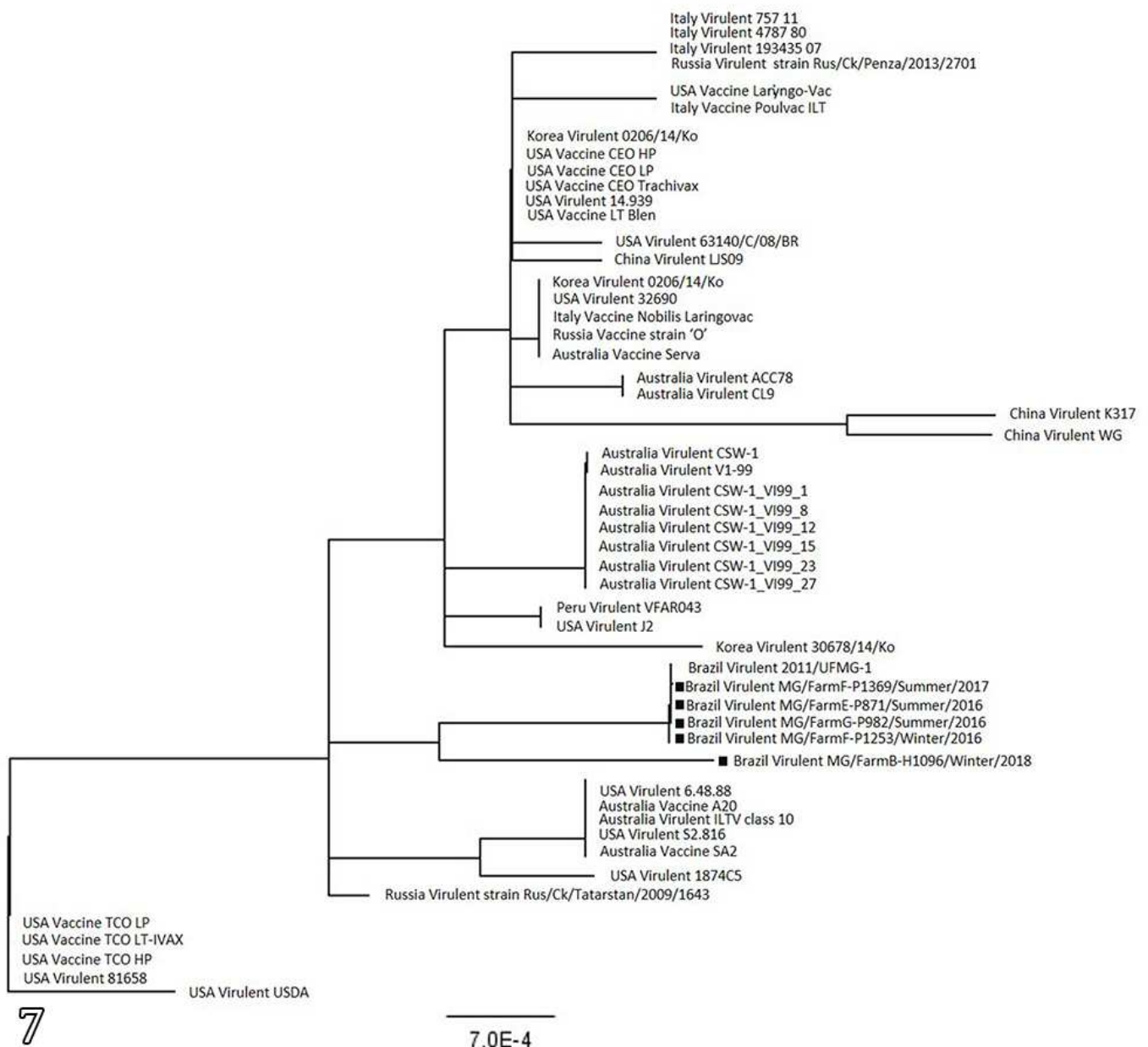


Fig.7. Dendrogram based on concatenated thymidine kinase (TK) and ICP4 genes. A square represents the Brazilian viruses sequenced in the present study.

It can be considered that prime/boost vaccination programs and the lesser influence of risk factors such as environmental (coldest Winter) and smaller population densities (numbers of chickens per cage and in the region) likely contributed to the gradual decrease in the proportion of infected chickens and reduced ILT outbreaks. Although these ancillary factors undoubtedly affected the welfare of the chickens, vaccination is very important. Previous modification on vaccination schedules made by two of the largest farms, applying only HVT-LT vaccination instead of the prime/boost vaccination (HVT-LT and FPV-LT), coincided with an increase susceptibility to ILT. The HVT-LT vaccine is known to induce long-lasting immunity. Still a prime/boost strategy is always superior to a single dose of ILT vaccination regardless of other parameters i.e., increased in-house population and low temperatures that happened in this region. Thus, stressful conditions may have occurred, reducing immunity and resulting in the reactivation, replication, and excretion of viral particles by chickens with latent infections (Hughes et al. 1989, 1991). Low temperatures are likely to increase the viability of viral particles in the environment, providing a more extended period for possible primo-infection of younger chickens (Dufour-Zavala 2008). In the present study, a statistically higher level of GaHV-1 infection (higher rate of PCR positivity) was detected in samples collected during the Winter. Low temperatures may also decrease the host immune response of these chickens. In cold months, other respiratory diseases in these flocks, such as mycoplasmosis, have a significantly higher frequency than ILT.

Experiments in chickens using HVT-LT and FPV-LT vaccines administered *in ovo* or subcutaneously achieved only partial protection (Johnson et al. 2010), reducing to some degree clinical signs and challenge of virus replication in the trachea (Vagnozzi et al. 2012). In the present study, farms initially using two doses of vectored vaccine, expressing different GaHV-1 glycoproteins, switched to single dose vaccination during a period of the study. A difference in the proportion of positive and negative animals among the vaccination protocols was observed. A lower proportion of positive chickens in the trachea by PCR (February 2016) was detected, suggesting better protection against GaHV-1, possibly associated with the complementary responses to the different vaccine formulations. The lower frequency of clinical disease in this period, despite the high rates of infection, suggests that prime/boost vaccination may influence the clinical disease control, but not the spread of the virus among chickens and between farms.

The detection of GaHV-1 DNA with primers specific for ICP4 and TK genes indicated circulation of wild-type field strains in chickens within the ILT affected region. The PCR assays were target specific for genes not incorporated in the vectored vaccine constructs. The fowl poxvirus vectored vaccines express gB and UL32 genes (Davison et al. 2006, Johnson et al. 2010, Coppo et al. 2013), and the *Meleagrid herpesvirus* (HVT) vaccines express glycoproteins D and I genes (Gimeno et al. 2011). As detected here by the PCR, a high frequency of positive chickens in an endemic area, usually without clinical disease and histologic lesions, may indicate latency and persistence of wild strains of GaHV-1 (Bagust et al. 2000, Williams et al. 1992). GaHV-1 can be reactivated from TG spontaneously or under stressful conditions (Bagust et al. 2000, Hughes et al. 1991, Thilakarathne et al. 2019). In the present study, GaHV-1 DNA was also detected in the TG

and trachea of healthy chickens from flocks without clinical or pathological evidence of ILT. The persistence of GaHV-1 in the trachea may be due to infected macrophages. Macrophages are present in the tracheal mucosa, as reported by Krunkosky et al. (2018) and susceptibility of macrophages to GaHV-1 infection was reproduced by Calnek et al. (1986).

Our limited genetic analysis showed that a virulent field virus circulates in chickens despite the vectored vaccination programs. Viral DNA sequences obtained by active and passive surveillances collected in 2016 and 2017 were closely related to those sequences analyzed from 2011 to 2013 (Couto et al. 2015). However, a sequence obtained from passive surveillance in 2018 revealed a unique SNP not seen in samples from previous years. This datum may suggest that a virulent field strain continues to circulate in the region. Also, the evolution of a new viral strain, with possibly greater pathogenicity, likely contributed to the resurgence of the latest clinical outbreaks of ILT. It is important to note that the sequenced PCR products indicated that the GaHV-1 strains circulating in this region were of virulent origin and not related to vaccinal revertants of TCO and CEO. These results were comforting and expected since the use of live attenuated vaccine strains of GaHV-1 was not permitted for use in the region during this period, although used in the neighboring state of São Paulo. The complete ILTV genome would provide a better characterization of the circulating strains. However, the TK and ICP4 genes sequenced in our study are commonly used for ILTV characterization. Results of the current study suggest no phylogenetic relatedness of the obtained sequences to sequences from São Paulo State.

Serum antibody titers against GaHV-1 were detected in 50-60% of chickens. The prevalence of antibodies against GaHV-1 in these chickens was higher when compared with the prevalence found in other serological studies, such as in Australia (Sellers et al. 2004), and Bangladesh (Uddin et al. 2015). The results also indicated that there were chickens that did not develop humoral immunity, either by natural infection or by vectored vaccination against ILT. Humoral immunity to ILTV is not a good indicator of immunological protection (Guy et al. 1991, Blacker et al. 2011). Therefore, the concomitant use of histopathological examination was considered fundamental to diagnosis ILT, differentiating disease from subclinical, or persistent infection. It should also be emphasized the importance of using molecular techniques and sequencing, to define the origin and enable data on the evolution of circulating virus strains. There is a high prevalence of GaHV-1 infection in the studied region, although the rate of clinically affected chickens does not follow this proportion. In an endemic region, ELISA as a single test is not a reliable method for the diagnosis of ILT. However, histopathology enables the differential diagnosis of the disease, especially because of the presence of ILT pathognomonic lesions (Pirozok et al. 1957, Preis et al. 2013, Couto et al. 2015). The accuracy of histopathology tests increases when chickens are examined during the acute phase, typically in the first eight days of the disease, in which all respiratory tissues and conjunctiva are sampled, as validated in the present study for passive surveillance.

Regarding chicken breeds, different results in susceptibility were obtained comparing Hyline and Bovans lineages, although other factors might influence. Hyline chickens had the highest positivity to the virus. They were mostly from the largest farms, in houses at high proximity (about

5 to 10m) and closer to other farms and roads, with also the highest density of hens per house. The least frequently infected breeds (Bovans), were not housed in the largest and densely populated farms. Results of the current study suggest that genetic breeders might influence the resistance to the GaHV-1 infection, corroborating with the study of Poulsen et al. (1998). Although the population density is well known to increase the susceptibility to respiratory infections.

The high level of GaHV-1 detection indicated an increase in viral circulation within the region. Three types of farms were characterized in the study (1) with no ILT cases, (2) having sporadic cases, and (3) farms with frequent outbreaks. High viral circulation generally is favored by biosecurity failures, and viruses can be spread by the airway (Dufour-Zavala 2008), or mechanical carriers (Kingsbury & Jungherr 1958). The proximity to other farms and the direction of the wind highly increases the risk of GaHV-1 transmission. Also, viral excretion by the tracheal epithelium may be increased in stressful situations, such as at the beginning or peak of laying, or by mistakenly introducing latently infected chickens, thereby infecting susceptible chickens (Hughes et al. 1989). Most of the farms in the region studied had biosecurity measures such as disinfection of the equipment, vehicles, and transportation boxes, employee exclusivity, and conditional entering for visitors. They should hold a 5-day interval from farm to farm and no recent contact with other poultry or poultry products, including litter. However, the execution of the effective biosecurity measures in most of these multiple-age farms, seen as a single unit, due to the risk spatially and temporally to spread pathogens (Halvorsen 2011), is the biggest problem in the region. It has been challenging to control the circulation of pathogens among farms in the region, as they were built before the consolidation of biosecurity practices. The producers in the region also raise chicks and pullets at close proximity to the layer-farms. The region is economically dependent on egg production, and it is not feasible to depopulate for complete sanitary cleaning (fallow period). The layers are kept in production for about 100 weeks. The proximity to the urban/periurban center and villages may also present biosecurity risks, considering a large number of backyard chickens in the region. GaHV-1 circulating also was demonstrated in these chickens (Preis et al. 2014), contributing to the status of an outbreak zone.

This study's main limitation was the impairment of the cohort study designed caused by the inability to ensure unchanged sanitary protocol. The initial sampling was changed as a non-vaccinated farm was closed. The two more populated farms that adopted the two vaccinations schedule changed for hatch vaccine only (HVT-LT). Also, other respiratory diseases were present. However, the passive surveillance strict data collection, and diagnosis and a prudential study period have validated our results, regardless of changes during the study. These results represent a real situation for the use of mitigation strategies in a large- layer production region.

CONCLUSIONS

The prevalence of positive chickens detected by PCR targeting the laryngotracheitis virus (LTV) ICP4 gene was significantly lower in chickens receiving recombinant vaccines expressing different GaHV-1 glycoproteins at day-old and 21-35 days of age when compared to chickens receiving only turkey

herpesvirus (HVT-LT). However, no significant difference was observed when compared chickens that received prime/boost vaccination and chickens vaccinated with fowl poxvirus (FPV-LT) or non-vaccinated. Additionally, viral persistence in the trachea was not impeded by any different vaccination protocols, although a decrease in the detection rate of GaHV-1, especially in the trigeminal ganglia (TG), was observed in the last periods of study. Nevertheless, the absence of outbreaks when all chickens from most farms were vaccinated, and the risk factors were lower suggesting that prime/boost using viral-vectored recombinant vaccines favored to reduce the infectious laryngotracheitis (ILT) incidence.

PCR of the tracheal mucosa enabled the detection of a higher percentage of GaHV-1 positive chickens in the absence of lesions, suggesting the persistence of the virus in the mucosa.

The partial genetic analysis revealed that the sequences from this study are different in comparison with São Paulo strains, another region that experienced ILT outbreaks. The field virus continued to circulate in the area with probably a high selective pressure driven by the number of cases, availability of hosts and transmissibility, and other factors, and a different genetic strain had emerged in 2018. The complete genome sequencing and pathogenicity studies of the new strain in the region would help understand the GaHV-1 evolution in the region.

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