Natural concurrent infections associated with infectious laryngotracheitis in layer chickens

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Primary Audience: Broiler Managers, Veterinarians, Poultry Scientists

SUMMARY

Layer chickens with severe respiratory disease belonging to farms with chickens infected by gallid herpesvirus (GaHV-1) were evaluated. Samples of nasal turbinates/sinuses and trachea from 31 chickens were subjected to histopathological analysis, which showed that 22.6% of chickens had lesions suggestive of co-infection, most frequently by GaHV-1 or *Mycoplasma* spp. PCR analysis showed the presence of at least two respiratory pathogens in 61.2% of chickens. Partial sequencing was performed for identification of the intergenic spacer region (IGSR) and cytadhesin 2 (mgc2) gene of the *Mycoplasma gallisepticum* genome. Analysis of IGSR revealed the involvement of two field and one vaccine strains. However, analysis of mgc2 from the same samples revealed five different strains of *M. gallisepticum,* four of which were field strains and one of which was a vaccine strain. These results demonstrate that combined molecular and histopathological analysis is a reliable and conclusive method for diagnosis of respiratory diseases in chickens since some etiologic agents can remain latent or maintain chickens as carriers. The results further confirm the importance and feasibility of the molecular characterization of *M. gallisepticum* genes to differentiate between vaccine and field strains involved in cases of respiratory disease in chickens.

Key words: layer chicken, respiratory disease, GaHV-1, *Mycoplasma* spp, mgc2, IGSR, histopathology

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DESCRIPTION OF PROBLEM

Respiratory tract infections in chickens cause major economic losses for the poultry industry worldwide. The losses occur due to decreases in productivity, weight loss, and egg production as well as increases in mortality and expenses for diagnostic tests, vaccines and antibiotic treatments. Such infections are favored by confinement systems that promote the development and spread of infectious agents [1].

A variety of pathogens are associated with respiratory infections in chickens, including viruses such as gallid herpesvirus 1 (GaHV-1) and diphtheric form of avipoxvirus (APV) and bacteria such as *Mycoplasma* spp. and *Avibacterium paragallinarum* [2]. These respiratory pathogens are especially problematic because they can cause a single infection or a combination of diseases [3]. The severity of

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lesions is often exacerbated and prolonged by concurrent infections [4], immunosuppression induced by mycotoxin, or infection with infectious bursa disease virus (IBDV) [5], chicken anemia virus (CAV) [6] and avian reovirus (ARV) [7, 8].

The rapid and early detection of respiratory disease etiology are essential for effective control and reduction of damage. However, it becomes challenging due to the similarity of the respiratory clinical signs and gross lesions. Moreover, more than one agent may be involved in the process, needing additional histological and microbiological analyses for the final diagnosis [2, 3]. Etiologic detection by PCR is an effective diagnostic tool, though it should be combined with histopathological assessment of lesions for confirming the disease because of carrier or latency status characteristic for some agents [2], and to account for the regular use of live vaccines against mycoplasmosis and infectious bronchitis [9].

Infectious laryngotracheitis (ILT) is a highly contagious, viral respiratory disease that causes severe problems for the poultry industry worldwide [10]. The etiological agent of ILT is gallid herpesvirus 1 (GaHV-1), a member of the Herpesviridiae family that can induce a state of latency in carrier birds [11, 12]. Diagnosis of the disease is based on confirmation by one or more laboratory tests, with histopathology being the fastest and most common method [13].

Among avian mycoplasmoses, *M. gallisepticum* infections are responsible for notably high morbidity and economic impact. With the increased utilization of live *M. gallisepticum* vaccines, more accurate methods are required for tracking the source of infection and to differentiate the field and vaccine strains. Sequencing methods have been introduced as a new tool in molecular epidemiological studies of these pathogens [14]. The sequencing of a larger number of genetic targets using multilocus sequence typing (MLST) facilitates discrimination between different strains. *M. gallisepticum* can be distinguished by sequencing of the intergenic spacer region (IGSR), located between the 16S and the 23S rRNA genes, and the cytadhesin (mgc2) gene [15].

Available live, attenuated vaccines used for the control of *M. gallisepticum* include the strains MG F, ts-11, 6/85 [16, 17], and MG-70 [18]. However, the 6/85 strain is not used in Brazil.

Fowlpoxvirus is common in free-range chickens in Brazil, and layer chickens are routinely vaccinated against avipoxvirus using live, attenuated strains, which are usually introduced in two doses. Nevertheless, reports have described outbreaks of more virulent strains [19].

The occurrence of co-infection of the respiratory tract of chickens has rarely been studied by combined histological and PCR analysis. We report the occurrence of GaHV-1 and co-infections in naturally infected chickens, describing the histological changes associated with the molecular detection. In addition, we performed the phylogenetic and molecular analysis of *M. gallisepticum* based on partial sequences of IGSR and mgc2 gene.

MATERIALS AND METHODS

Samples, Necropsy and Histopathology

Tissue samples from layer chickens with respiratory disease were selected and collected (n = 31) from six farms with populations ranging from 100,000 to 2,800,000 birds within a region with a total of approximately 8 million layer chickens. The farms are located in the mountainous Mantiqueira region (22° S; 44° W) south of Minas Gerais, Brazil. The farms in this area were placed under quarantine by the governmental poultry agency due to the widespread occurrence of ILT. The samples were obtained from chickens ranging from 20 to 70 wk of age that presented severe respiratory disease and had been diagnosed with ILT. Chickens were sampled bimonthly throughout the period of March 2011 to February 2012. Between May 2012 and September 2013, new samples were taken to monitor the occurrence of disease caused by GaHV-1 and included chickens with and without clinical presentation of respiratory disease.

The chickens were evaluated for clinical signs and were then euthanized by cervical dislocation in accordance with recommendations from Ethics Committee of the University (Protocol 204/2011). After necropsy and gross evaluation of the organs, samples were collected from

Table 1. Vaccination programs (Group 1 and Group 2) employed in layer chickens with respiratory disease in Minas Gerais, Brazil.

Chickens	Disease	Age (days)
Group 1 One farm	Avian pox; Marek's disease	1
	Newcastle disease; Infectious bronchitis	7
	Swollen head syndrome	21
	Newcastle disease; Infectious bronchitis	30
	Infectious coryza	35
	Swollen head syndrome	42
	Avian pox	60
	Newcastle disease; Infectious bronchitis	70
	Egg drop syndrome; Infectious bronchitis; Infectious coryza (trivalent oil-emulsion); Swollen head syndrome	105
Group 2 Five farms	Infectious bursal disease; Marek's disease	1
	Newcastle disease; Infectious bronchitis	7
	Swollen head syndrome	20
	Infectious bronchitis; Newcastle disease	28
	Infectious coryza; *Mycoplasma*; *Salmonella* Enteritidis	35
	Swollen head syndrome	60
	Inactivated oil-emulsion combined Newcastle disease; Infectious bronchitis	70
	Egg drop syndrome; Infectious bronchitis; Swollen head syndrome; Infectious coryza; Newcastle disease and *Salmonella* Enteritidis	105

turbinates and paranasal sinuses, larynx, trachea, lung, brain and ganglia of the trigeminal nerves. Samples were fixed in neutral 10% buffered formalin for 72 h and then transferred to 70% ethanol. For histopathological analysis, samples were dehydrated using a series of increasing ethanol concentrations, cleared in xylene, embedded in paraffin, sectioned at 4-μm thick, stained with hematoxylin and eosin (H&E), and evaluated by light microscopy. The larynx and cranial part of the trachea were transversely cut, while the distal part of the trachea was sectioned longitudinally to enable identification of the cranial and distal segments of the trachea during histological analysis. The samples showing bacterial colonies during histopathological analysis were selected for special staining (Good pasture) [20] to characterize the morphology and dye affinity of the bacteria.

The analyzed chickens were collected from farms employing different immunization protocols, which are detailed in Table 1.

DNA Extraction and PCR

The paraffin-embedded tissue from nasal turbinate/sinuses and trachea of 31 commercial laying hens collected in 2011 were assessed. Twenty 5-μm-thick serial sections from each tissue were obtained, placed in DNase-free 2-mL microtubes, and deparaffinized with xylene before proceeding with the DNA extraction. A QIAamp DNA Kit (QIAGEN, Valencia, CA) was used according to the manufacturer's instructions. DNA was quantified immediately after extraction using a spectrophotometer (NanoDrop ND-1000, Thermo Scientific, Wilmington, DE) and the samples were frozen at –20°C. DNA was also extracted from samples collected in 2012 and 2013 while monitoring chickens for GaHV-1 infection and from a sample of the MG-70 vaccine (BIOVET).

The extracted DNA was quantified and subjected to PCR using primers specific for different respiratory pathogens (Table 2). PCR testing for GaHV-1 was performed according to Preis et al. [21]. For detection of *M. gallisepticum* DNA, primers recommended by the OIE were used [9], and *Mycoplasma synoviae* detection was performed according to Lauerman et al. [22]. For PCR detection of *A. paragallinarum* DNA, the primers and protocol were based on those described by Chen et al. [23]. PCR reactions were performed in a volume of 25 μL (PCR Master Mix Promega) using 200 ng of DNA sample. As an internal control of the viability and quality of DNA, negative samples were tested for

Table 2. Sequences of primers used for molecular diagnosis of different respiratory pathogens.

Respiratory pathogens	Primer	Sequence (5′ → 3′)	PCR product size	Reference
GaHV-1	Foward Reverse	CCTTGGTTCGGGATGAAACC TTCATTACCTCCAGCGGTTCC	237 bp	Preis et al., 2013
Mycoplasma gallisepticum	MG-14F	GAGCTAATCTGTAAAGTTGGTC	185 bp	OIE 2008.
	MG-13R	GCTTCCTTGCGGTTAGCAAC		
Mycoplasma synoviae	MS-1	GAAGCAAAATAGTGATATCA	207 bp	Lauerman et al., 1993.
	MS-2	GTCGTCTCCGAAGTTAACAA		
Avibacterium paragallinarum	N1	TGAGGGTAGTCTTGCACGCGAAT	500 bp	Chen et al., 1996.
	R1	CAAGGTATCGATCGTCTCTCTACT		

Table 3. Sequences of primers used for sequencing the IGSR and mgc2 gene of *Mycoplasma gallisepticum*.

Amplified gene	Primer	Sequence (5′ → 3′)	PCR product size	Reference
IGSR	IGSR F	ACTGATAGCTTTTCGTACAGCACG	812 pb	Callison et al., 2006
	IGSR R	CATCGGGACATTCTCCAGGTAGCA		
mgc2	mgc2 F	GCTTTGTGTTTCTCGGGTGCTA	824 pb	Ferguson et al., 2005
	mgc2 R	CGGTGGAAAACCAGCTCTTG		

beta actin [24]. Solutions containing PCR Master Mix reagents, ultrapure water, and primers and lacking a DNA sample template were used as a negative control. As a positive control for GaHV-1, a field sample that had been previously confirmed positive for GaHV-1 by PCR and sequencing was used. The positive control for *M. gallisepticum* and *Avibacterium paragallinarum* were extracted from the MG-70 (Biovet, São Paulo, Brazil) and Coryza Oily 3 (Fort Dodge, São Paulo, Brazil) vaccines, respectively. The vaccine DNA extractions were performed on 200-μL vaccine samples by adsorption to silica according Boom et al. [25]. The positive control DNA for *M. synoviae* was obtained by DNA extraction using silica from 200 μL of antigen for rapid agglutination tests (Laudo Laboratório Avícola, Uberlândia, Brazil).

Sequencing

Total DNA from six samples tested positive for *M. gallisepticum* by PCR, including three collected in 2011, one in 2012, two in 2013, and DNA from vaccine MG-70 were used for partial sequencing of IGSR and mgc2. Amplification of partial IGSR was based on the methodology described by Callison et al. [26] and yielded an 812-bp product. Primers and PCR conditions for partial mgc2 amplification were the same as described by Ferguson et al. [27] and generated a product of 824 bp (Table 3).

The amplified DNA products were extracted from agarose and purified using a commercial kit (Invisorb® DNA Extraction Spin Kit). Each sample was amplified three times and sequenced bi-directionally by capillary electrophoresis using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Foster City, CA) on an ABI 3130 Genetic Analyzer automated sequencer (Applied Biosystems). Using SeqScap® (version 2.5) software, the sequences were analyzed for quality, and once the consensus sequences were obtained, MEGA version 5.1 software was used for alignment with reference sequences from the GenBank database (NCBI) using the BLASTn platform. Phylogenetic trees were generated using the neighbor-joining distance method coupled with

the Kimura 2-parameter model with bootstrap analysis of 1,000 replicates.

Accession Numbers of Nucleotide Sequences

The following sequences published in GenBank (accession number in parentheses) were used for comparison with IGSR: COL/68938/CK08 (KC247840); ECU/77025/CK09 (KC247842); GUA/79672/CK09 (KC247844); PAN/68630/CK08 (KC247845); VEN/69993/CK08 (KC247847); USA/K6001B/CK07 (KC247849); USA/65099/PF08 (KC247850); EGY/67240/CK08 (HQ143380); ISR/K3868/CK95 (HQ143384); JOR/111/CK09 (KC247857); SPA/5742–3/CK09 (KC247858); IND/38825/CK05 (KC247859); THA/3/CK08 (KC247860); RSA/22/CK10 (KC247830); RSA/66/CK12 (KC247831); USA/F/CK58 (HQ143383); ts-11 (KC247864); 6/85 (KC247865).

The following sequences published in GenBank (accession numbers in parentheses) were used for comparison with the mgc2 gene: COL/68938/CK08 (KC247873); ECU/77025/CK09 (KC247875); GUA/79672/CK09 (KC247877); PAN/68630/CK08 (KC247878); VEN/69993/CK08 (KC247880); USA/K6001B/CK07 (KC247882); USA/65099/PF08 (KC247883); EGY/67240/CK08 (HQ143372); ISR/K3868/CK95 (HQ143377); JOR/111/CK09 (KC247890); SPA/5742–3/CK09 (KC247891); IND/38825/CK05 (KC247892); THA/3/CK08 (KC247893); RSA/22/CK10 (KC247866); RSA/66/CK12 (KC247868); USA/F/CK58 (KC247897); ts-11 (KC247898); 6/85 (KC247899).

RESULTS

History and Clinical Signs

In all of the farms where chickens were severely affected by respiratory disease, increase in the week mortality rate was observed, reaching an average of 2%. On one farm with a population of approximately 150,000 chickens, the mortality rate reached 10%, and there was a significant reduction in egg production. The clinical signs were observed mainly in chickens that were between 20 and 35 wk old, which presented with coughing, sneezing, swelling of the paranasal sinuses and nasal discharge. Other signs included apathy, conjunctivitis, and cyanosis.

Gross Lesions and Histopathology

The turbinates and paranasal sinuses presented hyperemia, thick mucus, and fibrinous exudates, characteristic of moderate acute rhinitis and sinusitis. Chickens that presented a prolonged disease evolution had marked increases in caseous exudates, determining intense paranasal sinus and facial swelling. The trachea of most chickens presented moderate to marked mucous or fibrinous inflammation.

Of the total chickens examined, 77.4% (24/31) showed histological changes that were suggestive of infection by only one respiratory pathogen, while 22.6% (7/31) showed histopathological findings that were suggestive of co-infection. Of the total chickens assessed, 61.3% (19/31) presented histological lesions that were consistent with GaHV-1 infection, 45.2% (14/31) had lesions consistent with infection by *Mycoplasma* spp., 12.9% (4/31) had lesions that were consistent with *Avibacterium* spp., and 3.2% (1/31) of chickens showed lesions that were characteristic of the diphtheric form of avipoxvirus infection.

In chickens that presented histological changes consistent with lesions induced by more than one etiologic agent, 57.1% (4/7) showed lesions that were compatible with GaHV-1 and *Mycoplasma* spp. co-infection, 14.3% (1/7) showed lesions that were consistent with GaHV-1 and avipoxvirus, 14.3% (1/7) showed lesions that were compatible with GaHV-1 and *Avibacterium* spp. co-infection, and 14.3% (1/7) showed lesions that were compatible with *Avibacterium* spp. and *Mycoplasma* spp. co-infection.

The typical ILT lesions observed in the respiratory mucosa were characterized by fibrinous exudates, necrosis, and desquamation with syncytial formation containing intranuclear inclusion bodies. The typical lesions of mycoplasmosis were observed in the mucosa of turbinates,

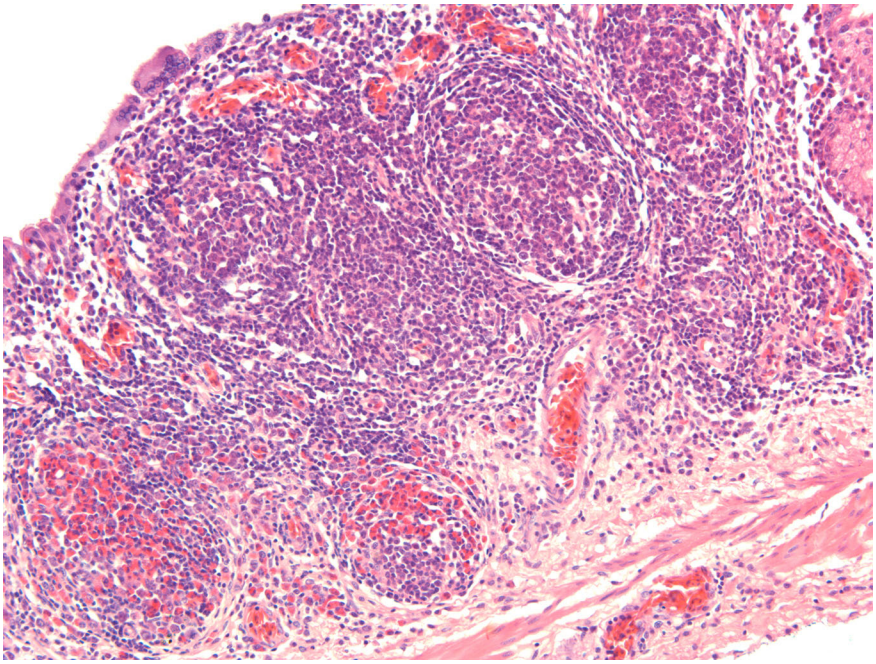

Figure 1. Lung of a chicken presenting marked thickening of bronchial mucosa characterized by lymphocytic inflammatory infiltrate with follicular-like formation. The epithelium presented loss of cilia, desquamation and syncytial cells containing intranuclear inclusion bodies. Layer chicken naturally co-infected by *Mycoplasma* spp. and GaHV-1. H&E, 400×.

larynx, trachea, and bronchi and were characterized by intense thickening of the lamina propria by infiltration of lymphocytes, in a follicular-like formation, in addition to many plasma cells and histiocytes. The surface epithelium showed multifocal to coalescing or diffuse flattening, absence of cilia, and a decrease in the number of goblet cells and mucous glands in the lamina propria. In chickens with marked lesions, the epithelium became cubic or flattened without cilia. Lungs also presented bronchial lymphoid tissue hyperplasia in addition to a diffuse infiltrate of lymphocytes and plasma cells in the lamina propria. In four chickens, characteristic lesions for both *Mycoplasma* infection and GaHV-1 were observed (Figure 1).

Chickens that presented lesions suggestive of infectious coryza showed changes in turbinates and paranasal sinuses and mild lesions in the larynx, trachea, and lungs. The lesions were characterized by marked necrocaseous and heterophilic rhinitis and sinusitis. The paranasal sinuses were filled with amorphous eosinophilic material containing cellular debris and intralesional bacterial colonies. In the larynx and trachea, multifocal to coalescing heterophilic infiltrate was observed in the lamina propria, with multifocal necrosis of epithelial cells. In the lungs, there was heterophilic and histiocytic inflammatory infiltrate in the parenchyma and also in the lamina propria of the bronchi. Sections of paranasal sinuses stained by Goodpasture revealed numerous bacterial colonies containing gram-negative bacilli.

Lesions characteristic of avipoxvirus were observed in the epithelium of the larynx and proximal trachea. There were lymphoplasmacytic laryngitis and tracheitis associated with hyperplasia and squamous metaplasia of the epithelium, which showed numerous intracytoplasmic eosinophilic inclusion bodies (Bollinger inclusions) in epithelial cells (Figure 2).

Moderate to intense lesions indicative of mycoplasmosis and laryngotracheitis were found mainly in the proximal segment of the trachea, and mild to moderate lesions were observed in the distal segment. No changes were observed in the brain or trigeminal ganglion in any of the chickens.

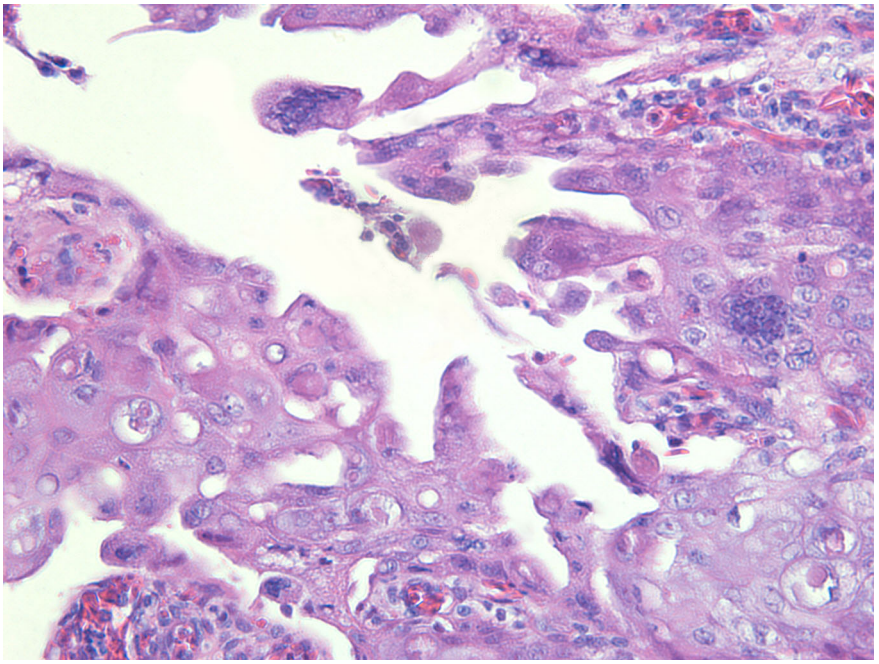

Figure 2. Trachea presenting metaplasia of the mucosa characterized by squamous epithelial hyperplasia with several eosinophilic intracytoplasmic inclusion bodies and syncytial cells with intranuclear inclusion bodies. Layer chicken naturally co-infected by GaHV-1 and *Avipoxvirus*. H&E, 400×.

PCR

The expected fragment sizes for GaHV-1 (237 bp), *M. gallisepticum* (185 bp), *M. synoviae* (207 bp), and *A. paragallinarum* (500 bp) were amplified. Chickens were considered to be positive for these agents when DNA was found either the trachea or nasal turbinates/sinuses. The highest percentage of positivity was observed for GaHV-1 at 74.2% (23/31), followed by 48.4% (15/31) for *M. synoviae*, 38.7% (12/31) for *A. paragallinarum*, and 38.7% (12/31) for *M. gallisepticum*. Most chickens presented with co-infection (61.2%, or 19/31 chickens) by more than one pathogen, with 38.7% (12/31) positive for two pathogens, 12.9% (4/31) positive for three pathogens, and 9.6% (3/31) positive for all four surveyed agents. However, 38,7% (12/31) of the chickens presented with a single infection.

Partial Sequencing of M. gallisepticum IGSR

For the partial sequencing of the IGSR, six amplicons positive for *M. gallisepticum* were used. Three amplicons derived from 2011 samples (2011/1, 2011/2, and 2011/3), one from 2012 (2012/1), two from 2013 (2013/1 and 2013/2), and one from the commercial vaccine (MG-70 BIOVET®), totaling seven samples. A fragment of 812 bp (position 321,490 to 322,280 bp), located in the IGSR between the 16S and 23S rRNA genes of the *M. gallisepticum* genome, was also amplified.

The sequences obtained showed considerable nucleotide variation, with various mutations, insertions and deletions. The 2011/3 sample showed the greatest variation, with two exclusive strain mutations that were not observed in any other sample sequenced in this study or previously submitted to GenBank. These mutations included a guanine-to-adenine change at position 364 and a guanine-to-thymine change at position 459 (Table 4).

Samples 2011/1 and 2011/2 were identical to each other, and showed 100% identity with a North American sample (KC247850). The MG-70 vaccine showed 100% identity with the Indian (KC247859) and Spanish (KC247858)

Table 4. Nucleotide sequence of positions 112 to 635 in the IGSR in Minas Gerais strains, vaccine strains and other published *Mycoplasma gallisepticum* strains. The 2011/3 strain presented the most variance, with two unique mutations at the 364 and 459 sites of IGSR.

Posição no gene	112	142	148	162	195	197	225	227	230	233	238	239	251	257	263	275	281	282	283	286	311	317	337	342	351	352	357	363	364
Brazil - 2011/3 strain	T	C	G	C	G	-	G	A	C	C	A	G	A	A	C	C	T	G	A	A	-	T	C	A	T	A	T	C	A
Brazil - 2011/2 strain	.	.	.	.	.	-	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	C	.	G
Brazil - 2011/1 strain	.	.	.	.	.	-	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	C	.	G
Brazil - 2012 strain	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	G
Brazil - MG-70 strain	.	.	.	T	.	-	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	C	.	.	C	.	.	.	G
KC247865 - U.S. 6/85	.	.	.	T	.	A	.	T	G	.	.	T	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	T	G
KC247864 - Australia ts-11	.	.	.	T	.	A	.	T	G	.	.	T	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	G
KC247860 - Thailand	.	.	.	T	.	A	.	T	G	.	.	T	.	.	G	T	.	C	G	T	A	.	.	.	-	G	.	.	G
KC247859 - India	.	.	.	T	.	-	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	C	.	.	C	.	.	.	G
KC247858 - Spain	.	.	.	T	.	-	.	.	.	.	.	.	G	.	.	.	.	.	.	.	A	C	.	.	C	.	.	.	G
KC247857 - Jordan	.	.	A	T	.	A	A	T	G	.	.	T	.	.	G	T	C	C	.	T	A	.	.	.	-	G	.	.	G
KC247850 - U.S.	.	.	.	.	.	-	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	.	.	-	.	.	C	.	G
KC247849 - U.S.	.	.	.	.	.	A	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	G	.	.	G
KC247847 - Venezuela	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	G
KC247845 - Panama	A	.	.	.	.	-	.	.	.	.	T	.	.	.	.	T	.	.	.	.	A	.	T	.	.	.	.	.	G
KC247844 - Guatemala	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	T	G	-	.	.	.	G
KC247842 - Ecuador	A	A	.	.	.	C	.	.	.	.	.	.	G	.	.	T	.	.	.	.	A	.	.	.	-	.	.	.	G
KC247840 - Colombia	.	.	.	T	.	A	.	T	G	.	.	T	.	.	G	T	.	C	G	T	A	.	.	.	-	G	.	.	G
KC247831 - South Africa	.	.	.	T	.	A	.	T	G	.	.	T	.	.	G	T	.	C	.	T	-	.	.	.	.	G	.	.	G
KC247830 - South Africa	.	.	.	.	T	A	.	T	G	.	.	.	.	C	.	.	.	.	.	.	G	.	.	.	.	.	.	T	G
HQ143384 - Israel	.	.	A	T	.	A	A	T	G	.	.	T	.	.	G	T	C	C	.	T	A	.	.	.	-	G	.	.	G
HQ143383 - U.S. F strain	A	.	.	.	.	C	.	.	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	.	.	G
HQ143380 - Egypt	.	.	.	T	.	A	.	T	G	.	.	T	.	.	G	T	.	C	.	T	A	.	.	.	-	G	.	.	G
Posição no gene	**380**	**386**	**388**	**393**	**397**	**402**	**407**	**409**	**410**	**424**	**428**	**432**	**459**	**466**	**467**	**468**	**481**	**482**	**488**	**511**	**517**	**529**	**532**	**544**	**635**				
Brazil - 2011/3 strain	T	T	C	C	G	A	G	C	T	T	C	G	T	T	G	C	C	T	T	T	A	G	G	T	A				
Brazil - 2011/2 strain	.	.	.	T	.	.	A	G	C	.	T	.	G	.	.	.	.	C	.	.	G	.	.	.	.				
Brazil - 2011/1 strain	.	.	.	T	.	.	A	G	C	.	T	.	G	.	.	.	.	C	.	.	G	.	.	.	.				
Brazil - 2012 strain	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	C	.	G	.	A	A	.				
Brazil - MG-70 strain	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	.	.	.	.	.	A	.				
KC247865 - U.S. 6/85	.	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	A	.	.	.				
KC247864 - Australia ts-11	C	.	.	.	T	.	.	.	.	.	.	C	G	A	T	.	T	.	.	.	G	.	.	.	.				
KC247860 - Thailand	.	.	.	.	T	.	.	.	.	.	.	C	G	A	T	.	T	.	.	.	G	.	.	.	.				
KC247859 - India	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	.	.	.	.	.	A	.				
KC247858 - Spain	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	.	.	.	.	.	A	.				
KC247857 - Jordan	.	.	.	.	T	.	.	.	.	.	.	A	G	A	T	.	T	.	.	.	G	.	.	.	.				

Table 4. *continued.*

Posição no gene	112	142	148	162	195	197	225	227	230	233	238	239	251	257	263	275	281	282	283	286	311	317	337	342	351	357	363	364
KC247850 - U.S.	.	.	.	T	.	.	A	G	C	.	T	.	G	.	.	.	.	C	.	.	G	.	.	.	.	.	.	.
KC247849 - U.S.	C	.	.	.	T	.	.	.	.	.	.	C	G	A	T	.	T	.	.	.	G	.	.	.	.	.	.	.
KC247847 - Venezuela	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	C	.	G	.	A	A	.	.	.	.
KC247845 - Panama	.	.	T	T	.	.	.	.	.	C	.	.	G	.	.	.	T	C	C	G	G	.	.	A	G	.	.	.
KC247844 - Guatemala	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	C	.	G	.	A	A	.	.	.	.
KC247842 - Ecuador	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	C	.	G	.	A	A	.	.	.	.
KC247840 - Colombia	.	.	.	.	T	.	.	.	.	.	.	C	G	A	T	.	T	.	.	.	G	.	.	.	.	.	.	.
KC247831 - South Africa	C	.	.	.	T	.	.	.	.	.	.	C	G	A	T	.	T	.	.	.	G	.	.	.	.	.	.	.
KC247830 - South Africa	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	C	.	.	G	A	.	.	.	.	.	.
HQ143384 - Israel	.	.	.	.	T	.	.	.	.	.	.	A	G	A	T	.	T	.	.	.	G	.	.	.	.	.	.	.
HQ143383 - U.S. F strain	.	G	T	T	.	.	.	.	.	C	.	.	G	.	.	.	.	C	C	.	G	.	.	A	.	.	.	.
HQ143380 - Egypt	.	.	.	.	T	.	.	.	.	.	.	C	G	A	T	.	T	.	.	.	G	.	.	.	.	.	.	.

(-)deletion.

strains. Finally, the sample 2012/1 showed 100% identity to strains described in Venezuela (KC247847) and the North American vaccine F strain (KC143383).

The phylogenetic tree divided the strains into different groups, each with a *bootstrap* greater than 60 (Figure 3). Strains 2013/1 and 2013/2 showed no amplification of IGSR. The sequences obtained in this study have been deposited in the GenBank database under the following accession numbers: KJ019166 (2011/1), KJ019167 (2011/2), KJ019168 (2011/3), KJ019169 (2012/1) and KJ019170 (MG-70).

Partial Sequencing of M. gallisepticum mgc2

The same DNA extracts used for the amplification of the IGSR were used for detecting the mgc2 gene. All samples showed amplification, and again, significant nucleotide variation was found among strains. However, no exclusive mutation was found in mgc2 in these strains (Table 5). Nucleotide analysis of mgc2 sequences yielded the formation of two main groups. Strains 2013/1, 2013/2, 2011/1, and MG-70 were all identical to North American (KC247882) and Spanish (KC247891) strains. Both 2011/2 and 2011/3 strains showed 100% identity between them and with one South African strain (KC247868). However, these strains differed from strains from Guatemala (KC24877), Venezuela (KC247880), South Africa (KC247866) and the Australian vaccine strain ts-11 (KC247898) by only one nucleotide cytosine-to-thymine change at position 593 of the gene. Strain 2012/1 showed 100% identity with the North American vaccine F strain. The phylogenetic tree based on the mgc2 sequences divided the strains into different groups, each with *bootstrap* greater than 60 (Figure 4).

The sequences obtained in this study were deposited into the GenBank database with the following accession numbers: KJ019171 (MG-70), KJ019172 (2011/1), KJ019173 (2011/2), KJ019174 (2011/3), KJ019175 (2012/1) KJ019176 (2013/1) and KJ019177 (2013/2).

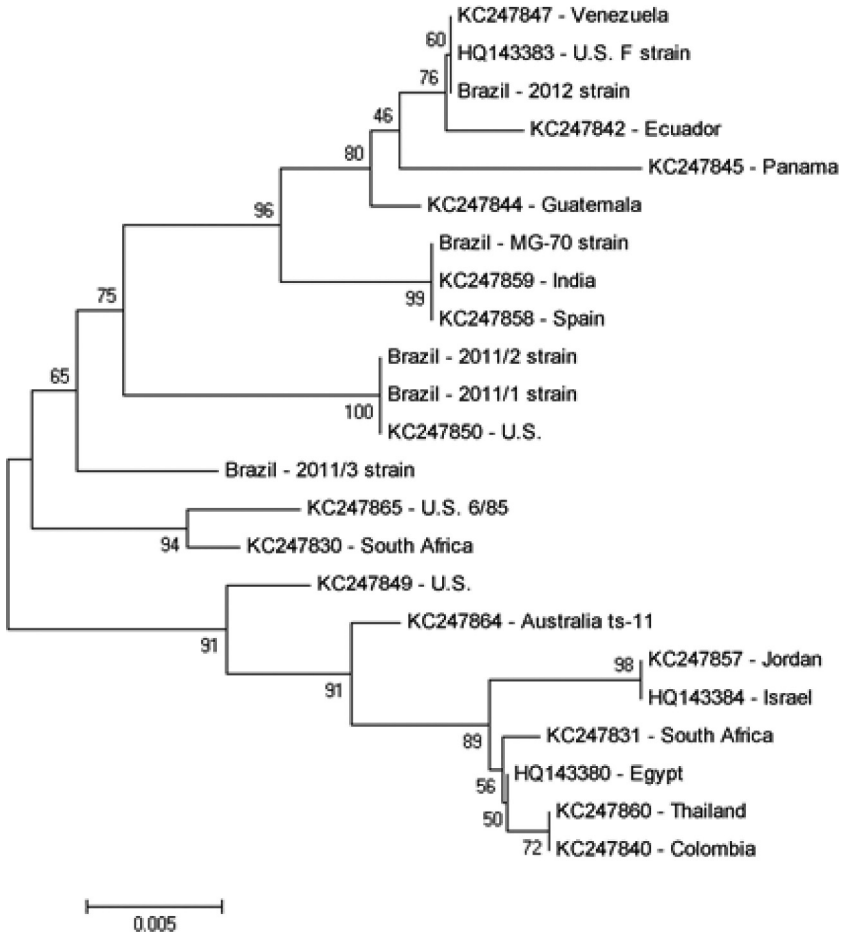

Figure 3. Phylogenetic tree generated from nucleotide sequences of a fragment of IGSR from *M. gallisepticum* field and vaccine strains. Method: *Neighbor-joining* with *bootstrap* analysis of 1,000 repetitions using Kimura 2.

DISCUSSION

The association between different respiratory pathogens in this study likely leads to increased mortality in infected chickens. Co-infections or secondary infections in diseases initiated by viral agents may provide a more serious condition, leading to increases in the mortality rate [4, 28]. However, the environment and variation of virulence of the pathogens may also influence disease severity and the mortality rate, as described for GaHV-1 [29, 30] *M. gallisepticum* [31], and *A. paragallinarum* [32].

Mixed infections are particularly challenging to diagnose because identification of the etiology may require multiple methodologies [33]. However, the detection of the etiology may not be sufficient for complete the diagnosis of the disease and/or cause of death. The aforementioned respiratory infections may develop a latent state, such as GaHV-1 [34], or a carrier status, such as *M. gallisepticum* [35]. Furthermore, the presence of live, attenuated vaccine strains may result in positive results in a PCR reaction, as described for mycoplasmosis [18]. Thus, the association of these findings with histopathological analysis is essential to establish the relationship between cause and effect and to allow the definitive diagnosis. It is important to highlight the frequent failure to detect positive samples by PCR or culture and isolation, even when the histopathological lesions are strongly indicative of a particular agent. The lack of identification may occur due to low antigen concentrations in the samples, which is common for chronic infections [36, 37, 38].

Table 5. Nucleotide sequence of mgc2 gene from the Minas Gerais strains, vaccine strains and other published *Mycoplasma gallisepticum* strains.

Posição no gene	494	498	501	504	507	508	510	519	525	526	527	530	531	537	543	549	561	570	571	582	585	588	593	600	606	611	612	624	631
Brazil - 2011/1 strain	G	T	C	A	G	G	C	C	G	C	C	C	T	A	A	A	C	C	C	A	A	G	C	A	A	G	A	C	C
Brazil - 2011/2 strain	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	T	.	G	.	.	.	.
Brazil - 2011/3 strain	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	T	.	G	.	.	.	.
Brazil - 2012 strain	.	.	T	.	.	.	T	.	.	.	.	.	.	C	G	G	.	G	.	T	G	.	.	C	G	.	G	.	.
Brazil MG-70 strain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brazil - 2013/1 strain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Brazil - 2013/2 strain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC247899 - U.S. 6/85 strain	.	.	.	G	.	.	.	.	.	T	.	T	.	.	G	.	.	.	.	.	-	-	-	-	-	-	-	-	-
KC247897 - U.S. F strain	.	.	T	.	.	.	T	.	.	.	.	T	.	C	G	G	.	G	.	T	G	.	.	C	G	.	G	.	.
KC247898 - Australia ts-11 strain	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.
KC247868 - South Africa	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	T	.	G	.	.	.	.
KC247866 - South Africa	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.
KC247873 - Colombia	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.
HQ143372 - Egypt	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC247875 - Ecuador	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	.	.	T	.
KC247891 - Spain	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC247877 - Guatemala	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.
KC247892 - India	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	-	-	-	-	-	-
HQ143377 - Israel	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	T
KC247890 - Jordan	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	A	T
KC247878 - Panama	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC247893 - Thailand	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC247883 - U.S.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	-	-	-	-	-	.	C	.	.	.
KC247882 - U.S.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC247880 - Venezuela	.	G	.	.	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	.	.	.	.	.	G	.	.	.	.
Posição no gene	**633**	**636**	**648**	**654**	**674**	**696**	**702**	**725**	**737**	**738**	**739**																		
Brazil - 2011/1 strain	C	A	A	G	C	T	A	C	T	G	C																		
Brazil - 2011/2 strain	.	.	.	.	.	C	.	.	C	.	.																		
Brazil - 2011/3 strain	.	.	.	.	.	C	.	.	C	.	.																		
Brazil - 2012 strain	A	T	T	.	.	.	.	.	.	C	T																		
Brazil MG-70 strain	.	.	.	.	.	.	.	.	.	.	.																		
Brazil - 2013/1 strain	.	.	.	.	.	.	.	.	.	.	.																		
Brazil - 2013/2 strain	.	.	.	.	.	.	.	.	.	.	.																		
KC247899 - U.S. 6/85 strain	-	-	-	.	.	C	.	.	C	.	.																		
KC247897 - U.S. F strain	A	T	T	.	.	.	.	.	.	C	T																		
KC247898 - Australia ts-11 strain	.	.	.	.	.	C	.	.	C	.	.																		
KC247868 - South Africa	.	.	.	.	.	C	.	.	C	.	.																		

Table 5. *continued.*

Posição no gene	494	498	501	504	507	508	510	519	525	526	527	530	531	537	543	549	561	570	571	582	585	588	593	600	606	611	612	624	631
KC247866 - South Africa	.	.	.	.	.	C	.	.	C	.	.																		
KC247873 - Colombia	.	.	.	.	.	.	.	A	.	.	.																		
HQ143372 - Egypt	.	.	.	.	.	.	.	.	.	.	.																		
KC247875 - Ecuador	.	.	.	A	.	C	.	.	C	.	.																		
KC247891 - Spain	.	.	.	.	.	.	.	.	.	.	.																		
KC247877 - Guatemala	.	.	.	.	.	C	.	.	C	.	.																		
KC247892 - India	-	-	-	.	.	.	.	.	.	.	.																		
HQ143377 - Israel	.	.	.	A	T	C	.	.	C	A	.																		
KC247890 - Jordan	.	.	.	A	T	C	.	.	C	A	.																		
KC247878 - Panama	.	.	.	.	.	.	.	.	.	.	.																		
KC247893 - Thailand	.	.	.	A	T	C	.	.	C	A	.																		
KC247883 - U.S.	A	.	-	.	.	C	.	.	.	.	.																		
KC247882 - U.S.	.	.	.	.	.	.	.	.	.	.	.																		
KC247880 - Venezuela	.	.	.	.	.	C	.	.	C	.	.																		

GaHV-1 infection associated with secondary infection by *A. paragallinarum* was described by Mohamed et al. [4]. The authors suggest that mixed infections can result in a shorter period of incubation and increased mortality in natural outbreaks, indicating synergism of the respiratory agents. In the present study, histopathology results showed that the frequency of disease with co-infection was lower than indicated by PCR results. Thus, it is clear that presence of the antigen does not necessarily result in a disease state, as the chicken may act as a silent carrier of one or more pathogens.

Previous studies based on serology and isolation demonstrated the involvement of *Escherichia coli* and *A. paragallinarum* infections in GaHV-1, demonstrating the mixed character of respiratory tract infections in birds. Such pathogens often find favorable conditions for infection in hosts due to immunosuppression caused by the primary infectious agents, such as the case for infectious bursal disease virus or infectious anemia virus [39] or secondary infection to respiratory viral infections such as low pathogenic influenza virus [28]. Live, attenuated vaccines such as that for infectious bronchitis virus can also facilitate respiratory infection by *E. coli* [40, 41]. High concentrations of ammonia and dust also promote respiratory distress, which can result from unsanitary conditions, inadequate management, and high population density [42, 43]. Moreover, seasonal variation, weather, and parasitic infections can be associated with compromised immune response [44].

Mycoplasma spp. infections in the laying hens of this study were likely a result of multiple factors. The release of chickens from vaccination-free areas into farms with vaccinated chickens creates a higher likelihood of disease. Biosecurity failures or deficient vaccination programs may also play a role. *M. gallisepticum* is known for its pathogenic synergism with other respiratory pathogens (viruses or bacteria) such as *A. paragallinarum* [45, 46] and *E. coli* [40, 47]. Interactions between *M. gallisepticum* and GaHV-1 similar to those observed in this study have been previously reported [48]. *M. synoviae* causes subclinical respiratory infection, although most pathogenic strains cause clinical respiratory disease [38]. Failures in biosecurity and poor vaccination programs allow the

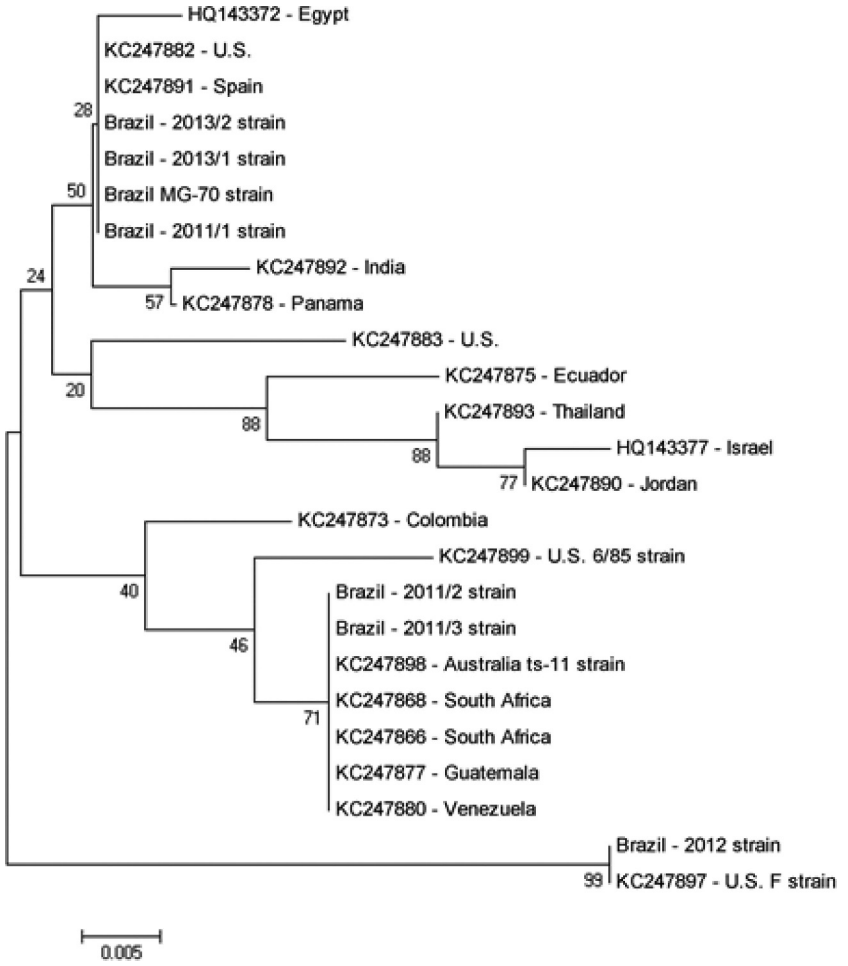

Figure 4. Phylogenetic tree generated from nucleotide sequences of a fragment of *mgc*2 gene from *M. gallisepticum* field and vaccine strains. Method: *Neighbor-joining* with *bootstrap* analysis of 1,000 repetitions using Kimura 2.

persistent presence of mycoplasmosis, predisposing the chickens to infection by additional respiratory agents.

Virulent avipoxvirus in its diphtheric form produces gross lesions in the oral and respiratory mucosa that are easily confounded with ILT [49]. In this study, a chicken presented histological lesions that are compatible with both agents, including intranuclear and intracytoplasmic inclusions bodies that were sometimes observed in the same cell. Recently, new cases of co-infection involving *Avipoxvirus* and GaHV-1 have been reported [50].

Chickens can be asymptomatic carriers [2] requiring a combination of molecular detection and histopathology for a diagnosis, which shall demonstrate the etiology and corresponding lesions. It is additionally necessary to characterize the pathogenicity or differentiating between vaccine (attenuated strains) and field strains by sequencing [18, 15]. The sequencing of multiple regions, particularly the IGSR and a fragment of the mgc2 gene, allows differentiation of *Mycoplasma* strains [15]. Analysis of the nucleotide alignment of IGSR sequences in this study allowed the identification of three different strains. Of the three strains, two were characterized as field strains and one originated from a vaccine (2012/1) that was used for layer chickens in Brazil and is identical to the F strain of the United States. Curiously, there was no IGSR amplification for any of the samples collected in 2013, which is possibly a result of a mutation or deletion in the target region.

Nevertheless, a fragment of mgc2 gene was also analyzed for the same samples and showed the presence of four different field strains and one vaccine strain. The alignment of the mgc2 gene-fragment sequences with sequences published in GenBank again showed 100% identity between strain 2012/1 and the vaccine F strain. The vaccine strain was found in a chicken with histopathological lesions indicative of mycoplasmosis, suggesting the possibility of pathogenic reversion, considering the severity of the lesions [51]. However, although the F strain infection may lead to increased inflammatory infiltrate in the lamina propria of the respiratory mucosa, infection by a vaccine strain would not result in the formation of large lymphocytic aggregates and metaplasia of the ciliated epithelium [52], which were observed in the 2012/1 strain infection.

In five chickens, sequencing of *M. gallisepticum* demonstrated the presence of field strains that are genetically unrelated to the vaccine strains. The three 2011 strains were from chickens that belonged to a farm rearing chickens without vaccination for mycoplasmosis, being potentially field strains. In this case, the histopathological lesions were moderate to intense. Strains 2012 and 2013 were obtained from farms in which chickens were vaccinated with *M. gallisepticum* F and MG-70 strains. However, the high variability of *M. gallisepticum* strains found in chickens between 2012 and 2013 that were vaccinated for *M. gallisepticum* suggests that vaccination does not guarantee cross-protection against different strains [15] and may enable co-infection of field and vaccine strains.

CONCLUSIONS AND APPLICATIONS

1. The incidence of respiratory disease with multiple etiologies may be a common condition in laying hens, mainly due the presence of multi-age chickens present in high population density.
2. Histopathology is a fundamental diagnostic technique that is useful for directing the molecular confirmatory tests, which when applied alone will not differentiate carriers or asymptomatic individuals.
3. It has been shown that vaccination with *M. gallisepticum* vaccine strains in the evaluated farms did not guarantee the necessary protection against different strains of *M. gallisepticum*, which presented high genetic variability.
4. This study highlights that genetic profiling is important for differentiating the genotypes of *M. gallisepticum* vaccine and field strains. Furthermore, it can clarify the evolutionary progression of this pathogen and the influence of vaccinations.

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