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Original Article

Characterization and Differentiation of Chicken Mycoplasma Isolates Using 16S-23S Intergenic Spacer Region Sequencing

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ABSTRACT

The objective of this study was to identify the species and characterize the genetic relationships among mycoplasma isolates from commercial layer hen flocks using 16S–23S rDNA intergenic spacer region (IGSR) sequencing. Twenty-one isolates were obtained from samples collected from commercial layer flocks in four Brazilian states: São Paulo, Minas Gerais, Rio de Janeiro and Espírito Santo. The isolates were recovered from the São Paulo, Rio de Janeiro and Espírito Santo states. Eleven isolates were originated from tracheal swabs, five from shell gland swabs and five from ovary fragment collection. The 16S–23S rDNA IGSR of isolates were amplified by PCR, and the obtained products were subsequently sequenced. The consensus of each isolate was compared to the available sequences using Nucleotide BLAST® to determine the mycoplasma species. A phylogenetic analysis of the *Mycoplasma gallisepticum* (MG) sequences was performed. Pairwise analyses showed homologies of 99% to 100% with the previously characterized sequences listed in GenBank®. Four *Mycoplasma gallinaceum* were isolated from three flocks and seven *M. pullorum* isolates were obtained from a single flock. The other 10 isolates were all identified as MG and were obtained from four flocks. The 16S-23S rDNA IGSR sequencing was a good method to identify *Mycoplasma* species isolated from field samples, providing fast and reliable results at relatively low costs. The results were also satisfactory for the single-locus sequence typing of MG isolates.

INTRODUCTION

Mycoplasma infections are highly prevalent among poultry flocks, and three species are considered to be pathogenic: *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Mycoplasma meleagridis* (MM). The economic losses attributed to mycoplasmosis are due to decreases in egg production and quality, poor hatchability, poor feed efficiency, high mortality rates and high carcass condemnation rates on slaughter (Kleven, 2008; Nascimento *et al.*, 2005; Nascimento; Pereira, 2009). These microorganisms prefer the mucous and serous membranes of birds, causing respiratory, joint and urogenital pathologies. There are several strains of MG, MS and MM, which are phenotypic and genotypically different, that have diverse degrees of pathogenicity, virulence and immunogenicity (Kleven, 2008; Nascimento *et al.*, 2005). The transmission of mycoplasmas can occur horizontally by aerosols; by sexual contact or artificial insemination; by direct contact with contaminated birds and indirect contact through people, other animals, food, water and equipment; or by vertical egg transmission (Kleven, 2008; Nascimento & Pereira, 2009). In addition to the notably pathogenic mycoplasma, another 20 species are also known to infect birds (Kleven, 2008). These species are regarded as nonpathogenic species; however, some cases have been reported in

which these species have caused diseases (Ganapathy *et al.*, 1998; Kleven *et al.*, 1978; Yagihashi *et al.*, 1993; Moalic *et al.*, 1997; Gomes, 2013; Silva *et al.*, 2014).

The identification of *Mycoplasma* species using conventional techniques can be laborious and complex, requiring the use of biochemical and serological tests, with specific serum for each species. PCR is widely used for identification of pathogenic mycoplasmas, such as MG (Nascimento *et al.*, 1991; García *et al.*, 2005) and MS (Lauerman *et al.*, 1998; Hong *et al.*, 2004). For more rapid results, multiplex PCR is also available (Wang *et al.*, 1997; Fraga *et al.*, 2013). However, identification of isolates other than pathogenic species in not practical using regular PCR.

Recently, sequencing became more accessible for smaller facilities and with lower costs, thus can be used not only for research, but also in a regular basis by diagnostic laboratories. Ferguson *et al.* (2005) described a method with high discriminatory power amongst MG isolates by sequencing four regions of the bacterium that encode surface proteins. Subsequently, Raviv *et al.* (2007) obtained similar results by sequencing the 16S-23S rDNA intergenic spacer region, using as a single-locus sequence typing (SLST) tool for MG isolate differentiation in diagnostic cases and epizootiological studies. Using the same method, Ramírez *et al.* (2008) found that the 16S–23S rDNA intergenic spacer regions (IGSR) of avian mycoplasmas could be used to differentiate species because this region of the DNA has low inter-species homology, with a maximum percent of homology of 90.5% and a minimum of 7.9%.

The objective of this study was to identify the species and characterize the genetic relationships among mycoplasma isolates from commercial layer hen flocks using a 16S–23S rDNA IGSR sequencing method.

MATERIAL AND METHODS

Samples were obtained from commercial layer flocks in four Brazilian states. The number of flocks sampled were five in São Paulo and Minas Gerais, two in at Rio de Janeiro and four in at Espírito Santo. From each flock, five birds were necropsied to obtain swabs from trachea, ovary and shell gland. All animal collection procedures were approved by the Ethics Committee on the Use of Animals of the Universidade Federal Fluminense under number 199, dated May 17, 2012. Isolation of mycoplasmas was performed using Frey medium (Frey *et al.*, 1968) in agar and broth. Samples were incubated until 21 days, when chance in color and formation of egg-shaped colonies in agar were identified for positive samples.

DNA was extracted from 1 mL of media that showed mycoplasma growth by a color change using the phenol-chloroform method (SAMBROOK; FRITSCH, 1989). The 16S–23S rDNA IGSR of the isolates were amplified by PCR, as described by Ramírez *et al.* (2008), using the forward primer 5´-CGT TCT CGG GTC TTG TAC AC-3´ and the reverse primer 5´- CGC AGG TTT GCA CGT CCT TCA TCG-3´. Sequencing was conducted at Fundação Oswaldo Cruz, RJ, Brazil, and the sequence was determined for each strand of DNA using an Applied Biosystems 3730xl DNA Analyzer. The resulting chromatograms were examined, and the forward and reverse complimented sequences of all isolates were compared to produce a consensus sequence using Lasergene SeqMan software (Version 7.2.1; DNASTAR, Australia).

The consensus of each isolate was compared to those available in GenBank® using Nucleotide BLAST® software (NCBI, 2017) to determine the mycoplasma species. The samples used for MG phylogeny included Brazilian isolates obtained by Couto *et al.* (2015) in Minas Gerais state, Brazil, with the following accession numbers: KJ019166 (UFMG 2011-1); KJ019167 (UFMG 2011-2); KJ019168 (UFMG 2011-3); KJ019169 (UFMG 2012); KJ019170 (MG 70); samples from other countries with the following accession numbers were used: HQ143383 (MG F USA); JQ770172 (MG S6); KC247865 (MG 6-85); JN935873 (MG PG31); KC247863 (MG USA-R-CK60); and KC247864 (MG TS-11). The IGSR isolate and reference sequences were aligned using Clustal-W, and a dendrogram was constructed using the neighbor-joining method with 1000-bootstrap replicates. Phylogenetic analyses were conducted using the MEGA version 6 software program (Tamura *et al.*, 2013).

RESULTS AND DISCUSSION

It was obtained 22 isolates, being 11 isolates originated from the trachea, five from the ovary and five from shell gland. The isolates were recovered from seven different flocks in São Paulo, Rio de Janeiro and Espírito Santo (Table 1).

A pairwise analysis carried out to compare the nucleotide sequences allowed the identification of species with 99% to 100% homologies to previously characterize sequences on GenBank®. Nonpathogenic mycoplasmas were isolated from three flocks: one from São Paulo and two from Rio de Janeiro. In Flock 2, *M. pullorum* was isolated from four hens and from all sampling sites (trachea, shell gland and ovary). In addition, *M. gallinaceum* was found in the shell gland

of one bird from Flock 2. The only mycoplasma isolated from Flocks 4 and 5 was *M. gallinaceum* (Table 1). These species, although not considered pathogenic, were able to colonize different parts of the hen. The interaction of *M. gallinaceum* with MS to exacerbate synovitis has been shown (Yagihashi *et al.*, 1993). Also, the synergistic effect of *M. gallinaceum* with MS was related to a higher prevalence of Infectious Coryza (Gomes, 2013). Evidence that *M. pullorum* and *M. gallinarum* can act as pathogens has been previously reported (Moalic *et al.*, 1997; Silva *et al.*, 2014), but the interactions of these species with MS or MG have not been studied.

Table 1 – The avian mycoplasma isolates used in the present study, Brazilian state of origin, flock identification, site sampled in the bird, species identified, identification of the sample in the laboratory and GenBank accession number of the sequences obtained.

Flock location (State)	Flock ID	Hen number	Sampling site	Species	Sample ID	GenBank accession number
São Paulo	Flock 1	2	Trachea	*M. gallisepticum*	LSA UFF 001	KT824804
	Flock 2	2	Trachea	*M. pullorum*	LSA UFF 002	KT824805
	Flock 2	2	Shell gland	*M. gallinaceum*	LSA UFF 003	KT824806
	Flock 2	2	Ovary	*M. pullorum*	LSA UFF 004	KT824807
	Flock 2	3	Trachea	*M. pullorum*	LSA UFF 005	KT824808
	Flock 2	4	Trachea	*M. pullorum*	LSA UFF 006	KT824809
	Flock 2	4	Ovary	*M. pullorum*	LSA UFF 007	KT824810
	Flock 2	5	Shell gland	*M. pullorum*	LSA UFF 008	KT824811
	Flock 2	5	Ovary	*M. pullorum*	LSA UFF 009	KT824812
	Flock 3	1	Trachea	*M. gallisepticum*	LSA UFF 010	KT824813
	Flock 3	4	Trachea	*M. gallisepticum*	LSA UFF 011	KT824814
Rio de Janeiro	Flock 4	4	Trachea	*M. gallinaceum*	LSA UFF 012	KT824815
	Flock 5	1	Trachea	*M. gallinaceum*	LSA UFF 013	KT824816
	Flock 5	2	Trachea	*M. gallinaceum*	LSA UFF 014	KT824817
Espírito Santo	Flock 6	1	Shell gland	*M. gallisepticum*	LSA UFF 015	KT824818
	Flock 6	1	Ovary	*M. gallisepticum*	LSA UFF 016	KT824819
	Flock 6	3	Ovary	*M. gallisepticum*	LSA UFF 017	KT824820
	Flock 6	5	Shell gland	*M. gallisepticum*	LSA UFF 018	KT824821
	Flock 7	1	Trachea	*M. gallisepticum*	LSA UFF 019	KT824822
	Flock 7	2	Trachea	*M. gallisepticum*	LSA UFF 020	KT824823
	Flock 7	2	Shell gland	*M. gallisepticum*	LSA UFF 021	KT824803

All other 10 isolates were identified as MG and were obtained from two flocks in São Paulo and two flocks in Espírito Santo. All isolates from Espírito Santo flocks clustered together with UFMG2012 and MG F vaccine strain, suggesting recovery of the vaccine strain.

The samples isolated from Flock 3 were similar to MG S6 but were also closely related to MG 70 vaccine strain, developed in Brazil, which was used in that flock. The sample isolated from Flock 1 was similar to MG 6/85 vaccine strain, despite the use of the MG 70 vaccine strain in that flock (Figure 1). This could be due to contamination from other flocks that had received the MG 6/85 vaccine. Isolates from different States were included in different clusters (Figure 1), implying a spatial distribution. Figure 2 shows the nucleotide differences among the strains examined in this work.

Although avian mycoplasmosis has special importance in the Brazilian national poultry health program, just few studies were carried to estimate prevalence of MG and MS in commercial layer hen flocks. Due to regular vaccination of the flocks, wild type MG prevalence is relatively low, not found using

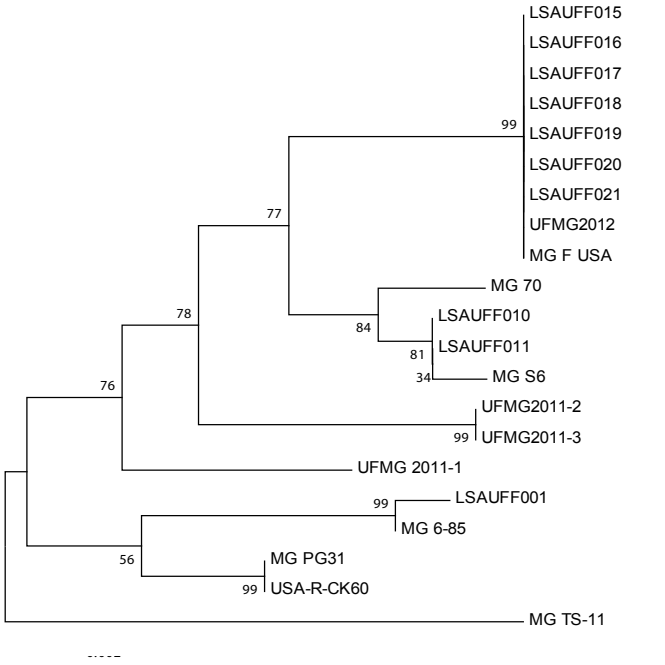

Figure 1 – Dendrogram of the *M. gallisepticum* isolates from Brazil, with vaccine and reference strains, constructed by Clustal-W alignment of the IGSR sequences by the neighbor-joining method with 1000-bootstrap replicates using the MEGA 6.0 software program (http://www.megasoftware.net).

Figure 2 – A representation of the differences among *M. gallisepticum* Brazilian isolates, vaccines and other strains available in GenBank.

IGSR position	67	81	110	126	160	177	191	193	194	220	223	226	229	235	245	247	253	271	279	295	296	297	298	307	313	336	337	338	339	340	345	346	347	348
LSAUFF001	-	G	T	A	T	T	G	A	A	-	T	G	C	T	C	A	C	C	A	A	C	T	A	G	T	T	G	A	T	T	T	T	T	-
LSAUFF010	-	.	.	G	C	.	.	-	.	-	A	C	.	G	A	.	A	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	C	-
LSAUFF011	-	.	.	G	C	.	.	-	.	-	A	C	.	G	A	.	A	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	C	-
LSAUFF015	A	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
LSAUFF016	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
LSAUFF017	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
LSAUFF018	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
LSAUFF019	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
LSAUFF020	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	T
LSAUFF021	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
MG 6-85	-	.	.	G	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
MG 70	-	.	.	G	.	.	.	-	.	-	A	C	.	G	.	G	A	.	.	.	.	.	.	A	C	.	.	.	.	.	.	.	C	-
MG F USA	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
MG PG31	-	.	.	G	C	-	T	.	-	-	A	C	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	-	.	-
MG S6	-	.	.	G	C	.	.	-	.	-	A	C	.	G	A	.	A	.	.	G	.	.	.	A	C	.	.	.	.	.	.	.	C	-
MG TS-11	-	.	.	G	.	.	.	.	.	-	.	.	.	.	.	.	A	.	.	.	.	.	.	A	.	.	.	.	.	.	-	-	.	-
UFMG 2011-1	-	A	.	G	C	.	.	-	.	-	A	C	.	G	.	.	A	.	.	-	-	-	-	A	.	.	.	.	.	.	.	.	-	-
UFMG2011-2	-	.	.	G	C	.	.	-	.	-	A	C	T	G	.	.	A	.	.	.	.	.	.	A	.	-	-	-	-	-	.	.	.	-
UFMG2011-3	-	.	.	G	C	.	.	-	.	-	A	C	T	G	.	.	A	.	.	.	.	.	.	A	.	-	-	-	-	-	.	.	.	-
UFMG2012	-	.	A	G	C	.	.	C	.	A	A	C	.	G	.	.	A	T	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	-
USA-R-CK60	-	.	.	G	C	-	T	.	-	-	A	C	.	G	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	-	.	-

IGSR position	349	354	360	361	377	385	390	394	399	402	404	406	407	414	420	424	428	439	453	460	461	473	475	476	482	494	495	509	521	524	536	630	631	650
LSAUFF001	A	T	T	G	T	C	C	G	T	A	G	C	T	T	T	C	G	-	G	T	G	A	C	C	T	T	-	G	A	G	T	T	A	-
LSAUFF010	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	.	-	-	A	G	.	A	.	.	-
LSAUFF011	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	.	-	-	A	G	.	A	.	.	-
LSAUFF015	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
LSAUFF016	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
LSAUFF017	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
LSAUFF018	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
LSAUFF019	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
LSAUFF020	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	-	-	-
LSAUFF021	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
MG 6-85	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-
MG 70	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	.	.	-	A	G	.	A	.	.	-
MG F USA	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
MG PG31	.	.	.	.	.	.	.	.	A	.	.	.	.	-	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-
MG S6	.	.	C	.	.	-	T	.	A	G	.	.	.	.	C	.	.	-	.	.	.	.	.	.	.	-	-	A	G	.	A	.	.	-
MG TS-11	G	.	C	.	C	.	.	T	A	.	.	.	.	.	.	.	C	T	.	A	T	-	T	T	.	.	T	.	G	.	.	.	.	-
UFMG 2011-1	.	.	C	A	.	.	.	.	A	.	.	.	-	.	.	.	.	-	T	.	.	.	.	T	.	.	-	A	G	.	.	.	.	A
UFMG2011-2	.	C	C	.	.	.	T	.	A	.	A	G	C	.	.	T	.	-	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	-
UFMG2011-3	.	C	C	.	.	.	T	.	A	.	A	G	C	.	.	T	.	-	.	.	.	.	.	.	.	.	-	.	G	.	.	.	.	-
UFMG2012	.	.	C	.	.	-	T	.	A	.	.	.	.	.	C	.	.	-	.	.	.	.	.	.	C	.	-	.	G	A	A	.	.	-
USA-R-CK60	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	-	.	.	.	.	.	.	-

Positions where nucleotide differences exist in one sequence are denoted with the ambiguity setter code, according to the IUB.

PCR by Buim *et al.* (2009), Mettifogo *et al.* (2013) and Barros *et al.* (2014), 5% (Teixeira *et al.*, 2015) and isolated from 2,67% of the flocks by Santos (2015). On the other hand, since flocks are not vaccinated for MS in Brazil, prevalence is estimated to be very high, with 79,31% (Buim *et al.*, 2009), 68% (Mettifogo *et al.*, 2013), 83,33% (Barros *et al.*, 2014), 45% (Teixeira *et al.*, 2015) and 93,34% (Santos, 2015). However, isolation of pathogenic mycoplasmas may be difficulted by rapid growth of commensal strains, such as *M. gallinarum* and *M. gallinaceum* (Kleven, 2008). Thus, rapid identification of isolates is desirable for diagnostic purposes.

Considering the laborious and time-consuming work of biochemical and serological species confirmation tests (immunoperoxidase/immunofluorescence and growth inhibition) and the difficulty of performing specific PCR for the 23 avian mycoplasma species, the 16S-23S rDNA IGSR sequencing was shown to be a good alternative method to identify isolated strains species, providing fast and reliable results at a relatively low cost. The results were also satisfactory for the SLST of MG isolates, yielding in this work clusters related to the origin of the strains.

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