Molecular detection and characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* strains in backyard poultry in Italy

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ABSTRACT *Mycoplasma gallisepticum* (**MG**) and *Mycoplasma synoviae* (**MS**) represent the most important avian *Mycoplasma* species in the poultry industry, causing considerable economic losses. In Italy, the presence of MG or MS has been investigated especially in commercial poultry farms. To our knowledge, no systematic investigations on MG or MS presence using highly specific diagnostic assays have been performed in backyard poultry. The aim of this study was to detect and molecularly characterize MG and MS strains in 11 backyard poultry flocks located in different regions of Italy. Tracheal swabs were collected and DNA was extracted. For MS, a PCR targeting a *vlhA* gene fragment was performed, and typing and subtyping was attempted. The presence of MG was investigated by a screening PCR, then MG typing by gene-targeted sequencing (**GTS**). All the amplicons were sequenced, then MG and MS dendrograms were constructed. All the flocks examined resulted Mycoplasma positive: 5 out of 11 (45.45%) were MG and MS positive, 3 (27.27%) were MG positive, and the remaining 3 (27.27%) were MS positive. The MS detections were assigned to types C, D, and F. All strains of type D belonged to subtype D1 and 2 unknown subtypes were identified. A MS sequence showed peculiar characteristics, which did not allow assignment to a known MS type or subtype. MG GTS analysis identified 6 MG strains belonging to 5 subclusters circulating in Italian backyards chicken flocks. The results of this study provide evidence of a risk for commercial poultry farms, especially in areas where backyard and commercial farms are close, suggesting the implementation of biosecurity measures.

Key words: backyard poultry, Italy, *Mycoplasma gallisepticum*, *Mycoplasma synoviae*

INTRODUCTION

Mycoplasma gallisepticum (**MG**) and *Mycoplasma synoviae* (**MS**) represent the most important avian *Mycoplasma* species worldwide in the poultry industry, causing considerable economic losses (Raviv and Ley, 2013). MG causes chronic respiratory disease in chickens and sinusitis in turkeys. MG disease is characterized by respiratory rales, coughing, nasal discharge and conjunctivitis, and infraorbital sinusitis in turkeys. Increased carcass and downgrading condemnation caused by aereosacculitis, decreased growth and egg production and increased medication costs, make MG one of costliest infection diseases (Raviv and Ley, 2013).

MS is mostly considered to occur as a subclinical upper respiratory infection, which can progress to respiratory disease with air sac lesions, when combined with Newcastle disease or infectious bronchitis, and to infectious synovitis when it becomes systemic (Lockaby et al., 1999). In the last decade, the importance of MS seems to be increasing. The emergence of MS strains that affect egg shell quality with typical eggshell apex abnormalities and decreased egg production (Feberwee et al., 2009; Catania et al., 2010, 2016a) as well as arthropathic and amyloid-inducing strains (Kleven et al., 1975; Landman and Feberwee, 2001) have been reported. Control of *Mycoplasma* has generally been based on eradication of the organisms from breeder flocks and the maintenance of a *Mycoplasma*-free status in the breeders and their progeny by implementation of biosecurity.

In Italy, the presence of MG or MS has been investigated especially in commercial poultry farms (Catania et al., 2010, 2016a). A case of MS infection in the lesser flamingo (*Phoeniconaias minor*) in a zoo of Northern Italy was also reported (Catania et al., 2016b). To our knowledge, no systematic investigations on MG or MS

Table 1. Examined flocks with MG and MS PCR results.

Year	Flock	Region	MS (type-subtype)	MG
2016	1	Lombardy	+(F2)	+
2016	2	Lombardy	−	+
2016	3	Emilia Romagna	−	+
2016	4	Tuscany	+(D1)	+
2017	5	Sicily	+(D1)	+
2017	6	Campania	+(n.a.)	+
2017	7	Emilia Romagna	+(n.a.)	−
2017	8	Friuli Venezia Giulia	+ (C7)	−
2017	9	Lazio	+(n.k.)	+
2017	10	Umbria	−	+
2017	11	Lazio	+(n.a.)	−

n.a.: not available.
n.k.: not known.

presence using highly specific diagnostic assays have been performed in backyard poultry.

The aim of this study was to detect and molecularly characterize MG and MS strains in backyard poultry flocks in different regions of Italy.

MATERIALS AND METHODS

Samples

Between January 2016 and December 2017, eleven backyard poultry flocks (1 to 11) located in different areas of Italy (Northern, Central and Southern Italy) were investigated for MG and MS presence (Table 1). The main breeds were Sussex, Robusta Lionata, and Cocincine Millefiori, reared for ornamental purposes or consumption of meat. Anamnesis reported a low or moderate morbidity of respiratory signs (sneezing, nasal discharge, and foam in the eye or rattle breathing) in adult birds, but this was severe in chicks with mortality up to 100%. None of these flocks were vaccinated against MG or MS, except flock 10, where a killed MG vaccine was used.

In total, 100 birds were randomly selected and sampled (8 to 10 per flock). Tracheal swabs were collected from only the symptomatic birds and then stored at −20°C until processing as a pool for each flock.

DNA Extraction

Pools of tracheal swabs were eluted in 2 mL of sterile PBS. Total DNA was extracted from elutions using the DNA Mini kit (Qiagen, Germany) according to the manufacturer's instructions, then tested using PCR for MS and MG.

Detection and Typing of Mycoplasma Synoviae

With respect to MS, a PCR targeting a 430 bp fragment of the *vlhA* gene coding for an abundant immunodominant surface protein was performed. The target region included tandem repeats that encode proline-rich repeats (**PRR**) and the highly polymorphic RIII region, allowing the typing and subtyping of MS strains (Benčina et al., 2001). The forward primer *vlhA*-F (5′-GATGCGTAAAATAAAAGGAT-3′) (Moscoso et al., 2004) and the reverse primer *vlhA*R2 (5′-AGTAACCGATCCGCTTAATGC-3′) (Hammond et al., 2009) were used. A previously characterized MS sample was included as positive control in all PCRs.

Detection and Typing of Mycoplasma Gallisepticum

The presence of MG was investigated using a PCR targeting a 237 to 303 bp fragment of the proline-rich domain of the *mgc*2 (**pr-mgc2**) adhesin-encoding gene (Moscoso et al., 2004). In order to type DNA samples, the gene-targeted sequencing (**GTS**) assay targeting portions of putative cytadhesin genes (*pvpA, gapA* and *mgc2*) and an uncharacterized hypothetical surface lipoprotein-encoding gene (designated coding DNA sequence MGA_0319) (Ferguson et al., 2005) was performed.

In particular, 590, 824, and 332 bp fragments of the MGA_0319, *mgc*2, and *gap*A genes were amplified according to Ferguson et al. (2005). With regard to the *pvp*A gene, forward pvpA3F (5′-GCCAMTC CAACTCAACAAGCTGA-3′) and reverse pvpA4R primers (5′-GGACGTSGTCCTGGCTGGTTAGC-3′) (Ferguson et al., 2005) or modified forward pvpA3F (5′-GGYAGTCCTAAGTTATTWGGTC-3′) (Liu et al., 2001) and pvpA4R (Ferguson et al., 2005) primers, amplifying a 497 or 702 bp fragment, respectively, were used. Ts-11 vaccine strain was included as positive control in all PCRs.

Sequencing and Phylogenetic Analysis

The *vlhA* MS and the GTS MG amplicons were sequenced in both directions by a commercial sequencing service (Macrogen Europe). The obtained sequences were edited and assembled using Bioedit software. Nucleotide identity of the *vlhA* sequences was determined using the nucleotide BLAST algorithm with GenBank database (http://www.ncbi.nlm.nih.gov) (Johnson et al., 2008).

Dendrograms were generated by the neighbor-joining method using Maximum Likelihood model, by Molecular Evolutionary Genetics Analysis (MEGA 6) (Tamura et al., 2013). Bootstrap values were calculated based on 1,000 replicates and considered significant when >70. In addition to the sequences obtained in this study, published corresponding sequences of 34 MS reference strains and previously pubblished Italian strains were included in the phylogenetic analysis (Supplementary Table 1). For MG sequences, the dendrogram was constructed with only MG samples for which all four genes have been successfully sequenced. The MG sequences in GTS analysis were compared with published strains including reference and vaccine strains (Supplementary Table 2).

Table 2. Accession numbers of MS strains submitted to GeneBank database.

Strain	Accession number
IT/MS675/ck/16	MH727587
IT/MS705/ck/16	MH807700
IT/MS801/ck/17	MH807701
IT/MS804/ck/17	MH807702
IT/MS879/ck/17	MH807703

RESULTS AND DISCUSSION

All flocks examined were *Mycoplasma* positive when detected using PCR. Of 11 flocks, 5 (45.45%) were MG and MS positive, 3 (27.27%) were MG positive, and the remaining 3 (27.27%) were MS positive (Table 1).

With respect to MS sequence analysis, the *vlhA* amplicons were successfully sequenced except those from flocks 6, 7, and 11. The MS strains analyzed were named as follows: IT/MS675/ck/16 (flock 1), IT/MS705/ck/16 (flock 4), IT/MS801/ck/17 (flock 5), IT/MS804/ck/17 (flock 8), IT/MS879/ck/17 (flock 9). The nucleotide sequences were submitted to the GenBank database (Table 2). The comparison of the sequences with GenBank showed a percentage of similarity ranging

from 92 to 100%. According to the length of the PRR region (Benčina et al., 2001), three known MS types, C (96 nt), D (69 nt), and F (108 nt), were detected in flocks 1 (F), 4 (D), 5 (D), and 8 (C) (Figure 1). Interestingly, the *vlha* sequence of the IT/MS879/ck/17 strain (flock 9) showed mutations in the PRR region, which meant this flock could not be assigned to a known MS type or subtype (Figure 2). With regards to the RIII region, the known subtype D1 was detected in flocks 4 and 5, and two unknown subtypes, termed C7 and F2, were identified in flocks 8 and 1 (Figure 1). Based on the dendrogram, *vlha* sequences of MS strains detected in the study clustered with Spanish, Japanese (IT/MS675/ck/16) and Italian MS strains (IT/MS705/ck/16 and IT/MS801/ck/17) except for the *vlha* IT/MS879/ck/17 sequence, which formed a completely separate branch from other Italian and reference strains (Figure 3).

With respect to the MG sequence analysis, of the 11 samples examined, 8 were pr-*mgc*2 positive. All the corresponding GTS amplicons were successfully sequenced, except the MGA_0319 gene from flocks 9 and flock 10 samples. The MG strains analyzed with GTS were named as follows: IT/MG675/ck/16 (flock 1),
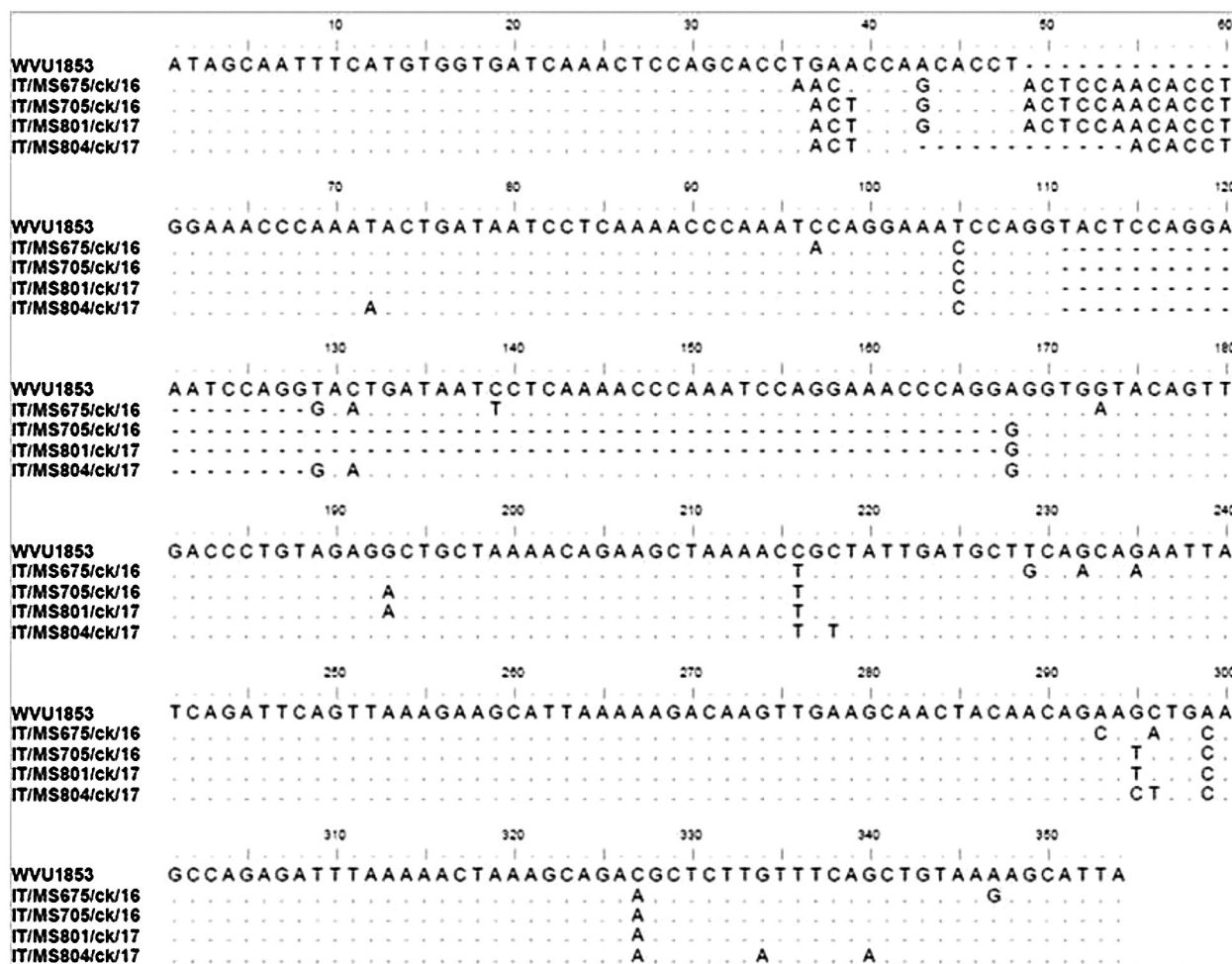

Figure 1. Nucleotide sequences of the PRR region of *vlhA* gene of MS strains detected in the study and reference strain WVU1853. Only residues that differ from the sequence of WVU1853 are shown.

Figure 2. Nucleotide sequences of the PRR region of *vlhA* gene of IT/MS879/ck/17 strain detected in the study and reference strain WVU1853. Only residues that differ from the sequence of WVU1853 are shown.
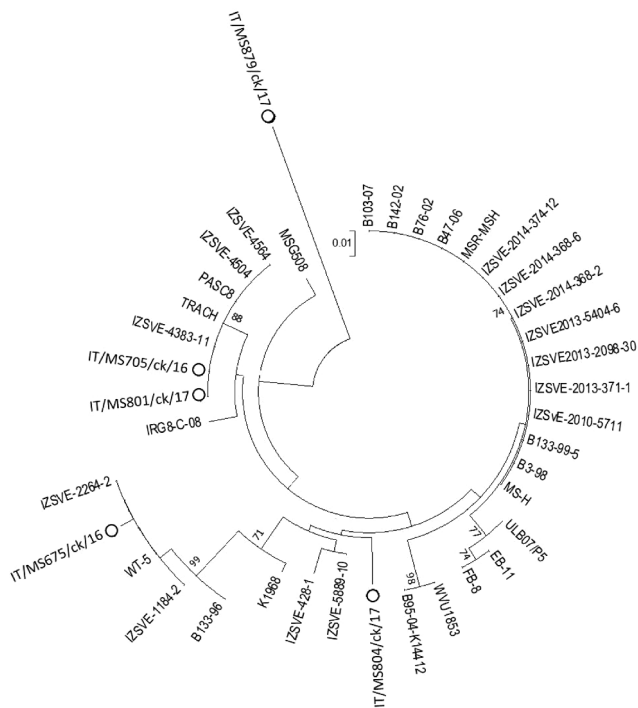

Figure 3. Phylogenetic tree based on the alignment of nucleotide sequences of *vlhA* genes of MS strains detected in the study (circle) and reference strains. Only bootstrap values >70 are reported.

IT/MG690/ck/16 (flock 2), IT/MG704/ck/16 (flock 3), IT/MG705/ck/16 (flock 4), IT/MG801/ck/17 (flock 5), IT/MG802/ck/17 (flock 6), IT/MG879/ck/17 (flock 9), and IT/MG880/ck/17 (flock 10). Nucleotide sequences were submitted to the GenBank database (Table 3).

Since IT/MG879/ck/17 and IT/MG880/ck/17 sequences were not included in the dendrogram, they were analysed using the nucleotide BLAST algorithm with the GenBank database. Sample IT/MG879/ck/17 showed a percentage of nucleotide similarity of 99% with ts-11 vaccine in *gapA*, 100% with ts-11 vaccine and Australian (K2966) strain in *mgc2* and 92% with German 2591/13CK strain in *pvpA*. Sample IT/MG880/ck/17 showed a percentage of nucleotide similarity of 99% with ts-11 vaccine, Australian (K2966), South Africa, Brazilian (2011/UFMG2) MG strains in *mgc2*, 99% with ts-11 vaccine, Australian (K2966), South Africa, USA, North America MG strains and Brazilian (2011/UFMG2) MG strains in *gapA* and 95.3% with German 2591/13CK strain in *pvpA*.

The dendrogram obtained from sequences of the MGA_0319, *mgc*2, *gap*A, and *pvp*A genes showed that MG strains belong to five different subclusters (Figure 4). IT/MG704/ck/16 (flock 3) and IT/MG690/ck/16 (flock 2) clustered with ts-11 and 6/85 vaccine strains, respectively. Similarities observed between IT/MG704/ck/16 and ts-11 was of 92.3% and between IT/MG690/ck/16 and 6/85 was of 94.8%.

IT/MG705/ck/16 (flock 4) and IT/MG801/ck/17 (flock 5), formed a cluster separately from all other analyzed strains, showing a high degree of similarity (99.6%) one with each other. However, the flocks 4 and 5 were geographically distant and no epidemiological link was reported between them.

Despite continued efforts to keep the poultry industry *Mycoplasma*-free the circulation of MS or MG strains in Italy have been reported in previous works, especially

Table 3. Accession numbers of MG strains submitted to GeneBank database.

	Accession numbers			
Strain	*gap*A	MGA_0319	*Mgc*2	*pvp*A
IT/MG675/ck/16	MH807704	MH807705	MH807706	MH807707
IT/MG690/ck/16	MH807708	MH807709	MH807710	MH814582
IT/MG704/ck/16	MH807711	MH807712	MH807713	MH807714
IT/MG705/ck/16	MH807715	MH814597	MH814590	MH814585
IT/MG801/ck/17	MH814594	MH814596	MH814589	MH814584
IT/MG802/ck/17	MH814593	MH814595	MH814588	MH814583
IT/MG879/ck/17	MH814591	/	MH814586	MH814580
IT/MG880/ck/17	MH814592	/	MH814587	MH814581
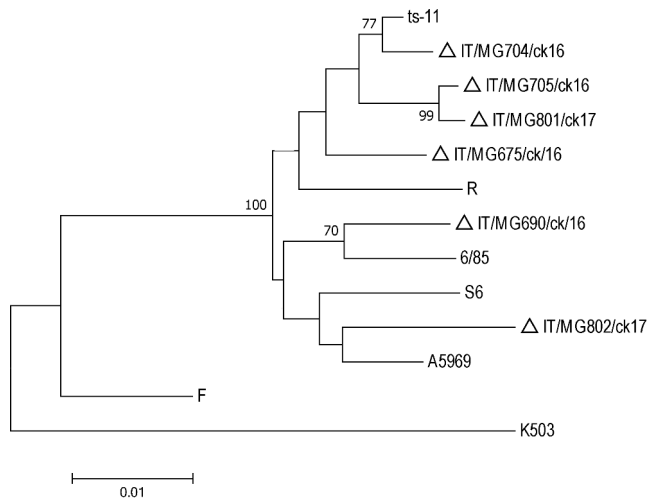
Figure 4. Phylogenetic tree based on the alignment of nucleotide sequences of *gap*A, MGA_0319, *mgc*2, and *pvp*A genes of MG strains detected in the study (triangle) and reference strains. Only bootstrap values >70 are reported.

focused on commercial poultry farms (Catania et al., 2010, 2016a),

Our study focused on backyard poultry farms, highlighting MS and MG occurrence in flocks distributed in various Italian regions. MG and MS DNA was detected in birds of different ages and breeds in association with respiratory signs, suggesting a potential clinical role, although other causes of respiratory disease cannot be excluded.

Based on the description of the *vlhA* fragment, A, C, D, E, F, G, and H types of MS have been detected in Italy (Moronato et al., 2014). In this study, the detection of MS sequences with peculiar molecular characteristics (IT/MS879/ck/17) or new subtypes (C7, F2) evidenced the potential role of backyard chickens as source of new *Mycoplasma* strains.

Phylogenetic analysis of MS *vlhA* sequences showed high nucleotide similarity with corresponding sequences of MS strains from Italy and European or extra-European countries, except IT/MS879/ck/17 sequence, which showed only 92% similarity with MS strains from several countries, including Italy. The dendrogram confirmed the peculiarity of IT/MS879/ck/17 being phylogenetically distant from other known MS strains.

With respect to MG sequences, GTS analysis allowed observation of 6 MG strains in a restricted geographical area such as Italy, indicating the ability of this method to differentiate among Italian MG strains.

The presence of the MS and MG sequences showing high nucleotide similarity with corresponding sequences from Italian or foreign *Mycoplasma* strains is not surprising considering the features of the farms tested, which commonly introduce animals from Italian farms and from European or extra-European countries, and participate in Italian or foreign exhibitions.

Haesendonck et al. (2014) and Derksen et al. (2018) reported that the backyard poultry flocks could act as reservoir or amplifier for poultry respiratory diseases serving as a continuous source of infection for industrial chickens. The present study detected the circulation of MS and MG in backyard poultry farms, confirming the potential role of this type of breeding to spread pathogens to commercial poultry production, especially in densely poultry-populated areas where backyard and commercial farms are close. Whereas the principles and practices of on-farm biosecurity may be familiar to commercial farmers, hobbyists and backyard farmers may not be aware of the steps required to keep infectious diseases out of their flock and prevent their spread to close farms.

The results of this study suggest that backyard chickens should be checked periodically to investigate the status of *Mycoplasma* infection. Moreover, the implementation of biosecurity measures in backyard poultry farms are needed. More exhaustive studies including attempts at isolation, in vivo pathogenicity studies and molecular analysis may be useful to better investigate the molecular profile and the potential epidemiological role of *Mycoplasma* strains circulating in backyard poultry farms.

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.psj.2019.12.020.

Supplementary Table S1. Accession numbers of MS sequences retrieved from GenBank used in the analysis.
Supplementary Table S2. Accession numbers of MG sequences retrieved from GenBank used in the analysis.

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