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# First molecular investigation to detect avian *Mycoplasma* species in clinical samples from laying-hen farms in Tunisia

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## Abstract

**Background** Avian mycoplasmas are known pathogens, which cause severe economic losses in poultry flocks. PCR is a rapid, sensitive, and less expensive diagnostic tool than culture for the identification of mycoplasmas in poultry farms. The objective of this study was to determine by PCR the presence of *Mycoplasma* spp., *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), and *Mycoplasma pullorum* (MP) in laying hens located in the Sfax region, in the South of Tunisia.

**Results** A total of 781 tracheal swabs were collected from 13 laying-hen farms without clinical signs at the date of sampling. MP was detected by a newly described specific PCR assay. The prevalence calculated from PCR results at the flock level was 100% for *Mycoplasma* spp., 0% for MG, 84.6% for MS and 61.5% for MP. The overall prevalence at the animal level was 38.7% for *Mycoplasma* spp., 0% for MG, 25% for MS and 6.4% for MP. The overall prevalence of 100% of avian mycoplasmas in laying-hen farms (38.7% prevalence at the animal level) shows an alarming situation.

**Conclusions** These results underline the importance of monitoring the emergence and spread of *Mycoplasma* strains in farms in order to decrease economic losses due to mycoplasmoses.

**Keywords** *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma pullorum*, PCR, Hen

## Background

Respiratory diseases are the leading cause of poultry morbidity worldwide. Avian mycoplasmas are highly contagious poultry pathogens that can cause acute and chronic diseases and are a major problem for all bird populations [1].

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Avian mycoplasmosis was first described as a poultry respiratory disease in turkeys in 1926 and chickens in 1936 (reviewed by Charlton and collaborators [2]). About 120 different species of *Mycoplasma* have been identified to infect various organisms, of which only about 20 are adapted to birds [3]. *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Mycoplasma meleagridis* (MM), and *Mycoplasma iowae* (MI) are of major concern and have the greatest impact on the poultry industry [4, 5].

MG is the most pathogenic species, responsible for Chronic Respiratory Disease (CRD) in chickens and infectious sinusitis in turkeys. CRD is characterized by respiratory rales, coughing, nasal discharge, air sacculitis,

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swelling of infraorbital sinuses and conjunctivitis in commercial poultry [6]. MS can cause respiratory problems, infectious synovitis, typical eggshell apex abnormalities, and reduced egg production in chickens and turkeys [7]. MI and MM are specific pathogens of turkeys, although isolation from chickens has already been described [4, 8]. These *Mycoplasma* infections have a significant economic impact on poultry production, resulting in a 10-20% decrease in productivity and in a 5-10% increase in embryo mortality [9].

*Mycoplasma pullorum* (MP) can be isolated from laying hens [10, 11]. The pathogenic role of MP in chicken and turkey remains unclear despite several studies. Kleckner [12] showed that inoculation of two strains of MP isolated from chicken tracheal exudates into the sinuses or trachea of turkey poults did not induce lesions. Inoculation into the thoracic air sacs and tendon regions of the hock or leg in chicken or turkey did not result in aerosacculitis lesions. However, MP was isolated from turkey embryos from flocks with low hatchability in France [13] and was found to be pathogenic to chicken and turkey embryos [13, 14]. Inoculation of turkey poults with MP in the air sacs and infraorbital sinuses resulted in moderate aerosacculitis lesions [13].

Other *Mycoplasma* species, including *Mycoplasma (M.) iners, M. gallinarum, M. gallinaceum, M. lipofaciens and M. glycophilum* are isolated from poultry. These species are not considered to be pathogens of high concern to the poultry industry due to their very low or no pathogenicity risk [15].

Culture is the gold standard method for the detection of *Mycoplasma* species, but it is difficult due to their fastidious nature and can take 3-4 weeks. Serological testing is much faster and less expensive but has many disadvantages due to nonspecific and cross-reactions [16]. As an alternative to culture and serology, molecular methods are used to specifically detect *Mycoplasma* species by PCR, which is a widely used procedure and a faster and less expensive diagnostic tool than culture methods [17].

Avian mycoplasmoses are regulated diseases in Europe and the USA [18, 19] causing heavy economic losses in poultry farms. MG and MS are also listed as notifiable diseases for the World Organization for Animal Health (WOAH) [20]. However, despite the continued development of the poultry industry in Tunisia, very little information regarding epidemiological data on the prevalence of avian mycoplasmas in laying-hen flocks is available in Tunisia. Only one old study reported a seroprevalence of 52.4% for MG and 28.6% for MS in layers [21]. Production of laying hens in 2021 in the Sfax region was 3,926,415, representing 59.3% of national production whereas broiler production represented only 7.8%. The breakdown of table egg production by region showed that 62% of this production was located in southern Tunisia, particularly in the Sfax region, with production of 1,863 million units in 2020 (Groupement Interprofessionnel des Produits Avicoles et Cunicoles (GIPAC), [22]), of which 97% were produced intensively, while 7% corresponded to local production (rural farms). Thus, PCR tests to detect *Mycoplasma* genus [23], MS [24] and MG [25] were performed on samples from laying-hen flocks in this region to obtain data on *Mycoplasma* prevalence. A PCR assay to detect specifically MP was developed in the present work.

## Materials and methods

### Field sample collection

This study was conducted from March to April 2022 in the Sfax region, located in the southeast of Tunisia and concentrating the largest part of layer farms.

In total, 781 tracheal swabs from 13 farms (12 commercial and one rural farms) of non-vaccinated laying hens of 18 to 106 weeks of age from different geographical areas of the Sfax region were tested for the presence of *Mycoplasma* species (Fig. 1; Table 1). Hens had been treated once or twice (except the rural farm) with antibiotics (tylosin, tylvalosin or tiamulin) as a preventative measure, particularly when chicks or pullets have been moved into their new buildings, but these treatments were carried out at least two weeks before the tracheal samples were taken.

Tracheal samples were collected with a cotton swab by veterinarians during official controls of farms and in collaboration with Veterinary Services (Regional Agricultural Development Commission, Sfax, Tunisia). Swabs were placed into 2.0 mL of Frey's broth medium [26] supplemented with antimicrobials (2.5 µg/mL of Amphotericin B (Sigma-Aldrich, St. Quentin Fallavier, France), 1 mg/mL of Ampicillin (Sigma-Aldrich) and 7.5 µg/mL of Colistin (Sigma-Aldrich)) to obtain initial suspensions. These suspensions were vortexed after a 60-90 min incubation at 37 °C ± 2 °C. From each sample in Frey's medium, a 500 µL aliquot was taken for DNA extraction and the rest was stored at <-70 °C.

### DNA extraction

DNA was extracted from clinical samples by a simple method following the protocol described by Ben Abdelmoumen and collaborators [27] with minor modifications. Briefly, 500 µL of broth sample was centrifuged at 14,000 x *g* for 30 min. The pellet was suspended in 100 µL of phosphate-buffered saline (PBS) and 100 µL of non-ionic detergent mixture solution (0.45% Nonidet P-40, 0.45% Tween 20, and 100 g/mL proteinase K) and incubated at 60 °C for 1 h, boiled for 15 min, and then centrifuged at 14,000 x *g* for 15 min. The supernatant was stored at -20 °C until use.

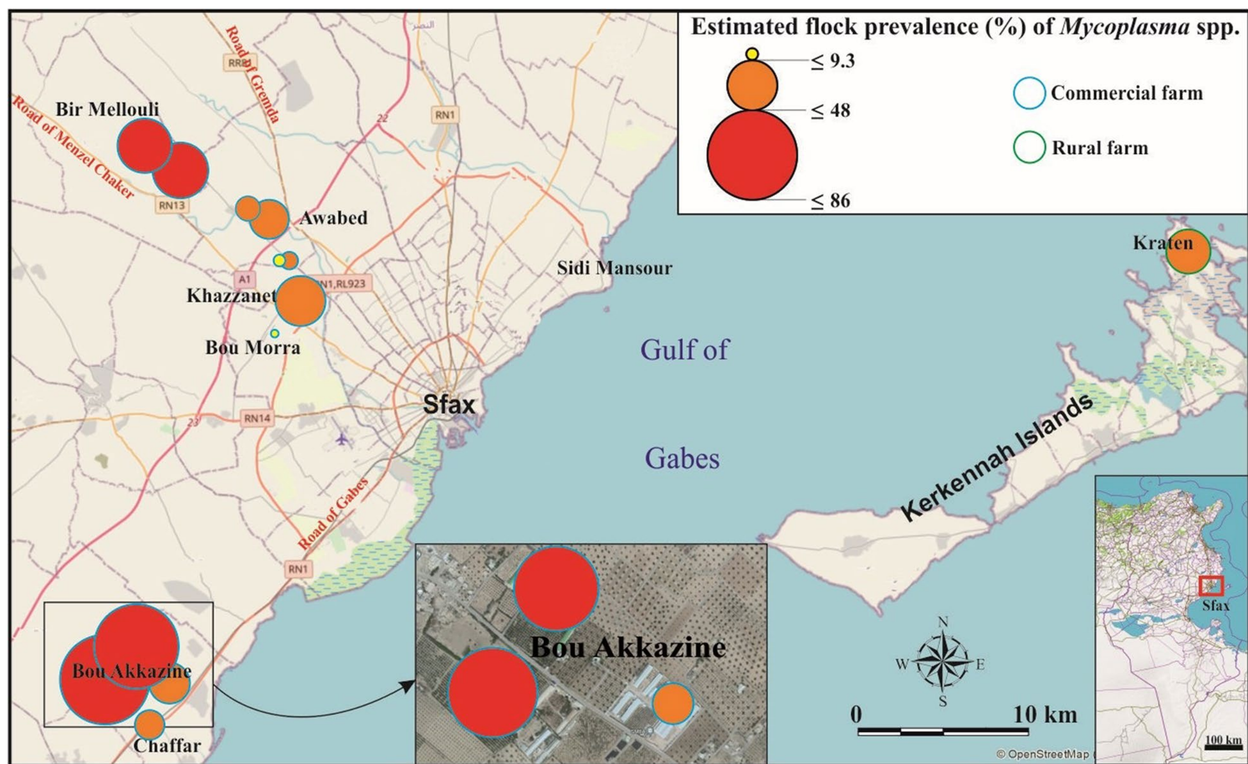


**Fig. 1** Map of the region of Sfax (Tunisia) showing all farm locations and the percentage of *Mycoplasma* spp.-positive tracheal samples per farm

**Table 1** Information on tracheal swabs collected in commercial and rural layer flocks in the Sfax region (Tunisia)

| Case Number | Production system[a] | Layer age (in weeks) | Total number of layer per flock | Number of samples | PCR results[b] | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | *Mycoplasma* spp. | MG | MS | MP |
| E1 | C | 46 | 25700 | 75 | 36 (48%)[c] | 0 (0%) | 19 (25.3%) | 0 (0%) |
| E2 | C | 83 | 32900 | 35 | 5 (14.3%) | 0 (0%) | 4 (11.4%) | 1 (2.9 %) |
| E3 | C | 83 | 12000 | 43 | 4 (9.3%) | 0 (0%) | 2 (4.7%) | 1 (2.3%) |
| E4 | C | 74 | 28500 | 72 | 4 (5.5%) | 0 (0%) | 0 (0%) | 0 (0%) |
| E5 | C | 106 | 20544 | 66 | 24 (36.4%) | 0 (0%) | 9 (13.6%) | 3 (4.5%) |
| E6 | C | 58 | 7000 | 64 | 13 (30.3%) | 0 (0%) | 9 (14.1%) | 0 (0%) |
| E7 | C | 50 | 20500 | 56 | 15 (26.8%) | 0 (0%) | 14 (25%) | 1 (1.8%) |
| E8 | C | 45 | 29500 | 50 | 43 (86%) | 0 (0%) | 31 (62%) | 0 (0%) |
| E9 | C | 46 | 15000 | 52 | 27 (51.9%) | 0 (0%) | 17 (32.7%) | 3 (5.8%) |
| E10 | C | 68 | 64000 | 67 | 25 (37.3%) | 0 (0%) | 0 (0%) | 13 (19.4%) |
| E11 | C | 73 | 48000 | 50 | 40 (80%) | 0 (0%) | 40 (80%) | 7 (14%) |
| E12 | C | 18 | 8000 | 57 | 30 (52.6%) | 0 (0%) | 26 (45.6%) | 0 (0%) |
| E13 | R | different ages | ND | 94 | 36 (38.3%) | 0 (0%) | 24 (25.5%) | 21 (22.3%) |
| TOTAL infected hens | | | | | 302/781 (38.7%) | 0/781 (0%) | 195/781 (25%) | 50/781 (6.4%) |
| TOTAL infected flocks | | | | | 13/13 (100%) | 0/13 (0%) | 11/13 (84.6%) | 8/13 61.5% |

[a] C: commercial farm; R: rural farm

[b] MG: *Mycoplasma* (*M.*) *gallisepticum*; MS: *M. synoviae*; MP: *M. pullorum*

[c] PCR results are presented as number of positive results (percentage of positive results)

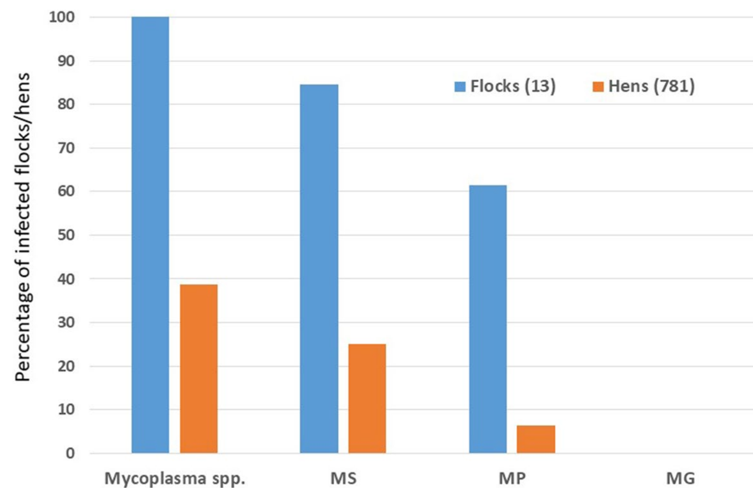


**Fig. 2** Detection of *Mycoplasma* spp., MS, MP and MG by specific PCR in tracheal samples collected from 13 laying-hen farms (781 hens) in the Sfax region of Tunisia. MS: *Mycoplasma* (*M.*) *synoviae*; MP: *M. pullorum* ; MG: *M. gallisepticum*

#### *Detection of* Mycoplasma *spp., MG and MS by PCR*

The presence of *Mycoplasma* spp. DNA in each sample was detected by a 16S rRNA genus-specific PCR as previously described [23] in a final volume of 50 µL. Briefly, the PCR mixture contained 2 mM of $MgCl_2$ (Biobasic, Markham, Canada), 5 µL of 10X PCR buffer (Biobasic), 400 nM of primers (Bio Basic) (Table 2), 200 µM of each desoxyribonucleotide triphosphate (dNTP) (Biobasic), 1.25 units of Taq polymerase (Biobasic), 10 µL of DNA samples and nuclease-free water (Bio Basic) to reach the final volume of 50 µL (Table S1). PCR amplification was performed using an automated thermal cycler (VWR we Enable science UNO 96, Berkshire, England) as described in Table 3. Standard samples of MS WVU1853 strain (ATCC 25204) and MG S6 (ATCC 15302) were used as positive controls and a DNA-free mixture reaction (with nuclease-free water) was used as negative control under the same conditions.

Samples that tested positive for the *Mycoplasma* genus were then tested using species-specific primers.

The presence of MS and MG in samples was detected by MS-specific [24] and MG-specific [25, 28] PCR assays in a final volume of 50 µL. Briefly, the reaction mixture contained 5 µL of 10X PCR buffer (Roche, Sigma-Aldrich, Saint-Quentin-Fallavier, France), 2 mM of $MgCl_2$ (Bio-Rad, Marnes-la-Coquette, France), 400 nM of MS-specific primers or 800 nM of MG-specific primers (Sigma-Aldrich) (Table 2), 200 µM of each dNTP (Eurobio, Les Ulis, France), 1.25 U of Roche Taq DNAPolymerase for MS or 1 U of Roche Taq DNA Polymerase (Sigma-Aldrich) and 5 µM of tetramethyl ammoniun chloride (TMAC) (Sigma-Aldrich) for MG, 5 µL of DNA samples and nuclease-free water (Eurobio) to reach the final volume of 50 µL (Table S1). Standard samples of MS WVU1853 strain (ATCC 25204) and MG S6 (ATCC 15302) were used as positive controls and a DNA-free mixture reaction (with nuclease free water) was used as negative control under the same conditions.

PCR amplifications were performed using a T100™ thermal cycler (Bio-Rad) as described in Table 3.

**Table 2** Primer sequences used for the detection and identification of *Mycoplasma* species

| Targeted species | Primer sequences[a] | Target gene | Product size[b] | Reference |
|---|---|---|---|---|
| *Mycoplasma*-genus | FW: 5′-GGGAGCAAACAGGATTAGATACCT-3′<br>RV: 5′-TGCACCATCTGTCAGTCTGTTAACCTC-3′ | 16S rRNA | 280 bp | [23] |
| *M. gallisepticum* | FW: 5′-TAACTATCGCATGAGAATAAC-3′<br>RV: 5′-GTTACTTATTCAAATGGTACAG-3′ | 16S rRNA | 330 bp | [25] |
| *M. synoviae* | FW: 5′-GAGAAGCAAAATAGTGATATCA-3′<br>RV: 5′-CAGTCGTCTCCGAAGTTAACAA-3′ | 16S rRNA | 207 bp | [24] |
| *M. pullorum* | FW: 5′-ACCCTTTAGTTTGGGATAACGACT-3′<br>RV: 5′-TTTACAACCCGAAGCCGTCA-3′ | 16S rRNA | 304 bp | Developed in this study |

[a] *FW* forward primer, *RV* reverse primer

[b] *pb* base pair

**Table 3** Conditions for PCR assays

| Targeted species | Initial denaturation | Nb cycles | Amplification | | | | Reference |
|---|---|---|---|---|---|---|---|
| | | | Denaturation | Annealing | Extension | Final extension | |
| *Mycoplasma*-genus | 95 °C/5 min | 35 | 94 °C/30 s | 58 °C/30 s | 72 °C/1 min | 72 °C/5 min | [23, 28] |
| *M. gallisepticum* | 90 °C/3 min | 35 | 95 °C/15 s | 60 °C/20 s | 75 °C/15 s | | |
| | | 1 | 95 °C/15 s | 60 °C/45 s | | 75 °C/5 min | [28] |
| *M. synoviae* | 94 °C/5 min | 35 | 94 °C/1 min | 50 °C/1 min | 72 °C/2 min | 72 °C/5 min | [24] |
| *M. pullorum* | 94 °C/5 min | 35 | 94 °C/1 min | 50 °C/1 min | 72 °C/2 min | 72 °C/5 min | This study |

**Development of a MP-Specific PCR Assay.**

Representative sequences of MP 16S rRNA were selected in GenBank (https://www.ncbi.nlm.nih.gov/genbank) and aligned with 20 different species of avian *Mycoplasma* using the ClustalW software available on the NPS@ website (https://npsa-pbil.ibcp.fr) to ensure the absence of significant homology with other *Mycoplasma*-species sequences. Species-specific primers for the detection of MP were designed and submitted to the online BLASTn tool available on the NCBI website (https://blast.ncbi.nlm.nih.gov) for final selection (Table 2). PCR primers were designed to exhibit optimal biophysical properties with no dimer formation with Primer3Plus (https://primer3plus.com). The selected MP-specific primers were subjected to PCR standardization to optimize hybridization, specificity and sensitivity.

PCR assays were performed using dreamTaq Green PCR Master mix (Fisher Scientific, Illkirch, France) according to the manufacturer's instructions. Briefly, a 25 µL reaction mixture contained 12.5 µL of dreamTaq PCR Master Mix, 400 nM of each primer (Sigma-Aldrich) (Table 2), 2 µL of DNA sample to be tested and nuclease-free water (Eurobio) to reach the final volume of 25 µL (Table S1). Nucleic acid extracts for optimization tests were quantified by spectrophotometry (Qubit fluorometer, Invitrogen by Life Technologies, Saint-Aubin, France): 50 ng of DNA and 10 µL of nuclease-free water were added to the mixture. Amplification, using a T100™ thermal cycler (Bio-Rad) was performed as described in Table 3 with hybridization for 40 s using a temperature gradient of 54 to 55 °C.

The specificity of the MP primers was confirmed with strains of MP, other avian *Mycoplasma* species and other bacteria (Table 4), as well as with positive and negative field samples stored in the ANSES laboratory. To determine the sensitivity of the PCR assay to detect MP, concentrations of 100 ng to 10 fg of DNA were used as template DNA.

After optimization, the protocol described in Table 3 was selected, with a hybridization at 54.6 °C.

**Table 4** *Mycoplasma* and other bacterial used to establish the specificity and sensitivity of the *Mycoplasma pullorum*-specific PCR

| Species | Number of strain tested | Origin/accession number |
|---|---|---|
| *M. pullorum* | 1 | CKK, ATCC 33553 |
| | 18 | Field isolates |
| *M. gallisepticum* | 1 | ATCC 15302 |
| | 3 | Field isolates |
| *M. synoviae* | 3 | Field isolates |
| *M. anatis* | 1 | Field isolate |
| *M. cloacale* | 1 | Field isolate |
| *M. columbinasale* | 1 | Field isolate |
| *M. columbinum* | 1 | ATCC 29257 |
| *M. columborale* | 1 | Field isolate |
| *M. gallinaceum* | 1 | Field isolate |
| *M. gallopavonis* | 1 | Field isolate |
| *M. glycophilum* | 1 | Field isolate |
| *M. imitans* | 1 | ATCC 51306 |
| *M. iners* | 1 | ATCC 19705 (PG30) |
| *M. iowae* | 1 | ATCC 33552 (I695) |
| | 1 | Field isolate |
| *M. lipofaciens* | 1 | Field isolate |
| *M. meleagridis* | 1 | Field isolate |
| *Acinetobacter baumannii* | 1 | Field isolate |
| *Enterococcus faecalis* | 1 | ATCC 29212 (CIP 103214) |
| *Escherichia coli* | 1 | ATCC 25922 (CIP 7624) |
| *Klebsiella pneumoniae* | 1 | Field isolate |
| *Ornithobacterium rhinotracheale* | 1 | Field isolate |
| *Pseudomonas aeruginosa* | 1 | Field isolate |
| *Riemerella anatipestifer* | 1 | ATCC 11845 (CIP 82.28T) |
| *Salmonella* Corvallis | 1 | Field isolate |
| *Salmonella* Pullorum | 1 | ATCC 9120 |
| *Staphylococcus aureus* | 1 | ATCC 25923 (CIP 7625) |

**Detection of Amplified DNA**

The PCR products were detected by electrophoresis at 120 V (*Mycoplasma* spp.), 115 V (MG) or 125 V (MS, MP) on a 2% agarose gel in Tris-Borate-EDTA buffer (TBE) (90 mM Tris, 90 mM borate, 2.5 mM EDTA pH 8.0). Amplified products were detected by ultraviolet

transillumination (Quantum ST4 device and VisionCapt software, Vilbert Lourmat, Fisher Scientific, Illkirch-Graffenstaden, France) with ethidium bromide staining (2 mg/L, Fisher Scientific). A GeneRuler 1 kb DNA Ladder (Fisher Scientific) was used as a molecular size standard: the expected sizes of *Mycoplasma* spp., MS, MG, and MP amplicons were 280 pb, 207 bp, 330 bp and 304 bp, respectively.

## Results

### *Development of the* Mycoplasma pullorum*-specific PCR test*

The primer sequences for the new MP-specific PCR, designed on the 16S rRNA gene and the amplicon size are summarised in Table 2. The best amplification results were obtained with a temperature of 54.6 °C, with a sensitivity up to 10 pg (Figure S1).

After optimizing PCR conditions, primer specificity was tested: no cross amplification was observed when testing the microorganisms listed in Table 3. A clear and well-defined specific band of approximately 304 bp was visualized only from MP strains or MP-positive samples, and not from the other *Mycoplasma* or bacterial species tested (Figures S2 and S3).

### Prevalence of Avian Mycoplasma Species in Layer Farms

For detection of *Mycoplasma* spp. in the samples from laying hens, the genus-specific PCR method was used. All thirteen sites (12 commercial and one rural farms) visited in the Sfax region were tested positive for *Mycoplasma* spp. (Table 1, Fig. 1), giving a prevalence of *Mycoplasma*-infected flocks of 100%. Out of the 781 samples collected, 302 (38.7%) samples were PCR-positive for *Mycoplasma* spp. Prevalence of positive tracheal swabs varied between farms, from 9.3% to 86.0% (Table 1, Figs. 1 and 2).

Using species-specific PCR assays, 11/13, 8/13 and 0/13 flocks were tested positive for MS, MP and MG, respectively, giving a prevalence of 84.6%, 61.5% and 0%. At the animal level, out of 781 tracheal swabs collected, 195 (25.0%) and 50 (6.4%) samples were positive for MS and MP, respectively and all swabs were negative for MG (Table 1, Fig. 2).

Finally, co-infection with MS and MP was observed in 25 hens (3.2%) belonging to five (38.5%) flocks (Table 1).

## Discussion

This study provides for the first time molecular data on the presence of different avian *Mycoplasma* species in layer farms in the Sfax region (South of Tunisia), which is the most productive poultry district in Tunisia [22].

To our knowledge, it is the first time that such a prevalence study is performed in Tunisia on layer flocks with PCR. Results showed a prevalence at flock level of 100%, 84.6%, 61.5% and 0% for *Mycoplasma* spp., MS, MP and MG, respectively; and a prevalence at animal level of 38.7%, 25.2%, 6.4% and 0%.

Our results are not in accordance with results of the only Tunisian study reporting a much higher flock prevalence of 52.4% for MG and a lower prevalence of 28.6% for MS in layers [21]. However, this previous study was based on serology and not PCR. Moreover, the Tunisian poultry production has evolved since 1997 and controls and biosecurity measures have been put in place on breeder farms to limit infections by MG, the most pathogenic species of avian mycoplasmas.

There is very high variability in results reported in the literature regarding avian mycoplasma infections in poultry farms worldwide [29]. Most prevalence studies performed in other North African neighbouring countries were carried out on samples taken from flocks showing clinical signs (which leads to an overestimation of the prevalence of pathogenic bacteria) and can hardly be compared to our study performed on flocks without apparent clinical signs. Two recent Egyptian studies calculated an individual prevalence of 40% for MG [30] and 50% for MS [31] in layer farms. A third one determined that 57.6% of the isolates were MG and 30.7% were MS in diseased and apparently healthy layers, but PCR assays were only carried out on isolates after culture and not directly on samples like our study, which may underestimate the percentage of infected hens [32]. An Algerian study, based on serology and isolation of mycoplasmas by culture, showed that 100% of laying hen flocks were MG-positive (serology) and MG was isolated by culture in 2.1% of hens [33]. Sid and colleagues [34] also showed that MG was endemic in Algeria. Finally, a study in Sicily showed an individual prevalence of 12.5% for MG and 23.2% for MS, and a flock-level prevalence of 28 to 40% for MG and 42.8 to 44% for MS [35]. Even if the techniques and methods used differ, the comparison of these studies seems to show that the prevalence of MG in layer flocks in Tunisia (0% at flock and individual levels) is significantly lower than in neighbouring countries. These results are in accordance with those reported in France or in Belgium for MG in layer flocks (0 and 0.2%, respectively; [36, 37]. The absence of MG in the farms visited in the Sfax region, even in hens from the rural farm, might be explained by the constant control measures adopted in breeding farms in Tunisia against this well-known pathogenic *Mycoplasma*, thus leading to a reduction of vertical transmission and of the presence of MG in the environment (horizontal transmission), and consequently to a reduction in the frequency of MG infections in commercial and rural flocks. It would be interesting to carry out another study including both commercial flocks without apparent clinical signs and others with respiratory clinical

signs to determine if MG can be isolated from diseased flocks. This low prevalence might also be due to the preventive and curative antimicrobial treatments applied during the hens' lifespan. In fact, an antibiotic treatment can reduce the number of live mycoplasmas present in the trachea and reduce the chances of detecting them by PCR. However, other mycoplasmal species were also detected in tracheal swabs despite these treatments.

The MS prevalence of 25.2% at the animal level determined in our study agrees with that reported in Sicily (23.2%) and in Egypt (30.7%) [32, 35] by PCR also. The very high prevalence at flock level (84.6%) is also in accordance with several studies in France (68%), Germany (75.0%) and Spain (95.0%) [36, 38, 39]. However, lower flock prevalences were found in Poland (29%) and Sicily (42.8-44%) [35, 40]. The persistence of this high level of MS contamination on farms may be explained by the recognized high capacity for vertical and horizontal transmission of MS [7]. MS infection in breeding flocks of laying hens can promote vertical transmission of the infectious agent to subsequent chicks [15]. In addition, MS can remain viable for long periods of up to 41 days on fomites (feathers, food or droppings) [41] and is able to infect other birds introduced in this MS-contaminated environment [42]. This can lead to the infection of new flocks introduced in the farm, especially if the cleaning-disinfection and emptiness steps are not well respected and in the case of multiple-age farms. Furthermore, laying hens remain on farms for long periods, at various stages of production. They are therefore more likely to be infected by different pathogens that can negatively affect their immune defences and make them more vulnerable to outbreaks of infection.

MS has been considered less important than MG in poultry for many decades. One of the main characteristics of MS infection is that it is mostly asymptomatic, resulting in a chronic subclinical upper respiratory infection [5], with generally little effect on the performance of layers [36]. However, it could promote infections by other *Mycoplasma* species, bacteria or viruses, and eventually have a negative effect on the host's immune defences [43, 44]. There is an increased consciousness to generate MS-free poultry [44, 45]. In countries with a well-developed poultry industry, the primary breeding stocks are free of MG and MS and the commercial breeding stocks are free of MG (compulsory slaughter of infected breeding stocks). In addition, studies documenting the high worldwide prevalence of MS (as documented in this study) stress the need for an update on its prevention and control. MS control and eradication programmes have been implemented for a long time in the USA, and the Dutch poultry industry implemented a mandatory control and an eradication programme for MS in 2013 [44]. However, control and eradication programmes are not compulsory in other European and many other countries. The MS-infection status of Tunisian breeding flocks is not known, which suggests that another study should be carried out, this time on breeding flocks instead of commercial ones to have data on the MS prevalence. Given its vertical and horizontal transmission, its faster dissemination than MG [5] and its persistence in the environment [10, 15, 42], the prevalence of this *Mycoplasma* species is likely to increase, resulting in greater economic losses for the local poultry sector.

PCR testing for *Mycoplasma* spp. showed a prevalence of 38.7% (302/781) at the animal level, and a flock prevalence of 100%. Few studies reported results about *Mycoplasma*-genus prevalence: most studies only consider MG, MS or both species. Such a high prevalence was already described in Germany with 100% of *Mycoplasma*-infected flocks [38]. Differences in prevalence of *Mycoplasma* spp. observed between farms (Table 1, Fig. 1) might be due to less strict biosecurity and hygiene measures (poorly carried out cleaning/disinfection, duration of emptiness not respected) or contamination of hens via fomites, insects, rodents or staff working on site in the most infected farms. The presence of farms with much lower levels of contamination than others (5.5 versus 86.0% for the most distant values) suggests that it could be possible to improve significantly the situation with respect to mycoplasmosis in layer farms by informing and training all farm personnel about the importance of biosecurity and hygiene measures.

Among the 302 *Mycoplasma*-positive samples, only 195 samples were MS-positive. This suggested that other *Mycoplasma* species were probably present, and that there was a need to develop further specific PCRs to detect the presence of other *Mycoplasma* species. Since MP was found to be frequently associated with MS in laying hens by Cisneros-Tamayo and collaborators [11], we developed in the present study a rapid PCR test with high specificity and sensitivity to investigate the prevalence of MP in laying hens. Assays carried out on the samples with this new PCR revealed a MP prevalence of 6.4% (50/302) at the animal level and of 61.5% at flock level. Among the 50 MP-positive samples, 25 were co-infected with MS. This result is consistent with those of Cisneros-Tamayo and colleagues [11] who detected MP in 56.0% of the samples collected on commercial laying hen MS-positive flocks in France. To the best of our knowledge, no other study reported data on prevalence of MP in layers in Tunisia and other countries. However, other studies reported detection of this species in chickens [10], partridges, pheasants, turkeys [13, 46] and pigeons [10].

Finally, it should be noted that, in most farms (10/13, 76.9%), the percentage of tracheas infected with *Mycoplasma spp.* is higher than those infected by MS and/or MP. This suggests the presence of other *Mycoplasma*

species that were not identified during this study with the three specific PCR assays. Other species can indeed be isolated from hens, such as *M. gallinaceum*, *M. glycophilum*, *M. iners* or *M. gallinarum* [4, 47]. Additional studies may be conducted on samples stored during this study to try to isolate and identify these species.

This study was carried out on a limited number of laying-hen flocks and in a single region of Tunisia (the Sfax region bringing together most layer flocks). Furthermore, this study was carried out on farms which did not present apparent clinical signs, which could lead to an underestimation of the prevalence of mycoplasmas, especially for MG which is the most pathogenic species. Other studies could be planned to confirm these figures, to sample farms with apparent clinical signs, and to extend it to other types of flocks (broilers, turkeys, breeding farms) and other regions of Tunisia.

## Conclusion

Our results indicate that the *Mycoplasma spp.*-, MS- and MG-specific PCR assays are accurate tools for the molecular diagnosis of *Mycoplasma* infections in layer flocks directly from clinical samples. And the MP-specific PCR developed in the present study is reproducible and sensitive enough to detect concentration of 10 fg of MP DNA in clinical samples and may be used in the future for further epidemiological investigations of MP spread in poultry flocks.

The comparison of the prevalence values of the different species of mycoplasmas to that of other countries suggests that MG infections are well controlled in layer farms in the Sfax region of Tunisia, thanks to the control and eradication programmes for this species at the level of breeding flocks, or thanks to antibiotic treatments in layer farms which help keep *Mycoplasma* infections low. However, the rate of *Mycoplasma*-positive samples (38.7%) or flocks (100%) showed a worrying situation. Lack of biosecurity measures or hygiene in some farms may be one of the main reasons for the high prevalence of mycoplasmas in the Sfax region. The adoption of better hygiene and biosecurity practices, particularly in farms with high *Mycoplasma* prevalence, is recommended as a control strategy for mycoplasmosis in Tunisian farms.

### Abbreviations

| | |
|---|---|
| CRD | Chronic Respiratory Disease |
| dNTP | Desoxyribonucleotide triphosphate |
| M. | *Mycoplasma* |
| MG | *Mycoplasma gallisepticum* |
| MI | *Mycoplasma iowae* |
| MM | *Mycoplasma meleagridis* |
| MP | *Mycoplasma pullorum* |
| MS | *Mycoplasma synoviae* |
| PBS | Phosphate-buffured saline |
| TBE | Tris-Borate-EDTA buffer |
| TMAC | Tetramethyl ammoniun chloride |
| WOAH | World Organization for Animal Health |

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-024-04311-5.

Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Supplementary Material 4.

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### Authors' contributions

RG, AB and LK conceived and designed the research. SS and SF conducted experiments and analyzed data. AS provided clinical samples. SS and AB wrote the manuscript. All authors read and approved the manuscript.

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### Availability of data and materials

The datasets generated during the current study are available from the corresponding author upon reasonable request.

## Declarations

### Ethics approval and consent to participate

Samples were collected by veterinarians during official controls of poultry farms for mycoplasmoses (*Mycoplasma gallisepticum* and *Mycoplasma synoviae* are regulated diseases under regular surveillance according to Tunisian decree number 2200-2009 [48]) and in collaboration with Veterinary Services (Regional Agricultural Development Commission, Sfax, Tunisia). According to the Directive 2010/63/EU, ethical approval was not required for this study because samples were collected during "non-experimental agricultural practices" (article 1, 5a) by veterinarians with the consent of the farmers during official controls.

### Consent for publication

Not applicable.

### Competing interests

The authors declare no competing interests.

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