Diagnosis of *Mycoplasma* from Starlings Lungs

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Abstract

Starlings have tremendous economic and environmental impacts because they can spread pathogens to livestock and poultry. These birds act as mechanical and biological carriers for different types of pathogens from and to their original habitat. The goal of this study was to ascertain the presence of *Mycoplasma gallisepticum* (MG) in the starlings’ lungs and confirm their diagnosis using the PCR technique. We altered the supplements that were added to *Mycoplasma* culture media by using calf serum instead of horse serum and sulphadimidine plus trimethoprim with nystatin instead of thallous acetate. Eighty-five starlings were bought from hunters in the spring of 2019, and their lungs were harvested and divided into two portions, one for *Mycoplasma* cultivation and the other for DNA extraction. Fifty-nine (69.4%) samples were positive for *Mycoplasma* colonies, thereby yielding accurate results using alternative supplements in the culture media. PCR revealed the presence of *Mycoplasma* in 78.8% lung samples, while MG was detected in only 43.3% of the positive samples, indicating the presence of other species of *Mycoplasma* too. The current study is the first of its kind not only in Iraq but also in the world, investigating the presence of MG in the lungs of starling birds. This study revealed that MG is significantly prevalent in starlings and also suggests that other *Mycoplasma* species may be present in starlings.

Keywords: Starlings, *Mycoplasma gallisepticum*, PCR, culturing, calf serum.

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INTRODUCTION

Starlings (Sturnus vulgaris Linnaeus) are migratory birds that visit our country every spring from Europe and Central Asia. These birds migrate in large swarms and at the end of spring, return to their original habitats. As a result of this annual migration, these birds serve as mechanical and biological carriers for different types of pathogens from and to their original habitat<sup>1-2</sup>. During this long migration, they pass through many countries and therefore, have a prominent role in transporting pollutants and microorganisms from and to these countries. There are local and international studies attributing the transmission of various microorganisms such as bacteria, viruses, and others, to these birds<sup>3-8</sup>. Starlings have considerable economic and the environmental effects because they can spread pathogens to livestock and poultry by contaminating the fodder while invading fields to obtain food<sup>6-7,9</sup>, as well as polluting drinking water meant for livestock. These birds were placed in the Invasive Species Specialist Group’s list of “100 of the World’s Worst” biological invaders<sup>10</sup>.

*Mycoplasma* is a microorganism that causes many diseases in animals, such as infections of the respiratory system and joints, abortion, and mastitis. The economically important mycoplasmas in birds and poultry are *Mycoplasma gallisepticum*, *M. synoviae*, *M. meleagridis*, and other less important species like *M. iowae*, *M. anatis*, *M. cloacae*, and *M. gallinarum*.<sup>11-12</sup> *M. gallisepticum* causes chronic respiratory disease (CRD) in chickens (mainly in broiler chickens) and infectious sinusitis in turkeys<sup>12-13</sup>. Although CRD is a moderate respiratory disease, it becomes severe due to infections by synchronous pathogens. The disease is characterized by sneezing, coughing, rales, and nasal influx, and has a high morbidity and low mortality. Infectious sinusitis in turkeys is manifested by a swollen face and tacky exudate in the infraorbital sinuses.<sup>12-13</sup> *M. synoviae* is the causative agent of infectious synovitis in chickens and turkeys. The disease is a moderate respiratory disease, manifested by synovitis in chickens at 4-6 weeks of age and in turkeys at 10-12 weeks of age, bulging of the footpads and hocks, yellowish viscous exudate in the bursa (a fluid-filled sac) of the keel and hock and wing joints, and air sacculitis in case of concurrent infection with IB virus<sup>12-13</sup>. The aim of this study was to detect the presence of *Mycoplasma* species, mainly *M. gallisepticum*, in the lungs of starlings to report if these birds play a role in the transmission of these pathogens to chickens and turkeys.

MATERIALS AND METHODS

Samples

Eighty-five apparently healthy starling birds were bought from hunters in Mosul city in the spring of 2019. Their lungs were harvested under aseptic conditions and divided into two portions, one for *Mycoplasma* culture and the other for DNA extraction.

Culture Media preparation

The liquid and solid media were prepared using brain heart infusion (BHI) broth as a base to which agar agar was added for the solidification of liquid media. After autoclaving the media, the following supplements were added: yeast extract (10%), calf serum (20%) (14-16), 3 mL of penicillin (100,000 IU/mL) for inhibiting gram-positive bacteria, and 3 mL of sulphadimidine 20% + trimethoprim 4% and nystatin for inhibiting the growth of gram-negative bacteria and fungi, respectively (instead of thallous acetate).

Bacterial isolation

A third of the lung was placed in BHI broth and incubated at 37°C for 7 days within a candle jar (17). Next, 0.1 mL of each broth sample was spread on BHI agar plates and incubated under the same conditions for 14 days<sup>12,17,18</sup>. Each solid plate was examined under a dissecting microscope to detect colonies with a fried egg appearance. Post examination, the *Mycoplasma* colonies were cultivated in BHI broth and stored at -20°C for subsequent DNA extraction<sup>12,16</sup>.

DNA extraction

DNA extraction was performed from both, lung tissue and *Mycoplasma* colonies (19-22). *Mycoplasma* genomic DNA was extracted from lung tissue and *Mycoplasma* colonies using the gSYNC™ Geneaid kit (Korea) following the manufacturer’s instructions. PCR was used to detect the polymorphisms of the 16S rRNA gene using forward and reverse primers. To extract DNA from *Mycoplasma* colonies, the frozen BHI broths were thawed at room temperature and centrifuged at 2500 g for 30 min<sup>16</sup>, after which 100µL was taken from the sediment and subjected...
to the same procedure used for extraction of DNA from lung tissue\textsuperscript{24-26}.

**DNA Amplification**

Two coupled PCR primers were used (Table 1) that included genus-specific (Universal) and species-specific primers for the detection of genus *Mycoplasma* and *M. gallisepticum*\textsuperscript{27-28}. Primers were synthesized by Bioneer Co. (Korea). The specificity of each PCR primer couple was confirmed using avian *Mycoplasma* strains.

PCR was performed using a total volume of 25\(\mu\)L comprising: 5\(\mu\)L of extracted DNA, 1\(\mu\)L of reverse and forward primer each, 2\(\mu\)L of MgCl\(_2\), 6\(\mu\)L ddD.W., and 10\(\mu\)L of 2.5’ Mastermix solution (ready to use).

Thermocycler programs are mentioned in Tables 2. Electrophoresis was performed using 10\(\mu\)L of the reaction solution on a 2% agarose gel with 0.8\(\mu\)L of ethidium bromide stain solution (10

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**Table 1.** Primers used to detect genus and Species of *Mycoplasma gallisepticum* strain

| Primer       | Sequence (5′-3′) Product |
|--------------|--------------------------|
| Detection of Genus *Mycoplasma*<br>**MYCO.-F** | **GGG-AGC-AAA-CAC-GAT-AGA-TAC-CCT** |
| Detection of species *M. gallisepticum*<br>**GALLI.-F** | **TGC-ACC-ATC-TGT-CAC-TCT-GTT-ACC-CTC** |
| **GALLI.-R** | **GAG-CTA-ATC-TGT-AAA-GTT-GGT-C** |
| Detection of species *M. gallisepticum*<br>**MYCO.-R** | **GCT-TCC-TTG-GGG-TTA-GCA-AC** |

**Table 2.** PCR thermocycler program for detection of Genus *Mycoplasma* and species *M. gallisepticum*

| Cycle | Temp. °C for *Mycoplasma* | Temp. °C for *M. gallisepticum* | Time | Stage                  |
|-------|---------------------------|-------------------------------|------|------------------------|
| 1     | 95                        | 95                           | 5min. | Initial DNA denaturation |
| 1     | 95                        | 95                           | 20sec. | DNA denaturation       |
| 30    | 59                        | 53                           | 30sec.  | Primer annealing       |
| 1     | 72                        | 72                           | 30sec.  | Primer extension       |
| 1     | 72                        | 72                           | 5min.   | Final extension        |
| 1     | 4                         | 4                            | "      | Cooling                |

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Fig. 1-3. Mycoplasmas colonies under dissecting and 10x light microscopes.
mg/mL). DNA bands were detected at 245-312 nm using a UV transilluminator (Biometra, Germany).

RESULTS
All 85 samples were cultivated in modified culture media to detect Mycoplasma colonies. Fifty-nine (69.4%) samples were positive for observable Mycoplasma colonies (Table 3, Fig. 1-3). Organisms from all fifty-nine BH broths (positive for Mycoplasma colonies) belonged to the Mycoplasma genus, according to the PCR results (Fig. 4), while 24 of these 59 samples (40.7%) were identified as M. gallisepticum (Fig. 5).

Lung samples
According to the PCR results, sixty-seven samples represented organisms from the Mycoplasma genus (78.8%), and among these, 29 samples were M. gallisepticum (43.3%) (Table 4).

DISCUSSION
Starlings are migratory birds that play a major role in spreading infectious agents from one region to another. They are responsible for the transmission of infectious microorganisms in livestock and poultry farms via contamination of feed, causing economic losses (4, 29-31). The goal of the present study was to detect the presence of Mycoplasma, mainly, Mycoplasma gallisepticum, in the lungs of starlings.

Culture media isolation revealed that Mycoplasma was present in 69.4% of the starlings. This is a high rate of isolation for these organisms, which makes these birds significant carriers for Mycoplasma species.

The rate of detection of Mycoplasma directly from the lungs as per PCR results (87.8%) was higher than the detection rate via culturing technique, suggesting that the detection and

Table 3. Results of culturing of starlings lungs

| Samples | No. | Mycoplasma | % |
|---------|-----|------------|---|
| Lungs   | 85  | 59         | 69.4|

Table 4. Presence of Mycoplasma in starlings lungs according to PCR results

| No.  | Lungs | Mycoplasma | %  | M. gallisepticum | %  | %  |
|------|-------|------------|----|------------------|----|----|
| 85   | 67    | 78.8       | 29 | 43.3*            | 34.1** |

* Rate of M. gallisepticum from the positive Mycoplasmal samples
** Rate of M. gallisepticum from the total lungs samples
The diagnosis of *Mycoplasma* species may be carried out directly from the tissues, especially lungs, without the need for culturing techniques, which take at least 14 days, thus reducing the time needed for the diagnosis of *Mycoplasma* infections\(^{16,20,22}\).

The current study used sulphadimidine–trimethoprim plus nystatin for the first time instead of thallous acetate as an inhibitor for the growth of gram-negative bacteria and fungi, and it provided excellent results for isolation without contamination. Also, this study is one of the few studies\(^{14-16}\) to use calf serum instead of horse serum, providing ideal conditions and results for the cultivation of *Mycoplasma*. This could point towards a new trend in *Mycoplasma* research where researchers abandon the use of horse serum and use alternatives such as calf serum.

*M. gallisepticum* represented 43.3% of the total *Mycoplasma* spp. detected by the PCR technique (Table 4), which represents a high prevalence of this organism. This result reveals that the other *Mycoplasma* spp. (56.7%) are also present in the starlings’ lungs, which may include one or more of the following: *M. synoviae*, *M. meleagridis*, *M. iowae*, *M. anatis*, *M. cloacae*, and *M. gallinarum*\(^{13}\).

The rate of directly detecting of *M. gallisepticum* from lungs by using PCR is higher than the rate of detecting by culturing; this indicates the possibility of diagnosing these bacteria from tissues directly without the culturing of samples, which involves long periods of time for the cultivation of *Mycoplasma* (at least 14 days).

References to the isolation or detection of *M. gallisepticum* from lungs of starling birds are nonexistent, though there are reports about other *Mycoplasma* species in starlings, like *M. sturni*, which was isolated from the conjunctiva of European starlings\(^{32-33}\). Most scientific articles mention the isolation and diagnosis of *M. gallisepticum* from finches and other species of birds (34-39), making this study the first to have detected and isolated *M. gallisepticum* from starlings in general and from their lungs in particular.

In conclusion, the results of this study elucidate a high presence of *M. gallisepticum* in the lungs of starlings, and this may be the only study that confirms the presence of these bacteria in the lungs of these birds. It also proves the possibility of using alternative supplements to be added to the culture media for *Mycoplasma*, in terms of alternate serum and antimicrobial types. Further, the current study has demonstrated the possibility of diagnosing *Mycoplasma* directly from tissues without the need for culturing.

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The authors declare that there is no conflict of interest.

AUTHORS’ CONTRIBUTION
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY
All datasets created or investigated during this study are involved in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.

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