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VETERINARY AND ANIMAL PRODUCTION - RESEARCH PAPER

Detection of *Mycoplasma* spp. in free-living seabirds

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Abstract
This study aimed to investigate the presence of *Mycoplasma* spp. and identify the species of mycoplasma isolates obtained from seabirds found on Brazilian coastal beaches. Tracheal and cloacal swab samples were collected from 50 seabirds rescued by three conservation and marine animal rehabilitation centers located in Brazil. The tracheal and cloacal samples were subjected to mycoplasma culture and the isolates were identified through PCR. A "Mollicutes-specific" 16S rRNA PCR reaction was employed for triage. Four species-specific PCR reactions were used to detect *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, *Mycoplasma meleagridis*, *or M. gallinarum*. The Mollicutes positive and species negative samples were submitted do 16S rRNA sequencing. Eighteen (36%) of 50 seabirds tested positive for mycoplasma by culture. In the PCR for the genus, 28 (56%) of 50 seabirds were positive for *Mycoplasma* spp., with 13 (26%) detected in the trachea, one (2%) in the cloaca, and 14 (28%) in both sites. In the species-specific PCR, *M. gallisepticum* was detected in 17.8%, and *M. meleagridis* in 17.8%. Both species were detected in 14.3%. Of the isolates not characterized at species level, we obtained ten sequences and they were divided into three clusters. The first cluster was closely related to *M. meleagridis*, the second to *M. synoviae*, and the third grouped *M. tully*, *M. gallisepticum*, and *M. imitans*. Four and five of nine species of seabirds studied had mycoplasma detected by culture or PCR, respectively. Mycoplasmas were found in the majority of the animals studied, with the highest prevalence proportionally found in *Sula leucogaster*, and the lowest in *Fregata magnificens*. The phylogenetic analysis identified *Mycoplasma* spp. adapted to aquatic birds.

Keywords Marine birds · *Mycoplasma* spp · Phylogenetic analysis · Mycoplasmosis · Seabirds · Penguin

Introduction

The occurrence of infectious diseases in wildlife can lead to the death of individuals, reducing genetic diversity, causing population decline, and thus posing a significant risk to endangered species. Additionally, these diseases can contribute to endemic infections in domestic animals and to the introduction, reintroduction, and maintenance of pathogens in the natural environment [1]. Mycoplasma species with pathogenic potential in wild birds have been reported in various species of free-ranging and captive birds [2–4].

Mycoplasmas are the smallest known prokaryotes and are associated with respiratory [5], joint-related [6], reproductive [7–9] and ocular diseases [10, 11]. Additionally, they create favorable environments for the development of secondary infections [12]. *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), and *Mycoplasma meleagridis* (MM) are the main species isolated in birds and included

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in notification list of the World Organization for Animal Health and the Brazilian Ministry of Agriculture and Livestock [13, 14].

In seabirds, more specifically, penguins and pelicans, various species of mycoplasmas have been described. *M. sphenisci* was the first mycoplasma species described in *Spheniscus demersus* and already detected in a fecal sample of *Pygoscelis adeliae*. *M. lipofasciens* was isolated from an *Eudyptes pachyrhynchus*, *Mycoplasma tullyi* sp. nov (MT) from *Spheniscus humboldti*, and microorganisms from the Micoplasmataceae family were observed in King Penguins (*Aptenodytes patagonicus*). All these agents were isolated from different sites; however, of the described species, only *M. sphenisci* and *M. lipofasciens* caused disease [4, 15–18]. *Mycoplasma* spp. was detected through PCR in *Pelecanus onocrotalus*, that arrived in Cape Town, South Africa [19] 49 animals. Despite these reports, studies focused on the prevalence of *Mycoplasma* spp. in marine birds are scarce.

Therefore, the aim of this study was to investigate the presence of *Mycoplasma* spp. and identify the species of isolates obtained from seabirds found on the beaches of the Brazilian coast.

Material and methods

Ethics statement

This project was submitted and approved under number 2723170122 (ID 001378) by the Ethics Committee on Animal Use (CEUA) of the Federal University (UFF) and by the Authorization and Information System on Biodiversity (SISBIO-ICMBio) under number 63456.

Samples

Tracheal and cloacal material from 50 free-living, debilitated marine birds of both sexes, with or without symptoms compatible with mycoplasmosis, were collected by using sterile swabs. These birds were rescued from three rehabilitation centers located in Rio de Janeiro, São Paulo, and Espírito Santo states, Brazil. The birds belong to 9 different species: *Spheniscus magellanicus* ($n=22$), *Fregata magnificens* ($n=9$), *Sula leucogaster* ($n=9$), *Sula dactylatra* ($n=2$), *Larus dominicanus* ($n=1$), *Puffinus gravis* ($n=1$), *Thalasseus acuflavidu* ($n=2$), *Thalasseus maximus* ($n=1$), *Phalacrocorax brasilianus* ($n=3$).

The collected material was placed in tubes containing 3.0 ml of liquid Frey medium [20] and sent under refrigeration to the NAL/UFF Laboratory for mycoplasma isolation and to the Laboratory of Molecular Epidemiology- LEpidM/UFF for molecular analysis.

Isolation of *Mycoplasma* spp. by culture

From each animal, an aliquot of 0.2 mL of the collected sample was inoculated into 1.8 mL of liquid Frey medium. Subsequently, serial dilutions were performed and the 10^{-5} dilution was inoculated onto plates containing solid Frey medium. All samples were incubated at 36 °C ± 1 °C under microaerophilic conditions. The verification of typical mycoplasma colony growth, resembling fried eggs on solid medium, was performed under a stereoscopic microscope at 40 × magnification every 48 h for a period of up to 21 days. Plates with colonies exhibiting mycoplasma-like characteristics on the solid medium were examined using Dienes staining and a digitonin sensitivity test to confirm that they were *Mycoplasma* spp.

Once identified as *Mycoplasma* spp., the colonies were purified three times according to the method described by Brown and collaborators (2007) [21] for strain isolation and subsequent identification by PCR.

DNA extraction and PCR

Briefly, 500 µL aliquots of the tracheal and cloacal swab samples immersed in Frey medium, as well as the isolated cultures from each animal, underwent DNA extraction using the adapted phenol–chloroform method [22]. The extracted DNA was quantified and its quality assessed using a spectrophotometer (Biodrop®). The samples were subjected to PCR for the Mollicutes class, and primers complementary to the conserved sequence of the 16S rRNA gene were used [23]. DNA from MS ATCC 25204 was used as a positive control.

A "Mollicutes-specific" 16S rRNA PCR reaction was employed for triage. Four species-specific PCR reactions were used to detect *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, *or M. gallinarum* (Table 1). The Mollicutes positive and species negative samples were submitted do 16S rRNA sequencing. As a positive control for the PCR, strains of *M. gallisepticum* ATCC 19610 and *M. synoviae* ATCC 25204 were used. For the reactions involving *M. meleagridis* and *M. gallinarum*, the controls were obtained from the sample bank of the NAL and LEpidM Laboratories.

The reactions were carried out in a PTC-Thermal Cycler 100® (Bio-Rad Laboratories, Ltd., Hertfordshire, England). The primer sequences, amplification conditions, and sizes of the PCR amplicons are specified in Table 1.

Sequencing and phylogenetic analysis

Ten strains identified as *Mycoplasma* spp. by the digitonin test, Dienes staining, and tested negative for the species in the PCR, had the 16S rRNA region amplified and sequenced

Table 1 Primer sequences, amplification conditions, and amplicons size used in the present study

	Target	Primer sequence (5'-3')	Amplification conditions	Product Size (bp)
GPO3 MGSO	16S rRNA	F:GGGAGCAAACAGGATTAGATACCCT R:TGCACCATCTGTCACTCTGTTAACCTC	94 °C, 5′ 94 °C, 1', 55 °C 1', 72 °C 2', 72 °C 10'	280
M. gallisepticum	DUF3713 lipoproten	*F: GGATCCCATCTCGACCACGAGAAAA *R CTTTTCAATCAGTGAGTAACTGATGA **F: CGTGGATATCTTTAGTTCCAGCTGC **R: GTAGCAAGTTATAATTTCCAGGCAT	95 °C, 5′ 95 °C, 1', 55 °C 2', 72 °C 1', 72 °C 5' 95 °C, 5′ 95 °C, 1', 55 °C 2', 72 °C 1', 72 °C 7'	732 481
M. synoviae	16S rRNA	F: GAGAAGCAAAATAGTGATATCA R:CAGTCGTCTCCGAAGTTAACAA	94 °C, 1′ 94 °C, 301', 55 °C 30'', 72 °C 1', 72 °C 5'	207
M. gallinarum	16S-23S rRNA intergenic	F: ATAGCAGTTGGAAACTAT R: AGTTTACAACCCATAGGGCC	94 °C, 5′ 94 °C, 1', 55 °C 1', 72 °C 1', 72 °C 5'	297
M. meleagridis	16S rRNA	F: CGA GCG AAG TTT TTC GGA AC R: GGTACC GTC AGG ATA AAT GC	94 °C, 5′ 94 °C, 30'', 56 °C 30'', 72 °C 30'', 72 °C 10'	422
16S-start F*** 16S-550 R ***	16S rRNA	F: GAGAGTTTGATCCTGGCTCAGG R: CCCAATAAATCCGGATAACGCTTGC	94 °C, 3′ 94 °C, 30'', 57 °C 30'', 72 °C 1′30'', 72 °C 5'	550
16S-510 F *** 16S-1050 R ***	16S rRNA	F: GTGACGGCTAACTATGTGCCAGCAG R: GCTGACGACAACCATGCACC	94 °C, 3′ 94 °C, 30'', 57 °C 30'', 72 °C 1′30'', 72 °C 5'	540
16S-980 F*** 16S-end R ***	16S rRNA	F: CGAAGAACCTTACCCACTCTTGACATC R: GGTAATCCATCCCCACGTTCTCG	94 °C, 3′ 94 °C, 30'', 57 °C 30'', 72 °C 1′30'', 72 °C 5'	500

Bp base pair, *F forward*, *R reverse*, *Round 1, **Round 2, *** Primers for DNA Sequencing

a as previously described [18] (Table 1). The products from positive PCR assays were purified and sequenced. For amplicon purification, the QIAquick PCR Purification Kit (Qiagen) was used. The reactions were conducted by the PDTIS Sequencing Platform of the Oswaldo Cruz Institute (IOC/Fiocruz, Brazil) on an automated DNA sequencer, Applied Biosystems ABI Prism 3730 (Applied Biosystems®, Foster City, California, EUA).

The DNA sequences obtained were assembled using the Lasergene software (DNASTAR, Madison, USA). Subsequently, they were compared to sequences deposited in GenBank® using the Blast tool to attempt species-level identification. All sequences were deposited in GenBank® under accession numbers OR104955 to OR104964.

Phylogeny was inferred using the Maximum Likelihood method and the Kimura 2-parameter model [24]. The samples were compared with 16S rRNA sequences from other *Mycoplasma* species isolated from domestic and wild birds. Evolutionary analyses were conducted in MEGA X [25].

Results

Culture

Eighteen (36%) out of the 50 birds studied, tested positive for mycoplasma by culture. Regarding the anatomical site, typical mycoplasma colonies (Fig. 1) were observed in 13 (26%) samples from the trachea, one (2%) from the cloaca, and four (8%) in both sites.

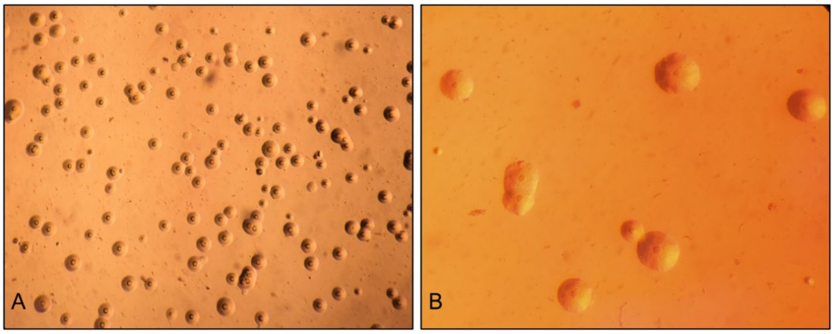

Fig. 1 **A** and **B** "Fried egg" shaped colonies observed from different samples under a stereoscopic microscope (40X)

PCR

Twenty eight (56%) out of the 50 birds studied tested positive for *Mycoplasma* spp., by PCR assays, with 13 (26%) in the trachea, one (2%) in the cloaca, and 14 (28%) in both sites.

In the species-specific PCR *M. gallisepticum* and *M. meleagridis* were equally detected in 17.8% (5/28) of the birds. Both mycoplasmas were detected in 14.3% (4/28) of the animals. In the remaining 50% (14/28) of the animals, it was not possible to characterize the mycoplasma species with the primers used.

Considering the nine species of marine birds studied, four were positive in isolation (*Spheniscus magellanicus*, *Sula leucogaster*, *Larus dominicanus* e *Phalacrocorax brasilianus*) and five were positive in PCR, specifically *Spheniscus magellanicus*, *Fregata magnificens*, *Sula leucogaster, Sula dactylatra*, *Larus dominicanus* e *Phalacrocorax brasilianus*.

Sequencing and phylogenetic analysis

The sequences obtained were assigned GenBank accession numbers from OR104955 to OR104964. The strains obtained in this study showed approximately 98% similarity to strains detected in other birds, mostly seabirds and were divided into three clusters. The first cluster is represented by strains closer to *M. meleagridis*, the second with strains more related to *M. synoviae* and the third cluster, more distant, grouped a single sample detected in this study with *M. tullyi, M. gallisepticum* e *M. imitans* (Fig. 2).

Discussion

Mycoplasmas are host-specific, but they have a great capacity to adapt to new hosts [26], and in their opportunistic nature, they can cause disease when birds are under conditions of stress. Currently, new mycoplasma species have

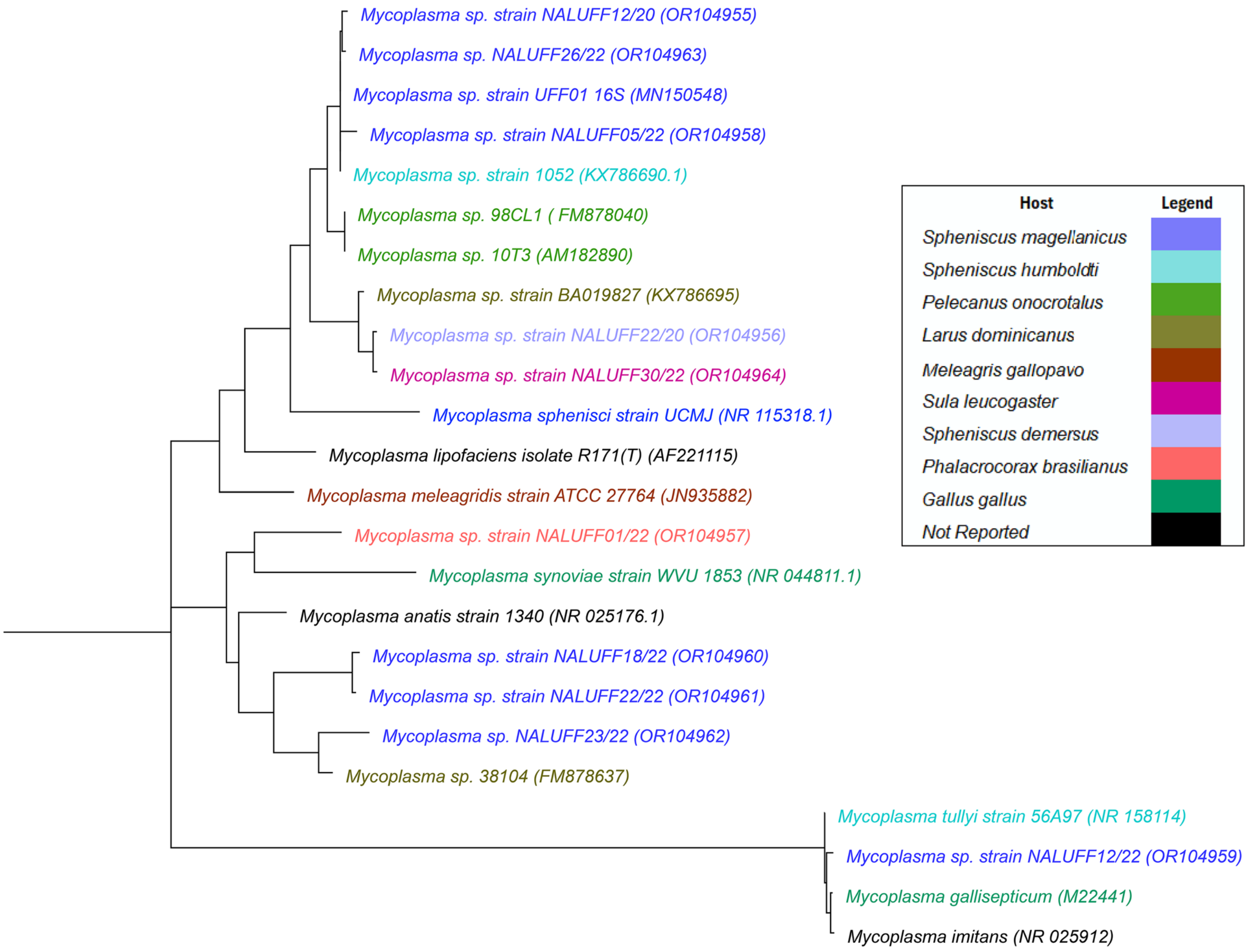

Fig. 2 - A phylogenetic tree inferred from the 16S rRNA sequences of Mycoplasma spp. strains isolated in this study (◆) and sequences of other avian mycoplasmas deposited in Genbank ®. The accession numbers of the strains are indicated in parentheses

been described [8, 18]. In this context, the results of this study contributed to the detection of mycoplasmas adapted to aquatic birds, with the possibility of being new mycoplasma species.

In this study, the presence of *Mycoplasma* spp. was confirmed in six species, distributed across five families of marine birds. Although most of the studied birds were weak and debilitated, they did not show signs of respiratory, joint, or gastrointestinal diseases.

The trachea was the site with the highest detection by both methods. However, the detection of mycoplasmas in the cloaca highlights that this site should be explored in health monitoring. Mycoplasmas are responsible for infertility in domestic birds [12, 27] and can be found in the semen of wild birds [8, 9]. These microorganisms can be a cause of failure in captive bird breeding programs and should be taken into consideration, especially when dealing with threatened bird species.

In this investigation, *M. gallisepticum* was detected by PCR only in penguins, a result similar to that found by [28], while *M. meleagridis* was found in both penguins and gannets. The detection of *M. gallisepticum* and *M. meleagridis* in marine birds is a cause for concern since both mycoplasma species are responsible for economic losses in industrial chicken and turkey production [29]. Furthermore, *M. gallisepticum* has already shown the ability to adapt to new species and become highly virulent [30–33].

In the *M. meleagridis* cluster, *M. lipofaciens* (AF221115), *M. spheniscі* (NR115318.1) were grouped with strains isolated from *Phalacrocorax brasilianus* (KX786695), pelicans (FM878040, AM182890), *Sphenisci humboldti* (KX786690.1), *Spheniscus magellanicus* (MN150548) and five strains from this study, isolated from *Spheniscus magellanicus* (OR104955, OR104958, OR104963), *Larus dominicanus* (OR104956) and *Sula leucogaster* (OR104964). Until now, only *M. lipofaciens* e *M. sphenisci* have been detected, respectively, in penguins with pneumonia and recurrent mucopurulent nasal discharge [4, 16]. However, these authors did not have data to infer the pathogenicity of *M. lipofaciens* and *M. sphenisci* in penguins. Sawicka-Durkalec et al. (2021) [34] obtained strains in *Larus canus* that were close to the strain KX786695, as well as sequences found in a *Larus dominicanus* and a *Sula leucogaster*. These authors report that *Mycoplasma* spp. may be part of the normal microbiota of seagulls, as these microorganisms are present in healthy birds. Our results corroborate with these authors, as the seagull in this study did not show signs of mycoplasmosis.

The strain OR104957 from the Cormorant and the strains OR104960, OR104961, and OR104962 obtained from *Spheniscus magellanicus* were grouped in a second cluster with proximity to *M. synoviae*, *M. anatis* and a strain obtained from *Larus dominicanus* (FM878637). Reports of *M. synoviae* in wild birds are infrequent. Parsons et al. (2016) [35] reported the presence of *Mycoplasma synoviae* antibodies in *Spheniscus demersus* and Sawicka-Durkalec et al. (2021) [34] reported the occurrence of *Mycoplasma* spp. in *Phalacrocorax hich* belongs to the *Phalacrocoracidae family,* which includes *Phalacrocorax brasilianus* in this study.

The strain NALUFF12/22 (OR104959) obtained from *Spheniscus magellanicus* was grouped in a more distant cluster, staying close to *M. gallisepticum*, *M. imitans*, and *M. tullyi*. *Mycoplasma tullyi* was described by Yavari et al. (2017) [18] as a species isolated from *Sphenisci humboldti*, related to *M. gallisepticum* and *M. imitans*.

In this research, the phylogenetic analysis detected *Mycoplasma* spp. adapted to aquatic birds, as these mycoplasmas had common ancestors with *M. anatis*, *M. sphenisci*, and greater similarity to mycoplasmas from aquatic birds, in addition to being considered species-specific [4, 9, 18].

Mycoplasmas were present in most of the bird species studied. This is the first report of *Mycoplasma meleagridis* in *Sula dactylatra*, *Sula leucogaster* and *Spheniscus magellanicus* from Brazil. The presence of mycoplasmas due their opportunistic nature should be considered in the rehabilitation programs for marine birds. Veterinarians and caregivers should adopt preventive measures to avoid the contamination of these birds in the recovery centers, which could make them agents of mycoplasma dissemination.

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Declarations

Competing interests The authors have no conflict of interest to declare.

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