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Investigation of *Mycoplasma* spp. in birds of the Rio de Janeiro Zoo by isolation and PCR[1]

Bárbara S.N. Magalhães[2]*, Virginia Léo A. Pereira[3], Thomas S. Dias[3], Leandro S. Machado[3], Mariane M. Silva[3], Elmiro R. Nascimento[3], Flavya Mendes-de-Almeida[2] and Nádia Regina P. Almosny[2]

ABSTRACT.- Magalhães B.S.N., Pereira V.L.A., Dias T.S., Machado L.S., Silva M.M., Nascimento E.R., Mendes-de-Almeida F. & Almosny N.R.P. 2020. **Investigation of *Mycoplasma* spp. in birds of the Rio de Janeiro Zoo by isolation and PCR.** *Pesquisa Veterinária Brasileira 40(3):210-215*. Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: barbaraneil@hotmail.com

Brazil is one of the countries with the most abundant avifauna in the world. The confinement of birds associated with close contact with other animals and humans favor the spread of agents of respiratory diseases. Among them, mycoplasmas can cause asymptomatic or apparent disease that manifests in birds by coughing, sneezing, rales, conjunctivitis, ocular and nasal discharge. Several described mycoplasmas cause disease in birds, especially *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS). The diagnosis of *Mycoplasma* spp. can be done by clinical observation and laboratory analysis. Molecular diagnosis by PCR was boosted by its speed, sensitivity, and low cost of agent isolation techniques that take up to 21 days to complete. This study aimed to verify the occurrence of *Mycoplasma* spp. in birds of the Rio de Janeiro Zoo (Rio Zoo), by isolation and PCR. Of the total 635 birds from the Rio Zoo, 81 were studied for detection of *Mycoplasma* spp., when taken for routine health assessment exams. These birds belonged to the following orders: Psittaciformes (45), Accipitriformes (18), Galliformes (7), Piciformes (5), Strigiformes (4), Falconiformes (1) and Cariamiformes (1), all individuals already identified by microchip or leg-ring. There was no isolation of mycoplasmas in any of the samples tested, whereas, in the PCR, 62.96% (51/81) were positive, with 1.96% (1/51) identified as MG and 19.61% (10/51) as MS, representing 1.23% (1/81) and 12.34% (10/81) of the total population studied. PCR was shown to be a more effective technique than isolation in the detection of *Mycoplasma* spp. in birds. It was possible to detect mycoplasmas in birds from Riozoo with no clinical respiratory signs, with higher MS prevalence than MG. The positivities for *Mycoplasma* spp., MS, and MG were different among the orders studied, being the highest occurrence in birds of prey, followed by Galliformes and Piciformes. The presence of MG and MS in birds of Rio de Janeiro Zoo confirms the circulation of these agents and the need for further studies on the dissemination of mycoplasmas in zoos for the epidemiological analysis of these bacteria in these places.

INDEX TERMS: Investigation, *Mycoplasma* spp., birds, Rio de Janeiro, Brazil, zoo, captive.

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[2] Graduate Program in Veterinary Medicine, Clinics and Animal Reproduction, Faculdade de Veterinária, Universidade Federal Fluminense (UFF), Rua Vital Brazil Filho 64, Niterói, RJ 24230-340, Brazil.

[3] Departamento de Saúde Coletiva Veterinária e Saúde Pública, Universidade Federal Fluminense (UFF), Rua Vital Brazil Filho 64, Niterói, RJ 24230-340.
*Corresponding author: barbaraneil@hotmail.com

RESUMO.- [**Investigação de *Mycoplasma* spp. em aves do Zoológico do Rio de Janeiro por isolamento e PCR.**] O Brasil é um dos países com maior avifauna do mundo. O confinamento de aves associado ao contato próximo a outros animais e seres humanos favorece a disseminação de agentes etiológicos causadores de doenças respiratórias. Dentre eles, os micoplasmas podem causar doença assintomática ou aparente que se manifesta em aves por espirros, estertores,

conjuntivite, corrimentos oculares e nasais. São diversos os micoplasmas descritos causadores de doença em aves, com destaque para *Mycoplasma gallisepticum* (MG) e *Mycoplasma synoviae* (MS). O diagnóstico de *Mycoplasma* spp. pode ser feito pela observação clínica e análises laboratoriais. O diagnóstico molecular pela Reação em Cadeia da Polimerase (PCR) ganhou impulso por sua rapidez, sensibilidade e baixo custo em relação às técnicas de isolamento do agente que levam até 21 dias para conclusão do gênero *Mycoplasma*. Objetivou-se verificar a ocorrência da infecção por *Mycoplasma* spp. em aves no Zoológico do Rio de Janeiro (Rio Zoo), por isolamento e PCR. Do plantel de 635 aves do Rio Zoo, foram estudadas 81 para detecção de *Mycoplasma* spp., quando contidas para exames rotineiros de avaliação da condição de saúde. Essas aves eram pertencentes às ordens Psittaciformes (45), Accipitriformes (18), Galliformes (7), Piciformes (5), Strigiformes (4), Falconiformes (1) e Cariamiformes (1), todas já identificadas por microchip ou por anilha. Não houve isolamento de micoplasmas em nenhuma das amostras testadas, enquanto na PCR, 62,96% (51/81) foram positivas, sendo 1,96% (1/51) identificadas como MG e 19,61% (10/51) como MS, representando 1,23% (1/81) e 12,34% (10/81) da população total estudada. A PCR demonstrou ser uma técnica mais efetiva que o isolamento na detecção de *Mycoplasma* spp. em aves. Foi possível detectar micoplasmas nas aves do Riozoo sem sinal clínico respiratório, tendo MS maior prevalência do que MG. As positividades para *Mycoplasma* spp., MG e MS foram diferentes entre as ordens de aves estudadas, sendo a maior ocorrência nas aves de rapina, seguida dos Galliformes e dos Piciformes. A presença de MG e MS nas aves do Rio de Janeiro Zoo confirma a circulação destes agentes e a necessidade de mais estudos sobre a disseminação de micoplasmas em zoológicos para análise epidemiológica dessas bactérias nesse local.

TERMOS DE INDEXAÇÃO: Investigação, *Mycoplasma* spp., aves, zoológico, Rio de Janeiro, Brasil, PCR, cativeiro.

INTRODUCTION

The birds are the most studied and valued group of animals in the world with more than 11,000 different species and an extraordinary variety. Some occur in abundance, and others represented by a few remaining individuals. It is estimated that in Brazil, there are from 1600 to 1900 bird species, more than 10% of them endemic, which makes it to be considered one of the most avifauna countries in the world (Lewinsohn & Prado 2005). To present these different bird species to the public, zoos around the world have facilities for the maintenance of these captive animals. However, the confinement of animals, associated with their contact with visitors and breeders, make the transmission and dissemination of pathogens a specific risk in these places (Cubas 2008, Loria et al. 2008). Among these agents are those that cause respiratory diseases, with emphasis on mycoplasmas that can cause apparent or subclinical disease in birds (Nascimento & Pereira 2009). Its spread occurs directly or indirectly horizontally through people, other animals, feed, water, and fomites. In addition to this pathway, vertical transmission via egg or venereal may occur through mating or artificial insemination (Stipkovits & Kempft 1996, Nascimento & Pereira 2009).

Clinical signs commonly seen in wild and captive birds are sneezing, rales, eye and nasal discharge, unilateral conjunctivitis, or bilateral or not accompanied by enlargement of the infraorbital sinus and may be associated with chronic infections (Phalen et al. 2006). Among the main species of the genus *Mycoplasma*, stand out *M. gallisepticum* (MG) and *M. synoviae* (MS) which, cause an apparent or asymptomatic respiratory condition. But other species such as *M. gypis*, *M. vulturii*, *M. gallinarum*, *M. gallinaceum*, *M. iners* and *M. corogypsi* have been described as causing disease in birds (Poveda et al. 1990, Panangala et al. 1993, Fischer et al. 1997, Oaks et al. 2004).

To reduce the risk of these infections in bird populations and to assess the sanitary control of poultry, it is crucial to know and monitor these mycoplasmas in the environment by epidemiological surveys and laboratory tests associated with the development and implementation of strict standards and procedures in livestock (Vilani 2006). The mycoplasmic diagnosis can be presumptive with clinical and epidemiological, anatomopathological, serological, and etiological evaluations by agent isolation or PCR (Nascimento et al. 2005, Umar et al. 2017). Because these microorganisms are tedious, requiring enriched culture media, and they are slow to grow exponentially, bacteriological isolation has been used in conjunction with molecular techniques. PCR is fast, sensitive, requires a low laboratory cost, and does not allow the need for isolation from clinical specimens (Islam et al. 2011). Therefore, the objective was to verify the occurrence of *Mycoplasma spp.* in birds of different orders at the "Zoológico do Rio de Janeiro" (Rio Zoo) by isolation and PCR.

MATERIALS AND METHODS

The project was submitted and approved under no. 1017 by "Comissão de Ética no Uso de Animais" (CEUA) of "Universidade Federal Fluminense" and by "Sistema de Autorização e Informação em Biodiversidade" (SISBIO-ICMBio) under number 59538-1. In addition, the project was registered in "Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado" (SISGEN), no. A4E34FC.

Material collection. From the 635 birds of Rio Zoo, 81 were studied, belonging to the order Psittaciformes (45), Accipitriformes (18), Galliformes (7), Piciformes (5), Strigiformes (4), Falconiformes (1) and Cariamiformes (1) (Table 1), all already identified by microchip or washer. The animals were manually restrained for routine health assessment and samples collected of swab tracheal, which were conditioned in microtubes containing modified Frey's liquid medium (Nascimento 2000). The microtubes containing the swabs were kept refrigerated until the time of laboratory processing.

Isolation of *Mycoplasma* spp. An aliquot of 0.2mL of the collected sample was inoculated into 1.8mL of the modified Frey liquid medium. Serial dilutions were made until 10^{-5}, and the dilutions 10^{-3} and 10^{-5} were seeded on plates containing modified Frey solid medium (Nascimento 2000). All samples were incubated at 37°C under microaerophilic and observed for 21 days under a 100x magnification stereoscopic microscope (Razin et al. 1998).

DNA extraction. A 500µl aliquot of the collected sample was submitted to DNA extraction by the phenol-chloroform adapted method (Sambrook & Russell 2006). Each sample was then homogenized and centrifuged at 13,500rpm at 10°C for 20 minutes. After centrifugation, the supernatant was discarded, and 400µL of Tris Ethylenediaminetetraacetic acid (TE) dextrose, 30µl 10% sodium dodecyl sulfate (SDS) and 30µl proteinase K 240µg/µl were added to the pellet. The sample was taken to the thermal block at 50°C for

30 minutes with a subsequent ice bath for 5 minutes. Subsequently, 500µL of phenol was added to the samples, homogenized by inversion for 15 minutes, and then centrifuged at 13,500rpm at 10°C for 30 minutes. The supernatant was removed and added to a new microtube with the same volume of chloroform, followed by gentle homogenization for 3 minutes and centrifugation under the conditions already described. The supernatant was removed, added to a 1ml microtube of ethyl alcohol, and precipitated "overnight." The precipitated DNA was centrifuged at 13500rpm at 10°C for 20 minutes and the pellet after drying; it was resuspended in 100µL

Table 1. Order, species, common name and number of birds of the Rio de Janeiro Zoo evaluated for the detection of *Mycoplasma* spp.

Order	Species	Common name	Number	Total
Psittaciformes	*Amazona aestiva*	Real parrot	9	
	Amazona ocrocephala	Puffin parrot	1	
	Ara ararauna	Canary macaw	11	
	Ara macao	Scarlet macaw	3	
	Ara chloropterus	Red macaw	3	45
	Anodorhynchusleari	Lear's macaw	4	
	Guaruba guarouba	Ararajuba	10	
	Pyrrhura frontalis	Red-fronted tiriba	1	
	Pionus maximilliani	Green parrot	3	
Accipitriformes	Amadonastur lacernulatus	Pigeon hawk	3	
	Geranoaetus albicaudatus	White-tailed hawk	6	
	Leptodon cayanensis	Gray-headed hawk	1	18
	Heterospizias meridionalis	Leather-tailed hawk	4	
	Rupornis magnirostris	Carijó hawk	4	
Falconiformes	*Milvago chimachima*	Yellow-tailed hawk	1	1
Galliformes	*Pavo muticus*	Peacock	2	
	Crax alector	Black porcupine	2	
	Crax fasciolata	Pinum curassow	1	7
	Chrylophus pictus	Canary pheasant	1	
	Pavo cristatus	Blue peacock	1	
Piciformes	*Ramphastos toco*	Toucan toco	5	5
Strigiformes	*Strix huhula*	Black owl	1	4
	Pseudoscops clamator	Eared owl	3	
Cariamiformes	*Cariama cristata*	Seriema	1	1
TOTAL				81

Table 2. Primer oligonucleotides for PCR for detection of avian *Mycoplasma* with their sequences, amplified product size, and reference

"Primers"	Sequence	Product	Reference
Mspp GPO3	5´GGGAGCAAACAGGATTAGATACCCT3´	270 pb	Van Kuppeveld et al. (1992, 1993)
Mspp MGSO	5´TGCACCATCTGTCACTCTGTTAACCTC3´		
MG-f	5´CGTGGATATCTTTAGTTCCAGCTGC3´	481 pb	Nascimento et al. (2005)
MG-r	5´GTAGCAAGTTATAATTTCCAGGCAT3´		
MS-f	5´GAGAAGCAAAATAGTGATATCA3´	207 pb	Lauerman etal. (1993)
MS-r	5´CAGTCGTCTCCGAAGTTAACAA3´		

Mspp f and Mspp r = *Mycoplasma* spp., MG-f and MG-r = *Mycoplasma gallisepticum,* MS-f and MS-r = *Mycoplasma synoviae.*

of TE buffer, quantified in Biodrop Touch® (Biochrom) and stored at -20°C until PCR.

PCR. The extracted DNA was submitted to PCR for the detection of *Mycoplasma* spp., *Mycoplasma gallisepticum* (MG), and *Mycoplasma synoviae* (MS) respectively, according to Van Kuppeveld et al. (1992, 1993), Nascimento et al. (2005) and Lauerman et al. (1993). The PCR for detection of *Mycoplasma* spp. was performed in 25µl final volume containing 2µl isolated DNA, 1 × PCR buffer (10mM Tris-HCl, pH 8.0 and 50mM of KCl), 2mM MgCl2, 0.2mM deoxynucleotide triphosphate (dNTP), 0.2mM forward and reverse primers and 1U Taq polymerase. For MG detection the reaction contained: 1X PCR buffer; 2mM MgCl2; 0.2mM dNTP; 0.2nmol of each specific primer (Table 2); 1U Taq Polymerase (Ludwig, Brazil) and extracted DNA totaling 25µl. The MG PCR was performed under the following conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 1 minute, 55°C for 2 minutes, and 72°C for 1 minute, with a final phase of 72°C for 5 minutes. The MS ATCC 25204 and MG ATCC 129 S6 strains were used as positive controls and as ultrapure water negative control. For MS the reaction contained 1X PCR buffer (10mM Tris-HCl, pH 8.0 and 50mM KCl); 1.5mM MgCl2; 0.2mM deoxyribonucleotide triphosphate (dNTP); 0.2nmol of each specific primer (Table 2); 1U Taq Polymerase, and extracted DNA, totaling 25µl. MS PCR was performed under the following conditions: 94°C for 1 minute, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final phase of 72°C for 5 minutes.

Electrophoresis. The amplicons obtained in PCR were applied in 1.5% agarose gel, submerged in Tris-Borato-EDTA Buffer (TBE), and then submitted to electrophoretic run at 94V for 40 minutes. After the electrophoretic run, the gel was stained with ethidium bromide, and visualization of the amplicons was performed under ultraviolet light in a transilluminator.

Statistical analysis. Descriptive statistics were performed to obtain the percentages for *Mycoplasma* spp., MG, and MS for order taxonomic of birds. The Mann-Whitney non-parametric test was used to differentiate between the percentages obtained, which uses the median for comparative effects, with a significance level of 5% using the Bioestat 5.3® software (Ayres et al. 2007).

RESULTS AND DISCUSSION

Of the 81 birds studied, none showed clinical respiratory signs at the time of collection. All birds were negative for isolation for *Mycoplasma* spp., while PCR for *Mycoplasma* spp. detected 62.96% (51/81) of birds 1.96% (1/51) for MG and 19.61% (10/51) for MS, respectively representing 1.23% (1/81) and 12.34% (10/81) of the poultry total population studied (Table 3). In the statistical analysis by the Mann-Whitney test, the differences in percentages obtained between the orders about *Mycoplasma* spp. and MS, were significant ($p<0.05$), excluding the occurrence of MG because there was a single record during the study. Asymptomatic infections are the most common in mycoplasma-infected animals because these agents can escape of host immune system and remain dormant (Razin et al. 1998). Besides, the presence of low pathogenicity strains may cause a mild or inapparent clinical picture (Lecis et al. 2010).

The Falconiform and Cariamiform orders had only one individual tested in each of them, both being PCR positive for *Mycoplasma* spp., and the falconiform identified as MS positive. Without considering these specimens, the highest percentage of positive results for *Mycoplasma* spp. was observed in the Accipitriformes order, with 88.89% (16/18), 22.22% (4/18) identified as positive for MS. Then, in Galliformes, 71.43% (5/7) of positive birds were found, with 14.29% (1/7) characterized with MS; Piciform, 60.00% (3/5) for *Mycoplasma* spp. and 20.00% (1/5) for MS; Psittaciformes, 53.33% (24/45) for *Mycoplasma* spp., 2.22% (1/45) for *M. gallisepticum* (MG) and 6.67% (3/45) for MS. The order of lowest occurrence was Strigiformes with 25.00% (1/4) for Mycoplasma spp., but the species was not identified.

The results found for Psittaciformes contrast the studies of Silva et al. (2016) and Carvalho et al. (2017) when assessing the presence of *Mycoplasma* spp. in asymptomatic parrots under human care. Silva et al. (2016), when analyzing 85 parrots of different species from a zoo in Pernambuco, reported the presence of *Mycoplasma* spp. in 16.47% of the birds, however these mycoplasmas were not identified as MG or MS. Carvalho et al. (2017), when evaluating 300 samples of CETAS parrots, commercial and conservation breeding, observed positivity for *M. gallisepticum* (MG) in 21.6% (16/74) in CETAS, 15.7% (19/121) in commercial breeding, and 6.7% (7/105) in conservationist, while for MS the occurrences were 2.7 % (2/74) in CETAS, 0.0% (0/121) in commercial breeding and 1.9% (2/105) in conservationist. MG was still described with a high occurrence by Gomes et al. (2010) in parrots from the seizure of trafficking with the positivity of 85.4%.

Table 3. Isolation and PCR detection of *Mycoplasma* spp., *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by the order of the birds of Rio de Janeiro Zoo

Order	Isolation	PCR		
	Mspp.*	Mspp.*	MG**	MS***
Psittaciformes	0/45 (0%)	24/45 (53.33%)	1/45 (2.22%)	3/45 (6.67%)
Accipitriformes	0/18 (0%)	16/18 (88.89%)	0/18 (0.00%)	4/18 (22.22%)
Falconiformes	0/1 (0%)	1/1 (100.00%)	0/1 (0.00%)	1/1 (100.00%)
Galliformes	0/7 (0%)	5/7 (71.43%)	0/7 (0.00%)	1/7 (14.29%)
Piciformes	0/5 (0%)	3/5 (60.00%)	0/5 (0.00%)	1/5 (20.00%)
Strigiformes	0/4 (0%)	1/4 (25.00%)	0/4 (0.00%)	0/4 (0.00%)
Cariamiformes	0/1 (0%)	1/1 (100.00%)	0/1 (0.00%)	0/1 (0.00%)
TOTAL	0/81 (0%)	51/81(62.96%)	1/81 (1.23%)	10/81(12.3%)

* Mspp.= *Mycoplasma* spp., ** MG = *Mycoplasma gallisepticum*, *** MS = *Mycoplasma synoviae*.

Lecis et al. (2016) analyzed samples of 62 birds of prey from two Wildlife Centers and a Veterinary Hospital in Italy, obtaining positivity for *Mycoplasma* spp. in 41.9% (26/62) of them. In our study, we analyzed samples of three orders of birds considered of prey, Falconiformes, Accipitriformes, and Strigiformes in which we found positivities ranging from 25.00% to 88.89%, that is, with a mean positivity of 56.95%, with the prevalence obtained being higher than that presented by other authors. This difference may be due to the proximity between the nurseries, which may have favored or predisposed to mycoplasma infections (Kleven & Fletcher 1983). Lecis et al. (2016) observed clinical symptoms compatible with mycoplasmosis in a positive individual; in the present study, such occurrence was not observed, despite the high frequency of mycoplasma positive birds. The high frequency found in asymptomatic prey corroborates other studies where a high frequency of mycoplasma was observed in birds of prey with mild or inapparent clinical presentation (Lierz et al. 2008, Lecis et al. 2010, Ziegler et al. 2019).

For Kleven (1998), there may be a preference for MG and MS for Galliformes. However, in our study, the positivity for *Mycoplasma* spp. in this order was 71.43% (5/7), none positive for MG, and 14.29% (1/7) identified as MS in the wild Galliformes tested. Michiels et al. (2016) tested 15 wildlife Galliformes in Belgium, but none were positive; however, Haesendonck et al. (2014) demonstrated a high prevalence of 76.3% for MG and 36.0% for MS in Galliformes reared for ornamental purposes in the same country, suggesting that these birds may serve as a reservoir for these agents.

The high prevalence of *Mycoplasma* spp. in birds of the zoo, observed in this study, is important because birds with or in the subclinical state contribute to the maintenance of mycoplasmas in environments. Besides, its transmission occurs by diffusion in the form of aerosol or droplets, being the upper respiratory tract and conjunctiva the main entry doors of the pathogen (Nascimento & Pereira 2009, Stipkovits & Szathmary 2012). These agents are capable of causing respiratory signs, as well as severe reproductive disease in birds (Carnaccini et al. 2016), constituting a significant challenge for the conservation and rearing of wild and captive birds, because in addition to impairing rehabilitation, causes high mortality of embryos as well as young and adult birds (Gomes et al. 2010).

CONCLUSIONS

PCR was more effective than the isolation technique in detecting *Mycoplasma* spp. It was possible to detect mycoplasmas in birds of different orders without a clinical respiratory sign, obtaining *Mycoplasma synoviae* (MS) more prevalent than *Mycoplasma gallisepticum* (MG). The positivities for *Mycoplasma* spp., MG, and MS were different among the studied orders, being the highest occurrence in birds of prey, followed by Galliformes and Piciformes.

The presence of MG and MS in the birds of Rio Zoo confirms the circulation of these agents and the need for further studies on the dissemination of mycoplasmas in zoos for epidemiological analysis of these bacteria in this location.

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Conflict of interest statement. - The authors have no competing interests.

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