http://dx.doi.org/10.1590/1678-4162-12815

Arq. Bras. Med. Vet. Zootec., v.75, n.6, p.1182-1187, 2023

Communication

[*Comunicação*]

Detection of *Mycoplasma* in dead psittacine embryos

[Detecção de micoplasma em embriões de psitacina mortos]

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Captive breeding of avian species, both for commercial and conservation purposes, demands healthy breeders for viable healthy progenies. Commercially raised pets must be healthy for market viability and considering the conservation projects, sufficient and health progeny numbers are required, especially for the endangered species. Native Brazilian species, endangered or extinct from nature, have been reproduced in captivity and the obtained progenies have enabled their return to nature, as demonstrated for *Amazona aestiva* (Lopes, 2016), *Aburria jacutinga, Crax blumenbachii*, and *Pauxi mitu* in Brazil (Avelar and Simpson, 2014; Silveira, 2017).

Both natural and artificial incubation may be impaired by embryonic disease, despite artificial incubation may enable the hygienic control of eggs and the elimination of parental/nest transmission (Smith, 2019). Species of *Mycoplasma* spp. are amongst agents capable of causing embryonic and hatchling mortality (Kleven, 2008), and although most species were described in chickens and turkeys, 17 species were found in wild avian hosts, including *Mycoplasma buteonis, M. corogypsi, M. falconis, M. gypis* and *M. sturni* (Luttrell and Fischer, 2007). However, *M. gallisepticum* (*MG*) was isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis in the surroundings of poultry houses (Ley *et al.*, 1996), which indicated spill over from chickens to passerines. *MG*, *M. synoviae* (*MS*) and *M. meleagridis* (*MM*) are long been known for causing high economic losses to the poultry industry (Kleven, 2008), and for this reason monitored for eradication in breeder flocks, as determined by the National Avian Health Program (*Programa Nacional de Sanidade Avícola*- PNSA) (Brasil, 2009), considering that vertical transmission may generate infected progenies (Lay and Yoder, 1996).

Despite its potential importance, few studies have been dedicated to evaluating embryonic infection by *Mycoplasma* in pet or wild birds. We describe the investigation of avian *Mycoplasma* in embryos of exotic or native psittacine species (Aves: Psittaciformes) reproduced by artificial incubation.

Forty-nine incubating eggs of Psittaciformes (Table 1) were studied. At the hatchery of commercial exotic pet (n=32) or conservation native breeder (n=17) facilities, eggs were candled and those discarded due to embryonic mortality, were saved for evaluation. According to the time of embryonic death, eggs were classified as of early, intermediate, or late mortality. One dead-in-shell hatchling (*Pionus maximiliani*) of a commercial facility was also evaluated. The embryonic tissues of late mortality embryos were collected separately for DNA extraction. DNA purification was performed by thermal extraction (Avian…, 2019) for yolk or colony samples or using sodium iodide-silicon dioxide for embryonic tissues (Boom *et al., 1990*). Qualitative and quantitative analyses of total DNA were performed (NanoVue ®, GE Healthcare, UK) prior to evaluation.

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†in memoriam; we miss our loving professor
Submitted: July 21, 2022. Accepted: November 29, 2022.

PCR reactions were performed in a thermocycler (Axygen-Maxygene, USA) for a universal *Mycoplasma* spp. reaction, using the oligonucleotides F5'-ACACCATGGGAGYTGGTAAT-3' and R3'-CTTCWTCGACTTYCAGACCCAAGGCAT–5' (12), with a 370-500 bp product, for amplifying part of the 16S ribosomal RNA gene. For the diagnosis of *MG*, the oligonucleotides MG-14F 5'-GAGCTAATCTGTAAAGTTGGTC-3' and MG-13R 5'-GCTTCCTTGCGGTTAGCAAC-3' (Avian…, 2019) were used for the detection of a 185 bp product, representing part of the cytoadhesin protein gene. Electrophoresis of products was performed at 100V/ 40 min (Life Technologies, Gaithersburgh, MD, USA), revealed by GelRed® (Biothium, CA, USA) and visualized in a UV transilluminator (Hoefer, San Francisco, CA, USA). The molecular mass of products was estimated by comparing with a 100 bp ladder (Promega, Fitchburg, WI, USA).

Attempts for the cultivation of *Mycoplasma* from one hatchling were performed in agar media containing 15% horse serum, and added with thalium acetate and penicillin, and incubated in microaerophilic atmosphere (Ley *et al.*, 1996). Colonies were visualized in a stereo microscope (Olympus SZX7, Japan).

Eggs of the commercial breeder, including both exotic and native species, were shown to be 37.5% (12/32) positive for *Mycoplasma* spp., being detected in the vitelline sac/yolk (47.3%), in the embryo carcass (26.3%), in the brain (15.8%) or in the head (10.5%). All embryos (17/17) of the conservation facility, all of which Brazilian native, were detected positive in diverse embryonic tissues (brain, liver, lung, whole embryo, yolk). The *Mycoplasma* spp. positive eggs were further tested for *MG*, with none returning positive. The detailed results are shown according to host species in Table 1. The bacterial isolation was successful from one dead hatchling of *Pionus maximiliani* (Fig. 1) and its identification as *Mycoplasma* spp. was confirmed by PCR.

Considering the dead embryos of the exotic species, *Mycoplasma* spp. was not detected in samples (0/5) of *Eclectus roratus*, despite it was detected in 4/26 (15.4%) eggs of *Psittacula krameri* (brain, embryo carcass, head, yolk), and in the *Lorius lory* egg sampled (yolk).

Among the eggs of the native species, *Mycoplasma* spp. was detected in the yolk of 2/4 (50%) eggs of *Amazona aestiva*, also detected in all (2/2) embryos of *Anodorhynchus hyacinthinus* (brain, lung, liver, yolk), detected in 2 (n=3) (66%) eggs of *Ara chloropterus* (chorio-allantoic liquid, yolk), and in one (1/2) of *Aratinga jandaya* embryos (brain). A single egg of *Psittacara leucophthalmus* (yolk tested) or *Pyrrhura cruentata* (embryo carcass tested), were available, and both were shown positive. In contrast, the single egg of *Pionus menstruus* was tested negative (yolk).

It was interesting to note that, according to the time of embryonic death, mortalities were distributed along the entire incubating period, in contrast to previously described with chicken embryos infected with *MG*, characterized by late mortality (Kleven, 2008).

Samples were obtained from both exotic and native psittacine embryos which died during the incubation or at hatch, and the detection of *Mycoplasma* in different tissues suggests a systemic embryonic infection. A determining role of the infection in the embryonic deaths was indicated by the evaluations of vital organs, such as brain, liver, or lung. In addition, its presence in the vitelline sac/yolk of several embryos would suggest ovarian infection of the breeders, enabling vertical transmission (Ley, 1997). Late embryonic death (at hatching) was detected in *P. maximiliani*, shown positive both by PCR and bacterial isolation, with *Mycoplasma* spp. detected in the allantoic fluid (AF), in agreement to previously shown that 2.3% chicken embryos from *M. synoviae* infected hens may contain AF infection (Benčina *et al.*, 2005).

Table 1. Evaluation of native and exotic psittacine eggs or hatchlings for *Mycoplasma* DNA by PCR

Species	Common name	Number of positive/total	Biological sample	*Mycoplasma* spp.	*Mycoplasma gallisepticum*	IUCN status*
Amazona aestiva	Blue-fronted amazon	2/4	Vitelline sac/yolk	+	-	LC
Amazona ochrocephala	Yellow-crowned amazon	1/3	Vitelline sac/yolk	+	-	LC
Anodorhynchus hyacinthinus	Hyacinth macaw	2/2	Brain, lung, liver, vitelline sac/yolk	+	-	VU
Ara chloropterus	Red-and-green macaw	1/3	Chorio-allantoic fluid; vitelline sac/yolk	+	-	LC
	Red-and-green macaw	1/3	Embryo carcass, head	+	-	
Aratinga jandaya	Jandaya parakeet	1/2	Brain	+	-	LC
Eclectus roratus	Eclectus parrot	0/5	Embryo carcass	-	-	LC
Lorius lory	Black-capped lory	1/1	Vitelline sac/yolk	+	-	LC
Pionus menstruus	Blue-headed parrot	0/1	Vitelline sac/yolk	-	-	LC
Pionus maximiliani	Scaly-headed parrot	1/1	Hatchling	+	-	LC
Psittacara leucophthalmus	White-eyed parakeet	1/1	Vitelline sac/yolk	+	-	LC
Psittacula krameri	Rose-ringed parakeet	1/26	Brain	+	-	LC
		1/26	Embryo carcass	+	-	
		1/26	Vitelline sac/yolk	+	-	
		1/26	Vitelline sac/yolk and head	+	-	
Pyrrhura cruentata	Ochre-marked parakeet	1/1	Embryo carcass	+	-	VU

*LC, least concern; VU, vulnerable. (https://www.iucnredlist.org/).

The detection of *Mycoplasma* spp. DNA was successful in all native species, except for the only *Pionus menstruus* egg tested, and included the detection and isolation in one dead hatchling of *Pionus maximiliani*. However, all embryos were tested negative for *MG*, results which were somewhat surprising, taking into consideration that a previous study found high rates of detection of *MG* in mortalities at a triage center (Gomes *et al.*, 2010). However, the psittacines of such previously tested premises were not retested here. Also, the protocols employed by Gomes *et al.* (2010) included broth enrichment (FREY), methodology not performed here, which could result in differences. The high indexes of *MG* detection previously described (Gomes *et al.*, 2010) might possibly also be associated to the presence of *M. imitans* in the psittacines, and PCR products could only be differentiated using

restriction fragment analysis using *MseI* and *AseI* (Lierz *et al.*, 2008a) or sequencing. In addition, the somewhat better biosafety standards of the commercial or conservation facilities here tested, as compared to the triage center (Gomes *et al.*, 2010), may also play a role for differences.

In conservation facilities, different avian species and origins are regularly incorporated into the premises, without the possibility of an adequate quarantine. Possibly, the absence of *M. imitans* (Bradbury et al., 1987), in our samples, may have resulted in *MG* negativity. Comparing results to previously described occurrence (Gomes et al., 2010), the risk conditions included differences in the psittacine populations, management and biosecurity. However, considering the commercial breeder, quarantine may be a common precaution.

Few studies were found regarding embryonic infection in psittacines. A twenty-five percent positivity for *Mycoplasma* was detected in Germany in psittacines with respiratory disease (Lierz and Hafez, 2008), but in none of the clinically normal. These findings might indicate that the clinical evaluation and retirement of psittacine breeders from breeding, as based on clinical signs, would be useful for reducing the risk of producing infected embryos, in contrast to chicken (Kleven, 2008) and raptors (Lierz *et al.*, 2008ab), which may have 100% subclinical infection.

The lack of previous studies in Brazilian psittacine embryos or young, did not allow local comparisons. However, captive psittacines were tested (cloaca, conjunctiva, and palate) in Recife (Pernambuco, Brazil) and shown 6.6% positive for *Mycoplasma* spp., but none for *MG* or *MS* (Silva *et al.,* 2016). These results corroborate with our findings regarding the negativity to *MG*, although a lower level of infection was detected, possibly related to differences in transmission conditions. In contrast, a blue-fronted *Amazon* parrot individual with severe respiratory disease was described with mixed *MG* and *MS* infections (Gomes *et al.,* 2012).

Wild passerines species mortalities by various causes were studied in the USA and mycoplasmas were associated to conjunctivitis (González-Astudillo *et al.,* 2016). Egg transmission of mycoplasmas was previously investigated in an infected breeder geese flock with low hatchability (51%) due to embryonic mortality, with *MG* found in 13.9% and *MS* in 15.2% and mixed infection in 16% of eggs (Benčina *et al.,* 1988). Fifteen avian species were evaluated for the presence of *Mycoplasma*, showing *M. cloacale* in the cloaca of 12 specimens of wild and domestic ducks and domestic geese (Bradbury *et al.,* 1987), potentially spreading through feces, although not associated with disease. Species of Anseriformes (*Anas platyrhynchos, Anas rubripes, Anas strepera* and *Aythya va!isineria*) were also investigated for *Mycoplasma*, being 37% detected with *M. cloacale* and 18% identified with *M. anatis*, with also unspecified isolates (Goldberg *et al.,* 1995). Previous studies using bacterial isolation found 52% infected by *Mycoplasma* (Benčina *et al.,* 1987), evaluating chickens, chicken embryos, turkeys, ducks, geese, pigeons and Japanese quail and embryos. *M. anatis* was isolated only from ducks and geese, *M. columbinum, M. columbinasale* and *M. columborale* detected only in pigeons, and *M. meleagridis* and *M. gallopavonis* only in turkeys, *M. lipofaciens* detected in a turkey and a duck. *M. cloacale* was detected in one chicken, turkey and a duck and *M. gallinarum* in a turkey. *M. synoviae* was the most frequently isolated (41.8%) and *M. gallinarum, M. gallinaceum, M. pullorum, M. glycophilum* and *M. lipofaciens* were the least detected. Considering host range, *M. synoviae* and *MG* had similarly wide host spectrum.

Isolation was successful for one hatchling of scaly-headed parrot. Typical colonies (Fig. 1) were collected and confirmed as *Mycoplasma* spp. by PCR, but not *MG*. Isolation is considered difficult, and obstacles might be associated with *post mortem* degradation or antibiotic treatment, both common conditions in the study.

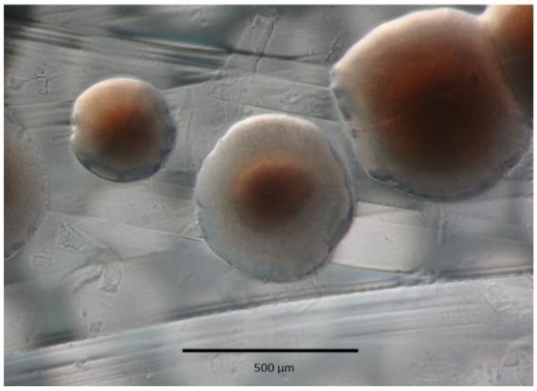

Figure 1. Isolated colonies of *Mycoplasma* spp. grown of the conjunctiva of a scaly-headed parrot (*Pionus maximiliani*) hatchling on agar media containing 15% horse serum added with thalium acetate and penicillin, in a microaerophllic atmosphere.

Mycoplasma spp. but not *M. gallisepticum*, was detected in ¼ of dead psittacine embryos and the infection may have a role in part of the embryonic deaths, as well as post hatch viability. The regular monitoring for *Mycoplasma* of avian flocks, especially breeders during the reproduction season, possibly ideally mandatory, including of pet facilities, exotic or native, and of conservation avifauna, is considered essential for the successful production of healthy embryos and progenies.

Keywords: Psittaciformes, *Mycoplasma* spp. vertical transmission, embryonic mortality

RESUMO

Quarenta e nove ovos embrionados de Psittaciformes com embriões que morreram durante a incubação foram examinados, provenientes de criatório comercial de aves de espécies exóticas ou nativas, ou provenientes de instituição de conservação de espécies da avifauna. Os ovos foram classificados, de acordo com o momento da morte, em mortalidade precoce, intermediária ou tardia. Conforme a idade embrionária, embriões inteiros ou tecidos embrionários foram coletados para extração de DNA e cultivo bacteriológico em ágar contendo acetato de tálio, soro equino e penicilina. Entre os embriões de espécies exóticas, 37,5% (12/32) foram detectados positivos para Mycoplasma *spp. Considerando os embriões das espécies nativas, 52,4% foram detectados positivos (11/21). O DNA de* Mycoplasma *spp. foi detectado em um filhote de* Pionus maximiliani *morto na eclosão. Testes adicionais dos embriões e das colônias por PCR, com protocolo específico para* M. gallisepticum, *não revelaram nenhum resultado positivo. As implicações da presença de* Mycoplasma *na viabilidade embrionária e de filhotes de espécies de aves comerciais ou de conservação são discutidas.*

Palavras-chave: Psittaciformes, *Mycoplasma* spp. transmissão vertical, mortalidade embrionária

REFERENCES

AVELAR, A.R.M.; SIMPSON, J.G. Rearing the extinct in the wild Alagoas curassow Pauxi mitu for future reintroduction programmes. *Int. Zoo Yearbook*, v.48, p.29-38, 2014.

AVIAN mycoplasmosis (Mycoplasma gallisepticum, Mycoplasma synoviae) In: *Manual of diagnostic tests and vaccines for terrestrial animals*. Paris: OIE, 2008. p.482-486. Available in: <http://www.oie.int/fileadmin/ Home/eng/Health_standards/tahm/2.03.05_%20 AVIAN_MYCO .pdf>. Accessed in: 20 Aug. 2019.

BENČINA, D.; NARAT, M.; BIDOVEC, A.; ZORMAN-ROJS, O. Transfer of maternal immunoglobulins and antibodies to Mycoplasma gallisepticum and Mycoplasma synoviae to the allantoic and amniotic fluid of chicken embryos. *Avian Pathol.*, v,34, p.463-472, 2005.

BENČINA, D.; DORRER, D.; TADINA T. Mycoplasma species isolated from six avian species. *Avian Pathol.*, v.16, p.653-664, 1987.

BENČINA, D.; TADINA, T.; DORRER, D. Natural infection of geese with Mycoplasma gallisepticum and Mycoplasma synoviae and egg transmission of the mycoplasmas. *Avian Pathol.*, v.17, p.925-928, 1988.

BOOM, R.; SOL, C.J.A.; SALIMANS M.M. *et al*. Rapid and simple method for purification of nucleic acids. *J. Clinic. Microbiol.*, v.28, p.495-503, 1990.

BRADBURY, J.M.; VUILLAUME, A.; DUPIELLET, J.P. *et al*. Isolation of Mycoplasma cloacale from a number of different avian hosts in Great Britain and France. *Avian Pathol.*, v.16, p.183-186, 1987.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Manual de legislação*: programas nacionais de saúde animal do Brasil. Brasília-DF: MAPA, 2009. 440p.

GOLDBERG, D.R.; SAMUEL, M.D.; THOMAS, C.B. *et al.* The occurrence of mycoplasmas in selected wild North American waterfowl. *J. Wildlife Dis.*, v.31, p.364-371, 1995.

GOMES, A.M.; COSTA, L.L.; VILELA, D.A.R. *et al*. Detection of Mycoplasma gallisepticum in dead captive psittacines in Belo Horizonte, Brazil. *Braz. J. Poult. Sci.*, v.12, p.75-78, 2010.

GOMES, A.M.; ORTIZ, M.C.; CARVALHAES, A.G.; MARTINS, N.R.S. Mycoplasma gallisepticum e M. synoviae em papagaio verdadeiro (Amazona aestiva) com doença respiratória - relato de caso. *Rev. Clinic. Vet.*, v.98, p.104-108, 2012.

GONZÁLEZ-ASTUDILLO, V.; HERNANDEZ, S.M.; YABSLEY, M.J. *et al*. Mortality of selected avian orders submitted to a wildlife diagnostic laboratory (Southeastern Cooperative Wildlife Disease Study, USA): a 36-year retrospective analysis. *J. Wildl. Dis*., v.52, p.441-458, 2016.

HARASAWA, R.; UEMORI, T.; ASADA, K.; KATO, I. Sensitive detection of Mycoplasmas in cell cultures by using two-step polymerase chain reaction. Rapid diagnosis of mycoplasmas. *Fed. Eur. Microbiol. Soc. Symp. Series*, v.62, p.227-232, 1993.

KLEVEN, S.H. Mycoplasmosis. In: SAIF, Y.M.; FADLY, A.M.; GLISSON, J.R. (Eds.). *Diseases of poultry*. 12.ed. Ames: Blackwell Publishing Professional, 2008. p.845-856.

LEY, D.H.; BERKHOFF, J.E.; MCLAREN, J.M. *Mycoplasma gallisepticum* isolated from house finches (*Carpodacus mexicanus*) with conjunctivitis. *Avian Dis.*, v.40, p.480-483, 1996.

LEY, D.H.; YODER, H.W. Mycoplasma gallisepticum infection. In: CALNEK, B.W.; BARNES, H.J.; BEARD, C.W.; MCDOUGALD, L.R.; SAIF, Y.M (Eds.). *Diseases of poultry*. 10.ed. Ames: Iowa State University Press, 1997. p.194-207.

LIERZ, M.; HAFEZ; H.M. Mycoplasma species in psittacine birds with respiratory disease. *Vet. Rec.*, v.164, p.629-630, 2009.

LIERZ, M.; HAGEN, N.; LUESCHOW, D.; HAFEZ, H.M. Use of polymerase chain reactions to detect Mycoplasma gallisepticum, Mycoplasma imitans, Mycoplasma iowae, Mycoplasma meleagridis and Mycoplasma synoviae in birds of prey. *Avian Pathol.*, v.37, p.471-476, 2008a.

LIERZ, M.; OBON, E.; SCHINK, B.; CARBONELL, F.; HAFEZ, H.M. The role of mycoplasmas in a conservation project of the lesser kestrel (Falco naumanni). *Avian Dis.*, v.52, p.641-645, 2008b.

LOPES, A.R.S. *Avaliação de técnicas de manejo na sobrevivência e comportamento de papagaios-verdadeiros (Amazona aestiva, Psittacidae) translocados*. 2016. 126f. Dissertation (Mestrado em Ecologia de Biomas Tropicais) - Universidade Federal de Ouro Preto, MG.

LUTTRELL, P.; FISCHER, J.R. Mycoplasmosis. In: THOMAS, N.J.; HUNTER, D.B.; ATKINSON, C.T. *Infectious diseases of wild birds*. Iowa: Blackell, 2007. p.317-331.

SILVA, L.T.R.; SANTOS, S.B.; RAMEH-DE-ALBUQUERQUE, L.C. *et al.* Molecular detection and isolation of Mycoplasma spp. in psittacines in Pernambuco state, Brazil. Detecção molecular e isolamento de Mycoplasma spp. em psitacídeos no estado de Pernambuco, Brasil. *Arq. Bras. Med. Vet. Zootec*., v.68, p.113-118, 2016.

SILVEIRA, LF. A gaiola que salva. Extinto na natureza, o mutum-de-alagoas escapa do desaparecimento total graças a um projeto de criação da ave em cativeiro. *Ornitologia*, n.251, 2017. Available in: http://revistapesquisa.fapesp.br/2017/01/09/a-gaiola-que-salva/. Accessed in: 20 June 2022.

SMITH, T.W. Care and incubation of hatching eggs. 2004. Mississippi State University Extension Service. The Poultry Site. Available in: https://thepoultrysite.com/articles/care-and-incubation-of-hatching-eggs. Accessed in: 8 May 2019.