Chlamydia psittaci in captive blue-and-gold macaws (Ara ararauna) in a triage center of wild animals in Brazil*

Chlamydia psittaci em araras-canindé (Ara ararauna) cativas em um Centro de Triagem de Animais Silvestres no Brasil

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Abstract

Chlamydia psittaci infection stands out due to its zoonotic potential. It was detected in several orders, being Psittaciformes its main reservoir. Our objective was to investigate the occurrence of *C. psittaci* infection by clinical and environmental examination, as well as agent detection by Polymerase Chain Reaction (PCR) from choanal and cloacal swabs in 46 blue-and-gold macaws (*Ara ararauna*) allocated at the Triage Center of Wild Animals, Brazilian Institute of Environment and Renewable Natural Resources, Rio de Janeiro state, Brazil. The frequency of positive detection of the bacteria found was 50% (23/46) by cloacal swab. In this percentual, 26.09% (12/23) were also positive by choanal swab, being the probability of detection 2.83 times higher for cloacal swabs when compared to choanal ones ($p<0.05$). There was no association between body condition, temperature variations and PCR positivity for *C. psittaci*, being 34.78% of positive individuals asymptomatic to this disease. Therefore, our findings show agent's dispersion at the squad and high frequency of asymptomatic birds. Considering the possibility of contagion to workers and free living birds, the quarantine in wildlife conservation and support centers, as well as the care with hygiene and individual protection for humans who deal with the animals, should be strictly followed even in the absence of clinical signs.

Keywords: Chlamydiosis, birds, infection.

Resumo

Infecções por *Chlamydia psittaci* destacam-se devido ao seu potencial zoonótico. Tal agente foi detectado em diversas ordens, sendo Psittaciformes seu principal reservatório. Objetivando-se demonstrar a ocorrência de tal processo infeccioso, procedeu-se investigação clínica e ambiental, associada à detecção do agente etiológico por Reação em Cadeia da Polimerase (PCR), a partir de *swabs* de coana e cloaca de 46 araras-canindés (*Ara ararauna*) pertencentes ao Centro de Triagem de Animais Silvestres do Instituto Brasileiro de Meio Ambiente e Recursos Renováveis, Rio de Janeiro, Brasil. Obteve-se uma frequência de detecção de 50% (23/46) a partir de *swabs* de cloaca. Entre esses, 26,09% (12/23) foram também positivos por meio de *swab* de coana, sendo a probabilidade de detecção 2,83 vezes maior a partir de *swabs* de cloaca que de coana ($p<0.05$). Não houve associação entre condição corporal, variação de temperatura e detecção do agente por PCR, sendo 34,78% dos indivíduos positivos assintomáticos. Portanto, nossos resultados demonstram a dispersão do agente no plantel e alta frequência de assintomáticos. Devido à possibilidade de contágio para trabalhadores e aves de vida livre, a quarentena nos centros de suporte e conservação da vida silvestre, assim como os cuidados com higiene e proteção individual para com os trabalhadores que lidam com os animais devem ser seguidas rigidamente, mesmo na ausência de sinais ou sintomas nas aves.

Palavras-chave: clamidiose, aves, infecção.

Introduction

Chlamydia psittaci is a highly contagious zoonotic pathogen (Gerlach, 1994). It has been considered to be the main causative agent of chlamydiosis in birds, although there are two new *Chlamydia* species recently detected, *C. gallinacea* in breeding birds and *C. avium* in wild birds (Guo et al., 2016; Szymańska-Czerwińska and Niemczuk, 2016; Sachse et al., 2014).

This bacterium can cause systemic disease clinically evident in many mammals, reptiles, amphibians and birds. In the Avian class, *C. psittaci* was diagnosed by agent detection or serology,

in at least 469 species of free-living or company birds (Kaleta e Taday, 2003). In these animals, the agent produces a systemic infection, which can vary according to bacterial strain and host susceptibility, which also include the possibility of asymptomatic infection (Sareyyupoglu et al., 2007).

Admittedly, Psittacidae birds are the largest reservoir of *C. psittaci* (Harkinezhad et al., 2009) and the close contact between humans and representatives of the Psittacidae family (Sigrist, 2006), particularly as pets, justifies the study of *C. psittaci* in these birds. Besides its public health importance (Harkinezhad et al., 2009), it also plays a role in ecosystem health, since

there is the risk of environmental contamination when occurs the release of individuals held temporary in captivity (ICMBIO, 2004). Therefore, chlamidiosis monitoring by agent detection or serology is mandatory in cases of bird release or exchange of ones (IBAMA, 2008).

Among Psittacidae birds, the blue-and-gold-macaw (*Ara ararauna* Linnaeus, 1758) has been heavily commercialized (Birdlife International, 2012). In Brazil, when there is an illegal sale intercepting of these animals, they are sent to the Triage Centers of Wild Animals (IBAMA, 2012**)**. This procedure plays a role in screening and controlling of chlamidiosis, as overcrowding at institutions that receive them contributes to symptomatic appearance (Efe et al., 2006). The stress due to overpopulation in captivity predisposes to infection by *C. psittaci* and can contribute to the activation of latent infections and their spread in the squad (Raso et al., 2004).

Therefore, this work aimed to investigate the occurrence of *C. psittaci* in 46 macaws (*Ara ararauna*) allocated in the *Centro de Triagem de Animais Silvestres - CETAS* (Triage Center of Wild Animals), Rio de Janeiro state, Brazil, belonging to *Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis - IBAMA* (Brazilian Institute of Environment and Renewable Natural Resources).

Materiais and methods

We investigated 46 blue-and-gold macaws, *Ara ararauna* species (Birds: Psittaciformes), housed in five collective places (Figure 1) in CETAS / RJ (IBAMA), Seropédica city, Rio de Janeiro, Brazil, from July to October of 2011.

By physical contention, we accessed the following parameters: body condition (score), body temperature and specific inspection of natural cavities. The ophthalmic, nasal, hearing, oral and cloacal regions were examined. The body scores ranged from 01 to 05, corresponding to "cachectic", "thin", "normal", "overweight" and "obese", respectively, according to pectoral musculature evaluation. The temperature of the birds was measured using a digital thermometer with gold sensor (Geratherm◊), detection range from 32.0°C to 42.9°C, resolution of 0.1°C and a maximum error of + or – 0.2°C, according to the manufacturer.

The macroscopic features of faeces and urine aspects were evaluated through their visualization on brown paper previously placed on the floor under the roosts of enclosures.

Choanal and cloacal biological samples were performed by the use of swabs during oral and cloacal cavities inspection. Ninety-two samples were collected from adult birds independently of sex, being 46 choanal and 46 cloacal swabs. The swabs were stored at 4°C in glass vials containing 3 mL of absolute ethanol, until processing (Raso et al., 2006).

DNA extraction followed a previous protocol (Ishizawa et al., 1991) with modifications. Swabs in absolute ethanol were transferred to 15 mL plastic tubes and submitted to homogenization by vortex for 2 minutes. Subsequently, the ethanol with biologic material in suspension were transferred to 2 mL microtubes and centrifuged at 20000 x g (15000 rpm) for 30 minutes at 4°C. Then, with a precision pipette, the supernatant was discarded leaving 100 μL of ethanol, with the precipitate. In this content, 10 μg of glycogen (20 μg/mL, Invitrogen) and 300 μL of a solution containing 6M NaI, 13 mM EDTA, 0,5% N-lauroylsarcosina-sodium, pH

Figure 1: Collective squad of Ara ararauna (Aves: Psittaciformes) at Triage Center of Wild Animals of the Rio de Janeiro state, Brazilian Institute of Environment and Renewable Natural Resources, RJ, Brazil, 2011.

8.0, 26 mM Tris-HCl, were added. After homogenization, the microtubes were incubated in a water bath at   60°C for 15 minutes. It was added 400 μL of absolute isopropanol at -20°C with homogenization in a vortex, leaving the material resting for 15 minutes. After that, centrifugation was performed at 10000 x g (12000 rpm) for 5 minutes with subsequent discarding of supernatant. Addition of 1 mL of 40% isopropanol at the same temperature as the absolute one was done, followed by vortex shaking. It was centrifuged again at 10000 x g for 5 minutes, discarding the supernatant and, then, the microtube was left open to dry overnight in laminar flow hood. On the following day, the DNA was diluted in 50 μL of Milli-Q water and was taken to the water bath at 37°C for 1 hour to dissolve the pellet. Every 15 minutes the microtube was shaken for homogenization of its contents, returning after this to incubation. After dilution, the material was stored at -20°C until PCR analysis.

As positive control in this procedure, *C. psittaci* DNA was extracted from the quadruple feline vaccine (Felis QUANTUM®4 Intervet Schering-Plough) using the same extraction procedure described.

For PCR, it was followed previous technique (Sareyyupoglu et al., 2007), Laroucau et al., 2001). The primer pair used was CpsiA, 5'- ATGAAACATCCAGTCTACTGG-3' and CpsiB, 5'-TTGTGTAGTAATATTATCAAA-3', amplifying a fragment of

300 base pairs (bp) (Sareyyupoglu et al., 2007). The concentration of each component of PCR consisted of 1X PCR buffer, 3 mM MgCl$_2$, 200 μM of deoxynucleoside triphosphate (dNTPs), 1U *Taq* DNA Polymerase, 0.2% dimethyl sulfoxide (DMSO) and 2 μM of each primer plus 10 μL of the diluted DNA, resulting in a total volume of 50 μL, supplemented with Milli-Q water or ultra pure. For all reactions, it was included a negative control with ultra-pure water instead of clinical specimen.

The amplification reaction was performed in a MG96 + model thermocycler (Long gene®ScientificInstrumentsCo. Ltd.) using the following thermal cycles: an initial denaturation at 94°C for 2 minutes followed by 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 65°C for 2 minutes, with a final extension at 65°C for 10 minutes (Sareyyupoglu et al., 2007).

The PCR products were visualized on 6% polyacrylamide gel stained with silver nitrate (AgNO$_3$) solution after electrophoresis in 1x TBE 100V, with 18W and 49 MA for 1 hour.

For the statistical analysis, the study aimed the probabilistic frequency response, by conducting a survey, with the generation of the agent's frequency in the group evaluated. Data were analyzed by simple linear regression, multiple logistic regression, Fisher's Exact Test and Risk Analysis.

Results and discussion

### Frequency of C. psittaci in the macaws evaluated

From the tested birds, 50% (23/46) were PCR positive for *Chlamydia psittaci* DNA by cloacal swab. Among these cloacal positive birds, 26.09% (12/23) were also positive by choanal swab (Table 1).

Table 1: Polymerase Chain Reaction (PCR) from cloacal and choanal swabs in *Ara ararauna* (Aves: Psittaciformes), at the Triage Center of Wild Animals/ Rio de Janeiro / Brazilian Institute of Environment and Renewable Natural Resources, Brazil, from July to October 2011

PCR Results	Site of material collection for PCR	
	cloacal swab	choanal swab
Positive	23 (50%)	12 (26.09%)
Negative	23 (50%)	34 (73.91%)
Total	46 (100%)	46 (100%)

Fisher's Exact Test: p<0.05
OD = 2.833; IC (1.180 to 6.804)

The assessment in relation to sample collecting sites yielded significant difference (p<0.05) by Fisher's Exact Test. The probability of bacterium DNA detecting by cloacal swab was 2.83 times greater than a choanal swab under PCR: Odds Ratio = 2.83, CI (1.180 to 6.804). Positive DNA bands are presented in Figure 2. Also in this figure, some nonspecific bands can be visualized at cloacal swabs samples.
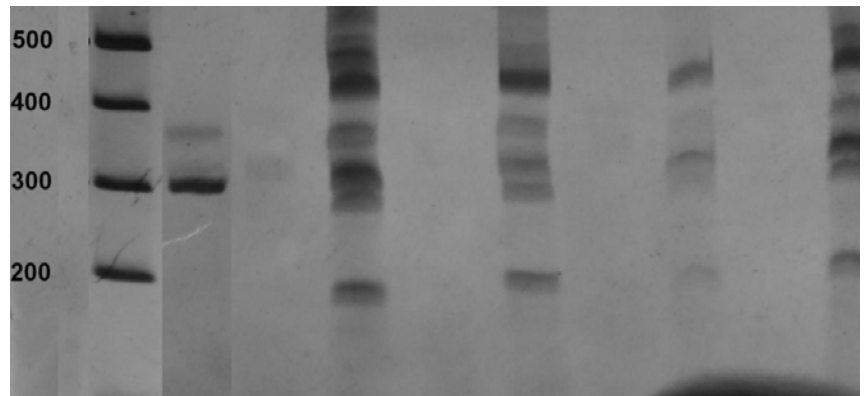
Figure 2: Positive bands of a DNA fragment of 300 bp of *Chlamydia psittaci* amplified by Polymerase Chain Reaction on 6% acrylamide gel stained using AgNO$_3$ solution. From left to right: 100 bp DNA ladder, positive control (300 bp) and negative results from coanal swabs intercalated with DNA bands from cloacal ones of blue-and-gold macaws (*Ara ararauna*; Aves: Psittaciformes) from Triage Center of Wild Animals of the Rio de Janeiro state, Brazilian Institute of Environment and Renewable Natural Resources, RJ, 2011. In the end, the negative control. Some nonspecific bands are also visualized at cloacal swabs samples.

The higher frequency of *C. psittaci* in cloacal swabs compared to choanal ones suggests longer time post exposure to the bacteria, once initial infections tend to stay primarily in the respiratory tract, being subsequently eliminated in feces (Andersen, 1996). These results corroborate to previous report (Santos et al., 2014; Raso et al., 2006) where *C. psittaci* semi-nested PCR detection in psittacine birds was more frequently in cloaca samples than oropharynx and tracheal ones. However, in another study (Andersen, 1996), it was found greater isolation rate of *C. psittaci* in cockatiels (*Nymphicus hollandicus*) from pharyngeal swabs (80.4%), while fecal and cloacal resulted in positivity of 45.1% and 37.3%, respectively.

It should be highlighted that as the agent has intermittent fecal shedding (Harkinezhad et al., 2009), the detection of bacterial DNA is naturally influenced by the post-exposure time and therefore negative individuals can also harbor the bacterium . Since *Ara ararauna* individuals were kept in collective places, it was expected that all birds had already been exposed to *C. psittaci*, once the transmission takes place by proximity, mainly by inhalation and sometimes by ingestion of contaminated material (Harkinezhad et al., 2009).

The prevalence of infection varies among free-living and captive birds, being higher when many birds are confined in small areas or when there are frequent changes in the flock (Sareyyupoglu et al., 2007). These features corroborate to the high positivity found in this study, since at the CETAS/IBAMA/RJ birds were allocated in collective places with turnover and constant arrival of new individuals, besides the proximity to free-living birds, with enclosures protected only by screens in their sides. Similar situation of collective infection it was reported in an outbreak of chlamydiosis in Psittacidae birds at CETAS / IBAMA/MG, where the birds were kept in collective aviaries with frequent arrival of rescued animals and proximity of pigeons and free-living birds (Eco et al., 2009).

Findings to the General Clinical Evaluation

Abnormal feathering was found in 13.04% (6/46) of the examined macaws, through visualization of areas with loss of feathers, and two of them were positives to *C. psittaci*. Body scores found ranged from 01 to 03, being 2.17% (1/46) the percentage

for cachectic, 47.83% (22/46) for thin and 50.00% (23/46) for normal. In fact, in birds, *C. psittaci* produces a systemic infection (Sareyyupoglu et al., 2007) and can show changes in plumage and weight loss (Gerlach, 1994). The plumage changes may have different etiologies: bacterial, viral, parasitic or behavioral (Cubas e Godoy, 2006). Despite the two *C. psittaci* positive macaws with plumage changes in this study, the multifactorial etiology of these findings do not allow to affirm their association with *C. psittaci* infection. In relation to the body condition, from the 23 *C. psittaci* positive macaws, 10 were regarded as thin, body score 02. However, 12 negative macaws also had score 02, which opens the possibility of coexistence of other factors acting together toward weight loss in birds. Therefore, the slimming of positive macaws may not be definitely associated to *C. psittaci*. The finding that 13 positive macaws had score 03 (normal), and the fact of many Psittacidae birds may not show clinical signs when chronically infected, corroborates this assertion (Harkinezhad et al., 2009).

The body temperature during contention ranged from 40 to 42.4°C, with an average of 41.40°C ± 0.62°C. The PCR positive individuals had an average of 41.39°C ± 0.58°C, and for negative ones, 41.41°C ± 0.67°C. Therefore, there was no suggestion of hyperthermia (Rodolakis e Mohamad, 2010), since in most birds the cloacal normal temperature is around 41.7 to 44.4°C (Greenacre e Lusby, 2010). Regarding the possibility of hypothermia (Gerlach, 1994), there were no substantial differences between the lower temperatures of positive and negative birds, with a difference of only 0.1°C between the lowest temperature recorded in the first group compared to the lowest in the second one. However, members of the Psittacidae family, when manually contained may raise its cloacal temperature in 2.3°C during the first 15 minutes of manipulation which yields significant difference in the first four minutes of containment (Greenacre e Lusby, 2010). So it would be expected higher temperatures in relation to those found in individuals whose measurements were below 41ºC.

It was observed an association between temperature (Y) and body score (X) risk factors (p <0.05) by simple linear regression: $Y = 40.86 + 0.22 (X)$, where the coefficient of determination (R^2) was 3.77%. The temperature worked as predictor, explaining 3.77% of body score variations. So as higher the temperature, higher is the score of the bird. However, there was no association among PCR positivity (presence of DNA bacterial in cloacal or choanal swab) and body score plus temperature (p> 0.05) by multiple logistic regression analysis.

No birds had clinical changes in nasal, oral or hearing cavities. The majority of macaws, 97.83% (45/46), had no change in clinical ophthalmic cavity. One individual presented a corneal scarring process, indicative of old injury, possibly not related to their current clinical status neither a Chlamydia infection. No one had notable changes in cloacal cavity, however, during the collection of the cloacal swab it was observed greenish-yellow cloacal content in three individuals; greenish-yellow cloacal mucous content in an individual and green one in another. Similar, macroscopic evaluation of faeces and urine for the group detected changes suggestive of chlamydiosis: green faeces only or green, watery and pasty faeces and increased amount of urate. All the changes found in faeces and urine had been described in the literature (Gerlach, 1994), as well as those evidenced in cloacal swabs, and *C. psittaci* DNA was detected in four out of five birds with changes showed in swabs.

In general, 34.78% (8/23) of *C. psittaci* positive macaws yielded no significant clinical changes. In fact, Psittacidae birds commonly exhibit a chronic form of the disease (Harkinezhad et al., 2009). In this context, Raso et al. 2002 and Grespan 2009 also showed asymptomatic infections; and it has been reported death without previous clinical signs in positive psittacines (Eco et al., 2009).

It should be noted that, since Psittacidae birds are the largest reservoir of *C. psittaci*, particularly under captive conditions (Harkinezhad et al., 2009), and being Brazil the richest country in these birds (Sick, 1997), it is expected higher prevalence of this disease in this country. Therefore, comprehensive studies are needed in relation to pet birds, wild populations and environmental contamination that they generate.

Ultimately, it becomes necessary to carry out further studies in favor of getting the real situation of the pathogen spread in Brazil, in order to assess what should be the best solutions and the optimal interventions to be performed. It must be emphasized the need of research on humans, with performance of differential diagnoses for respiratory and neurological diseases, as well as the need of study performance about the zoonotic transmission from pet and free-living birds, especially with those have been adapted to urban centers.

Conclusion

In this study, PCR was efficient in detecting *Chlamydia psittaci* in captive macaws (*Ara ararauna*), yielding a positivity of 50% in a premises of 46 macaws at CETAS / IBAMA, RJ. Detection from cloacal swabs was more indicated than choanal ones, with a probability of detection 2.83 times higher for cloacal site.

There was no relationship between body condition score, temperature variations and PCR positivity to *C. psittaci*. PCR positivity to *C. psittaci* was found also in asymptomatic macaws. Therefore, the quarantine in wildlife conservation and support centers, also the care with hygiene and individual protection for humans who deal with the animals, should be strictly followed even in the absence of clinical signs.

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