Chlamydia psittaci in free-living Blue-fronted Amazon parrots (Amazona aestiva) and Hyacinth macaws (Anodorhynchus hyacinthinus) in the Pantanal of Mato Grosso do Sul, Brazil

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Abstract

Chlamydia psittaci (C. psittaci) infection was evaluated in 77 free-living nestlings of Blue-fronted Amazon parrots (Amazona aestiva) and Hyacinth macaws (Anodorhynchus hyacinthinus) in the Pantanal of Mato Grosso do Sul, Brazil. Tracheal and cloacal swab samples from 32 wild parrot and 45 macaw nestlings were submitted to semi-nested PCR, while serum samples were submitted to complement fixation test (CFT). Although all 32 Amazon parrot serum samples were negative by CFT, cloacal swabs from two birds were positive for Chlamydia DNA by semi-nested PCR (6.3%); these positive birds were 32 and 45 days old. In macaws, tracheal and cloacal swabs were positive in 8.9% and 26.7% of the samples, respectively. Complement-fixing antibodies were detected in 4.8% of the macaw nestlings; macaw nestlings with positive findings were between 33 and 88 days old. These results indicate widespread dissemination of this pathogen in the two evaluated psittacine populations. No birds had clinical signs suggestive of chlamydiosis. To the best of our knowledge, this is the first report on C. psittaci in free-living Blue-fronted Amazon parrots and Hyacinth macaws in Brazil.

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1. Introduction

Chlamydia is one of the main infectious diseases in psittacine birds. Clinical signs include anorexia,
dyspnea, dehydration, diarrhea with yellowish-green urates, weight loss, conjunctivitis, rhinitis, and sinusitis (Gerlach, 1994). The severity of the disease depends on strain virulence, host factors, degree of exposure, stress and environmental factors. Many affected birds become chronically infected but show no clinical signs until stressed. Most carriers of *Chlamydophila psittaci* (*C. psittaci*) may shed the organism intermittently and represent a significant source of infection for humans and other birds (Fudge, 1996).

Seventy-two species of the Order Psittaciformes are found in Brazil, 17 of them are considered at risk (Galetti et al., 2002). The Hyacinth macaw (*Anodorhynchus hyacinthinus*) is the largest of all psittacine birds. Studies of Hyacinths estimated only three distinct populations: Pará and Amazonas (Brazil); Tocantins, Piauí, Maranhão and Bahia (Brazil), and the Pantanal wetland (including Brazil, Bolivia, and Paraguay). The largest of these three populations is located in the Pantanal (Munn et al., 1990; Guedes and Harper, 1995). The Blue-fronted Amazon (*Amazona aestiva*) is one of the world’s most popular species in captivity and has a relatively wide distribution (northwest Brazil, eastern Bolivia, northern Argentina, and southern Paraguay) (Seixas and Mourão, 2002). However, the progressive decrease of the natural habitat and the illegal exploration of these birds for the pet trade, both within Brazil and internationally, are factors that might have caused a decrease in the size of the natural population in the last years (Beissinger and Bucher, 1992). In the Pantanal, modifications in the habitat are largely due to the planting of pasture for cattle, while in other areas the habitat destruction is driven by population pressure and the colonization and clearing of land (Guedes and Harper, 1995).

Such facts have been contributing to the increase of research in conservation and management programs for those species in wild life (Guedes and Harper, 1995; Seixas and Mourão, 2003). Nevertheless, little is known about the health status of these animals in their natural habitat in Brazil. Several reports reveal that the Order Psittaciformes contains by far the most *Chlamydia*-positive bird species, 153 out of 342 (45%) (Kaleta and Taday, 2003). In Brazil, studies showed that 16–56% of apparently healthy captive parrots were positive for *C. psittaci* (Raso et al., 2002) and an outbreak with high mortality was reported in Amazon parrots (Raso et al., 2004). Although free-living psittacine birds are recognized as important reservoirs of this microorganism in nature (Brand, 1989), there are no previous studies reporting the occurrence of this agent in Brazilian wild psittacine birds. In the present study, *C. psittaci* was assessed by semi-nested polymerase chain reaction (semi-nested PCR) and by complement fixation test (CFT) in free-living nestlings of Blue-fronted Amazon parrots (*A. aestiva*) and Hyacinth macaws (*A. hyacinthinus*) in the Pantanal of Mato Grosso do Sul, Brazil.

### 2. Materials and methods

#### 2.1. Field survey and sampling

A survey was conducted in the sub-regions Miranda and Aquidauana (19°51′–19°58′S and 56°17′–56°24′W) at the Pantanal (Mato Grosso do Sul, Brazil). The Pantanal is a large sedimentary basin of approximately 139,000 km², situated within the drainage area of the upper Paraguay River Basin. The study area consists of a mosaic of floodplains, grasslands, savannas, scrub savannas, arboreal savannas, forests of riparian vegetation, and man-made pastures. Annual precipitations averages 1207 mm and the dry and wet seasons are well defined, with most rain between December and February (Seixas and Mourão, 2002). Free-living nestlings of Blue-fronted Amazon parrots (*A. aestiva*) and Hyacinth macaws (*A. hyacinthinus*) were evaluated for the presence of *C. psittaci* during the reproductive season (October–December) for two consecutive years.

The trees presenting nests were accessed by climbing equipments and ladders, marked with numbered metal plates and geographical coordinates. The birds were taken from the nest, carefully examined and sampled, and immediately returned to the nest. Birds’ age were estimated by Richards asymptotic linear model (Seixas and Mourão, 2003). In the Pantanal, parrots and macaws incubate their eggs for a period of 28 up to 30 days. Usually, Amazon parrots nestlings stay in their nests for 60 days, while nestlings of macaws do so for 107 days.

Tracheal and cloacal swab samples (*n* = 32 each) and blood samples (*n* = 30) were collected from 32 parrot nestlings in 21 nests. With regard to Hyacinth
macaws, tracheal and cloacal swab samples \((n = 45)\) and blood samples \((n = 42)\) were collected from 45 nestlings in 35 nests. No dead nestlings or adult birds were found, except for remains of carcasses left by predators in two nests.

### 2.2. Chlamydial DNA detection by semi-nested PCR

For *C. psittaci* DNA analysis by semi-nested PCR, tracheal and cloacal swabs were collected, placed into 1 mL of ethanol (100%) and stored at 4°C until analysis. Swab samples were vortexed for 2 min and then centrifuged at 20,000 \(\times\) g for 30 min at 4°C. The pellet was resuspended into 40 μL of buffer (0.1 M NaCl, 10 mM TRIS, 1 mM EDTA; 5% Triton \(\times 100\)) and 9 U proteinase K (Invitrogen, USA), incubated at 56°C for 90 min and then centrifuged at 2000 \(\times\) g for 2 min. DNA extraction was performed from supernatant using GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, USA), according to manufacturer’s instructions.

Primers flanking the conserved MOMP gene from Chlamydiaceae (Buxton et al., 1996) were used for PCR reaction. Primers A (5’CAGGATATCTTGCTTGGCTTTAA3’) and B (5’GCAAGGATCGCAAGGATC3’) produced a 260-bp fragment in the PCR, whereas primers B (5’GCAAGGATCGCAAGGATC3’) and C (5’TAGAGGTGAGTATGAAAAA-AACTC3’) amplified a 165-bp fragment in the semi-nested PCR. Amplification reactions contained 5 μL of the DNA template, 1 \(\times\) enzyme reaction buffer (Biotools, Spain), 0.2 mM of dNTPs (Amersham Pharmacia Biotech, USA), 0.2 μM of each primer (Life Technologies, Brazil), 1.25 U of DNA polymerase (Biotools, Spain) and sterile water to a final volume of 25 μL. PCR cycling conditions were 10 min at 94°C; 34 cycles at 94°C for 1 min, 54°C for 1 min and 72°C for 1 min; and a final extension at 72°C for 4 min. The semi-nested PCR reaction was similar, except that 2 μL of the amplified product was added and annealing was performed at 52°C for 1 min. Positive and negative control samples were included in each run. PCR and semi-nested PCR products were analyzed by electrophoresis in 1.5% agarose gels stained with ethidium bromide (0.5 μg mL\(^{-1}\)) and visualized under ultraviolet light.

### 2.3. Production and partial purification of chlamydial elementary bodies (EB)

*C. psittaci* strain S23/3 (kindly provided by Institute Zooprofilattico Sperimentale delle Venezie, Italy) was inoculated in the yolk sac of 7–8-day-old embrionated Specific Pathogen Free (SPF) eggs. The yolks were harvested and tested for presence of the agent by Gimenez staining (Andersen, 1998) and direct immunofluorescence test (IMAGEN Chlamydia Test, Dako Diagnostics Ltd., UK). Partial purification of the elementary bodies (EB) was carried out according to Perez-Martinez and Storz (1985) with modifications. Briefly, positive yolks were homogenized using sterile sand, resuspended in 1 mL sterile PBS (pH 7.4) and centrifuged at 900 \(\times\) g for 10 min at 4°C. The intermediate phase was transferred to a fresh tube, and an equal volume of PBS was added. The solution was inactivated at 60°C for 1 h and then centrifuged at 15,000 \(\times\) g for 60 min at 4°C. The pellet was resuspended in PBS and centrifuged at 40,000 \(\times\) g for 90 min at 4°C with a 35% (v/v) solution of contrast medium meglumine amidotrizoate urographin (Urografina 370, Schering AG, Germany). Elementary bodies were washed in PBS (40,000 \(\times\) g) for 60 min at 4°C, resuspended in 1 mL of sterile PBS pH 7.4 containing 0.1% sodium azide and stored at −70°C. The partially purified antigen was used in the complement fixation test (CFT) as described below.

### 2.4. Complement fixation test (CFT)

Complement fixation tests were carried out according to the method described by Bier et al. (1968), except that the tests were adapted to microplates (Raso et al., 2004). The optimal amount of chlamydial antigen was determined by checkerboard titration with a positive avian serum. Test sera were inactivated at 56°C for 30 min prior to the tests and diluted two-fold (1:8 to 1:1024) in triethanolamine buffer pH 7.4. Briefly, 25 μL of test sera and 25 μL of chlamydial antigen, followed by 50 μL of complement (50% hemolytic end point-two of complement) were added to each well and incubated overnight at 4°C. Subsequently, 25 μL of sensitized sheep red blood cells were added to each well and the plates were incubated for 30 min at 37°C. Controls for serum and antigen were included in every run as well
as for the complement and the hemolytic system. Finally, plates were centrifuged at 800 × g for 5 min and the degree of lysis was visually assessed. Samples showing more than 50% lysis at serum dilutions of 1:16 or higher in the presence of 2 U of complement were considered positive (titer ≥16).

3. Results

3.1. Blue-fronted Amazon parrot population

Sampled parrot nestlings were between 29 and 64 days old (Fig. 1) and did not show evidence of clinical disease. All 30 serum samples of parrot nestlings were negative by CFT, whereas only two cloacal samples were positive by semi-nested PCR (Table 1). The two positive parrots were 32 and 45 days old.

3.2. Hyacinth macaw population

Sampled macaw nestlings were between 21 and 96 days old (Fig. 2). Positive samples were detected in 8.9% of tracheal samples and 26.7% of cloacal samples taken from macaw nestlings (Table 1). Positive serum samples (4.8%) showed titers of 16. One of the positive nestlings by CFT was also positive by semi-nested PCR (tracheal swab), while the other showed negative results in all swabs samples (Table 2). No serum sample was collected from three nestlings; from these, one nestling showed negative results in all swab samples and two were positive (tracheal swabs) by semi-nested PCR (Table 2). Overall, 37.8% (17/45) macaws were positive by C. psittaci (semi-nested PCR and/or CFT), with age varying from 33 to 88 days (Fig. 3). Macaw nestlings

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**Table 1**

<table>
<thead>
<tr>
<th>Method</th>
<th>Amazon parrots</th>
<th>Hyacinth macaws</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tracheal semi-nested PCR</td>
<td>0/32 (0%)</td>
<td>4/45 (8.9%)</td>
</tr>
<tr>
<td>Cloacal semi-nested PCR</td>
<td>2/32 (6.3%)</td>
<td>12/45 (26.7%)</td>
</tr>
<tr>
<td>CFT</td>
<td>0/30 (0%)</td>
<td>2/42 (4.8%)</td>
</tr>
</tbody>
</table>

* Number of positive samples/number of tested samples (%).

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**Table 2**

<table>
<thead>
<tr>
<th>Method</th>
<th>CFT+</th>
<th>CFT−</th>
<th>CFT (ND)</th>
<th>Total samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>T− and C−</td>
<td>1</td>
<td>27</td>
<td>1</td>
<td>29</td>
</tr>
<tr>
<td>T+ only</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>C+ only</td>
<td>0</td>
<td>12</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>T+ and C+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total samples</td>
<td>2</td>
<td>40</td>
<td>3</td>
<td>45</td>
</tr>
</tbody>
</table>

T. tracheal semi-nested PCR; C. cloacal semi-nested PCR; +, Chlamyphila positive; −, Chlamyphila negative; ND, not done.
positive either by semi-nested PCR and/or by CFT showed no clinical signs of the disease in the physical examination.

4. Discussion

This study represents the first survey reporting the presence of C. psittaci in free-living birds in Brazil. All parrot nestlings were serologically negative; however, chlamydial DNA was detected in cloacal swabs. It is not surprising that there were no seropositive parrots under such conditions, since complement-fixing antibodies are, in general, only detected 7–10 days past the initial infection (Flammer, 1997). Moreover, young birds with initial infection usually do not produce detectable immune response (Andersen, 1998).

The elimination of Chlamydia by Hyacinth macaw nestlings via trachea or cloaca was confirmed by semi-nested PCR. Although the primary site of infection is generally the upper respiratory tract (Andersen, 1996), in this case, the macaw nestlings were shedding the microorganism more frequently through the cloaca. Only one of the serologically positive macaws was also positive by semi-nested PCR (Table 2). Therefore, this evidences an individual that is infected, sheds the microorganism actively and presents humoral immune response.

Adult psittacine birds actively feed their nestlings by regurgitation, in addition to this, the male regurgitates food to the female during egg incubation. Therefore, the oral route is a common transmission route, since food may be contaminated with secretions from the crop, nasal cavity and pharynx. In addition, the transmission of C. psittaci through regurgitated food, feces, exudates or egg in the nest is also important to maintain infection in some populations (Wittenbrink et al., 1993; Andre, 1994). Macaw nestlings stay longer in the nest than the parrot nestlings (Figs. 1 and 2) and are thus more susceptible to the contamination in this environment. This fact may be related to the largest number of positive samples in Hyacinth macaws. Approximately half of the positive samples were detected in 60-day-old or older macaws, particularly the positive serum samples (Fig. 3).

The few studies about C. psittaci in free-living psittacine refer to adult individuals, indicating that the prevalence of infection is highly variable among wild psittacine species that commonly intermingle (Brand, 1989). Similar results were also observed in our study, in which significant differences in C. psittaci prevalence was seen between the Amazon parrots (6.3%) and the Hyacinth macaw (37.8%) inhabiting the same geographical area, however, living in diverse ecologic niches and showing different behaviors. These results diverge from those found in other psittacine species. In Bolivia, no free-ranging Blue-fronted Amazon parrots were found to have chlamydial antibodies (Deem et al., 2005). Similarly, in Peru, no adults of wild parakeets (Aratinga weddellii and Brotogeris sanctithomae) showed chlamydial antibodies (Gilardi et al., 1995). In Australia, PCR and isolation of C. psittaci showed negative results in samples of conjunctiva, cloaca and coana taken from wild cockatoos (McElnea and Cross, 1999). Despite the occurrence in wild birds, there are few mortality reports caused by C. psittaci in natural habitats. Occasional deaths by chlamydiosis have been described in free-living doves, robins and gulls (Grimes et al., 1966; Gough and Bevan, 1983; Simpson and Bevan, 1989; Franson and Pearson, 1995). In our study no evidence of clinical disease or death due to chlamydiosis was found.

In fact, the presence of infection with no clinical sign of disease suggests a stable host–parasite relationship. Probably, balanced co-existence is the most common form of chlamydial infection and has
been observed not only in birds, but also in many other animal species (Storz and Kaltenboeck, 1993; Schachter, 1995). Nevertheless, when birds are captured and taken from their natural habitat, the balance is altered by diverse factors, mainly inadequate hygiene conditions, feeding and overpopulation during illegal pet trade. Birds in captivity are more susceptible to infection, and latent infections become apparent. Consequently, high mortality rates are occasionally observed in recently captured wild birds kept in captivity, as previously reported in younglings of Blue-fronted Amazon parrots that were being illegally commercialized in São Paulo, Brazil (Raso et al., 2004).

The present study showed the presence of *C. psittaci* in free-living psittacine birds in the Brazil, demonstrating the variable prevalence of positive findings among the two studied species. Although the host and the parasite seem to live in balance, such results must be considered an alert. Diverse interferences in the natural habitat or in the population of these birds might facilitate and/or change the latency of *C. psittaci*, predisposing to clinical disease or death of the birds or yet human contamination.

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