Rainbow lorikeet, an example of a psittacine bird.

**INTRODUCTION**

*Chlamydia psittaci* is a Gram-negative, obligate intracellular bacterium that is the aetiopathogenic agent of chlamydiosis in birds, especially Psittaciformes. The objective of the present study was to detect *C. psittaci* by means of semi-nested PCR among psittacine birds sold at pet markets and kept as pets in Salvador, Bahia, Brazil. Questionnaires were used to identify risk factors involved in the epidemiology of the disease. In addition, the management of birds and cages was observed at each location studied. The frequency of *C. psittaci* infection was 10.6% (33/311) in the psittacine birds studied. Birds kept in households were less frequently positive (3.4%: 5/148) than those at pet markets (17.2%: 28/163). Among the several factors analysed in the epidemiology of the disease, only population density (*P* = 0.001) and cage hygiene (*P* = 0.041) in birds at pet markets were significantly associated with *C. psittaci* infection. These results demonstrate the presence of *C. psittaci* infection in Psittaciformes kept as pets and held at pet markets in Salvador, Bahia, showing that this micro-organism is a public health concern. Control measures should be encouraged to prevent the spread of the agent among birds, as well as among employees and customers.

**Abbreviations:** EIA, enzyme immunoassay; snPCR, semi-nested PCR.

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**Risk factors associated with *Chlamydia psittaci* infection in psittacine birds**

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*Chlamydia psittaci* is the aetiological agent of chlamydiosis in birds, especially Psittaciformes. The objective of the present study was to detect *C. psittaci* by means of semi-nested PCR among psittacine birds sold at pet markets and kept as pets in Salvador, Bahia, Brazil. Questionnaires were used to identify risk factors involved in the epidemiology of the disease. In addition, the management of birds and cages was observed at each location studied. The frequency of *C. psittaci* infection was 10.6% (33/311) in the psittacine birds studied. Birds kept in households were less frequently positive (3.4%; 5/148) than those at pet markets (17.2%; 28/163). Among the several factors analysed in the epidemiology of the disease, only population density (*P* = 0.001) and cage hygiene (*P* = 0.041) in birds at pet markets were significantly associated with *C. psittaci* infection. These results demonstrate the presence of *C. psittaci* infection in Psittaciformes kept as pets and held at pet markets in Salvador, Bahia, showing that this micro-organism is a public health concern. Control measures should be encouraged to prevent the spread of the agent among birds, as well as among employees and customers.

**Abbreviations:** EIA, enzyme immunoassay; snPCR, semi-nested PCR.

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**INTRODUCTION**

*Chlamydia psittaci* is a Gram-negative, obligate intracellular bacterium that is the aetiopathogenic agent of chlamydiosis in birds and psittacosis in humans (Kuo et al., 2010; Stephens et al., 2009). The order Psittaciformes contains the greatest number of *Chlamydia*-positive bird species (Kaleta & Taday, 2003). Infected birds usually remain asymptomatic and may intermittently shed the agent in nasal secretions and faeces, especially when submitted to stress factors such as nutritional deficiency, prolonged transportation, overcrowding, temperature changes and/or reproduction. Many clinical signs seen in birds are non-specific, and can include nasal discharge, diarrhoea with greenish-yellow faeces, ruffled feathers, anorexia and conjunctivitis (Harkinezhad et al., 2009). Infected birds can be sources of infection for other avian species and humans.

Although notification of psittacosis is mandatory in most countries, the impact of this disease on human health is difficult to determine (Beeckman & Vanrompay, 2009). In Brazil, the Ministry of Health includes psittacosis in the list of work-related diseases (Ministério da Saúde, 2001). Thus, the disease is mostly an occupational risk, affecting employees such as those at poultry farms (Laroucau et al., 2009a; Verminnen et al., 2008), slaughterhouses (Laroucau et al., 2009b; Petrovay & Balla, 2008), pet stores (Saito et al., 2005) and zoos (Raso et al., 2010). In a previous study carried out in Brazil, it was observed that 4.7% (17/364) of people who worked in contact with birds presented with anti-*C. psittaci* antibodies (Raso et al., 2010). The authors suggested that prevention and control measures against *C. psittaci* should be implemented in such a work environment. In a case report of psittacosis involving the owners of a pet store in Japan, Saito et al. (2005) commented on the risk of occupational infection and the difficulty in differentiating the symptoms of psittacosis from those of other respiratory infections. Vanrompay et al. (2007) conducted a study in Belgium using PCR and demonstrated *C. psittaci* infection in 13% (6/46) of parrot owners. These data emphasize the importance of birds as a source of *C. psittaci* infection in humans, especially when living in close proximity.

In Brazil, *C. psittaci* infection in Psittaciformes has been detected in birds in breeding facilities, illegally traded birds and those in the wild, with positivity rates ranging from 6.3% to 56.1% (Raso et al., 2006; Raso et al., 2002, 2004).
The objectives of this study were to use semi-nested PCR (snPCR) to detect *C. psittaci* among Psittaciformes sold at pet markets and kept as household pets, and to use observation and questionnaires to identify factors that may be involved in the epidemiology of chlamydiosis.

**METHODS**

**Samples from birds.** Between March and July 2011, biological samples were collected from 311 Psittacidiformes of several species. This study was conducted in Salvador, the capital of the state of Bahia, Brazil. A licence for this study was obtained from the Brazilian Institute for the Environment and Renewable Natural Resources (Instituto Brasileiro do Meio Ambiente e Recursos Naturais Renováveis), along with approval from the Ethics Committee for Animal Use of the School of Veterinary Medicine and Animal Sciences, Federal University of Bahia (CEUA-MEV, protocol no. 15/2010).

The pet markets included in this study were based on unofficial information, as there is no official list of addresses of this kind of market. The households surveyed were located and included in a similar manner. In the pet markets that were visited, all of the cages that contained psittacine birds were examined. Three birds were randomly sampled from cages containing one to 10 individuals, while five birds were sampled from cages containing 10–20 individuals. When visiting households, samples were collected from all of the pet birds. The birds were restrained manually, and the procedure was carried out as quickly as possible to avoid stressing the animals. Samples were obtained from the oropharynx and cloaca with sterile swabs that were then placed in sterile microtubes, refrigerated and sent to the Veterinary Infectology Laboratory of the Federal University of Bahia. A total of 600 μl of 0.9 % saline solution was added to each microtube and the samples underwent homogenization for 5 min and centrifugation at 20,000 g for 30 min. Swabs were then removed from the solution and stored at –80 °C until subsequent DNA extraction.

**Epidemiological questionnaire.** In addition to observations carried out by the researchers, an epidemiological questionnaire was conducted at the pet markets and with each bird owner, with the objective of gathering information on the following issues: the origin of the bird, type of feed, contact with synanthropic birds, ventilation of the environment, cage density, hygiene, type of cleaning, use of personal protective equipment, knowledge about the chlamydiosis disease, history of disease occurrence among the birds and the people with whom they coexist, and observations of clinical signs suggestive of chlamydiosis among the birds.

**Molecular diagnosis.** DNA was extracted as described by Sambrook *et al.* (2001) using 250 μl of each sample. A total of 30 μl SDS (10 %) and 20 mg (proteinase K) ml⁻¹ (Invitrogen) was added, with subsequent overnight incubation at 56 °C. Then, 10 μl RNase (Invitrogen) was added and the resulting product was incubated at room temperature for 10 min. Phenol (Invitrogen), chloroform (Fmaia) and isooamyl alcohol (Vetec) were then added in the following respective proportions: 25:24:1. The resulting product was homogenized for 20 s in a centrifuge at 20,000 g for 10 min. After discarding the phenolic phase, chloroform and isooamyl alcohol were added (24:1), and the mixture was again homogenized by centrifugation at 20,000 g for 10 min. The aqueous phase was transferred to a new microtube, to which sodium acetate (3 M, pH 5.0, 10 % of the volume) and absolute alcohol (20 % of the volume) were added. The solution was incubated overnight at –20 °C and then centrifuged at 18,000 g for 5 min. A total of 1 ml of 70 % alcohol was added to the DNA pellet, which was then centrifuged at 18,000 g for 5 min. This procedure was repeated twice, always after removing the alcohol. Finally, the DNA pellet was eluted in 30 μl Tris EDTA (10:0.1) and stored at –80 °C.

The snPCR to detect *C. psittaci* DNA was carried out as described by Raso *et al.* (2006), with some modifications. The primers used were based on the major outer-membrane protein gene (MOMP) of the bacterium. The sequences were as follows: A (5’-CAGGACATCT-TGTCTGCCCATTAA-3’) and B (5’-GGAGAGCTCGCAAG ATC-3’) for the first reaction, yielding a product of 260 bp, and C (5’-TTAGAGGTGAATGAAAACCTC-3’) and B for the second reaction, which produced an amplicon of 165 bp. Reactions were carried in 25 μl volumes using 2 mM MgCl₂, 0.2 mM dNTPs, 1 U platinum Taq polymerase (Invitrogen) and 0.5 μM of each primer, and 5 μl genomic DNA for the first reaction and 1 μl amplicon for the second reaction. The reactions were carried out at 94 °C for 10 min, followed by 35 cycles at 94 °C for 60 s; 54 °C for 60 s (for the first reaction) and 52 °C for the second reaction); and 72 °C for 90 s, with a final extension at 72 °C for 4 min. Positive and negative control samples were included in each run (Raso *et al.*, 2006). The PCR products were stained with SYBR Gold solution (Invitrogen) and subjected to electrophoresis on 1.5 % agarose gel, in Tris/borate-EDTA buffer, together with a 100 bp molecular mass marker (Invitrogen). Reading was carried out in a transilluminator and results were photographed (Biometra).

**Statistical analysis.** Associations between the variables studied and the snPCR results were evaluated using a χ² test in SPSS version 19 (IBM), with a significance level of 5 %.

**RESULTS**

Samples from the oropharynx and cloaca of 311 Psittacidiformes were analysed for *C. psittaci* DNA. From this total, 257 birds were from exotic fauna (four genera) and 54 were from the native Brazilian fauna (seven genera) (Table 1). Among the 18 pet markets visited, 163 birds for sale were examined, of which 162 were exotic and one was native. Swabs from the oropharynx (*n* = 162) and cloaca (*n* = 163) were obtained from each animal. *C. psittaci* infection was detected at 50 % (*n* = 9/18) of the markets. A total of 148 birds were examined in the 65 households visited. Among these, 95 were exotic and 53 were native. Swabs were collected from the oropharynx (*n* = 146) and cloaca (*n* = 148) of these birds. *C. psittaci* infection was detected in 6.15 % (4/65) of the households. One of these households had two infected birds.

The overall frequency of *C. psittaci* in the psittacine birds was 10.6 % (33/311): 17.2 % (28/163) at the pet markets and 3.4 % (5/148) in household pet birds. Regarding the samples, *C. psittaci* DNA was detected in 9.3 % (29/311) of cloaca and 1.3 % (4/308) of oropharynx samples (Table 1). *C. psittaci* DNA was not detected simultaneously in samples from the oropharynx and cloaca of the same bird.

The frequency of responses to the questionnaires conducted in the pet markets and among pet owners is presented in Table 2.

Based on the variables studied and the snPCR results, significant associations between population density (*P* = 0.001) and cage hygiene (*P* = 0.041) and *C. psittaci* infection were detected for pet markets. Cages were characterized as...
F. Santos and others

overcrowded when they contained a number of birds per unit area greater than that recommended by Brazilian regulations (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, 2008). Only one bird, of the species *Melopsittacus undulatus*, presented with clinical signs (diarrhoea) that might be compatible with chlamydiosis and was positive for *C. psittaci* on PCR.

No significant associations were observed between the household variables studied and snPCR results. All of the Psittaciformes infected with *C. psittaci* that were kept as pet birds had access to synanthropic birds that fed on the scraps that fell from the cages of the Psittaciformes. Birds lived in individual cages in 60% (39/65) of the households, and there was no overcrowding in the remainder of the households.

In all the cases of infected Psittaciformes kept in households, cage cleaning consisted of removing faeces only. Gloves were used to remove bird faeces in only 1.5% (1/65) of households. There were no reports of clinical signs suggestive of chlamydiosis among the Psittaciformes kept in households. There was only one previous report of a clinical diagnosis of chlamydiosis in a bird kept as a pet, according to the owner of the bird, and *C. psittaci* DNA was detected on the molecular assay in the present study.

### DISCUSSION

The results demonstrate the presence of *C. psittaci* infection in 10.6% of Psittaciformes examined among those for sale at pet markets and kept as pets in Salvador, Bahia, Brazil. Until now, no scientific paper published in Brazil has investigated the presence of *C. psittaci* among psittacine birds at pet markets or kept in households. Previous studies have reported on the detection of *C. psittaci* in wild psittacine birds, at breeding facilities and in illegal trading. Raso et al. (2002) reported *C. psittaci* infection among birds of the *Amazona* genus from breeding facilities in the states of São Paulo (16.7%), Minas Gerais (22.2%) and Mato Grosso do Sul (56.1%). Later, Raso et al. (2004) investigated an outbreak of chlamydiosis among *Amazona aestiva* chicks that were seized from illegal animal traders, and recorded a mortality rate of 96.5% (56/58). A study of *C. psittaci* infection among *Anodorhynchus hyacinthinus* and *Amazona aestiva* nestlings in the state of Mato Grosso do Sul reported prevalences of 37.8% (17/45) and 6.3% (2/32), respectively (Raso et al., 2006).

Data on the occurrence of *C. psittaci* among psittacine birds sold at pet markets or kept as pets in other countries are scarce in the literature. In Australia, McElnea & Cross (1999), using dot blot PCR, reported an infection frequency of 23.3% (21/90) among several species of birds kept at commercial establishments. However, these authors did not mention how many psittacine birds were included in their sample. In Turkey, Çelebi & Ak (2006), using PCR, found that 34.4% (33/96) of birds in 22 commercial establishments and six households in different cities were infected by *C. psittaci*. According to these authors, in addition to being predominant in the sampling, parrots presented the greatest

<table>
<thead>
<tr>
<th>Bird location</th>
<th>Species</th>
<th>C. psittaci DNA detection</th>
<th>Total positive/total individuals, n/n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cloaca, n (%)</td>
<td>Oropharynx, n (%)</td>
</tr>
<tr>
<td>Pet market</td>
<td><em>Agapornis</em> spp.</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Lorius</em> spp.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Melopsittacus undulatus</em></td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td><em>Nymphicus hollandicus</em></td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Primolius auricollis</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>26/163 (15.95)</td>
<td>2/162 (1.23)</td>
</tr>
<tr>
<td>Household</td>
<td><em>Agapornis</em> spp.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Amazona aestiva</em></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Amazona amazonica</em></td>
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<tr>
<td></td>
<td><em>Ara ararauna</em></td>
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<td>0</td>
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<tr>
<td></td>
<td><em>Aratinga</em> spp.</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td><em>Brategeris triric</em></td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Melopsittacus undulatus</em></td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Nymphicus hollandicus</em></td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Pionus</em> spp.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Primolius auricollis</em></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal</td>
<td></td>
<td>3/148 (2%)</td>
<td>2/146 (1.36%)</td>
</tr>
<tr>
<td>Total positive</td>
<td></td>
<td>29/311 (9.3)</td>
<td>4/308 (1.3)</td>
</tr>
</tbody>
</table>

*Psittaciformes from the Brazilian fauna.*
number of positive results (45 %; 25/56). On the other hand, nested PCR is more sensitive and can improve diagnosis, as reported by Sachse & Hotzel (2003), who described a nested PCR assay targeting the \textit{omp1} gene as a good tool for diagnostic PCR. A study carried out in Belgium assessed occurrences of \textit{C. psittaci} among parrots with clinical signs suggestive of chlamydiosis. Chlamydiosis was confirmed in 25 % of cases (5/20) using nested PCR/enzyme immunoassay (EIA) (Harkinezhad \textit{et al}, 2007). Also in Belgium, Vanrompay \textit{et al} (2007) reported a \textit{C. psittaci} infection frequency of 19.2 % (59/308) in several psittacine reproduction centres, as assessed by nested PCR/EIA.

In the present study, \textit{C. psittaci} infection was detected more frequently in samples from the cloaca (9.3 %) than from the oropharynx (1.3 %). This may be attributed to variations in the stage of infection, reflecting a smaller number of birds in the initial stage of infection, when there are more bacteria in the oropharynx, and a predominance of chronic infection, characterized by intermittent elimination of the bacteria through the cloaca (Andersen, 1996). Raso \textit{et al} (2006) obtained similar results when investigating \textit{C. psittaci} infection in macaws (\textit{Anodorhynchus hyacinthinus}) in the Pantanal of Mato Grosso do Sul, recording positive results in 26.7 % (12/45) of cloaca samples and only 8.9 % (4/45) of tracheal samples. However, in a study involving the experimental infection of 133 cockatiels, Andersen (1996) observed the greatest positivity in pharynx samples (80.4 %), followed by 45.1 % in faecal samples and 37.3 % in cloaca samples. The greater concentration of positive results in oropharynx samples reinforces the affirmation that, in recent infections, there is greater detection of bacteria in this region. This author suggested that diversification of the sampling sites may increase the chance of detecting positive birds. Smith \textit{et al} (2011) recommended carrying out sampling on the same bird on alternate days, with the objective of increasing the chance of detecting \textit{C. psittaci}. In cases of persistent infection, birds tend to shed the agent intermittently and usually remain asymptomatic, which makes it difficult to identify infected birds. Several authors in Brazil have observed that birds infected with \textit{C. psittaci} are often asymptomatic (Raso \textit{et al}, 2006; Raso \textit{et al}, 2011). In the present study, only one bird (\textit{M. undulatus}) presented with diarrhoea (a clinical sign suggestive of chlamydiosis), and the infection was indeed confirmed by molecular diagnosis.

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|}
\hline
\textbf{Location} & \textbf{Question} & \textbf{Response} \\
\hline
\textbf{Pet market} & Bird origin & 94.5 % from a professional breeder \\
& & 5.5 % from their own breeding system \\
& Type of feed & 66.7 % seeds \\
& & 33.3 % seeds and fruits \\
& Presence of synanthropic birds & 72.2 % yes \\
& & 27.8 % no \\
& Ventilated environment & 83.3 % yes \\
& & 16.7 % no \\
& Overcrowded cages & 38.9 % yes \\
& & 61.1 % no \\
& Cage cleaning & 77.8 % yes \\
& & 22.2 % no \\
& Use of personal protective equipment & 22.2 % yes \\
& & 77.8 % no \\
& Knowledge about the disease chlamydiosis & 27.8 % yes \\
& & 72.2 % no \\
\hline
\textbf{Household} & Bird origin & 45.3 % pet markets \\
& & 41.2 % from other owners \\
& & 13.5 % undefined \\
& Presence of synanthropic birds & 52.3 % yes \\
& & 47.7 % no \\
& Ventilated environment & 87.7 % yes \\
& & 12.3 % no \\
& Cage cleaning & 67.7 % yes \\
& & 32.3 % no \\
& Type of cleaning & 52.3 % faces removal only \\
& & 18.5 % washing and disinfection \\
& & 29.2 % washing only \\
& Knowledge about the disease & 36.9 % yes \\
& & 63.1 % no \\
\hline
\end{tabular}
\caption{Responses to the epidemiological questionnaire conducted at 18 pet markets and among 65 owners of pet Psittaciformes}
\end{table}
According to the questionnaire conducted at the pet markets, the budgerigar (M. undulatus) and the cockatiel (Nymphicus hollandicus) were the most commonly commercialized species, but also presented with the greatest frequency of C. psittaci infection: 19% (12/63) and 21.2% (11/52), respectively. Three out of five birds that were kept in households and presented positive results in this study belonged to one of these two species (Table 1). In a study carried out by Raso et al. (2011) among cockatiels at private breeding facility in Brazil, the authors found that 90.6% (29/32) of the birds were infected with C. psittaci, suggesting that birds became infected at the breeding facility.

In the present study, the fact that fewer infected birds were kept in households (3.4%) than at pet markets (17.2%) suggests that the environment, management and hygiene conditions of the birds kept in households reduced the chance of infection. On the other hand, stress factors such as overcrowding (P=0.001) and inadequate cage cleaning (P=0.041) showed significant associations with C. psittaci infection among birds at the pet markets. Smith et al. (2011) stated that factors such as overcrowding and a dirty and stuffy environment, as well as transportation, changes in the diet and inadequate temperature predisposed birds towards C. psittaci shedding, thereby increasing the risk of infecting humans and other susceptible birds.

Dovc et al. (2005) tested people who were considered to have a high risk of C. psittaci infection, and observed that there was a higher frequency of positive cases among people who sold pet birds than among parrot owners: 18.2% and 9.5%, respectively. These authors suggested that the high frequency of positive cases among the pet store owners was due to the high bacterial load in the work environment, a result of greater elimination of C. psittaci by birds that were subjected to stress factors, which are common in this type of setting. Several factors, as well as their interactions, seem to contribute to a greater exposure of birds to C. psittaci infection at pet markets, such birds being traded at a very young age, when their immune system is still developing to face the pathogens present in this environment. Moreover, birds are often inadequately transported and, at pet markets, are usually not quarantined before being caged with birds already for sale, hindering any control of C. psittaci dissemination to susceptible birds and preventing any reduction in the risk of infection among the employees and customers of these sites.

According to Smith et al. (2011), daily removal of faeces and cage disinfection and washing are essential for maintaining hygienic conditions. In the present study, all of the infected psittaciformes lived in households in which the only cleaning procedure was the removal of faeces from the cage. In addition, and as an aggravating factor, personal protective equipment (e.g. gloves and masks to prevent infection from aerosols when cleaning cages) were only used in one household and at four stores. Matsui et al. (2008) investigated an outbreak of psittacosis in a bird park in Japan, and observed that employees had contact with sick birds without any protection, leading to a greater risk of infection with C. psittaci and demonstrating that lack of knowledge about the disease resulted in an underestimation of its risk and negligence regarding the use of preventive measures.

The current results show that Psittaciformes at pet markets and in households in Salvador, Bahia, are infected with C. psittaci. In addition, in view of the observed frequency of infection and hygiene conditions, this study suggests that pet markets that sell these birds play an important role in the epidemiology of chlamydiosis. Thus, control measures for this zoonosis in pet markets should include strict sanitary and environmental management, with the aim of reducing the risk of infection among birds, as well as among employees and customers.

ACKNOWLEDGEMENTS

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