# Molecular Detection of *Chlamydia psittaci* in blue-fronted amazons (*Amazona aestiva*) in Paraguay

*Detección molecular de Chlamydia psittaci en loros habladores (Amazona aestiva) en Paraguay*

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## ABSTRACT

Chlamydiosis constitutes a high-risk zoonosis for people who have direct contact with infected birds. In Paraguay, epidemiological data on avian chlamydiosis are still not sufficiently known, mainly due to the lack of knowledge of the disease or the difficulty of its diagnosis. The aim of this study was to determine the presence of *Chlamydia psittaci* by PCR nested (PCRn) in cloacal swabs from captive blue-fronted amazons (*Amazona aestiva*) admitted to the Consultorio de Animales Silvestres y Exóticos of the Facultad de Ciencias Veterinarias, Universidad Nacional de Asunción, in 2022. For this purpose, 35 animals were sampled, of which 5.71% (2/35) were positive for *Chlamydia psittaci*. The results of this work demonstrate the presence of the pathogen in blue-fronted amazons in Paraguay, being the first report of the pathogen in this species in the country, for which reason it is suggested that the pathogen be monitored in captive bird populations, as well as free living individuals.

**Key words:** Disease monitoring, psittacosis, surveillance, zoonosis.

## RESUMEN

La clamidiosis constituye una zoonosis de alto riesgo para las personas que tienen contacto directo con aves infectadas. En Paraguay, los datos epidemiológicos sobre clamidiosis aviar aún no son suficientemente conocidos, debido principalmente al desconocimiento de la enfermedad o a la dificultad de su diagnóstico. El objetivo de este estudio fue determinar la presencia de *Chlamydia psittaci* mediante PCR anidada (PCRn) en muestras cloacales de loros habladores (*Amazona aestiva*) bajo cuidado humano, trasladados al Consultorio de Animales Silvestres y Exóticos de la Facultad de Ciencias Veterinarias de la Universidad Nacional de Asunción en 2022. Para ello, se muestrearon 35 animales, de los cuales 5,71% (2/35) fueron positivos para *Chlamydia psittaci*. Los resultados de este trabajo demuestran la presencia del patógeno en loros habladores en Paraguay, siendo el primer reporte del mismo en esta especie en el país, por lo que se sugiere el monitoreo del patógeno en poblaciones de aves en cautiverio, como también en vida libre.

**Palabras clave:** Monitoreo de enfermedades, psitacosis, vigilancia, zoonosis.

## INTRODUCTION

*Chlamydia psittaci* is a highly pathogenic obligate intracellular gram-negative bacterium of the family Chlamydiaceae, capable of infecting a vast diversity of bird species (OIE 2012, cited in Rodríguez et al 2018, Ruiz Laiton 2020) and is therefore considered a pathogen of veterinary interest (Arraiz et al 2012). Chlamydiosis is also one of the main zoonoses of avian origin that implies a high risk both for owners of wild birds, as well as for those involved in trade, breeding and clinical practice, which also translates into a significant impact on public health (Furlanetto and Plácido 2020).

Currently, the term chlamydiosis is used to refer to the pathology in birds and the term psittacosis in humans (Rohde et al 2021), the latter being a notifiable disease in many countries (Martínez and Tinetti 2012). Data concerning psittacosis, coming from neighboring countries such as Brazil, indicate that the disease is endemic in that country (Afonso 2018); in Argentina the epidemiological and molecular characteristics of the agent are still not sufficiently known (Layun et al 2018) and in Paraguay to date there is little or no information and/or case reports, the only recorded report dates from 1945; since then no further information and/or reports of this zoonosis have been recorded (MSPBS 2015).

The reservoirs of the pathogen are mainly psittacine birds (Order Psittaciformes) and less frequently poultry, pigeons, canaries and seabirds (MSPBS 2015). The clinical presentation of the disease in animals varies, depending on the species involved, the age of the bird, the immunological status, the management conditions and the serotype of *C. psittaci*, ranging from an asymptomatic or subclinical infection to a severe systemic disease with high mortality (Raso 2014). Infected birds, even without clinical signs, shed the bacterium intermittently through feces and ocular and nasal secretions, and humans can become infected by exposure to these materials (Harkinezhad et al 2009, Balasmo et al 2017) with most cases in humans resulting from contact with infected pet birds (Afonso 2018).

Shedding of the agent can be triggered by stressors, including overcrowding, improper handling, nutritional deficiency, environmental changes, and prolonged transport (Anderson 2021). Prevalences from 6.3% (2/32) to 72% (17/32) have already been reported in blue-fronted amazons (*Amazona aestiva*) in Brazil and Argentina (Raso et al 2004, Raso et al 2006, Vilela et al 2019, Furlanetto and Plácido 2020, Santos et al 2023, Riccio et al 2024), proving the circulation of the pathogen in neighboring countries. The latter is one of the most widespread Amazon species in South America, found from northeastern Brazil to the southwest in Paraguay, eastern Bolivia and northern Argentina (Schaaf 2016). Although the blue-fronted amazon is categorized as Least Concern (LC), capture for the pet trade, and loss of habitat quality and quantity due to land-use change, are important threat factors for the species (Birdlife international 2019).

Due to the potential zoonotic impact of the pathogen, the aim of this study was to determine the presence of *Chlamydia psittaci* by PCRn in cloacal swabs from blue-fronted amazon (*Amazona aestiva*) admitted to the Consultorio de Animales Silvestres y Exóticos (Wildlife and Exotics Practice) of the Facultad de Ciencias Veterinarias, Universidad Nacional de Asuncion, in 2022.

## MATERIALS & METHODS

A sampling of 35 captive animals was performed, without distinction of sex, age, or presence of clinical signs, considering a confidence level of 95% based on the study conducted by Raso et al (2006). Sampling was performed at the Wild and Exotic Animals Practice in the Facultad de Ciencias Veterinarias, Universidad Nacional de Asuncion (FCV, UNA). The clinical assessment of each animal was carried out, recording the clinical findings in an individual clinical record. Cloacal samples were obtained by using sterile polypropylene swabs, and then introducing the swab into a microtube with 100 µL of lysis buffer, refrigerated at 4°C until processing.

For the handling of the animals, the legislation in force in Paraguay was taken into account (Law 96/92: "On Wildlife"; Law 4840/13: "On Animal Protection and Welfare"), and the professionals in charge of taking the samples are registered in the Registro Nacional de Vida Silvestre (National Wildlife Registry, in spanish) of the Ministry of Environment and Sustainable Development, as required by law.

Sample processing was performed at the Animal Biotechnology Laboratory Division, FCV, UNA, where genomic DNA was extracted from the cloacal swab samples using a commercial extraction kit (Thermo Scientific™). The DNA samples were subjected to a nested PCR protocol to detect a conserved region of the 16S rRNA gene of *Chlamydia psittaci* using the protocol described by Messmer et al (1997) to amplify an approximately 127 bp region of *C. psittaci*. For the first step, the sequence ACG GAA TAA TAA TGA CTT CGG was used in forward (5' → 3'), and the sequence TAC CTG GTA CGC TCA ATT in reverse (5' → 3'); and for the second step the sequence ATA ATG ACT TCG GTT GTT GTT ATT was used in forward (5' → 3'), and the sequence CGT CAT CGC CTT GGT GGG CTT in reverse (5' → 3') (Messmer et al 1997).

Both reactions were composed of 5 µL of Gotaq Green mastermix®, 200 nM of each partitioner and 2 µL of template DNA for the first reaction and 0.6 µL for the second reaction brought to a total volume of 10 µL, with nuclease-free water (Thermo Scientific©, USA). The thermal protocol was carried out as follows: 1 cycle of 95°C for 2 minutes, and 35 cycles of 94°C for 1 minute, 55°C for 30 seconds, and 72°C for 1 minute. Amplification was completed with a final extension at 72°C for 1 minute. The nested or inner PCR mixture was similar to the first reaction, except that it contained 1µl of the product of the outer PCR and 0.2 µM each inner primer, with identical cycling conditions. Previously sequenced DNA from a naturally infected bird was used as a positive control, and ultrapure DNAse-free water was used as a negative control. The products of the first and second PCR were visualized on 1.5% agarose gels (Agarose Gel Electrophoresis Kit - MB570) from HiMedia (HiMedia Laboratories, India) using electrophoresis in a BioRad chamber and source (V500/150) for one hour. For DNA visualisation, the gels were stained with ethidium bromide (0.5 mg/mL) in 1X TAE for 40 minutes and observed on a UVP Inc Digi Doc-It Darkroom (P/N 76-0311-01) ultraviolet light transilluminator with Drawer Assembly (P/N 98-0068-01). DNA extraction, PCR amplification, and electrophoresis were performed in three separate rooms to avoid cross-contamination.

To determine the presence or absence, and the species present, all DNA samples were analyzed by a nested PCR described by Messmer et al (1997). A positive result meant that amplicons presenting bands of a size of 436 bp for *Chlamydia* spp. and 127 bp for *C. psittaci* were observed in the samples tested. To confirm the products obtained by PCRn, a positive sample was randomly selected and sent to Macrogen (South Korea) for purification and subsequent sequencing using the Sanger method. The forward and reverse sequences were analyzed in the MEGA 11 program to obtain consensus sequences, and the percentage of identity was determined using the BLAST tool (Altschul et al., 1990).

**RESULTS & DISCUSION**

Of the 35 samples tested, 2 were positive for *Chlamydia psittaci* (5.71%) (Fig. 1), although one other sample was positive only for *Chlamydia* spp. (2.85%) (Fig 2). Sanger sequencing resulted in generated sequences that, due to insufficient length or quality, could not be further evaluated, although the sequence quality was sufficient for BLAST analysis, which confirmed *C. psittaci*. Most of the animals sampled presented a suboptimal body condition and tarnished plumage. The animals positive for *C. psittaci* presented apathy and digestive alteration (greenish-yellow feces). None of the animals positive for both *Chlamydia* spp. and *C. psittaci* presented visible respiratory or ocular alterations.

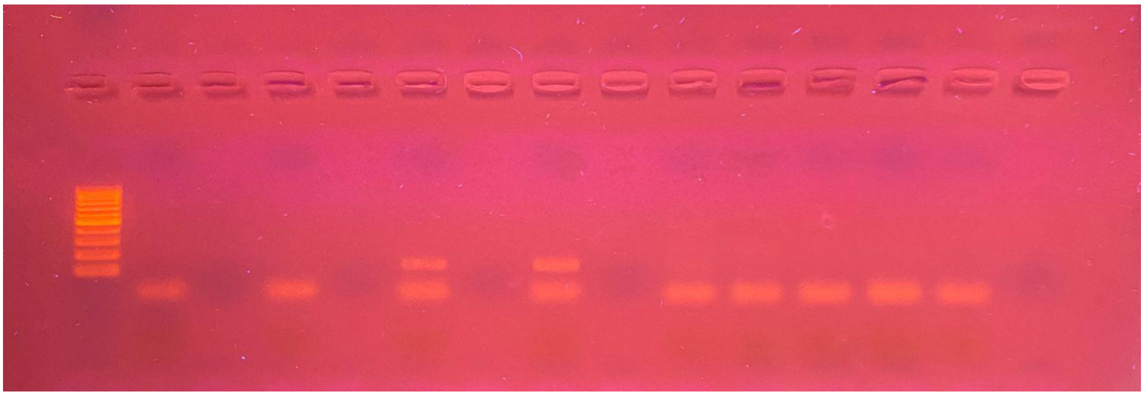

**Figure 1.** Results of 1.5% agarose gel electrophoresis of the second PCRn amplification for *C. psittaci*. Lane 1: molecular weight marker (ladder). Lane 2: negative control. Lane 3: negative sample. Lane 6: positive control (127 bp). Lane 8: positive sample. Lane 9 to 13: negative samples. (Photograph: Lorena Núñez)

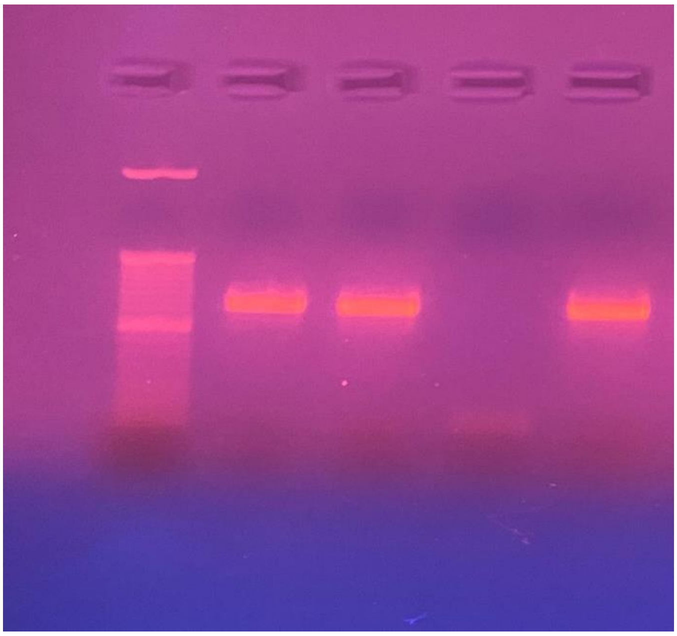

**Figure 2.** Results of 1.5% agarose gel electrophoresis of the first PCR amplification demonstrating the presence of a 436 bp band for *Chlamydia* spp. Lane 1: molecular weight marker (ladder). Lane 2 & 3: positive samples. Lane 4: negative control. Lane 5: positive control. (Photograph: Lorena Núñez)

Infections caused by *C. psittaci* have a worldwide distribution and appear to be more prevalent in tropical countries (Ruiz Laiton 2020). In the wild, chlamydiosis is mostly a disease of young birds, with incidence being less than 5% in free-ranging adult psittacines. After capture the incidence approaches 100%, due to the effect of poor nutrition, low growth, bird movement, overcrowding, change of environment and other stress factors and concurrent infections with other microorganisms (Raso et al 2004, Martínez and Tinetti 2012). The intermittent and prolonged shedding of the agent in the excretions of infected birds, with or without clinical manifestations, contributes to the condition becoming a public health issue, as it contributes to environmental contamination and its dissemination to other birds and/or humans (Piccoli et al 2021).

The positive results obtained with regard to the detection of *C. psittaci* are similar to a study carried out in the Pantanal of Mato Grosso do Sul, Brazil, which reported 2 positive cases of *C. psittaci* in 32 samples of blue-fronted amazons analyzed by semi-nested PCR (6.3%) (Raso et al. 2006). When interpreting the positive result, it should be considered that the presence of the pathogens DNA does not necessarily indicate that the bird has the disease. Therefore, PCR test results should be interpreted in the context of the complete clinical case and in relation to other laboratory tests to avoid misinterpretation (Raso 2014, Rohde et al. 2021).

In terms of digestive signs, *C. psittaci* produces a systemic infection and may show plumage changes and inadequate body condition (Gerlach 1994). However, inadequate body condition may also be due to other factors such as poor feeding, inadequate habitat, stress, among others. Plumage changes can have different etiologies such as bacterial, viral, parasitic or behavioral (Vasconcelos et al 2016).

Regarding the absence of respiratory signs, this may be because *C. psittaci* genotypes A, D and E/B cause severe respiratory symptoms, while genotype B has mild clinical manifestations or asymptomatic infections, although this does not rule out the possibility that these individuals may develop symptoms later and all genotypes should be considered potentially pathogenic to humans (Arraiz et al 2012).

The high number of negative results (33/35) for *C. psittaci* could have been due to several factors. Negative results do not exclude the possibility of the presence of the bacterium in the animals, but the bacterial load in the samples could have been below the sensitivity threshold of the method used (Afonso 2018). At the time of sample obtention, swabbing should be performed correctly, so as to exfoliate large amounts of cells from the cloacal mucosa. The correct obtaining of the material suggests an 8.15 times higher probability of detecting *C. psittaci* in DNA obtained through cloacal swabbing versus fecal sample (Ruiz Laiton 2020). Also, contamination with feces at the time of swab extraction may inhibit nPCR, due to the large amounts of metabolic compounds and polysaccharides (Ruiz Laiton 2020). For future work, further sampling per bird should be considered. Considering the intermittent elimination of *C. psittaci*, and the different detection sites according to the stage of the disease, serial collection of more than one clinical sample per birds over a period of 3 to 5 days is recommended (Afonso 2018). In this work, being a prospective study of consecutive cases, the samples were obtained from birds that attended the clinic on an outpatient basis, making serial collection difficult. Finally, the result may have been due to the fact that the birds did not have DNA compatible with *C. psittaci*.

The differences found between the samples positive to genus and negative to *C. psittaci* species are probably due to the fact that the primers used in the first genus PCR also amplify other species, which could indicate *Chlamydia* infection by other species. The presence of non-*psittaci Chlamydia* has been reported in free-living and captive birds, such as *C. avium*, *C. trachomatis*, *C. pneumoniae*, *C. pecorum* and *C. abortus* detected in passerines, psittaciformes, columbiformes, as well as in humans, showing that birds did not have DNA compatible with *C. psittaci*, but did have DNA compatible with the Chlamydiaceae family (Rodríguez et al 2018, Origlia et al 2014, Origlia et al 2023).

In birds, transmission and infection by the agent occurs primarily through the air, via inhalation of dried droppings, ophthalmic secretions, and respiratory tract secretions of infected birds (Piccoli et al 2021). Human infection with *C. psittaci* generally occurs when a person inhales organisms that have been aerosolized from dried droppings, respiratory tract secretions of infected birds, or mouth contact with the beaks and handling of plumage and tissues of infected birds (Balasmo et al 2017). Although it is usually a notifiable disease, it is suggested that *C. psittaci* infections in humans caused by parrots are underestimated due to the similarity of the clinical signs of psittacosis with those of pneumonia, making correct diagnosis and treatment difficult (MSPBS 2015, Rohde et al 2021).

In Paraguay, blue-fronted amazons, as well as other psittacines, are highly sought for and kept in captivity, although there is still no legal way to acquire any wild animal in the country (Vetter & Insfran, 2025). The presence of these birds, from unknown origin or background, in domestic situations and close proximity to humans, is a particularly risky situation considering the potential of disease transmission. Steps have to be taken to address a situation that involves not only zoonotic risk, but is also associated to illegal wildlife traffic and unregulated captive management of animals protected by current legislation, as the tracking and surveillance in these situations can be complex due to the amount of networks involved and the fear of reporting health issues because of the illegal background.

## CONCLUSION

This study confirms the presence of *Chlamydia psittaci*, detected by PCRn technique, in captive blue-fronted amazons in Paraguay, being the first report of the pathogen in *Amazona aestiva* for the country. These findings highlight the need for increased surveillance in avian populations due to the high number of captive psittacines in the country, and the zoonotic potential of *C. psittaci.*

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