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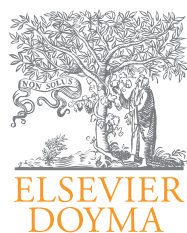
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BRIEF REPORT

Molecular evidence of *Chlamydomphila pneumoniae* infection in reptiles in Argentina

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KEYWORDS

Chlamydomphila pneumoniae;
Reptiles;
Phylogenetic Analysis;
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Abstract

In the central area of Argentina, the epidemiological and molecular characteristics of *Chlamydomphila pneumoniae* infections in reptiles are still unknown. A nested polymerase chain reaction of the *rpoB* gene was used to detect *C. pneumoniae* in cloacal swab samples from 19 reptiles at a recreational area. Eleven (57.89%) reptiles were positive; the sequencing and phylogenetic analysis confirmed the presence of this bacterium. Neither *C. pneumoniae* DNA in the caregivers' pharynges nor IgM antibodies anti-*C. pneumoniae* in their serum samples were detected; however, caregivers presented very high titers of IgG anti-*C. pneumoniae*. The detection of *C. pneumoniae* DNA in reptiles demonstrated the circulation of this agent in the recreational area and could be responsible for the exacerbated immune response of the personnel handling the reptiles, which suggests a potential zoonotic cycle. This is the first report of the detection of *C. pneumoniae* in reptiles in Argentina.

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PALABRAS CLAVE

Chlamydomphila pneumoniae;
Reptiles;
Análisis filogenéticos;
Respuesta inmunitaria;
Argentina

Evidencia molecular de la infección por *Chlamydomphila pneumoniae* en reptiles de Argentina

Resumen

En la región central de Argentina, las características epidemiológicas y moleculares de las infecciones por *Chlamydomphila pneumoniae* en reptiles son desconocidas. Para detectar *C. pneumoniae*, se usó la reacción en cadena de la polimerasa anidada que amplifica el gen *rpoB* en muestras de hisopado cloacal de 19 reptiles. Once (57,89 %) reptiles resultaron positivos. La secuenciación y el análisis filogenético corroboraron la presencia de esta bacteria. No se detectó ADN de *C. pneumoniae* en la faringe ni IgM anti-*C. pneumoniae* en el suero de los cuidadores; sin embargo, ellos presentaron títulos muy elevados de IgG anti-*C. pneumoniae*. La detección de ADN de *C. pneumoniae* en los reptiles demostró

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la circulación de este agente en el centro recreativo donde se realizó este estudio, lo que podría explicar la exacerbada respuesta inmunitaria en los cuidadores; este hallazgo sugiere la presencia de un potencial ciclo zoonótico. Se reporta aquí por primera vez la detección de *C. pneumoniae* en reptiles en Argentina.

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Chlamydia pneumoniae is a widespread pathogen responsible for upper and lower respiratory tract infections, in addition to a wide range of human and animal diseases⁹. The range of hosts known to be infected by *C. pneumoniae* has expanded to several species of reptiles, such as puff adders (*Bitis arietans*), flap-necked chameleons (*Chameleo dilepis*), green turtles (*Chelonia mydas*), Nile crocodiles (*Crocodylus niloticus*), green iguanas (*Iguana iguana*) and Burmese pythons (*Python molurus bivittatus*)^{1-3,7,9}. Lesions are varied and include histiocytic granulomas (puff adders), granulomatous inflammation (flap-necked chameleons), necrotizing enteritis (green iguanas), necrotizing myocarditis (green turtles), and proliferative pneumonia (Burmese pythons)⁷. However, in humans, *C. pneumoniae* infections range from asymptomatic to severe respiratory diseases, including pneumonia. Previous studies have reported seroprevalence values of 50% by the age of 20 years and 80% in the elderly³.

Genome characterization of the *C. pneumoniae* sequence detected in Australian reptiles and amphibians showed 99% homology with the human sequence, revealing their potential role in the zoonotic transmission from reptiles to humans¹¹. Recently, at a Recreation Center in Cordoba province, a specimen of reticulated python (*Python reticulata*) presented symptoms compatible with proliferative pneumonia, which led to the development of this investigation. The aim of this study was to describe the presence of *C. pneumoniae* in several species of reptiles in Córdoba and in their caregivers, and to evaluate the presence of specific antibodies against *C. pneumoniae* in the personnel.

We analyzed 27 cloacal swab samples from 19 reptiles at a recreational area using a specific nested-PCR for *C. pneumoniae*⁸. In May 2011, we obtained 1 cloacal swab from the reticulated python with symptoms compatible with proliferative pneumonia and 18 samples of reptiles living together. In December 2011, resampling of only 8 of these reptiles could be conducted.

The cotton swabs were placed in 1 ml sucrose-phosphate-glutamate (SPG) and 200 µl was subjected to DNA extraction using the Accuprep Genomic DNA Extraction Kit (BIONEER, Alameda, CA, USA) according to the manufacturer's instructions. DNA AR39 *C. pneumoniae* (ATCC53592) was used as positive control. Negative controls (deionized H₂O instead of template DNA) were included in all PCR reaction sets. First, 5 µl of DNA extract was used to amplify a fragment of RNA polymerase beta subunit gene (*rpoB*) using primers HL1 (GTT GTT CAT GAA GGC CTA CT)/ HR1 (TGC ATA ACC TAC GGT GTG TT) and N1 (AGT TGA GCA TAT TCG TGA GG)/ N2 (TTT ATT TCC GTG TCG TCC AG) as previously described by Mass et al.⁸.

Positive samples were sequenced to corroborate the species. For sequence analysis, the nested polymerase

chain reaction products were purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and subjected to direct nucleotide sequencing reaction in both directions using an ABI automatic sequencer (Applied Biosystems, Foster City, CA, USA). *C. pneumoniae* identification was confirmed by phylogenetic analysis.

Human pharyngeal swabs were also analyzed by a nested-PCR of *C. pneumoniae*⁸. Furthermore, sera from the 6 healthy caregivers were tested for *C. pneumoniae* antichlamydial immunoglobulin G (IgG) and IgM using the microimmunofluorescence assay (MIF), according to the manufacturer's instructions (Bion Enterprises®, France). In this test, *C. pneumoniae* elementary bodies were purified by removal of genus-reactive LPS. Since this technique has very low cross-reactivity with other *Chlamydophila* species, it is recommended for the differential diagnosis of these agents in humans.

The *rpoB* sequences obtained were submitted to the GenBank (accession numbers are shown in Table 1) and alignment using the ClustalX program was performed (Conway Institute UCD Dublin, Dublin, Ireland)¹². The maximum likelihood tree was constructed with the PhyML 3.0 software (Université de Montpellier, Montpellier, France)⁵. The model of nucleotide substitution for the data set analyzed was selected according to the Akaike Information Criterion implemented in the ModelTest 3.7 software (Universidad de Vigo, Galicia, Spain)¹⁰.

Table 1 Characteristics of *C. pneumoniae* positive samples analyzed

Positive samples	Host	Vernacular name	GenBank accession
ARG PK1	<i>Python molurus bivittatus</i>	Burmese Python	JX960449
ARG PK2	<i>Python molurus bivittatus</i>	Burmese Python	JX960447
ARG PK11	<i>Bothrops alternatus</i>	Big Yarará	JX960450
ARG PK14	<i>Micrurus pirracryptus</i>	Argentinian Coral Snake	JX960448
ARG PK20	<i>Trachemys scripta elegans</i>	Lagoon Turtle	JX960451
ARG PK21	<i>Phrynops hiliaris</i>	Side-necked turtle	JX960446

Among the cloacal swabs samples of 19 reptiles studied, 11 (57.89%) tested *C. pneumoniae* positive by nested-PCR [*Python reticulata* (n=2), *Python molurus bivittatus* (n=2), *Bothrops alternatus* (n=1), *Crotalus atrox* (n=1), *Trachemis scripta elegans* (n=2); *Phrynosoma hylaris* (n=2); *Micrurus pirracryptus* (n=1)]. DNA of *C. pneumoniae* was not detected in samples from the symptomatic reticulated python, probably because at the time of collection, this reptile was isolated and had already received specific antibiotic treatment. PCR products suitable for sequencing were obtained from 6 reptiles. Sequence analysis confirmed that the amplicons obtained belonged to *C. pneumoniae* (Fig. 1). These findings demonstrated that *C. pneumoniae* is capable of infecting several species of snakes and turtles, broadening the range of reptiles known to be infected by this primarily human pathogen. After confirmation of infection, reptiles were subjected to specific antibiotic treatment. All the caregivers' pharyngeal swabs tested negative for *C. pneumoniae*, excluding active human infection.

In the second visit to the recreational centre in December, all cloacal and pharyngeal swabs analyzed were negative for *C. pneumoniae*.

In terms of immune response, IgM antibodies anti-*C. pneumoniae* were not detected in the caregivers' sera tested in May. One of the caregivers who maintained sporadic contact with the animals presented IgG titers of 1:512. Three caregivers with periodic contact showed IgG titers of 1:2048. One susceptible caregiver [IgG (-)] was the

only worker who always used protection when handling the animals or cleaning their excreta. In December, the caregiver who used protective equipment remained susceptible while IgG levels remained elevated in the others (1:2048). The immune status of a new caregiver who had reported pneumonia in September showed high titers of IgG anti-*C. pneumoniae* (1:4096); however specific IgM was not detected.

The presence of *C. pneumoniae* strains in reptiles is important because this bacterium is a common cause of human pneumonia and bronchitis throughout the world; in addition, it has recently been associated with several chronic diseases, including coronary heart disease, Alzheimer's disease, and multiple sclerosis^{3,4,13}. Although *C. pneumoniae* DNA or specific IgM was not detected in the caregivers, the detection of *C. pneumoniae* DNA in the animals demonstrated the circulation of this agent in the recreational park and supports the exacerbated immune response in people handling the reptiles, which suggests a potential zoonotic cycle. Respiratory diseases and excretion of this bacterium in the reptiles were attenuated by specific antibiotic treatment. The implementation of biosecurity measures is essential to prevent transmission, as demonstrated by our results.

In our study, as well as in other reports in a range of reptiles (snakes, iguanas, chameleons) and amphibians (frogs, turtles), the relationship of our sequences with other reference data sets of *chlamydiae* indicates that

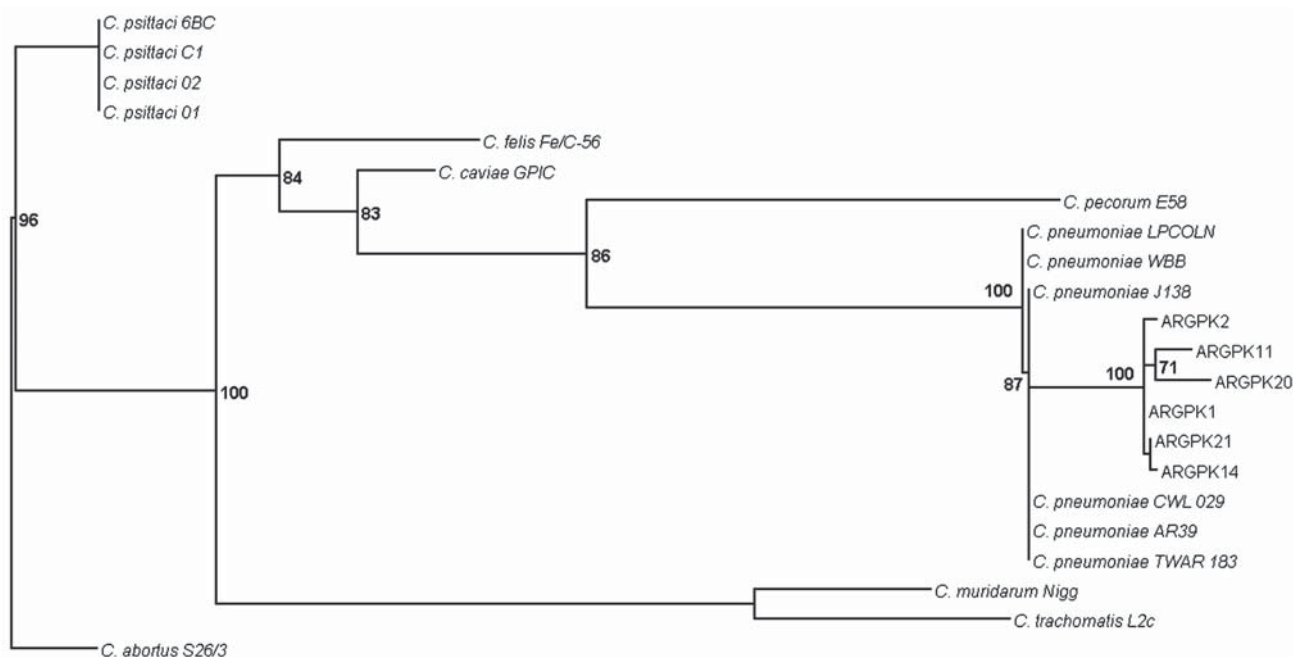


Figure 1 Maximum likelihood tree for the *rpoB* gene (441 nt) constructed using GTR+G as model of nucleotide substitution, with parameters suggested by ModelTest 3.7 (PhyML software). Sequences belonging to this study are initiated by ARG PK. Numbers above branches: bootstrap values over 2000 bootstrap pseudo replicates. Only bootstrap values >50% are shown at nodes. *C. abortus* S26/3 is used as out-group. *C. pneumoniae* LPCOLN: isolated from a koala. *C. pneumoniae* WBB: isolated from a bandicoot. *C. pneumoniae* TWAR 183, CWL029, AR39, J138: isolated from humans. *C. psittaci* 01: isolated from a pig. *C. psittaci* 02: isolated from cattle. *C. psittaci* C1: isolated from a sheep. *C. psittaci* 6BC: isolated from a parakeet. *C. felis* Fe/C-56: isolated from a cat. *C. caviae* GPIC: isolated from a guinea pig. *C. pecorum* E58: isolated from cattle. *C. muridarum* Nigg: isolated from a mouse. *C. trachomatis* L2c: isolated from humans. *C. abortus* S26/3: isolated from a sheep.

although our sequences belonged to the *C. pneumoniae* branch, they are quite distinct from the human TWAR strain (Fig. 1)^{1,6,11}.

Even though a limited amount of sequences were analyzed, we found that all *C. pneumoniae* sequences clustered among them and revealed segregation with sequences of human strains. Although transmission among humans is believed to occur from person to person through respiratory secretions^{3,13}, until more is known about the epidemiology, pathogenicity, and transmission of *C. pneumoniae* strains recovered from captive and wild populations of cold-blooded animals, their zoonotic potential must be considered. Moreover, because of the increasing ownership of reptiles as pets, caution must be exercised during direct handling of these animals.

Isolation and characterization of reptile *C. pneumoniae* strains are required to assess their clinical impact and possible zoonotic potential.

Ethical responsibilities

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this investigation.

Confidentiality of data. The authors declare that they have followed the protocols of their work centre on the publication of patient data and that all the patients included in the study have received sufficient information and have given their informed consent in writing to participate in that study.

Right to privacy and informed consent. The authors declare that no patient data appears in this article.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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V. Ré is a scientific member of CONICET, Argentina.

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