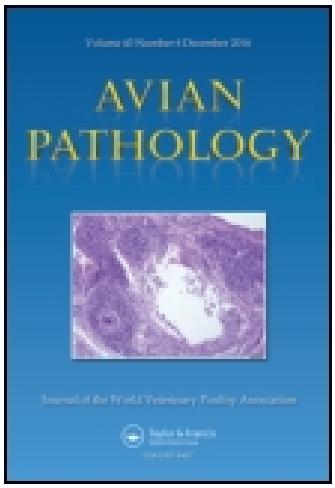

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Genetic diversity of Chlamydia among captive birds from central Argentina

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ORIGINAL ARTICLE

Genetic diversity of *Chlamydia* among captive birds from central Argentina

María C. Frutos[1*], Marina S. Monetti[1], Lucia Gallo Vaulet[2], María E. Cadario[3], Marcelo Rodríguez Fermepin[2], Viviana E. Ré[1], and Cecilia G. Cuffini[1]

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To study the occurrence of *Chlamydia* spp. and their genetic diversity, we analysed 793 cloacal swabs from 12 avian orders, including 76 genera, obtained from 80 species of asymptomatic wild and captive birds that were examined with conventional nested polymerase chain reaction and quantitative polymerase chain reaction. *Chlamydia* spp. were not detected in wild birds; however, four species (*Chlamydia psittaci*, *Chlamydia pecorum*, *Chlamydia pneumoniae* and *Chlamydia gallinacea*) were identified among captive birds (Passeriformes, $n = 20$; Psittaciformes, $n = 15$; Rheiformes, $n = 8$; Falconiformes $n = 2$; Piciformes $n = 2$; Anseriformes $n = 1$; Galliformes $n = 1$; Strigiformes $n = 1$). Two pathogens (*C. pneumoniae* and *C. pecorum*) were identified simultaneously in samples obtained from captive birds. Based on nucleotide-sequence variations of the *ompA* gene, three *C. psittaci*-positive samples detected were grouped into a cluster with the genotype WC derived from mammalian hosts. A single positive sample was phylogenetically related to a new strain of *C. gallinacea*. This report contributes to our increasing understanding of the abundance of *Chlamydia* in the animal kingdom.

Introduction

Chlamydiae are obligate intracellular bacteria responsible for important clinical and epidemiological implications worldwide, both in human and veterinary medicine (Kuo & Stephens, 2011).

Psittacosis is a well-known human disease caused by *Chlamydia psittaci* (Rodolakis & Yousef Mohamad, 2010), which is acquired from poultry and wild birds. Additionally, pet birds (primarily parrots) are still considered the primary transmitters (Andersen & Franson, 2007; Laroucau *et al.*, 2009). In wild birds, isolates of *C. psittaci* have been reported from more than 460 avian species, as well as from mammals such as hares and muskrats (Andersen & Varompay, 2003; Smith *et al.*, 2005). *C. psittaci* may produce clinical and/or subclinical infections in birds. Some infected birds may appear healthy and shed the organism intermittently. Shedding can be exacerbated by internal factors such as immune status, in addition to external factors like stress, reproductive activities, rearing of young pigeons, relocation, shipping, crowding and chilling (Smith *et al.*, 2005). The clinical signs vary greatly in severity and depend on the species and age of the birds, as well as the specific causative strains involved (Andersen & Varompay, 2000; Andersen & Franson, 2007; OIE, 2013). *C. psittaci* is excreted in the faeces and nasal discharge of infected birds. Typical human transmission pathways involve inhalation of infectious aerosols while handling infected animals, carcasses or tissues (Beeckman & Vanrompay, 2009). *C. psittaci* is classified into nine genotypes: A to F, E/B, M56 and WC. Sequence analysis of the outer membrane protein A (*ompA*) gene is one way to identify all of the already known genotypes and eventually new genotypes (Geens *et al.*, 2005). However, Sachse *et al.* (2008) have suggested adjustments and extensions to the current scheme, which include the introduction of subgroups to the more heterogeneous genotypes A, E/B and D, as well as six provisional genotypes representing so far untypable strains.

Chlamydial infections in birds count among the longest documented zoonotic infections, but recently several authors have suggested that the spectrum of diseases caused by such chlamydial infections may be much wider than realized (Zhang *et al.*, 1993; Berger *et al.*, 1999; Soldati *et al.*, 2004; Mitchell *et al.*, 2010; Sachse *et al.*, 2012).

Chlamydia pneumoniae has an extremely diverse host range; it has been reported in humans, horses, reptiles, amphibians and several Australian marsupials, including koalas and bandicoots, but has rarely been found in birds (Berger *et al.*, 1999; Hotzel *et al.*, 2001; Bodetti *et al.*, 2002; Jacobson *et al.*, 2004; Cochrane *et al.*, 2005; Mitchell *et al.*, 2010). *Chlamydia pecorum* is a pathogen found in ruminants, swine and koalas of several countries (Zhang *et al.*, 1993; Jackson *et al.*, 1999; Longbottom & Coulter, 2003). This species has also been detected previously in captive birds from Japan and in the central area of Argentina (Tanaka *et al.*, 2005; Frutos *et al.*, 2012a).

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Recently, Sachse *et al*. (2012) have found *C. pecorum* in urban pigeons for the first time in Germany.

While the recognized hosts are widely acknowledged as transmitters of chlamydial infections, the established knowledge of host restriction of certain chlamydial species is constantly modified by newer studies that report the detection of chlamydial species in animals that are not or rarely identified as host species. Therefore, this study intends to contribute to improving our understanding of the occurrence and genetic diversity of *Chlamydia* in captive and feral birds from Córdoba Argentina.

Materials and Methods

Samples. This study was conducted as a part of the surveillance programme for *Chlamydia* and *Arbovirus* approved and promoted by the Ministry of Environment of Córdoba province.

Cloacal swabs from 505 feral birds were collected during the period 2009 to 2012 from three different places—the Mar Chiquita bird sanctuary, Province of Cordoba (30°50′ S; 62°54′ W) *(n* = 254); Dean Funes city (35° 25′S; 64° 21′W) *(n* = 169); and rural areas of Vicuña Mackenna (33°56′S; 64°22′W) *(n* = 82)—and from 288 captive birds (76 samples from zoo parks and 212 households pets) from Córdoba city (31°;26′S; 64° 09′W), central region of Argentina (Supplementary data 1). These samples were stored at 4°C and transferred to the Institute of Virology, School of Medicine, National University of Córdoba, Argentina. In total, 12 avian orders including 76 genera and 80 species of feral and captive birds were studied. The captive birds had no clinical signs compatible with chlamydial infection.

The species and source of the samples are shown in Supplementary data 2.

DNA extraction. The cotton swabs were placed in 1 ml sucrose–phosphate–glutamate (Warford *et al.*, 1984) and 200 µl of this solution were subjected to DNA extraction using the Accuprep Genomic DNA Extraction Kit (BIONEER, Alameda, CA, USA) according to the manufacturer's instructions. DNA extracted from the L2/434Bu strain of *Chlamydia trachomatis* was used as a positive control. The extracted DNA was stored at 4°C.

Generic polymerase chain reaction for *Chlamydia* spp. DNA extract (5 µl) was used to amplify a fragment of 576 base pairs (bp) of the variable domains III and IV of the *ompA* gene of *Chlamydia* spp., using primers 191CHOMP (GCI YTI TGG GAR TGY GGI TGY GCI AC) and CHOMP 371 (TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC), as described by Sachse & Hotzel (2003).

Nested polymerase chain reaction for *C. psittaci* and *C. pecorum*. Two microlitres of templated DNA of generic polymerase chain reaction (PCR) were used for specific nested PCR for amplified *C. psittaci* and *C. pecorum* using primers 218PSITT (GTA ATT TCI AGC CCA GCA CAA TTY GTG) and CHOMP 336 (CCR CAA GMT TTT CTR GAY TTC AWY TTG TTR AT) for *C. psittaci* (404 bp) and primers 204 PECOR (CCA ATA YGC ACA ATC KAA ACC TCG C) and CHOMP 336 for *C. pecorum* (441 bp) (Sachse & Hotzel, 2003).

C. psittaci genotype identification was confirmed by sequence analysis using the *ompA* gene.

Hemi-nested PCR for *C. pneumoniae*. Based on the positive results of the generic PCR, we decided to investigate the presence of *C. pneumoniae*. Primers described by Campbell *et al*. (1998) and Mass *et al*. (1998) were used to amplify a fragment of the *rpoB* gene of *C. pneumoniae:* primers HL1 (GTT GTT CAT GAA GGC CTA CT) and HR1 (TGC ATA ACC TAC GGT GTG TT), and primers N1 (AGT TGA GCA TAT TCG TGA GG) and N2 (TTT ATT TCC GTG TCG TCC AG).

We modified the protocol and optimized a hemi-nested PCR by combining the primers HL1/N2 and N1/HR1 to amplify two fragments: 273 bp and 249 bp, respectively. These fragments overlapped one another and allowed us to obtain a 441 bp sequence of the *rpoB* gene of *C. pneumoniae*.

Real-time PCR targeting the 23S *rRNA* gene. In this study, a Chlamydiaceae-specific real-time PCR targeting the 23S rRNA gene was used only in positive nested PCR samples with the purpose of quantification (Everett *et al.*, 1999b). The quantitative PCR was carried out in a final volume of 25 µl containing 5 µl extracted DNA using an ABI Prism7000 thermocycler (Applied Biosystems, Buenos Aires, Argentina). The cycle threshold value (Ct) was calculated automatically. Each sample was examined in duplicate. The TaqMan test was able to detect as few as 1 inclusion-forming unit or elementary body, or seven targets (Everett *et al*., 1999b).

Sequencing. After the second step, products of the nested PCR were purified by gel electrophoresis using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and subjected to a direct nucleotide sequencing reaction in both directions using the internal (second-round) PCR primers by Macrogen, Inc. (Seoul, Korea).

Sequence analysis and dendrogram constructions using the *ompA* gene and the *rpoB* gene. The sequences obtained from regions *of the ompA* gene and *the rpoB* gene were edited and prepared with BioEdit v 7.0.9 (Hall, 1999) and subsequently aligned with ClustalX 2.12 (Larkin *et al.*, 2007), along with the sequences downloaded from the GenBank. Relatedness of newly characterized sequences was assessed by analysis with the 2.2.19 Basic Local Alignment Search Tool.

The dendrogram was constructed using the TreeExplorer module of the MEGA program 4 (Tamura *et al*., 2007) with the neighbour-joining method and the *p*-distance parameter. The branch support was evaluated by non-parametric bootstrapping with 1000 pseudo-replicas.

Results

A total of 793 samples were tested for *C. psittaci, C. pecorum* and *C. pneumoniae* using the nested PCR assay. None of the *Chlamydia* species was detected in feral birds; however, 17.4% (50/288) of the samples from captive birds were positive. Specific nested PCR confirmed the occurrence of *C. pneumoniae* in 48% (*n* = 24), *C. pecorum* in 22% (*n* = 11) and *C. psittaci* in 6% (*n* = 3) of the positive samples. Eleven (22%) birds presented mixed infections of *C. pneumoniae* and *C. pecorum* (Table 1). In our study, only one sample was negative when tested with specific nested PCR.

By sequencing and analysis of the genetic composition of *the ompA* gene and *the rpoB* gene, genetic diversity and associations among the detected positive samples of *Chlamydia* spp. were determined. The sequences obtained in this study were deposited in GenBank under the following accession numbers: JX399852 to JX399854 for the *ompA* region of the *C. psittaci*, JX399855 for the *ompA* region of *C. gallinacea*, JN016880 to JN016884 for the *ompA* region of *C. pecorum,* and JX645161 to JX645175 and JX649919 for the *rpoB* region of *C. pneumoniae*.

Genetic analysis of *the ompA* gene for *C. psittaci* revealed that bird samples (ARG AMB 208, ARG AMB 204 and ARG AMB 209) grouped in the genetic cluster represented for WC strains associated with mammal hosts (Figure 1). The Basic Local Alignment Search Tool analysis revealed that *ompA* sequences of sample ARG AMB P3 exhibited a degree of similarity close to a group of sequences of *Chlamydia gallinacea* found in the French psittacosis outbreak in France (GQ398033, GQ398036) (Sachse *et al*., 2014). The *ompA* sequence of *C. pecorum*-positive samples was highly homologous and shared more than 98% similarity between each other (Figure 1). Genetic analysis of *the rpoB* gene for *C. pneumoniae* revealed that bird samples grouped together in a separate cluster (Figure 2).

The comparison of Ct values with quantitative PCR demonstrated different levels of presence of *Chlamydia* in the cloaca. *Piranga*, *Paroaria* and *Sicalis* birds (Ct: 31) showed a higher level of occurrence than *Rhea*,

Table 1. *Occurrence of* Chlamydia *in 288 captive birds (pet in households,* n = *212; zoo parks,* n = *76).*

	Classification of the avian fauna			Chlamydial species (*n*)				
Habitat	Order	Family	Genus	*C. psittaci*	*C. pneumoniae*	*C. pecorum*	*C. pneumoniae/ C. pecorum*	Total
Pet in household	Anseriformes	Anatidae	*Anser*	–	1	–	–	1
	Galliformes	Phasianidae	*Gallus*	–	1	–	–	1
	Passeriformes	Emberizidae	*Cyanocompsa*	–	1	1	3	6
			Gubernatrix	–	–	1	–	1
			Diuca	1	–	–	–	1
			Paroaria	1	1	1	3	6
			Pheucticus	–	1	–	–	1
			Sicalis	–	1	1	–	2
		Thraupidae	*Piranga*	–	–	–	1	1
		Turdidae	*Turdus*	1	–	1	–	2
	Psittaciformes	Psittacidae	*Melopsittacus*	–	–	–	2	2
Total (*n,* %)				3 (12.5)	6 (25)	5 (20.8)	9 (37.5)	24 (11.3)
Zoo parks	Falconiformes	Falconidae	*Falco*	–	1	–	–	1
			Polyborus	–	1	–	–	1
	Piciformes	Ramphastidae	*Ramphartor*	–	2	–	–	2
	Psittaciformes	Psittacidae	*Agapornis*	–	6	–	–	6
			Cyanoliseus	–	1	–	–	1
			Nymphicus	–	1	4	–	5
			Psittacula	–	–	1	–	1
	Rheiformes	Rheidae	*Rhea*	–	5	1	2	8
	Strigiformes	Tytonidae	*Tyto*	–	1	–	–	1
Total (*n,* %)				–	18 (69.2)	6 (23.1)	2 (7.7)	26 (34.2)

Gubernatrix and *Pheucticus* (Ct: 38) (Ct ≤39; the r^2 linearity value from the linear regression was 0.9935 and efficiency = 10 [−1 / slope] − 1 = 99%).

Table 1 shows that *Chlamydia* spp. were predominantly detected among eight Passeriformes avian species. This included positive rates of 8.3% in the Turdidae family (2/24), 7.7% (17/220) in the Emberizidae family and 6.7% (1/15) in the Thraupidae family. In Psittaciformes birds, we detected *Chlamydia* in 19.7% (15/76) only in the Psittacidae family. However, in birds from the Falconidae family, we detected *Chlamydia* spp. in 28.6% (2/7). We also detected *Chlamydia* in 88.9% (8/9) of the birds from the Rheidae family and 1.2% (1/81) in Galliformes birds (Phasianidae family). In birds of the Piciformes order, Ramphastidae family, *Chlamydia* was found in 22.2% (2/9) of the subjects.

The table also shows that *C. pneumoniae* was the main species detected in captive birds. These bacteria were found in Passeriformes (*n* = 4), Psittaciformes (*n* = 8), Rheiformes (*n* = 5), Falconiformes (*n* = 2) and Piciformes birds (*n* = 2), and among one specimen of Strigiformes, Anseriformes and Galliformes birds. *C. pecorum* was detected in Passeriformes (*n* = 5) and Psittaciformes (*n* = 5), and one sample from Rheiformes birds. *C. psittaci* was detected only in three samples of Passeriformes birds. Mixed infections among *C. pneumoniae* and *C. pecorum* were detected in Passeriformes (*n* = 7), Psittaciformes (*n* = 2) and Rheiformes birds (*n* = 2), respectively. *C. gallinacea* was detected in a Passeriformes bird (*n* = 1).

Among household birds, we detected *C. psittaci* in 12.5% (3/24), *C. pecorum* in 20.8% (5/24), *C. pneumoniae* in 25% (6/24) and mixed infections in 37.5% (9/24) of the *Chlamydia*-positive samples. However, *C. psittaci* was not found in birds of zoo parks, while *C. pneumoniae* was detected in 69.2% (18/26), followed by *C. pecorum* in 23.1% (6/26). In addition, we detected mixed infections in 7.7% (2/26) of the samples. The new chlamydial agent *C. gallinacea* was detected in 4.1% (1/24).

Discussion

In this study, nested PCR and hemi-nested PCR were used to detect and genetically characterize *Chlamydia* spp. For the molecular detection of *C. pecorum* and *C. psittaci*, we used partial *ompA* gene amplification. The choice of the genomic region was based on the fact that it is a region widely associated with the genetic divergence of *Chlamydia* spp. (Poole & Lamont, 1992; Batteiger *et al*., 1996; Longbottom & Coulter, 2003; Sachse & Hotzel, 2003; Geens *et al*., 2005; Kaulfold *et al*., 2006; Laroucau *et al*., 2009; Pantchev *et al*., 2009). Amplification of the *rpoB* gene was used for *C. pneumoniae* detection, based on recommendations of the National Center for Infectious Disease Control (Dowell *et al*., 2001).

With these amplification strategies, a large panel of avian samples was examined, revealing the presence of *Chlamydia* spp. in an extended host range of captive birds (20 species of Passeriformes and non-Passeriformes birds); however, these bacteria were not detected in wild birds. While both wild and captive birds suffered stress at capture time, it is possible that the disruption of the ecological and environmental preservation in captive birds was the cause of the *Chlamydia* detection.

C. pneumoniae was the species most commonly detected in captive birds, followed by *C. pecorum* and *C. psittaci*. In addition, *C. gallinacea* and mixed infections of *C. pneumoniae*/*C. pecorum* were also found.

Among Passeriformes, mainly *Paroaria*, a high occurrence of a wide diversity of *Chlamydia* was found, along with higher levels of chlamydial presence. These results suggest

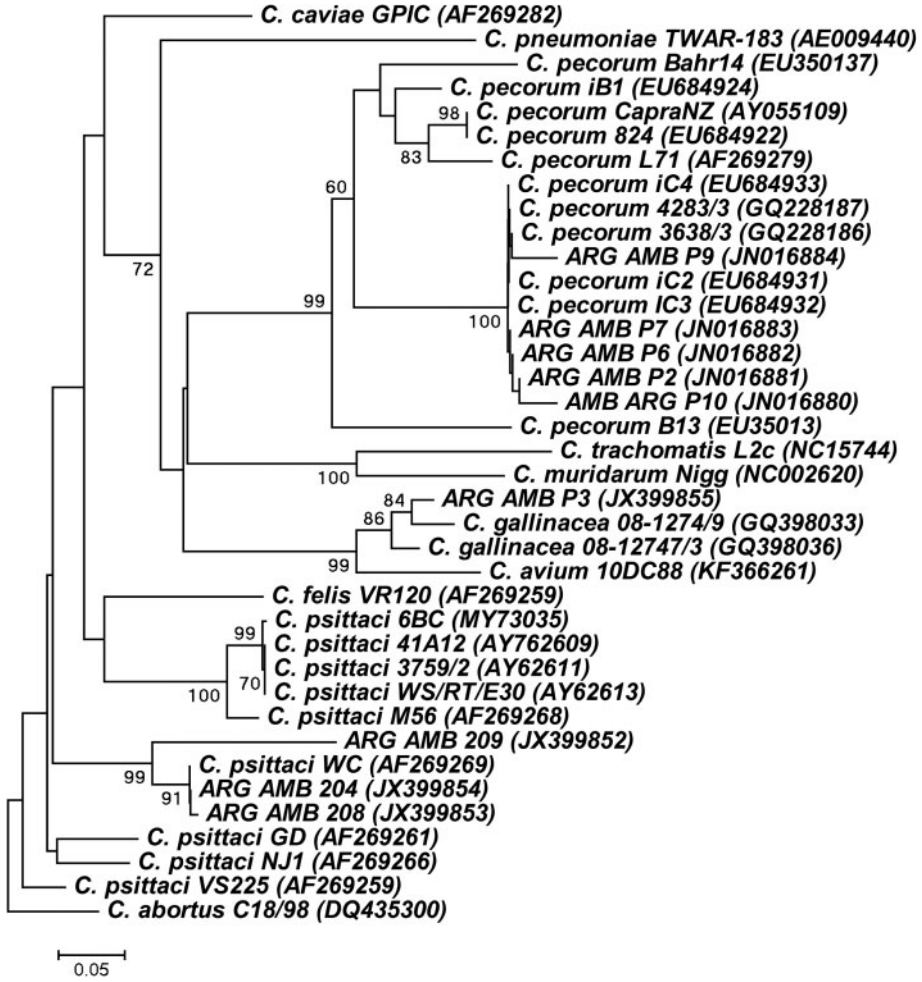

Figure 1. *Neighbour-joining dendrogram based on comparison of 404 bp of the* ompA *gene of* Chlamydia*. Samples that belong to this study have the prefix ARG AMB and GenBank accession numbers provided. Numbers above branches are bootstrap values as a percentage of 1000 pseudo replicates and only bootstrap values >60% are shown.* Chlamydia abortus *C18/98 was used as an out-group. Scale bar shows the percentage sequence diversity.*

their potential role as reservoirs of *Chlamydia* in our region. However, the occurrence of *Chlamydia* in Passeriformes is more surprising. Some researchers have not been able to demonstrate the presence of *Chlamydia* in Passeriformes (Prukner-Radovcic *et al*., 2005; Chahota *et al*., 2006), while others have reported frequencies of detection ranging from 0.8 to 54% (Hirai *et al*., 1983; Holzinger-Umlauf *et al*., 1997; Olsen *et al*., 1998; Kaleta & Taday, 2003; Dovc *et al*., 2005; Celebi & Ak, 2006; Madani & Peighambari, 2013).

C. psittaci is the most widely studied *Chlamydia* species among birds; it has been extensively reported that pigeons are the natural reservoirs, as well as the source of human infections (Fukushi *et al*., 1983; Haag-Wackernagel & Moch, 2004; Chahota *et al*., 2006; Yousef Mohamad & Rodolakis, 2010). However, samples from local pigeon yielded negative results. *C. psittaci* was detected only in Passeriformes birds (Emberizidae and Turdidae family); but did not appear to be associated with any clinical sign of disease in these birds. Thus, these birds could be subclinical carriers of *C. psittaci*. In addition, the molecular characterization revealed that the positive samples amplified in this study were closely related to the WC strain derived from mammalian genotypes (Everett *et al*., 1999a). This finding is in line with previous reports of our group (Frutos *et al*., 2012b) in which this *C. psittaci* genotype was detected among six of the nine human cases (Frutos *et al*., 2012b). These results indicate an important circulation of mammalian genotypes in Córdoba-Argentina, suggesting that mammals may represent an underestimated source of *C. psittaci* or that birds could carry strains associated with mammals.

The finding of *C. pecorum* in captive birds provided further evidence of its circulation in asymptomatic birds in our region (Frutos *et al*., 2012a); however, the epidemiological significance of *C. pecorum* is not clear at this stage. Further studies are needed to estimate the zoonotic role of this pathogen.

C. pneumoniae was the species most frequently detected, reflecting its endemic circulation in the local avifauna. Furthermore, the analysis of the amplified sequences showed a close association with the *C. pneumoniae* sequence previously isolated from human samples. Although *C. pneumoniae* has been described in several animal species, its zoonotic role remains unclear (Bodetti *et al*., 2002; Myers *et al*., 2009; Mitchell *et al*., 2010).

Another remarkable finding was the first detection of *C. gallinacea* in our region; this new species of *Chlamydia* had been previously found only in poultry (Gaede *et al*., 2008; Laroucau *et al*., 2009; Lemus *et al*., 2010; Sachse *et al*., 2014); however, in our study it was found in Ultramarine Grosbeak (Passeriformes, Emberizidae). Also, it is important to note that our study provides further evidence of the

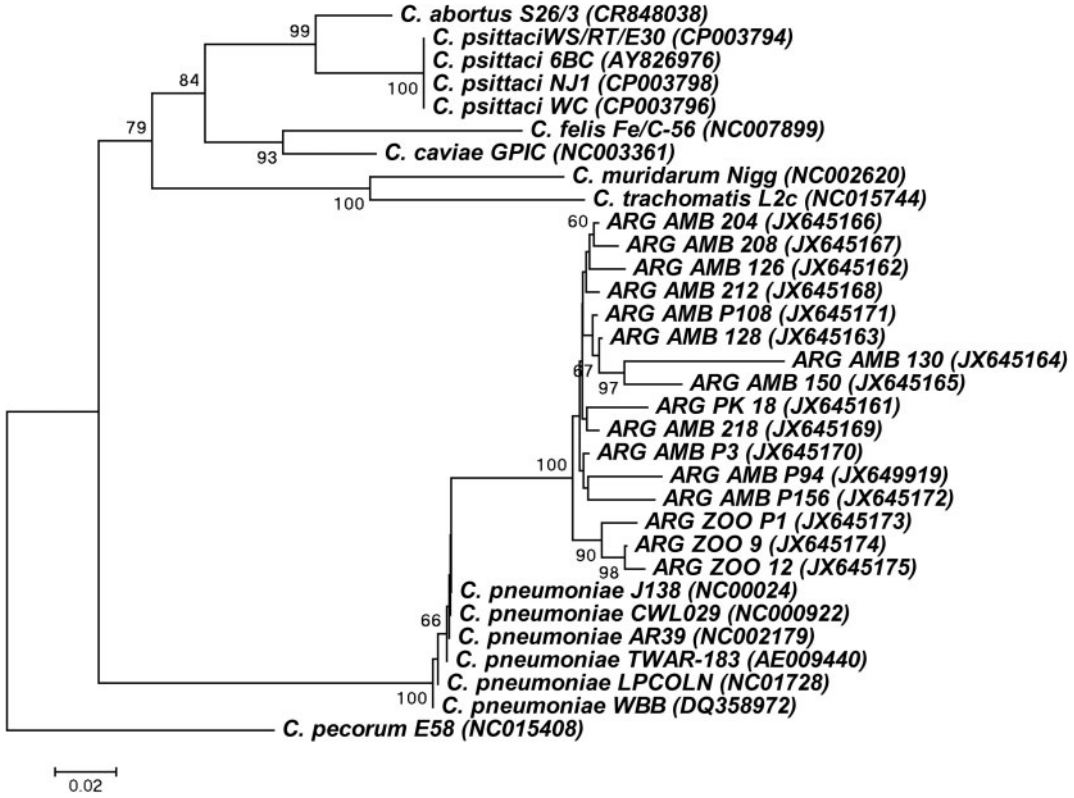

Figure 2. *Neighbour-joining dendrogram based on comparison of 441 bp of the* rpoB *gene of* Chlamydia*. Samples that belong to this study have the prefix ARG and GenBank accession numbers provided. Numbers above branches are bootstrap values as a percentage of 1000 pseudo replicates and only bootstrap values >60% are shown.* C. pecorum *E58 was used as an out-group. Scale bar shows the percentage sequence diversity.*

occurrence of mixed *Chlamydia* species among birds. This fact has also been reported by Tanaka *et al*. (2005) and Sachse *et al*. (2012).

The presence of *Chlamydia* in captive birds could represent a potential source of infection to caregivers, which emphasizes the need to implement biosecurity measures to mitigate the effects of a possible spread of infection. Several limitations of our study need to be considered. Isolation in cell cultures (CDC, 1998) was the preferred technique to confirm and corroborate the results obtained; however, this is not recommended in the case of *C. psittaci* because of the biological risks. On the other hand, it was not possible to isolate *C. pecorum* and *C. pneumoniae* in cell cultures, perhaps due to the lack of sterile and cooling conditions where the bird samples were collected.

In the literature, studies of bird chlamydial infections have usually been confined to the search for *C. psittaci*, so little is known about the presence of other *Chlamydia* species. The current findings confirm that our knowledge on the variety of chlamydial bird organisms is only partial.

In this study, positive samples were found mainly in two avian orders, namely Passeriformes and Psittaciformes; therefore, they should be taken into account when field studies are performed and their epidemiological importance should be considered.

This report is the first contribution to the identification and molecular characterization of *Chlamydia* spp. in captive birds of Argentina, and it contributes to improving our understanding on the abundance of *Chlamydia* in the animal kingdom.

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Supplemental data

Supplemental data for this article can be accessed *here*.

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