Enzootic Activity of Chlamydia in Farms Located in a Hotspot Area for Zoonosis Emergence

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Abstract

Chlamydias are obligated intracellular Gram-negative bacteria, considered important zoonotic pathogens, broadly present in several bird species and responsible for economic losses in animal production. We analyzed the presence of Chlamydial species with zoonotic risk in farm animals in a highly biodiverse area and with great human circulation, the Argentine, Brazil and Paraguay tri-border area. We surveyed nine farms in an area and nasally swabbed a total of 62 animals. DNA was extracted and specific PCR was performed to identify chlamydial species. We detected Chlamydia spp . in 6.5% (4/62) of the animals tested, positive samples belonged to cattle and none of them showed symptoms of respiratory disease nor had been diagnose with reproductive diseases. Specific nested PCR confirmed two samples belonged to C. pecorum and two to C. psittaci . We report for the first time Chlamydia circulation with zoonotic risk in the region. Surveys in birds and wild mammals could give a better understanding to know what Chlamydial species are circulating in the wild interface. The zoonotic potential should be taking into account as farm workers and the surrounding population could be silent carriers or have respiratory diseases being underdiagnosed, and therefore should be considered in the differential diagnoses.

Introduction

Chlamydias are obligated intracellular Gram-negative bacteria, which can cause serious infections both in humans and animals, such as birds and cattle. In the latter, it causes abortions, infertility, conjunctivitis, enteritis, respiratory diseases among others, thus leading to economic losses in productivity. In humans, it is associated with sexually transmitted infections (STIs) and respiratory diseases (Whittum-Hudson & Hudson AP. 2005; Mackern-Oberti et al., 2013)

They belong to the family Chlamydiaceae that includes only the genus Chlamydia; containing 15 known species (*C. trachomatis*, *C. avium*, *C. buteonis*, *C. caviae*, *C. felis*, *C. gallinacean*, *C. ibidis*, *C. muridarum*, *C. poikilothermis*, *C. serpentis*, *C. suis*, *C. abortus*, *C. psittaci*, *C. pecorum* and *C. pneumoniae*). The last three species have proven to be zoonotically relevant (Longbottom & Coulter 2003; Rohde et al., 2010; Staub et al., 2018) . *Chlamydia psittaci* is the most widely studied in avian and human populations and was first detected in 1879, in Switzerland, in a family that traded parrots from South America (Harris & Williams 1985). It causes human severe respiratory conditions that can lead to death. Infections in humans initiates by the inhalation of products from fluids of infected birds or by handling products derived from poultry (Vanrompay et al., 2007; Balsamo et al., 2017). In Argentina, reports of this disease dates from the beginning of the last century and new outbreaks are regularly recorded (Frutos et al., 2012a; Cadario et al., 2017).

Unlike *C. psittaci*, the description of *C. pneumoniae* and *C. pecorum* dates from the early 1990s. At first it was thought that *C. pneumoniae* only affected humans, but over time studies proved its zoonotic potential (Mitchell et al., 2010; Roulis et al., 2013) . In humans, it causes respiratory infections in adults and children, and at least 70% of the population may eventually become expose to this pathogen (Kuo et al., 1995).

Although its zoonotic potential is still under research, *C. pecorum* is an important pathogen of domestic livestock, mainly sheep and cows and is relevant to the conservation of koala (*Phascolarctos cinereus*) populations in Australia (Polkinghorne et al., 2013). Often asymptomatic, *C. pecorum* can cause polyarthritis, conjunctivitis, pneumonia, miscarriages, encephalomyelitis and gastrointestinal problems, thus, leading to economic losses (Jelocnik et al., 2015). Though studies are scarce in Argentina, it was detected in birds seized from illegal trafficking in the province of Córdoba (Frutos et al., 2012b). Nonetheless, it is necessary to expand the geographical scope of research and the range of hosts that can act as reservoirs.

In the last decade several studies were carried out on the circulation of pathogens with zoonotic relevance in the triple border between Argentina, Brazil and Paraguay (Rivero et al., 2017; Thomaz-Soccol et al., 2018; Valente et al., 2019). This region is a potential hotspot for zoonotic diseases emergence due to the massive interchange of people driven by trade and tourism activities around the Iguazu Falls (Scarpaci 2012). Thus, the area host several protected areas of remnants of Atlantic Forest with a high biodiversity of fauna and flora (Di Bitetti et al., 2003; Galindo-Leal & Câmara 2003). Moreover, in all three countries, land-use change has increased due to the expansion of agricultural activities, generating tensions over land tenure and increasing socio-economic inequalities among local residents (Galeano, 2012). Under this scenario, the aim of this work was to study the circulation of Chlamydias of zoonotic relevance in farm animals on the Argentine side of the triple border.

Materials And Methods

Study site and sample collection.

Field sampling was carried out in nine farms located in the province of Misiones, Argentina, in the border of Brazil and Paraguay (25°40'13"S 54°02'34"W) (Figure 1). This rural area limits with two major protected areas, Iguazu National Park in Argentina, and the Iguaçu National Park in Brazil. We involved local stakeholders by identification a first key stakeholder that then, allowed contact with the other owners in the area. We informed the farm owners involved about the project and that all information provided was anonymous and confidential (e.g., the exact location of their properties). Participation was voluntary and they could withdraw with no explanation. Finally, taking into account the guidelines of the International Society of Ethnobiology-ISE (2006), we informed the results and conclusions of the project (ISE 2006).

Field work was carried out in 9 farms from September to November 2018. Nasal swabs were obtained with brush-tipped swabs, making circular movements during 10 seconds, and placed them in a 1ml tube containing SPG (sucrose–phosphate–glutamic acid buffer).

Figure 1. Map illustrating the study area, the different land uses and farms sampled and those that tested positive for Chlamydia.

DNA extraction

We subjected 200 µl of sample swabs to DNA extraction using the DNeasy Blood & Tissue KitQIAGEN® (Cat No. /ID: 69506) (QIAGEN) commercial kit, following the manufacturer's specifications. 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.

The DNA obtained was first subjected for the detection of a 576 bp fragment of the variable domains II, III and IV of the ompA gene of Chlamydia. The process was performed according to Frutos et al., (2015). Oligonucleotides were selected according to Sachse and Hotzel (2003). The selected external oligonucleotides primers were 191CHOMP (GCI YTI TGG GAR TGY GGI TGY GCI AC) and CHOMP 371 (TTA GAA ICK GAA TTG IGC RTT IAY GTG IGC IGC). DNA fragments were amplified (5 µl) by adding 0.2 mM of the respective primers, 0.8 mM of dNTPs and 1 unit of enzyme Gotaq polymerase (Invitrogen, Life Technologies, Carisbad, CA) in final volume 50 µl. We used a *C. trachomatis* strain L2c (CP002024) from the Chlamydia laboratory of the "Dr. J. M. Vanella" Institute of Virology as positive control.

Nested-PCR for the determination of *C. psittaci, C. pneumoniae* and *C. pecorum*

Based on the positive results of the generic PCR we performed this procedure, as described by Frutos et al., (2015). We used 2 µl of PCR I product and 0.2 mM of the respective internal primers, 0.8 mM of dNTPs and 1 unit of enzyme Gotaq polymerase (Invitrogen, Life Technologies, Carisbad, CA) were added in final volume 50 µl.

Chlamydia psittaci strain VS225 (CP003793), *C. pecorum* strain 2047 (GQ228191) and *C. pneumoniae* strain TWAR 183 from the Chlamydia laboratory of the Institute of Virology "Dr. J. M. Vanella" were used as positive controls.

Visualization of the DNA fragments obtained by nested-PCR

Amplified DNA fragment were separate by electrophoresis in 1.5% agarose gel containing 0.5 ul/ml of ECO-Gel 20.000X Highway dye and visualized through an ultra-violet transilluminator (Sigma).

Sequencing and Phylogenetic analysis

PCR products were purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, US) and subjected to direct nucleotide sequencing Sanger reaction in both directions in Macrogen, Inc. (Seoul, Korea).

Alignment of nucleotide sequences was performed with MEGA 6.0 software package (Kumar et al., 2018). Phylogenetic trees were generated using the Maximum Likelihood method with MEGA 6.0 software package. A bootstrap re-sampling analysis was performed (1,000 replicates) to test tree robustness. The reference strains used for the phylogenetic trees were obtained from the NCBI GenBank Database.

Nine reference sequences of the ompA region of the *C. pecorum* genome (403 bp) were included in this analysis. The corresponding accession numbers, country of origin, host and clinical condition of these isolates are shown in Table 1.

Results

A total of 62 nasal swabs were obtained from two horses, nine pigs and 51 cows. *Chlamydia* spp. infection were obtained in 6.5% (4/62) of the animals sampled. All positive samples belonged to cattle from two of the nine sampled farms (Figure 1)). Specific nested PCR confirmed the occurrence of *C. pecorum* (n = 2) and *C. psittaci* (n = 2). In farm "A" 1 out of 10 animals tested positive for *C. psittaci* and in farm "J" 3 out of 7 animals tested positive, two for *C. pecorum* and one for *C. psittaci*. None of the samples were positive for *Chlamydia pneumoniae.*

Genetic diversity and relationships of isolated *C. pecorum* and *C. psittaci* strains were analyzed by sequencing ompA and rpoB genes. Obtained sequences were deposited in GenBank under the following accession numbers: MW888425 and MW888425 for the ompA region of the *C. psittaci,* MW888427 and MW888428 for the ompA region of the *C. pecorum.*

Chlamydia psittaci isolated strains (J006 and A003) grouped with strains previously described in both birds and mammals from different regions of the world as well as with strains identified in the central region of Argentina (Figure 2). *Chlamydia pecorum* strains (J005 and J008) grouped together with strains isolated from birds in Argentina and different mammals such as livestock and koalas (Figure 2).

Discussion

In cattle, *C. pecorum* and *C. psittaci* can generate subclinical infections or respiratory symptoms, encephalomyelitis or genital sexual-borne infections, affecting fertility, as well as miscarriages with great losses in production (Li et al., 2016; Barati et al., 2017). Although there are reports of *Chlamydia pecorum* in humans, its zoonotic potential is yet to be understood (Frutos et al., 2015). Some authors suggest that *C. pecorum* infection is endemic in livestock worldwide, but prevalence is yet unclear, since veterinary chlamydial diagnostics are limited to study genus only (Sachse et al., 2009; Walker et al., 2015).

Li et al., (2016) reported prevalences of several chlamydia species from both dairy and beef cattle production in China. Although in their research they did not test nasal swabs, they tested whole blood, vaginal swabs, feces and milk. In their study they reported high prevalence of *C. pecorum* in fecal samples, indicating *C. pecorum* as an endemic species optimally adapted to cattle, so it easily spreads throughout the intestinal tract. Our findings in nasal mucosa could indicate that *C. pecorum* it is not only present in intestinal tract; suggesting the aerial transmission, and support the idea of a prevalent pathogen in cattle worldwide. Moreover, several authors refer to the fact that *C. pecorum* may be asymptomatic in cattle, as our findings show (Reinhold et al., 2008; Poudel et al., 2012; Li et al., 2016).

In cattle, *C. psittaci* causes low milk productivity, respiratory disease, abortions, and its pathogenicity has been demonstrated experimentally (Borel et al., 2006; Ostermann et al., 2013; Van Loo et al., 2014). However, it is likely that *C. psittaci* infections in cattle occur in sporadic events, due to possible interactions with birds (Li et al., 2016). Li et al., (2016) reports a much lower prevalence (10%), comparing to *C. pecorum* (75%). In their study, sequenced strains had a genetic similarity close to pigeons. In that sense the circulation of *C. psittaci* is documented for several bird species in Argentina (Frutos et al., 2016). Although *C. psittaci* infection is mainly associated with birds, the findings of this work indicate that mammals are also a source of *C. psittaci* and may be carriers of strains associated with birds, as proposed by Frutos et al., (2014).

While our study was restricted to farm animals, it is important to highlight that the study site is located in the vicinity to an area of high wildlife conservation value, the Iguazu National Park in Argentina and the Iguaçu National Park in Brazil. To date we do not know which species of Chlamydia could affect the local fauna and what their relevance could be at the sanitary and conservation level. In the farms surveyed, many wild species frequent the area, and may have contact with domestic animals. This interaction (domestic/wild hosts) can result in the transmission of pathogens in both ways, by spillover from wildlife to domestic animals or vice versa. As an example, in Australia it has become a huge conservation issue for the koala (*Phascolarctos cinereus*) populations since the introduction of *C. pecorum* from infected livestock, causing in koalas ocular and reproductive disorders and thus, increasing mortality rates (Polkinghorne et al., 2013; Bachmann et al., 2014). Regarding *C. psittaci*, birds living in protected areas frequently visit farms, such as parrots and toucans, due to food availability (fruit trees, seeds and palms) and the scarce presence of predators. Cattle can be infected either by ingesting contaminated pastures by bird feces or seeds that they discard during the flight, making it difficult to control and eradicate the disease in the herds. In farming areas close to natural reserves, monitoring chlamydial species that affect wildlife can be an important tool to have a better understanding of the occurrence and potential of emergence of this pathogen in the area.

Regarding its zoonotic potential, it is necessary to analyze samples from farmers that may or may not have clinical manifestations of respiratory disease, being the infections under-diagnosed, and that are in close contact with the potential cattle reservoir of *Chlamydia* spp. In this study, the animals that tested positive and its owners did not show symptoms of respiratory disease, nor have they mentioned cases of recent abortions by the cattle.

This is the first study that detected the presence of two species of Chlamydia in the same study area in the triple border area of Argentina, Brazil and Paraguay: *Chlamydia pecorum* and *C. psittaci*, and the first report of *C. pecorum* in bovines for Argentina. The phylogenetic analysis of the strains detected in our study shows a genetic closeness to strains previously detected in central Argentina, which also indicates a regional clustering. Our study provides the basis to deepen the circulation of chlamydia in the area and to understand which animal species could be its hosts and amplifiers. The chlamydial species found are of zoonotic risk and therefore should be included as differential diagnoses in cases of respiratory symptoms in humans.

Monitoring birds and poultry in the proximity of the farms is strongly suggested to assess Chlamydial circulation. As in cattle, these diseases should be considered as differential diagnosis in spontaneous abortions or reproductive diseases. Therefore, it is necessary to extend surveillance and deepen studies on chlamydial species involved in animal pathology and their zoonotic potential, since information in the region is scarce.

Declarations

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Ethical approval: Guidelines for the care and use of animals were followed by the ICLAS Ethical Guideline for Researchers. The Argentine legislation did not require an ethics protocol committee to approve our work.

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate: All the authors consented to participate in this study.

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Tables

Table 1. Chlamydial strains used in partial ompA DNA sequencing in our study.

Chlamydia strain	Host	Clinical Signs	Country origin	GenBank accesion	Reference
C.psittaci	*Equus caballus*	Aborted foetus	Australia	KY287781	Jelocnik et al., 2017
	Melopsittacus undulatus	Systemic infection	Not reported	M73035	Kaltenboeck et al., 1993
	Amazona aestiva	Hepatic disease	Brazil	MH138293	Vilela et al., 2019
	Meleagris gallopavo	Nasal discharge	Belgium	AY762609	Geens et al., 2005
	Bos taurus	No clinical signs	Argentina	MW888425	This study
	Bos taurus	No clinical signs	Argentina	MW888425	This study
	Diuca diuca	No clinical signs	Argentina	JX399853	Jelocnik et al., 2017
	Paroaria coronata	No clinical signs	Argentina	JX399854	Kaltenboeck et al., 1993
	Bos taurus	Enteritis	USA	AF269269	Vilela et al., 2019
	epizootic	Not reported	USA	AF269268	Geens et al., 2005
	Bos taurus	Pneumonia	Germany	EU350138	Jelocnik et al., 2017
C.pecorum	*Bos Taurus*	Diarrhea	Japan	LC021422	Kaltenboeck et al., 1993
	Paroaria coronata	No clinical signs	Argentina	JN016882	Vilela et al., 2019
	Bos taurus	No clinical signs	Argentina	MW888427	This study
	Bos taurus	No clinical signs	Argentina	MW888428	This study
	Gubernatrix cristata	No clinical signs	Argentina	JN016884	Frutos et al., 2015
	Capra aegagrus hircus	No clinical signs	France	EU684933	Mohamad et al., 2008
	Bos taurus	Diarrhea	Japan	LC021419	Ohtani et al., 2015
	Capra aegagrus hircus	No clinical signs	France	EU684932	Mohamad et al., 2008
	Phascolarctos	Urogenital	Australia	KU214244	Legione et al.,

cinereus	infection	2016

Figures

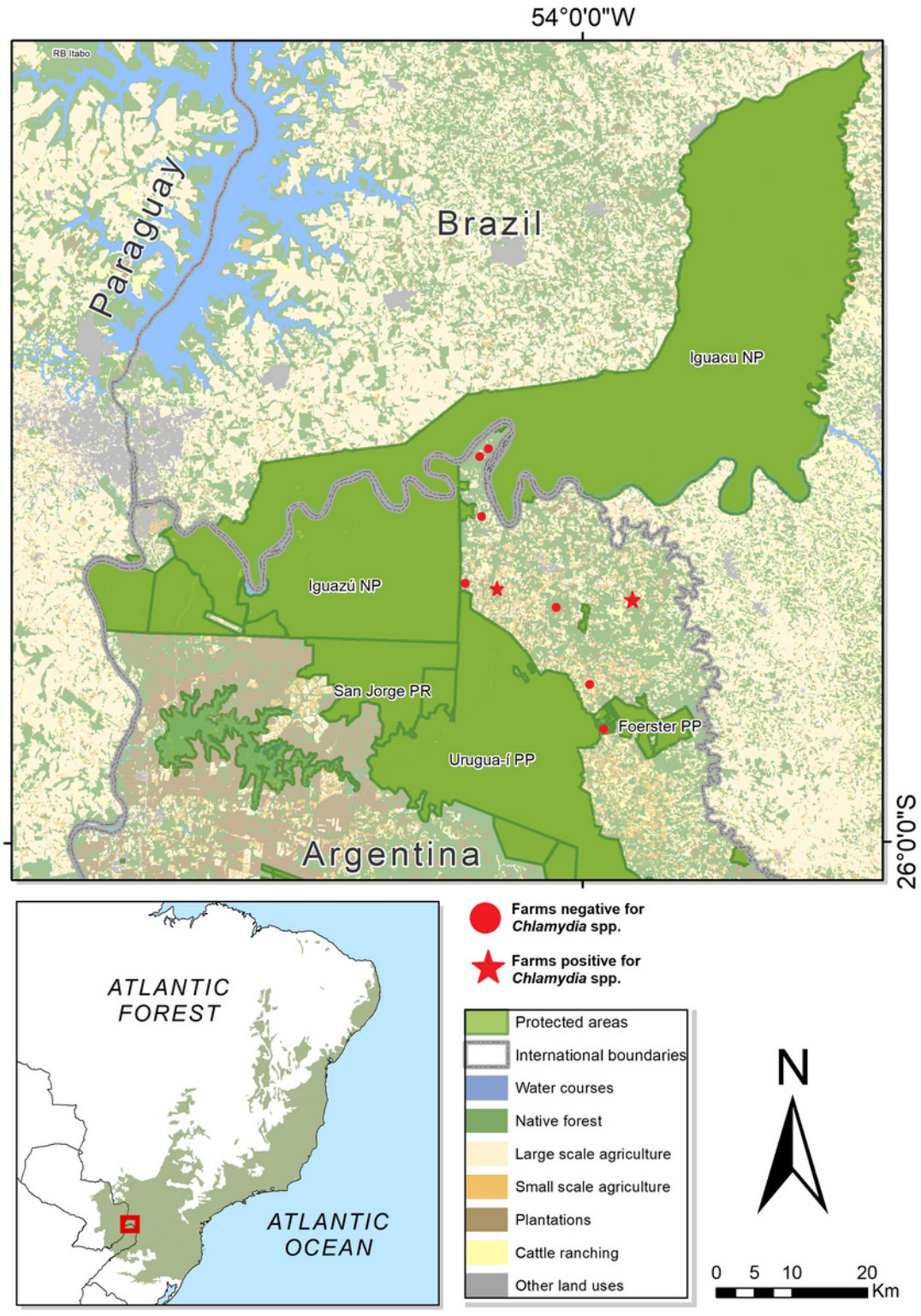

Figure 1

Map illustrating the study area, the different land uses and farms sampled and those that tested positive for Chlamydia.

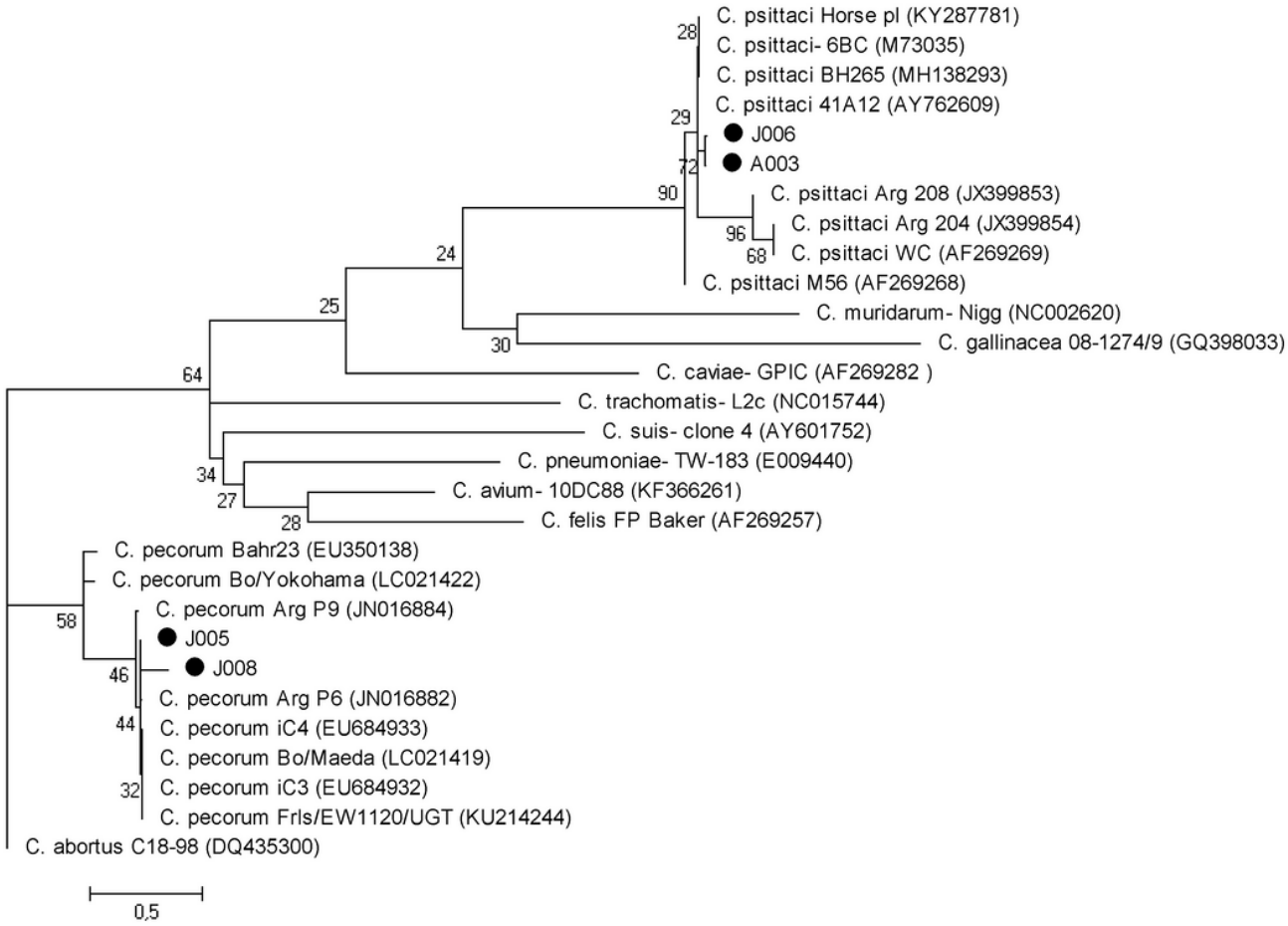

Figure 2

Numbers above branches are bootstrap values as a percentage of 1000 pseudo replicates with Maximum Likelihood method. C. pecorum C18-98 was used as a root group. Scale bar shows the percentage sequence diversity.