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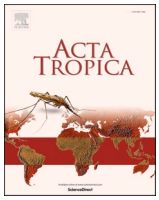

Serological and molecular survey of Q fever in the dog population of the Campania region, southern Italy

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ABSTRACT

Q fever is a re-emerging zoonosis whose epidemiological cycle in ruminants is well defined, while the role of other species (including pets) is still debated. In this study, the serological and molecular prevalence of *Coxiella burnetii* in a sample of dogs in the Campania region, southern Italy was evaluated. A seroprevalence of 5.97 % (16/268) was observed using a commercial multispecies ELISA, compared to only 2.7 % (5/197) at the molecular level. No risk factors correlated with higher levels of exposure except for the size of the animal (small dogs showed significantly higher seroprevalence). Positive samples were further evaluated for reactivity to phase I and II antigens using IFA and phase-specific ELISAs (for specific IgG detection). Two animals showed antibodies against both phases of infection, suggesting that *Coxiella burnetii* seroconversion in dogs follows similar dynamics to those observed in ruminants.

One of the five samples that showed positive results in real-time PCR was confirmed at the PCR endpoint and showed similarity with other *Coxiella* spp. strains detected in tick and dog samples when sequenced.

In this study, we demonstrated exposure to *Coxiella burnetii* for different categories of dogs in southern Italy, including pet dogs living indoors. Since reports of transmission of infection from pets to humans have been described in both rural and urban areas, careful surveillance of these species is also necessary. In the lack of additional information, comprehending the risk to humans requires monitoring of wild and domestic animal populations.

1. Introduction

Coxiella burnetii (*C. burnetii*) is a Gram-negative bacterium responsible for Q fever, a worldwide spread infection whose impact is mainly due to its zoonotic potential and economic losses to the farm industry (Guatteo et al., 2011). The main source of infection for humans are domestic ruminants (in particular small ruminants), which shed a large amount of *Coxiella* spp. into the environment with vaginal fluids and aborted fetuses (Guatteo et al., 2011). In humans (who acquire the pathogen through food or aerosol), the acute infection is characterized by flu-like syndromes, but the chronic form (typical in predisposed or immunosuppressed patients) can cause serious pneumonia and endocarditis (Angelakis and Raoult, 2010; Porter et al., 2011). Although the spread of infection in ruminants is often indicated as the cause of outbreaks in humans, there are numerous examples in the literature of outbreaks linked to other species (Angelakis and Raoult, 2010). *C. burnetti* has the potential to infect any mammal (e.g., cat, dog, rabbit, pig, wild boar, horse, rodent, birds, etc.) and is able to stimulate the host's humoral immune response and cause seroconversion, while active elimination has been demonstrated in a few species (Angelakis and Raoult, 2010).

Although reports of transmission of infection from pets to humans have been described in both rural and urban areas, little is known about the pathogenesis of Q fever in companion animals (Agerholm, 2013; Celina and Cerný, 2022). Reproductive disorders in dogs and cats have been documented, including dystocia, stillbirths, and abortions. Since the presence of *C. burnetii* DNA in pet fluids is sporadic, many investigations have suggested limited bacterial shedding (Agerholm, 2013).

Pets may acquire the disease by inhalation, ingestion (for example, contaminated placentas), or tick bites (however, it has been established for the latter pathway that several bites from infected ticks are required)

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(Ali et al., 2023; Celina and Cerný, 2022). For this reason, some categories of dogs, such as hunting dogs, stray dogs, shepherd dogs, etc., are predisposed to this infection. Scientific evidence suggests that during outbreaks in ruminants, exposed barn cats, and farm dogs, seroconverts and some fluids (such as vaginal secretions) can test positive in molecular testing (Bauer et al., 2021). Furthermore, infected pets are completely asymptomatic and, for this reason, could represent a potential threat to human and livestock health (Celina and Cerný, 2022). Pet-related Q fever outbreaks affect humans through interactions with parturient cats or dogs (Komiya et al., 2003). Reproductive issues in dogs and cats, such as dystocia, stillbirths, and neonatal mortality, have been linked to human Q fever cases however, *Coxiella* spp. detections have also been reported in normal birth (Ma et al., 2020).

The role of dogs in *C. burnetii* transmission to humans is unknown, and serological and molecular surveys in dogs have not previously been carried out in southern Italy. The aim of this study was to evaluate the exposure of different categories of dogs from the Campania region, southern Italy, to Q fever, highlighting the risk factors associated with higher seroprevalences. Moreover, we performed a molecular survey attempting to identify the DNA of the pathogen in the same blood samples.

2. Materials and methods

2.1. Sampling procedures

This study examined blood serum samples from 268 dogs belonging to different categories from the Campania region (Fig. 1, designed using EpiInfo and BioRender), Southern Italy (40°49′34″N 14°15′23″E). Campania is located between the Tyrrhenian Sea and the southern Apennines, with a surface area of 13,670.95 km^2. The sample size was determined using Thurshfield's formula for a theoretically ``infinite'' population, which included the following information: estimated Q fever prevalence (10 %), 95 % confidence interval (CI), and desired absolute precision (5 %). A total of 52 hunting dogs, 154 stray dogs, and 62 pet dogs were sampled. For each animal, a whole blood sample (for serum) was collected. Blood samples with anticoagulant (for DNA extraction) were also collected from 197 animals (Ferrara et al., 2024b). Samples were shipped to the Department of Veterinary Medicine and Animal Production of Naples under cold chain conditions. Each blood sample was kept at −80 °C, and each serum sample was stored at −20 °C before being analyzed. A questionnaire was used to collect information on each sampled animal (sex, breed, size, province, age, origin, lifestyle, and attitude). The animal study protocol was approved by the Institutional Ethics Committee of the Department of Veterinary Medicine and Animal production (Centro Servizi Veterinari), University of Naples, Federico II (PG/2022/0093420, 21st July 2022).

2.2. Serological assay and statistical analysis

Each serum sample was tested for the presence of anti-*Coxiella* antibodies using a commercial (indirect) multispecies kit (ID Screen® Q Fever Indirect Multi-species, catalog number FQS-MS-5P). This kit is commonly used in animal surveys (it includes coated phase I and II antigens and a multispecies anti-IgG conjugate). Briefly, samples diluted 1:50 were incubated in 96 plates and, after one hour and three washes, were incubated with 100 µl of a specific conjugate. After a further washing phase, the substrate (incubated for 15 min) and the stop solution were added. Finally, the plates were read using a spectrophotometer in order to obtain the optical density (OD) of each well. The cut-off calculation, useful for verifying the positivity of the sample, was calculated as described by the manufacturer's instructions. The correlation between dependent (ELISA outcome) and independent variables (obtained through the questionnaire) was assessed using Chi-square statistics. The independent variables were sex (male or female), age (≤2 years considered young, >2 and ≤6 years considered adults, <6 years considered old), province (Avellino, Benevento, Salerno, Caserta, Napoli), attitude (hunting and non-hunting), breed (mix or pure breed), origin (private or stray), and size (≤10 kg considered small, >10 and ≤25 kg considered medium, and >25 kg considered large/giant). A p-value lower than 0.05 was considered significant. MedCalc Statistical Software version 16.4.3 (MedCalc Software, Ostend, Belgium) and JMP

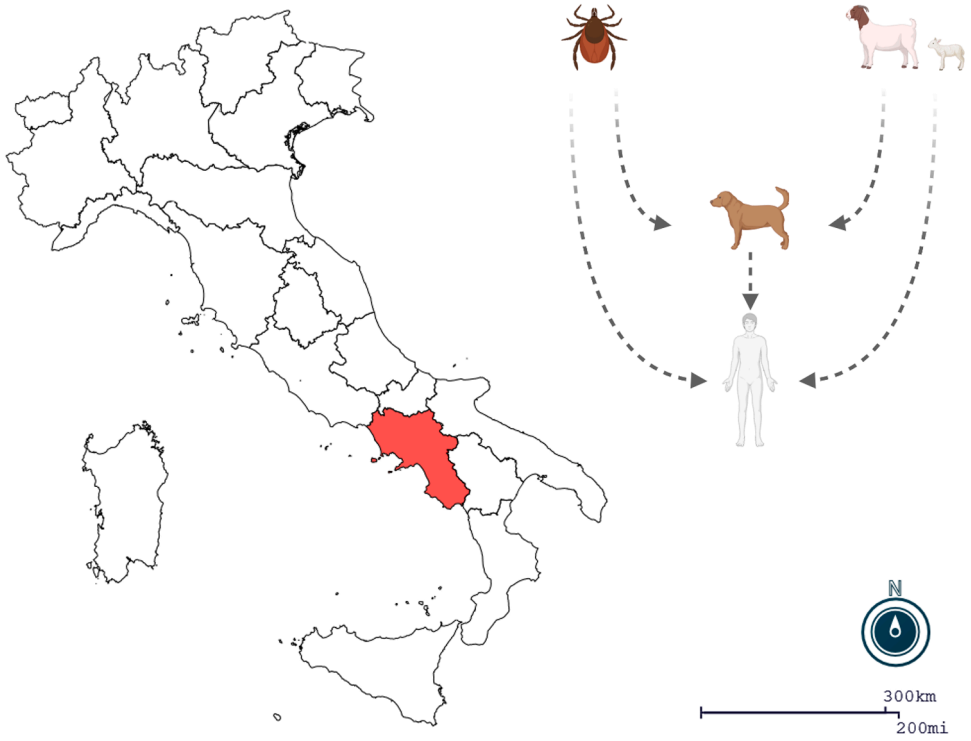

Fig. 1. Map of the study area compared to the Italian territory and infection dynamics of *Coxiella burnetii* in dogs.

version 14.1.0 (SAS Institute Inc.) were used for the statistical analysis.

2.3. Serological analysis

Positive samples were further tested in two phase-specific (indirect) ELISAs and a specific immunofluorescence assay that used phase I and phase II antigens separately and detect IgG. The phase-specific ELISAs (Anti-*Coxiella burnetii* phase 1 and 2 IgG, Euroimmun, Germany, catalog number EI 217a-9601-1G and EI 217a-9601-2G) were also multispecies assays (employing multispecies conjugates) and were employed in accordance with the manufacturer's instructions. Serum samples were diluted 1:101 in sample buffer and provided in each well of a 96-wells plate. After 30 min and 3 washing steps, the conjugate was incubated for 30 min. Further three washing steps preceded substrate incubation and stopping. Photometric measurement of the color intensity was read with a spectrophotometer in order to obtain the optical density (OD). The cut-off calculation, useful for verifying the positivity of the sample, was calculated as described by the manufacturer's instructions. This approach was effective in determining whether the response was directed towards phase I (chronic form) or phase II (acute form). For IFA preparation, *C. burnetii* inactivated cells (1 mg/ml) were diluted 1:5 in PBS 1X and approximately one μl was applied to the slide, then air dried and fixed with thirty μl of 3.7 % formalin diluted in PBS. Cells were washed three times in PBS, then blocked with 5 % BSA in PBS for 1 h at 37 °C. After washing three times with PBS, the antigen was incubated for 1 h at 37 °C in a wet chamber with dog sera (1:8 and 1:16) or human positive sera as a control (from the collection at the Biomedical Research Center, Slovak Academy of Sciences, Bratislava, Slovakia) with a dilution of 1:32 and 1:64 in PBS containing 2.5 % BSA. Samples were washed five times and incubated with rabbit anti-dog IgG conjugated with Alexa fluor 488 (Jackson ImmunoResearch Lab., USA) diluted 1:500 in PBS containing 2.5 % BSA for 1 h at 37 °C in a wet chamber in dark. After five times washing with PBS, the slide was dried and mounted with DAPI Vectashield mounting media with DAPI (Vector Laboratories) and viewed with fluorescence microscopy (Eclipse Ni, Nikon Japan).

2.4. Real-time PCR and sequencing

Genomic DNA was extracted from each blood sample using a commercial kit (DNeasy® Blood & Tissue, Qiagen). Extracted DNAs were quantified using a Nanodrop and used as a template in a real-time PCR previously described in the literature, targeting a 295 bp fragment of the transposase gene of *C. burnetii* (IS1111) (Klee et al., 2006). Primers and probes used in this protocol included Cox-F = GTCTTAAGGTGGG CTGCGTG, Cox-R = CCCCGAATCTCATTGATCAGC, TaqMan probe FAM-AGCGAACCATTGGTATCGGACGTT-TAMRA-TATGG (Klee et al., 2006). The thermal cycle included 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 30 s at 60 °C. The positive control of the amplification reaction was DNA extracted from *Coxiella* phase I obtained in a previous study (Ferrara et al., 2022b). The presence of the GAPDH gene sequence was tested in order to assess the individual sample's quality (de Sousa et al., 2017). The results were read using CFX96™ Real-Time PCR Detection System (Bio-Rad). An additional trans-PCR was conducted using the positive samples as templates and amplifying the partial transposase gene of IS element IS1111 with the use of the following primers: F: '5 -GTAACGATGCGCAGGCGAT-3′ and R: '5 -CCACCG CTTCGCTCGCTA-3′ (Das et al., 2014). The cycling conditions were as follows: an initial denaturation at 95 °C for 2 min, followed by five cycles of 94 °C for 30 s, 66–61 °C (the temperature was lowered by 1 °C in consecutive cycles) for 1 min, and 72 °C for 1 min. This was followed by 40 cycles of 94 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min. The final step of the reaction included a 10-min extension at 72 °C. To decrease the risk of sequencing artifacts, each PCR product was sequenced (by the Sanger method) at least twice with each primer. Sequences were submitted to NCBI to determine the most comparable reference sequences. The nucleotide sequences were deposited into the gene bank and provided accession numbers. The phylogenetic connection was analyzed using molecular evolutionary genetics software version 10 (MEGA 10) and the maximum likelihood (ML) approach.

3. Results

In this study, a prevalence of 5.97 % was observed by testing 268 dogs using a commercial ELISA (Table 1). No correlations were highlighted between the different risk factors studied (location, sex, age, breed, origin, attitude, and lifestyle) and the risk of exposure (Table 1). The only exception was the size of the animal, since small animals had a higher (13.2 %) seroprevalence than medium (3.4 %) and giant animals (2.1 %). All seropositive animals were further tested with two phase-specific ELISAs and with an indirect IFA. Antibodies against one of the two phases of infection were detected with a specific ELISA only in four dogs. Of these, two animals had a strong reactivity towards phase II, one animal had a mild reactivity (just above the cut-off) towards both phases, and one animal had a strong reactivity towards phases I and II. Different OD ratios were obtained with a cut-off of 1.1 for both phase I and II. There was an intense reaction against phase II (2.17 and 6.96) in two animals, and in a sample slightly above the cut-off in one animal (1.2 against phase I and 1.11 against phase II). A significant reactivity against both phases (10.9 against phase I and 8.85 against phase II) was seen in a single sample (Supplementary file 1). The IFA results confirmed the presence of specific antibodies (IgG) in only 2 animals (tested positive in both phase-specific ELISAs). The sample with the highest phase-specific ELISAs reactivity also reacted strongly to the two phases in IFA (Fig. 2).

Only 5 of the 190 blood samples tested were positive for real-time

Table 1
Univariate analysis of *Coxiella* risk factors in dogs.

Coxiella						
Factor	n	Positive	%	95 %CI	χ^2	*p*
Total	268	16	5.97	3.1–8.8		
Province						
Avellino	43	2	4.6	0–10.9		
Benevento	36	0	0	0		
Salerno	56	4	7.1	0.4–13.9	3.12	0.5
Caserta	67	5	7.4	1.2–13.8		
Napoli	66	5	7.6	1.2–14		
Sex						
Male	146	7	4.8	1.3–8.2		
					0.79	0.37
Female	122	9	7.4	2.7–12.		
Age						
Young	57	2	3.5	0–8.3		
Adult	129	9	7	2.6–11.4	0.85	0.65
Old	82	5	6.1	0.9–11.3		
Bred						
Mix	162	10	6.2	2.5–9.9		
					0.03	0.86
Specific bred	106	6	5.7	1.3–10.1		
Origin						
Stray	176	8	4.5	1.5–7.6		
					1.85	0.17
Housed	92	8	8.7	2.4–14.4		
Size						
Small	76	10	13.2	5.6–20.8		
Medium	145	5	3.4	0.5–6.4	9.8	**0.007**
Large/Giant	47	1	2.1	0–6.2		
Attitude						
Hunting	52	4	7.7	0.4–14.9		
					0.34	0.56
Non-hunting	216	12	5.6	2.5–8.6		
Life						
In	53	3	5.7	0–11.9		
					0.01	0.91
Outside	215	13	6	2.9–9.2		

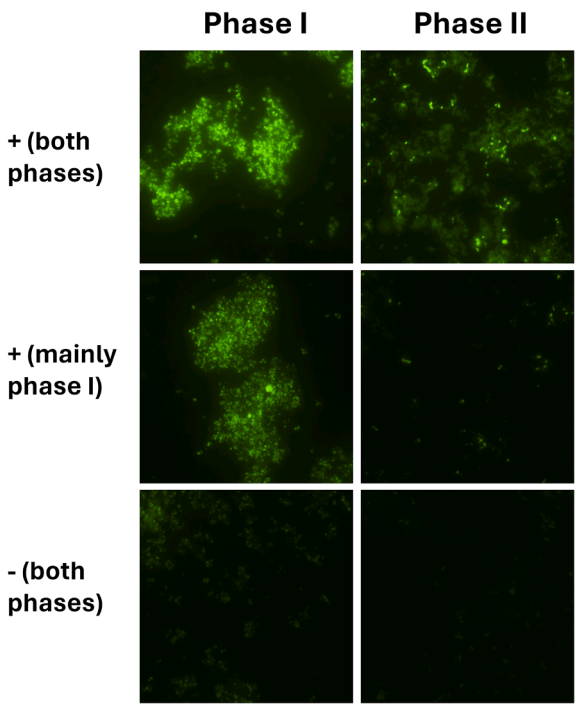

Fig. 2. An example of an immunofluorescence assay: The results of an immunofluorescence experiment with two positive and one negative serum. The first serum reacted intensely to phase I and II antigens, but the second serum reacted mostly to phase I antigens. The negative serum did not react with any antigens.

PCR, and only one positive sample resulted seropositive in ELISA. The threshold cycles (Ct) of amplification were generally high (between 34 and 38), while the positive controls amplified considerably earlier (17th or 19th cycle). However, it was not possible to confirm the positivity in end-point PCR for the majority of the samples, and therefore, the sequencing of the amplification products was successful only for one sample. The sequence obtained, of approximately 300 bp, was compared with those deposited in international databases and obtained from samples derived from dogs (blood, serum, or ticks) with a homology of 100 % (Figs. 3 and 4). The sample came from a 13-year-old male pet dog who lived outside, which resulted seropositive to multispecies ELISA but seronegative to IFA and phase-specific ELISA.

4. Discussion

The role of domestic and wild carnivores in the spread of *C. burnetii* is still widely debated (Agerholm, 2013). In this study, the Q fever serological and molecular prevalence was evaluated in the canine population of a region of southern Italy. The seroprevalence observed in the present study was lower than those described in other studies conducted in other European countries. For example, in France, a seroprevalence of 9.8 % was reported using IFA, and in Portugal, it was 9.5 % (Anastácio et al., 2022; Èl Boni et al., 1998.; Mohabbati Mobarez et al., 2017) using ELISA. The only evidence of *Coxiella* spp. circulation among pets in Italy revealed seroprevalences (using IFA) of 8.13 % and 8 % in Tuscany and Sicily, respectively (Ebani, 2020; Torina et al., 2007). Other studies describe lower exposures in Thailand (using IFA), Iran (in a systematic review), and Montenegro (using IFA) of 2.78 %, 0.55 %, and 1.16 %, respectively (Laušević et al., 2019; Mohabbati Mobarez et al., 2017; Saengsawang et al., 2022). Similarities and differences between the different studies cited resulted from the different types of tests used (IFA, ELISA, etc.), the type of population, the year, and the epidemiological scenario of the country taken into consideration. Most of the large-scale studies in dogs have been carried out in Australia. In this country, concern for this infection among animals is essential, and vaccination is suggested for veterinarians and other professionals who come into close contact with animals (Tozer et al., 2014). Several surveys revealed seroprevalence between 2 and 26 % (using both IFA and ELISA), often higher in dogs belonging to aboriginal communities (probably due to greater contact with reservoirs and a greater risk of exposure via arthropod vectors) (Ma et al., 2020; Orr et al., 2022; Shapiro et al., 2016). Another risk factor positively associated with higher exposure in Australia was the proximity (150 km) to recent human Q fever outbreaks

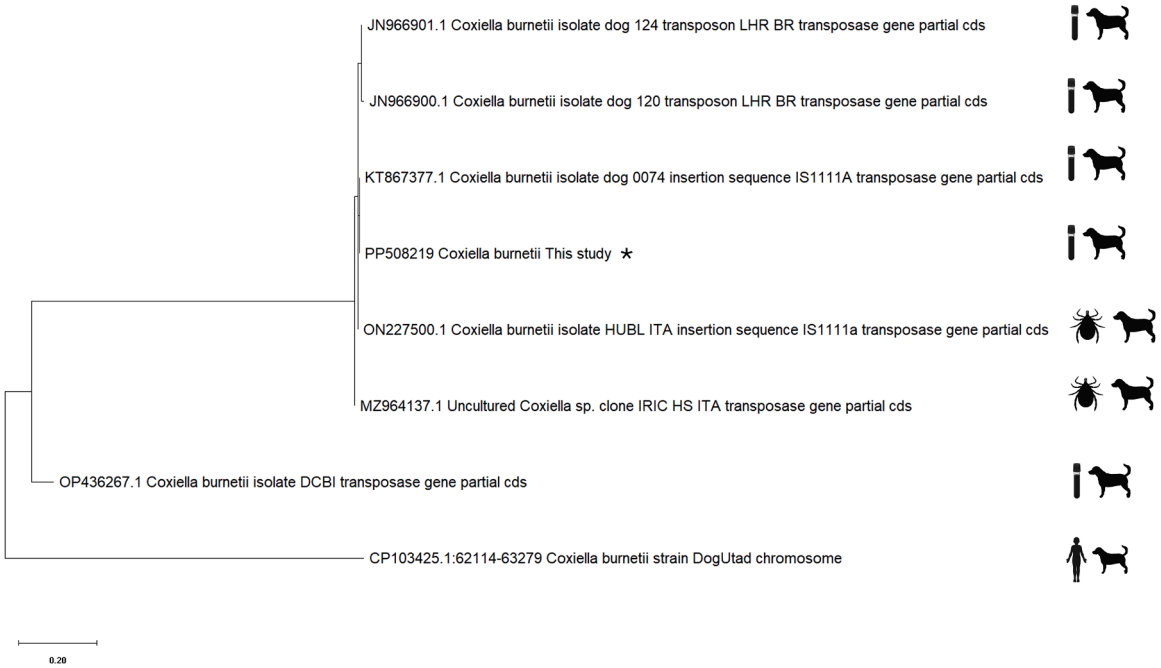

Fig. 3. Phylogenetic analysis of *Coxiella* spp. strains based on the partial IS1111 gene sequences identified in dogs. A maximum-like tree was constructed using MEGA software. The GenBank accession numbers and host. The *Coxiella* spp. strain characterized in this study is indicated with an asterisk. The scale bar indicates the number of substitutions per site.

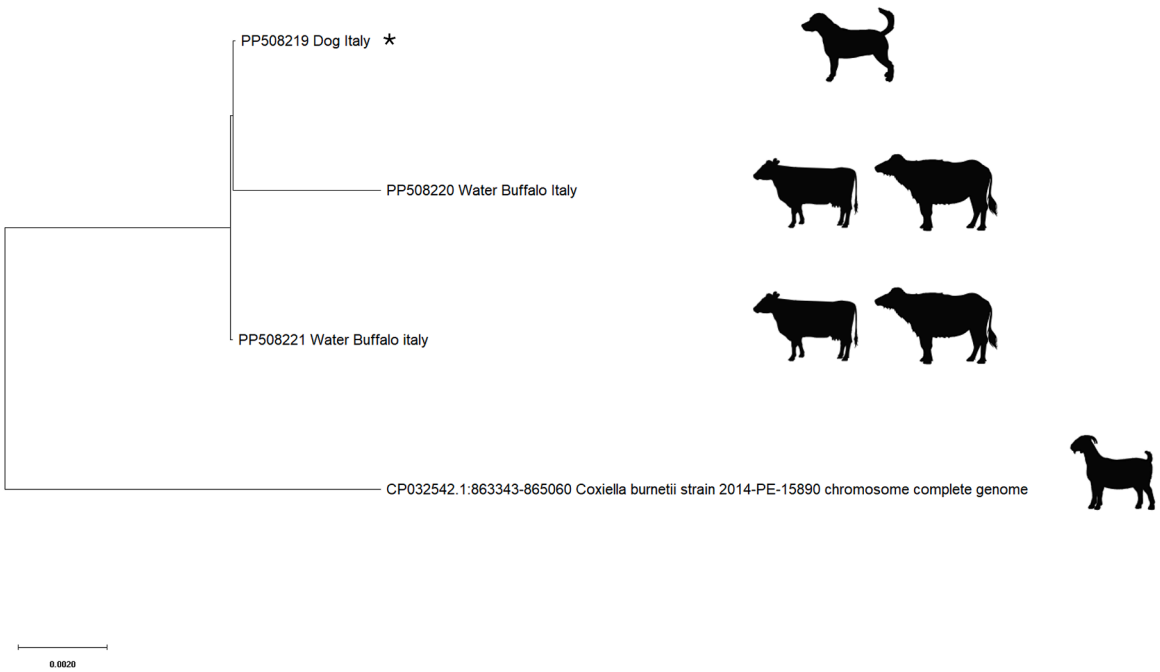

Fig. 4. Phylogenetic analysis of *Coxiella* spp. strains based on the partial IS1111 gene sequences identified in Italy. A maximum-like tree was constructed using MEGA software. The GenBank accession numbers and host are indicated. The *Coxiella* spp. strain characterized in this study is indicated by an asterisk. The scale bar indicates the number of substitutions per site.

(Ma et al., 2020). Variables found as risk factors in other studies (in dogs or other carnivores) identified attitude (hunting), location (rural areas), and age (in dogs older than 5 years) (Saengsawang et al., 2022). In our study, these variables were not confirmed as risk factors, while we found a significantly higher seroprevalence in small dogs. Furthermore, no differences were observed in the categories of dogs that were assumed to be more susceptible (such as hunting dogs, strays, or those that live outside) (Petruccelli et al., 2021). Only four and two animals (all hunting dogs) were positive when tested with the phase-specific ELISAs and IFA, respectively. This pattern is predictable since *C. burnetii* shares its antigenic profile with several common infections in dogs, including *Bartonella* spp and *Rickettsiae* spp. Commercial ELISA prevalences are typically overestimated due to cross-reactions with antibodies against the aforementioned microorganisms (Ferrara et al., 2023, 2022b). Although these methods were not validated in dogs, our findings represented the first-time application of a phase-specific test in this species. Any cross-reactions may have not only influenced the observed seroprevalence but also influenced the analysis of risk factors (small animals presented a greater risk of exposure for no apparent reason).

Evidence of Q fever has been described in several animal species in the study area of the present study (Campania region). For example, 14.3 % of cattle, 6.5 % of buffalo, and 4.1 % of pigs were seropositive in recent studies (Ferrara et al., 2024a, 2022a). A sample of wild boar reproductive organs (out of a total of 63) was positive in a recent molecular survey in the same study area (Ferrara et al., 2024c). All this evidence supports a large circulation of *Coxiella* spp., both at the sylvatic and synanthropic levels, to which the dog can be easily exposed.

Reports of molecularly positive findings are considerably uncommon in canine-derived samples (genital swabs, blood, ticks, or feces) (Kim and Seo, 2023; Ma et al., 2020; Oskam et al., 2017; Sgroi et al., 2022; Shapiro et al., 2017). When described, the positive rate of matrices (mainly blood) was between 2 and 10 % (Chitanga et al., 2018; Ghaoui et al. 2024). In our study, only one blood sample was confirmed by sequencing. The discrepancy we found between serology and molecular biology was expected given the assumptions behind the approaches, since serology shows previous exposures while molecular biology on blood reveals early infections. Similarly, it was assumed that some real-time positive samples gave negative results in sequencing. In fact, certain PCR methods are unable to distinguish between homologous sequences of *C. burnetii* and *Coxiella*-like endosymbionts (reported in ticks but also in animal blood) (Raele et al., 2015). However, evidence in the literature suggests interpreting molecular biology results with caution, especially if they are not confirmed by sequencing (Elsa et al. 2015).

The sequence obtained was compared with those deposited in international databases, obtaining 100 % homology with sequences obtained in Brazil using dog blood and in Italy from ticks (collected from dogs) (Mares-Guia et al., 2014; Sgroi et al. 2022). Furthermore, positive findings in real-time PCR are typically detected at high cycle threshold due to the low concentration of *Coxiella* spp. in the blood, which occurs only during the bacteremia that indicates early infections. This pattern appears to be totally different among wild carnivores, such as foxes. In addition to higher seroprevalence, wild carnivores frequently test positive for molecular assays (including genotyping), and the spleen has been described as the most appropriate matrix (Ebani et al., 2017; Medkour et al., 2020; Meredith et al., 2015). In a study conducted in Algeria, *C. burnetii* was also identified in the spleen of a dog, suggesting spleen localization even in this species (Bessas et al., 2016). It is unclear what explains the higher likelihood of finding serological and molecular positives in wild carnivores. The greater exposure might be attributed to closer interaction with reservoirs and/or increased activity of arthropod vectors on wild animals compared to domestic ones (although higher seroprevalences were not reported in stray dogs in our research). The sample confirmed positive by sequencing came from an animal seropositive by multispecies ELISA but negative by IFA and phase-specific ELISA. In the municipality where the dog lived, cattle (about 270) and a small number of sheep (7) were farmed (Banca Dati Nazionale dell' Anagrafe Zootecnica, https://www.vetinfo.it/j6statistiche/, accessed on March 18, 2024). The sequence we obtained differed by only one base from sequences identified in buffalo/cattle and by six bases from sequences identified in goats in central-southern Italy (Fig. 4). However, these sequences differed significantly from those found in the only full genome of *Coxiella* spp. isolated from dogs (associated with a human outbreak) (D'Amato et al. 2014).

Although there is no evidence that carnivores may exhibit symptoms from *Coxiella* spp. infection, it is widely known that they become infected and produce specific antibodies (Agerholm, 2013). It is unclear how these animals were exposed to the infection, with hypotheses including interaction with a contaminated environment, other infected domestic and wild animals (placentas, feces, etc.), ticks, and humans. The state of the art is unable to define what role these animals have in the transmission of the infection to humans, while occasional dog/cat-related human outbreaks have been reported (especially in Australia and Canada), and some studies have found higher exposures among dog owners (Buhariwalla et al., 1996; Duplaix et al., 2021; Skerget et al., 2003.). In the absence of other evidence, understanding the risk to humans in the present scenario (monitoring wild and domestic animal populations) is imperative.

5. Conclusions

The canine population of the Campania region was exposed to Q fever, finding a seroprevalence of 9 %, although probably overestimated due to the false positives (caused by cross-reactions with other bacteria) frequently described when commercial tests have been used. The analysis of risk factors did not identify variables correlated with greater risks of exposure (except for the size of the animal). Specific antibodies in IFA and phase-specific ELISA (IgG) were only found in 2 and 3 animals, respectively (all hunting animals). The results obtained with the molecular approach showed that five animals tested positive, even though only one of the sequencings was successful. In the lack of more information regarding the epidemiological importance of the dog in the transmission of Q fever to humans and other animals, it is necessary to employ extreme caution and continue to monitor the dissemination of this infection in pet populations.

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Ethics approval

The animal study protocol was approved by the Institutional Ethics Committee of Department of Veterinary Medicine and Animal production (Centro Servizi Veterinari), University of Naples, Federico II (PG/2022/0093420 20 July 2022).

CRediT authorship contribution statement

G. Ferrara: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **G. Flores-Ramirez:** Writing – review & editing, Resources, Methodology, Investigation. **K. Palkovicova:** Resources, Methodology. **F. Ferrucci:** Resources. **U. Pagnini:** Writing – review & editing, Visualization, Supervision, Investigation, Funding acquisition. **G. Iovane:** Supervision, Project administration, Funding acquisition. **S. Montagnaro:** Writing – review & editing, Supervision, Software, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared the data (Accession number) in the availability statement data.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2024.107299.

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