Original Article

Serological diagnosis and risk factors for *Coxiella burnetii* in goats and sheep in a semi-arid region of Northeastern Brazil

Diagnóstico sorológico e fatores de risco para *Coxiella burnetii* em caprinos e ovinos da região semiárida do nordeste do Brasil

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Abstract

Coxiella burnetii is a zoonotic agent transmitted mainly by small ruminants. In Brazil the disease has been classified as a notifiable disease since 2013, when human cases were reported. This study aimed to identify risk factors associated with the presence of anti-*Coxiella burnetii* antibodies in goats and sheep in a semiarid region of Northeastern Brazil. Sera of 412 goats and 403 sheep from municipality of Petrolina, Pernambuco, were examined by the Indirect Fluorescent Antibody Test (IFAT) against antigens of *C. burnetii*. Information about management variables (independent variables) that could be associated with the presence of the microorganism (dependent variables) were obtained from the supervisor of each farm. It was determined that 2.2% (9/412) of the goats and 2.1% (9/403) of the sheep had antibodies reactive to *C. burnetii*. The presence of anti-*C. burnetii* antibodies was associated with the dry area of the Sequeiro (a region in the northern part of the municipality of Petrolina) (P = 0.025), male sheep (P = 0.020), and intensive goat breeding (P = 0.005). This study therefore showed the presence of anti-*C. burnetii* antibodies in goat and sheep, confirming for the first time that this agent is likely circulating among goat herds in the Caatinga Biome, semi-arid of Brazil.

Keywords: Q fever, IFAT, small ruminants, zoonosis, Pernambuco.

Resumo

Coxiella burnetii é um agente zoonótico transmitido principalmente por pequenos ruminantes. No Brasil, a doença foi classificada como de notificação compulsória desde 2013, quando casos humanos foram relatados. O objetivo deste estudo foi identificar os fatores de risco associados à presença de anticorpos anti-*Coxiella burnetii* em caprinos e ovinos em uma região semiárida do Nordeste do Brasil. Este estudo envolveu um inquérito sorológico de 412 caprinos e 403 ovinos em fazendas do município de Petrolina, no estado de Pernambuco. Os soros foram examinados pela Reação de Imunofluorescência Indireta (RIFI) contra antígenos de *C. burnetii*. Informações sobre variáveis de manejo (variáveis independentes) que poderiam estar associadas à presença do microrganismo (variáveis dependentes) foram obtidas do proprietário de cada fazenda. Foi determinado que 2,2% (9/412) dos caprinos e 2,1% (9/403) dos ovinos tinham anticorpos reativos a *C. burnetii*. A presença de anticorpos anti-*C. burnetii* foram associados com a área seca do Sequeiro (região no norte do município de Petrolina) (P = 0,025), ovinos machos (P = 0,020) e criação intensiva de caprinos (P = 0,005). Este estudo, portanto, observou a presença de anticorpos anti-*C. burnetii* em pequenos ruminantes, confirmando pela primeira vez que este agente pode estar circulando em rebanhos caprinos no bioma Caatinga, semiárido do Brasil.

Palavras-chave: Febre Q, RIFI, pequenos ruminantes, zoonoses, Pernambuco.

Introduction

Coxiella burnetii, an obligate intracellular Gram-negative bacterium, is the causative agent of Q Fever. It was first classified in the order Rickettsiales, but was later reallocated to the order Legionellales (ANGELAKIS & RAOULT, 2010). The primary reservoirs of *C. burnetii* are sheep, goats, and cattle. Usually asymptomatic, the infection may cause spontaneous abortion at the end of gestation, as well as other reproductive failures. It can be excreted into the environment through vaginal secretions, birth products, urine, feces, semen, and milk. The agent can survive in soil for long periods and be carried by the wind over distances of up to 30 km (ELDIN et al., 2017).

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In Brazil, the presence of *C. burnetii* in humans has been reported by serological and/or molecular assays in the states of São Paulo (BRANDÃO et al., 1953; VALLE et al., 1955; SICILIANO et al., 2015), Rio de Janeiro (LAMAS et al., 2009, 2013; LEMOS et al., 2011, 2018; ROZENTAL et al., 2012; MARES-GUIA et al., 2016), Minas Gerais (RIEMANN, et al., 1974; COSTA et al., 2005, 2006) and Bahia (SICILIANO et al., 2008). Among three Brazilian states (Rio de Janeiro, Piauí, and Alagoas), seropositive reactions to *C. burnetii* have been identified in dogs, sheep and/or goats, and/or *C. burnetii* DNA has been amplified from goat milk or placenta, or dog blood (MARES-GUIA et al., 2014; GUIMARÃES et al., 2017; OLIVEIRA et al. 2018). Therefore, this zoonotic disease was included in Brazil's list of notifiable diseases in 2013 (BRASIL, 2013).

In Northeastern Brazil, extensive farming is normally used to raise small ruminants to produce meat and milk for the farmer's own consumption or for sale. The economic importance of these animals in the region is significant, despite their low productive and reproductive performance, which causes financial losses to farmers (ALENCAR et al., 2010; PEREIRA et al., 2013). Given the importance of research into the epidemiological aspects that contribute to the introduction of pathogens into small ruminant farms, the aim of this study was to identify the main risk factors associated with the presence of anti-*C. burnetii* antibodies in goats and sheep in a semi-arid region of the state of Pernambuco, northeastern Brazil, where to our knowledge, there has been no investigation on *C. burnetii*.

Material and Methods

Study area

The municipality of Petrolina is located in the state Pernambuco (-9.39416 Latitude and -40.5096 Longitude), Brazil. Petrolina is within the Caatinga Biome, where the semi-arid climate is typically hot and dry, with an annual average temperature of 25.7 °C (IBGE, 2015). Petrolina has an estimated ruminant population of 233,900 goats and 157,500 sheep, and the municipality covers an area of 4,561.874 km^2. Random visits were made to 26 farms located in five areas: Sequeiro, Capim, Pontal, Irrigation Projects, and Riverside areas.

Sampling procedures

A total of 815 healthy small ruminants (412 goats and 403 sheep), 6 to 120 months old, of different breeds (although most were crossbreeds) were sampled between March and September 2016. This sample size was previously estimated based on an estimated 50% prevalence, 5% absolute precision, and 95% confidence interval (THRUSFIELD, 1995). Approximately 25 animals were conveniently selected for blood collection at each farm. A farm was deemed positive when it presented at least one seropositive animal.

Blood samples from small ruminants were collected by jugular venipuncture and were centrifuged (3,600 rpm, 10 min), and the serum obtained from each animal was aliquoted into 1.5 ml tubes and stored at -20 °C until the time of examination. Geographic coordinates were obtained by GPS to create maps, using the ArcGis® program, pinpointing the location of seropositive and seronegative animals.

The farmers were asked to fill out a comprehensive questionnaire about possible risk factors for *C. burnetii*. The information collected included the general characteristics of the farms: species (goat or sheep), breed (pure or mixed breed), age (0-1, 1-4 and > 4 years), breeding system (intensive or extensive), type of corrals (covered or open), reproductive disorders (occurrence of spontaneous abortion, malformed offspring), sanitary aspects (corral cleaning frequency, presence of ectoparasites, availability of veterinary care) and the location (Sequeiro, Capim, Pontal, Irrigation Projects, and Riverside areas).

This study was approved by the Committee on Ethics and Deontology in Studies and Surveys (CEDEP-Univasf) under Protocol no. 0016/170516.

Detection of antibodies

The presence of anti-*C. burnetii* IgG antibodies in the sera of goats and sheep was assessed by the Indirect Fluorescent Antibody Test (IFAT) using crude antigens of *C. burnetii* strain At12 (PACHECO et al., 2013). For this purpose, strain At12 was cultivated in Vero cells and harvested when near 100% of the cells were infected. The infected cells were centrifuged at 12,000 g for 10 minutes and the pellet was washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), centrifuged again, and resuspended in PBS containing 0.1% sodium azide. Ten microliters of *C. burnetii*-infected cells were applied onto each well of 12 antigen slides. The antigens on the slides were air-dried and then fixed in acetone for 10 minutes. Slides were kept at -20 °C until used.

Animal sera were diluted in two-fold increments with PBS starting from a 1:64 dilution. Ten microliters of diluted sera were added to each well of the antigen slides. The slides were incubated at 37 °C for 30 min in a humid chamber. The slides were rinsed once, and then washed twice for 10 min per wash in PBS. The slides were incubated with fluorescein isothiocyanate-labeled rabbit anti-goat (dilution 1:1,000) and rabbit anti-sheep IgG (dilution 1:1,000) (Sigma, St Louis, USA), and washed as described earlier. The slides were mounted with buffered glycerin under coverslips. The slides were read using an ultraviolet microscope (Olympus, Tokyo, Japan) at 400x magnification. Samples were run in duplicates. Serum was considered to contain antibodies against *C. burnetii* if it reacted at a dilution of 1:64 (PACHECO et al., 2013), according to the protocol described by Reeves et al. (2006). Endpoint titers against *C. burnetii* were determined by testing two-fold serial dilutions of serum. Each slide contained a serum previously shown to be non-reactive (negative control) and a serum known to be reactive (positive control) (GUIMARÃES et al., 2017).

Risk factor analysis

The variables were organized for presentation in ascending or descending order in terms of scale of risk. When necessary, these variables were recategorized. The lower-risk category was used as baseline for comparison with the other categories. An initial exploratory analysis of the data (univariable) was conducted to select variables with $P \leq 0.2$ by the chi-square test or Fisher's exact test. The variables that passed this cut-off were then subjected to logistic regression (HOSMER & LEMESHOW, 2000), and variables presenting P-value < 0.05 were considered risk factors. The fit of the final model was ascertained by means of the Hosmer and Lemeshow test, and collinearity between independent variables was determined by correlation analysis. For variables with a strong collinearity (correlation coefficient of >0.9), one of the two variables was excluded from the multiple analysis, according to their biological plausibility (DOHOO et al., 1997). Confounding was assessed by monitoring the changes in the model parameters when adding new variables. If substantial changes (i.e., higher than 20%) were observed in the regression coefficients, this was considered as indicative of confounding. The calculations were performed using SPSS version 20.0 software.

Results

Anti-*C. burnetii* antibodies were detected in 2.2% (18/815) of the small ruminants, 2.1% (9/412) of which were goats and 2.2% (9/403) were sheep. At least one seropositive animal was identified at eight (30.8%) farms. Figure 1 presents the spatial location of positive and negative farms. Endpoint titers ranged from 64 to 4,096 for goats, and 64 to 65,536 for sheep (Table 1).

In the univariable analysis for *C. burnetii* in goats, the variables of breed, breeding system, type of installations, gestation phase abortion, disposal upon death, veterinary care, and location showed $P < 0.20$ and were significant and selected for the multivariate analysis, which indicated that intensive breeding was a risk factor (Table 2). In sheep, the variables selected ($P < 0.20$) in the univariable analysis were sex, breed, age, type of installations, installation cleaning frequency, fecal fate, preterm birth, weak offspring, deworming, and location (Table 3). Multivariate analysis indicated that male animals, and location (Sequeiro) were risk factors (Table 4). Both final models (for goats and sheep) presented a good fit (Hosmer and Lemeshow test: $P > 0.05$).

Table 1. Endpoint antibody titers against *Coxiella burnetii* in goats and sheep, Petrolina municipality, Brazil.

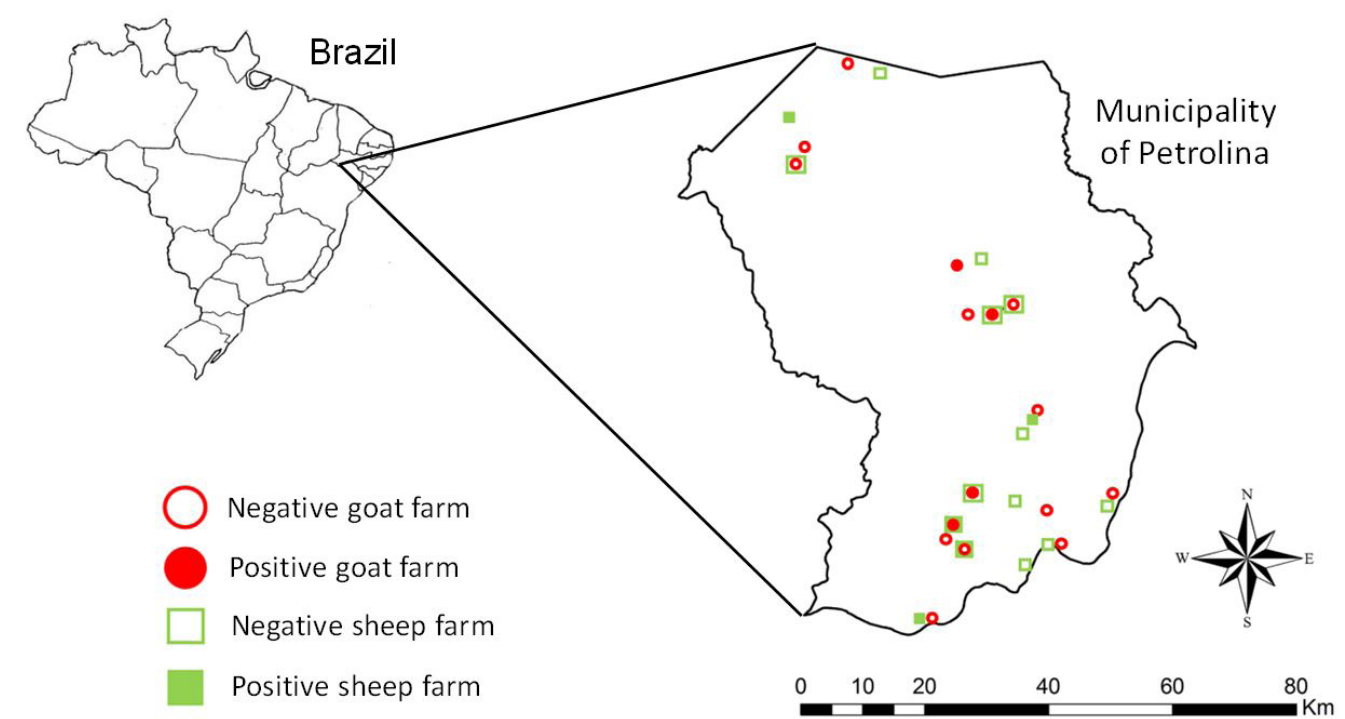| Titer	Goat (n)	Sheep (n)	Total
64	3	1	4
128	1	-	1
256	-	1	1
512	-	3	3
1,024	2	2	4
2,048	1	-	1
4,096	2	-	2
32,768	-	1	1
65,536	-	1	1

Figure 1. Map of Petrolina municipality of Pernambuco State, Brazil, indicating the location of 26 farms according to serological condition of animals.

Table 2. Univariable analysis for risk factors showing the variables associated (P < 0.20) with *Coxiella burnetii* seropositivity in goats from Petrolina municipality, State of Pernambuco, Brazil.

Variable	Category	N° total of animals	N°. of positive animals (%)	p-value
Breed	Mixed breed	361	2 (0.6)	< 0.001
	Pure	41	7 (17.1)	
Type of breeding	Intensive	25	5 (20)	< 0.001
	Extensive	377	4 (1.1)	
Type of corrals	Covered	25	5 (20)	< 0.001
	Descovered	377	4 (1.1)	
Gestation season abortion[a]	Outset	53	2 (3.8)	0.154
	Medium	71	1 (1.4)	
	Finale	100	0 (0)	
Animal destination in death[b]	Discard/burn	175	0 (0)	0.098
	Burn	152	4 (2.6)	
Veterinary assistance[c]	No	277	4 (1.4)	0.024
	Yes	75	5 (6.7)	
Area	Sequeiro, Pontal, Capim, River	281	3 (1.1)	0.024
	Irrigation projects	121	6 (5)	

[a] n = 224; [b] n = 327; [c] n = 352

Table 3. Univariable analysis for risk factors showing the variables associated (P < 0.20) with *Coxiella burnetii* seropositivity in sheep from Petrolina municipality, State of Pernambuco, Brazil.

Variable	Category	N° total of animals	N°. of positive animals (%)	p-value
Sex	Male	75	5 (6.7)	0.022
	Female	330	5 (1.5)	
Breed	Mixed breed	361	7 (1.9)	0.083
	Pure	44	3 (6.8)	
Age (years-old)	0 – 1	100	1 (1)	< 0.001
	1 – 4	291	5 (1.7)	
	> 4	14	4 (28.6)	
Type of corrals	Covered	373	8 (2.1)	0.183
	Descovered	32	2 (6.2)	
Frequency of installations cleaning[a]	Fortnightly	103	6 (5.8)	0.126
	Monthly	47	1 (2.1)	
	Bimonthly	79	1 (1.3)	
	Quarterly	54	0 (0)	
Fecal fate[a]	Sells	76	6 (7.9)	0.005
	Discard	28	1 (3.6)	
	Reuses	179	1 (0.6)	
Preterm birth[a]	No	156	7 (4.5)	0.078
	Yes	127	1 (0.8)	
Weak offspring[a]	No	130	7 (5.4)	0.026
	Yes	153	1 (0.7)	
Deworming[a]	No	1	1 (100)	0.028
	Yes	282	7 (2.5)	
Area	Sequeiro	78	5 (6.4)	0.071
	Pontal	77	2 (2.6)	
	Capim	40	1 (2.5)	
	Irrigation projects/River	210	2 (1)	

[a] n = 283

Table 4. Multivariable analysis (logistic regression) with the risk factors associated with *Coxiella burnetii* seropositivity in small ruminants from Petrolina municipality, State of Pernambuco, Brazil.

Risk fator	Regression coeficiente	Standard error	Wald	Odds ratio (IC 95%)	p-value
Goat					
Intensive breeding	3.168	1.123	7.960	23.7 [2.6 – 214.5]	0.005
Sheep					
Male animals	1.701	0.729	5.438	5.5 [1.3 – 22.9]	0.020
Area – Sequeiro	1.923	0.857	5.038	6.8 [1.3 – 36.6]	0.025

Discussion

While we found a relatively low seroprevalence for *C. burnetii* (<3.3%) among sheep and goat, it is noteworthy that half of the seropositive animals had high endpoint titers, varying from 1,024 to 65,536 (Table 1). High endpoint titers are suggestive of recent infection (GUIMARÃES et al., 2017; OLIVEIRA et al., 2018); however, we cannot discard cross-reactions with other unknown agents in some of our seropositive animals, especially those with low endpoint titers. Regardless, the overall low seroprevalence values in the present study are similar to the 2% (3/153) seroprevalence on sheep that was reported in another study from the Caatinga Biome (south of Piauí state) in northeastern Brazil (GUIMARÃES et al., 2017). In contrast, much higher seropositivity to *C. burnetii* has been reported among goats in the Agreste of the state of Alagoas (55.1%; 172/312) (OLIVEIRA et al. 2018), and in the Atlantic forest Biome of Rio de Janeiro state, where 66.6% (2/3) of sheep and 50% (5/10) of goats were seropositive (MARES-GUIA et al., 2014).

The 26 farms of this study are located 3 to 50 km apart from each other. Although the occurrence of anti-*C. burnetii* antibodies was detected in only a few animals, the farms that had seropositive animals are distributed throughout the municipality (Figure 1). Since *C. burnetii* can disperse via aerosol over distances of up to 30 km (ELDIN et al., 2017), the agent is likely to be widely distributed in this study area.

Frequent cleaning of facilities prevents bacteria from accumulating inside corrals. In this study, the highest seropositivity was found on farms where facilities were cleaned out every fortnight to remove manure, which the farmers sold, reused or discarded in the environment. The problem with discarding untreated manure is that it contains the microorganism, i.e., after contaminated products of miscarriage and urine become aerosolized, they contaminate other environments and therefore other animals and humans. A comparative study found that *C. burnetii* was transmitted to humans more frequently when they were in the proximities of contaminated manure than in that of farms with Q fever (HERMANS et al., 2014).

In the present study, animals were divided by age into groups of 0-1 year (young), 1-4 years (adults), and > 4 years old. Older animals showed a higher percentage of seropositivity to *C. burnetii*, corroborating Anastácio et al. (2013) and Jung et al. (2014), who reported significantly higher seropositivity on older sheep and goat, when compared to young animals in the flock. This higher seroprevalence was expected, due the more probability of

repeated contacts with the pathogen proportionally to life span (RIZZO et al., 2016).

An analysis of purebred, mixed and crossbred animals revealed a higher percentage of seropositivity among purebreds. In Indiana, USA, no significant difference in seropositivity was found between milk and meat goat breeds (BAUER et al., 2016). In Ontario, Canada, where intensive breeding is more common and the presence of the microorganism may be more prevalent, a higher prevalence of seropositivity was reported among milk goats (MEADOWS et al., 2015b). However, a study in Kenya carried out by Muema et al. (2017) found that animals raised in a pastoral nomadic system (similar to extensive farming) were three times more likely to test seropositive for *C. burnetii* than animals in other systems like mixed farming. This may be attributed to the wide-ranging mobility and contact between different pastoral herds during grazing and aggregation at common watering points; aggravated by the fact that *C. burnetii* persists and remains infectious in the environment for long periods.

In the present study, potential risk factors for *C. burnetii* infection were intensive farming for goats, and male sex and the Sequeiro location for sheep (Table 4). Extensive farming is a traditional livestock raising method in the region, which involves releasing animals into the native Caatinga (typical scrubland of the region) for the day and returning them to the corral at night. This method enables the animals to defecate, urinate or even abort during the daytime, leaving the products far removed from the homestead, thus minimizing the accumulation of the microorganism at the farm and thereby reducing the risk of infection among the animals. Intensive farming, which involves keeping animals permanently in the corral, is uncommon in this region. Such corrals usually have a roof and one solid wall to provide protection against strong winds. In this case, the microorganism remains in close contact with the animals for long periods of time. Thus, the proportion of seropositive animals is determined by the number of microorganisms distributed in the environment (MEADOWS et al., 2015a). Moreover, the risk of seropositivity is higher in poorly ventilated spaces (SCHIMMER et al., 2011). These facts lead us to assume that the low seropositivity found in this study may be associated with the extensive farming method practiced in the region.

Sequeiro is a region in the northern part of the municipality of Petrolina, where the farms most distant from the urban center are located. Although extensive farming predominates in this region, it was considered a risk factor here. The bacterium is not only resistant to high temperatures but can also survive in

the environment for long periods (SESHADRI et al., 2003). The corrals on these farms are enclosed by fencing and have no roofs, facilitating the passage of the wind. Also, the animals graze through the Caatinga and are liable to come into contact with other animals, especially wildlife. This fact represents a research opportunity to gain a better understanding about the pathways of transmission in the region.

Male animals with good characteristics are usually selected for reproduction at the farms. These males are not allowed to roam freely in the pastures, either because of their aggressiveness or to prevent possible unintended mating with females in estrus, and also to ensure that they live longer. Therefore, they are more susceptible to disease and/or to possible reinfection. However, Meadows et al. (2015a) states that the probability of infection in females is higher due there are generally more female animals on a property than males.

This study revealed the prevalence of anti-*C. burnetii* antibodies in small ruminants, suggesting that the agent circulates among goat and sheep herds in a semi-arid region of northeastern Brazil, and the possibility that goats and sheep act as reservoirs for the agent in the region. This study can also be seen as a starting point for further research aimed at gaining more in-depth knowledge about epidemiological factors, as well as improving diagnostic techniques to confirm the presence of active *C. burnetii* infection in animals and humans, adopting preventive measures for the productive and reproductive improvement of the herd, and preventing environmental contamination by products of animal origin, and consequently, dissemination of the disease in the Caatinga Biome of Brazil.

Acknowledgements

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