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Molecular detection of *Coxiella burnetii* in horse sera in Iran

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ABSTRACT

Coxiella burnetii is a zoonotic bacterium that can infect a wide range of animals including horses. However, its circulation dynamics in and through horses are still unclear. The aim of this study was to evaluate prevalence of *C. burnetii* and its genomic characteristics in horse sera samples in the North of Iran (Golestan Province). The samples were collected in 2018 and the age, sex, and breed of each animal were recorded. Nested-PCR was used to detect *C. burnetii* based on the presence of the transposable gene IS1111. The results showed that 7.50 % ($P < 0.05$; 95 % CI: 0.5 %–0.12 %) of the examined sera samples were positive for *C. burnetii*. Based on the resuls, prevalence of *C. burnetii* in the age groupof < Years 1–5 (p-value < 0.05, 95 % CI: 1 %–8 %) was less than the age group of > 6 years old (p-value < 0.05, 95 %, CI: 7 %–19.8 %). In previous studies, it was concluded that the horses' population in Golestan Province should be considered as an important factor in the epidemiology of Q fever and consequently in public health. Further studies should be implemented to evaluate if horses may be relevant indicators of zoonotic risk in urban and suburban endemic areas.

1. Introduction

Coxiella burnetii is a Gram-negative bacterium that causes Q fever in humans. It targets macrophage cells of body tissues (e.g., lymph nodes, spleen, lungs, and liver) and circulating monocytes [1]. Several genetic studies have been performed on *C. burnetii* strains showing that the chromosomes varied in size from 1.5 to 2.4 base pairs and were highly variable among different strains. Indeed, recent data showed that genetic variation had an apparent closer connection with the geographical source of the isolate with clinical presentation [2]. Furthermore, host factors are reasonably more important than genomic variation in developing acute or chronic disease [3]. at first it was detected as a rickettsia-like organism in the spleen and liver of mice treated with the urine of the slaughterhouse workers [3,4]. Human Q fever usually has a subclinical course or a mild course characterized by transient flu-like symptoms. Rarely, more severe conditions such as pneumonia, endocarditis, hepatitis, and miscarriage may develop. *C. burnetii* can infect a wide range of animals and Q fever is a known abortifacient in domestic ruminants [5]. The infection may cause abortion, premature delivery, and stillbirth, particularly in goats and sheep while such incidents seem to be rare in cattle [6]. The infection is highly prevalent in Danish cattle, where 60–80 % of dairy herds had antibodies in the bulk tank milk [7,8] and 4.1 % of slaughtered beef cattle were seropositive [9].

C. burnetii infection has been reported less frequently in several other domestic or wild mammals, including horses, rabbits, swine, camels, water buffalo, rats, and mice [3,10–12]. Due to the wide range of hosts for *C. burnetii*, horses may also become infected although the significance of the infection needs to be further investigated. Two older experimental studies performed in non-pregnant horses have shown that horses may develop fever, conjunctivitis, respiratory and gastrointestinal symptoms after inoculation [6].

C. burnetii infection can be demonstrated in different ways, depending on the type of sample and the purpose of the investigations. Serological tests are used especially for screening herds or flocks and to detect previous exposure to the bacterium but they are not appropriate for determining the infectivity status of individual animals. On the other hand, isolation of the causal agent in cell culture or embryonated eggs is laborious and time consuming, while its detection using stained smears has a low sensitivity. Consequently, several PCR based diagnostic assays have recently been developed to detect *C. burnetii* DNA in clinical samples. Techniques targeting genes of a substantial copy

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number, like the insertion sequence IS1111, were demonstrated to be especially sensitive [13,14]. Based on previous literatures, PCR is a well-approved laboratory method for diagnosing *C. burnetii*. Trans-PCR (transposon like element) is particularly sensitive to the detection of *C. burnetii* using primers targeting IS1111. Due to the presence of at least 56 copies of the insertion sequence in the genome, it increases the sensitivity of the diagnosis [15–17]. Trans-PCR technique used is capable of direct detection of *C. burnetii* in genital swabs, milk and fecal specimens in less than 6 h on various biological samples [13]. Although still controversial, PCR performed on serum sample may be used to diagnose acute Q fever in the first 2 weeks of the disease [2]. Comparison of the sensitivities of the two PCR methods was made using serially diluted *C. burnetii* pure genomic DNA. Our protocol enhanced sensitivity over the Trans PCR in detecting *C. burnetii* by 10- to 100-fold and it proved to be an effective tool for the diagnosis of animal coxiellosis [18].

Iran has about 155,000 horses. The main reason for choosing Golestan province for this study is the use of breeds in this province in terms of economic importance and its important use in equestrian competitions [19].

Although Q fever occurs infrequently in Iran, [20]. Also, according reports regarding the prevalence of Q fever among domestic and wild animals, Q fever is endemic in Iran. Q fever cases are not diagnosed in Iran for > 40 years due to a lack of diagnostic facilities and the relatively low level of awareness with in the Iranian health care system.

Although several studies have investigated *C. burnetii* infection in dairy cattle, sheep and goats in Iran ([21–23]), no studies reported on the occurrence of *C. burnetii* in horses. Recently, it showed a boom in the horse international trade in Iran. The risk of Coxiella *spp*. transmission to humans maybe increase after exposure to infected horses during horseback riding and horse keeping. This study aimed to investigate the prevalence of *C. burnetii* in horse serum by using Trans-PCR method (for the first time in Iran) in Golestan Province, Iran.

2. Materials and methods

2.1. Field sampling

2.1.1. Study area

This study was carried out in Golestan Province located in the north of Iran, [geographically located between 36° 50′ 21.48″ N, 54° 26′ 39.84″ E (https://tools.wmflabs.org)]. The climate of Golestan Province enjoys mild weather and a temperate climate most of the year. Geographically, it is divided into two sections: The plains, and the mountains of the Alborz range. (Fig. 1).

3. Sampling and data collection

3.1. Sera collection

In 2017–2018, Two hundred Blood samples were collected, in two geographical regions of Golestan Province (Kalaleh County (n = 121), Gonbad Kavus (n = 79). The study population represented 4 breeds. Most horses were Turkmen (n = 50) followed by Akhal-Teke (n = 51), Turkmen Dokhun (n = 50), and Yamut Turkmen (n = 49) horses. Sex distribution was (n = 100) mares and stallion (n = 100), geldings. Sampled animals were grouped into two different age groups (< Year 1–5) (n = 141) and over 6 years old (n = 59). Sera samples were taken immediately put in sterile tubes. The collected sera samples were placed on ice and transferred to the microbiology laboratory at the Faculty of Veterinary Medicine, Urmia, Iran.

A volume of 10 mL of blood sample was collected from the jugular vein of each horse into dry and Without EDTA containing tubes; serum was recovered after centrifugation (10 min, 3000 *g*) and frozen at −20 °C.

3.2. Coxiella burnetii DNA detection

3.2.1. Extraction and purification of DNA

3.2.1.1. Boiling. One milliliter of the pre-enriched sera was transferred to a micro centrifuge (Eppendorf, Germany) tube with a capacity of 1.5 mL. The cell suspension was centrifuged for 10 min at 14,000 × *g*. The supernatant was discarded carefully. The pellet was suspended in 300 μL of DNase-RNase free distilled water (DNA Zist ASIA) by vortexing (Fanavaran Sahand Azar co, Iran). The tube was centrifuged at 14,000 × *g* for 5 min, and the supernatant was discarded carefully. The pellet was suspended in 200 μL of DNase-RNase-free distilled water by vortexing. The micro centrifuge tube was incubated for 15 min at 100 °C and immediately chilled on ice. The tube was centrifuged for 5 min at 14,000 × *g* at 4 °C. The supernatant was carefully transferred to a new micro centrifuge tube and incubated again for 10 min at 100 °C and chilled immediately on ice. An aliquot of 5 μL of the supernatant was used as the template DNA in the PCR.

The procedure was performed according to the method outlined by B. Malorny http://www.pcr.dk/DNA-purification.htm) [24].

During DNA extraction procedure, Water DEPC-Treated (Santa Cruz, USA) was used as Negative Control of Extraction. (*C. burnetii* standard Nine Mile strain RSA 493) was used as Positive Control of DNA amplification procedure. In this research bacterial DNA extracted from sera directly, because coxiella cannot growth on usual microbiological media and need specific cell cultures in level 3 labs at least.

4. Laboratory analyses

4.1. Nested-PCR for the molecular identification of C. burnetii

The Nested-PCR protocol, amplifying a fragment of 203bp of the IS1111 insertion sequence and described. The primers for the trans-PCR and nested-PCR which were used in this study previously described by Parisi et al. (Parisia et al., 2006) and [13] respectively. Primer sequences for detection of C. burnetii IS1111 gene by nested PCR, Touchdown and Trans-PCR conditions and Nested-PCR conditions showed in (Table 1 and 2) respectively.

There were some modifications in Touchdown and Nested PCR conditions that described by (Parisia et al., 2006) and ([13]a). Taq DNA Polymerase Master Mix RED (Amplicon, Denmark) was used. The PCR reaction was prepared in 25 μl volume comprising 5 μl of extracted DNA, 50 Pico moles of each primer (Trans 1 & Trans 2), 12.5 μl of master mix. The touchdown PCR was used to optimize and reduce contamination and inhibitors, as well as increasing the specificity and sensitivity of the reaction. The touchdown and Trans-PCR thermal programs were defined in the thermal cycler device (Quanta Biotech, England) as described previously ([13]a).

For the nested-PCR stage, PCR reaction was prepared as Trans-PCR which described previously except for the DNA template, which 2.5 μl of 1:100 diluted PCR product from the first stage was used. The thermal cycling condition was applied according to Parisi et al. procedure (Parisia et al., 2006). The PCR products of both stages were electrophoresed on a 2% agarose gel containing safe stain and then visualized using Ingenious Gel Documentation (Syngene Bio Imaging, UK).

Due to the current situation regarding Covid-19 outbreak and the closure of research centers, we could not be able to send any samples abroad to do sequencing on obtained DNA.

5. Data analyses

The obtained data were statistically analyzed by the Chi-square test using SPSS software Ver. 22 (SPSS Inc., Chicago, IL). The P value < 0.05 was considered significant.

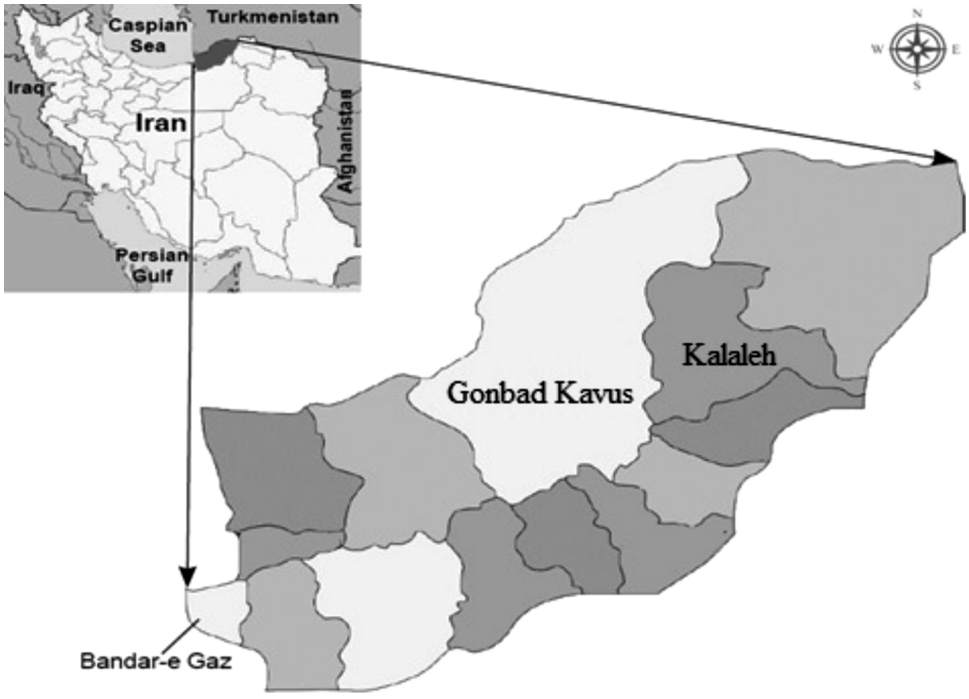

Fig. 1. Schematic map of the study areas, Golestan Province, Iran.

Table 1
Primer sequences for detection of **C. burnetii** IS1111 gene by nested PCR.

Protocol	Primer Name	Sequence 5′——3′	PCR product size (bp)
Trans-PCR	Trans 1	TATGTATCCACCGTAGCCAGTC	687
	Trans 2	CCCAACAACACCTCCTTATTC	
nested-PCR	261F	GAGCGAACCATTGGTATCG	203
	463R	CTTTAACAGCGCTTGAACGT	

Table 2
Touchdown, Trans-PCR and Nested-PCR conditions °C/no. of Second (s)/ Minutes (m).

Time	Temperature				Phase
120^{s}	$95^{°C}$				Pre Denaturation
	Cycle	Time	Temperature	Denaturation	Touchdown PCR
	5	30^{s}	$94^{°C}$		
		1	$66-61^{°C}$	Annealing	
		1	$72^{°C}$	Elongation	
	Trans-PCR Cycle Temperature				Denaturation
30^{s}	$94^{°C}$				
30^{s}	$61^{°C}$				Annealing
60^{s}	$72^{°C}$				Elongation
	End Cycle				
10^{m}	$72^{°C}$				Final Elongation
Nested PCR					
3^{m}	$94^{°C}$				Pre Denaturation
35	Cycle				
30^{s}	$94^{°C}$				Denaturation
45^{s}	$54^{°C}$				Annealing
60^{s}	$72^{°C}$				Elongation
10^{m}	$72^{°C}$				Final Elongation

6. Results

Of the two hundred horse serum samples tested for Nested-PCR, 15 serum samples were positive for *C. burentii* bacterium and molecular prevalence (7.50 %) (P < 0.05; 95 % CI: 0.5 % –0.12 %) was obtained. Of the 15 molecularly positive samples, 8 (4%) serum samples belong to stallion (p-value < 0.05, 95 %, CI: 4 %–15 %). And also 7 (3.5 %) serum samples were Mares specimens (p-value. < 0.05, 95 %, CI: 3.4 %–14 %). there was no significant difference between stallions and mares in infection with *C. burentii*. Also, the rate of infection among the age group > 6 years old (p-value < 0.05, 95 %, CI: 7 %–19.8 %) is higher than the age group < Year 5 − 1 (p-value < 0.05, 95 % CI: 1 %–8 %). The results of this study showed that there was a significant difference between age and level of pollution. In this study, there was no significant difference between the study areas. Statistical analysis of the data including Sex, Age and Region as epidemiological factors involved in Q fever infection showed in (Table3).

Agarose gel image of amplified fragment of *C. burnetii* IS1111 gene (203 bp) using nested-PCR, illustrated in (Fig. 2).

7. Discussion

Q fever is a zoonotic illness which has expanded across the world. *C. burnetti* could be isolated from a broad range of animals including farm animals (e.g. cattle, sheep and goats), wildlife, and arthropods. It distributes globally. The transmission of this disease occurs mainly via inhalation of aerosols contaminated with *C. burnetii*. Bacterial excretion (delivered placentas, fetal fluids, vaginal discharges, feces, urine, and milk) from infected animals contaminates environment [14]. As *C. burnetii* is a bacterium with unique characteristics in terms of persistence in the environment and hosts [3], gathering information on the

Table 3
Statistical analysis of the research results (Sex, Age and Region).

Variable	Category	Freq.	PCR-Positive (%)	95 % CI
Participant		200	15(7.5 %)	(5%–12 %)
Sex	Mares	100	8(8%)	(4 %–15 %)
	stallion	100	7(7%)	(3.4 %–14 %)
Age group	< Year5 − 1	100	3(3%)	(1 %–8 %)
	> 6 years old	100	12(12 %)	(7 %–19.8 %)
Region	Kalaleh	116	10(8.62 %)	(5 %–15 %)
	Gonbad Kavus	84	5(6%)	(2.6 %–13 %)

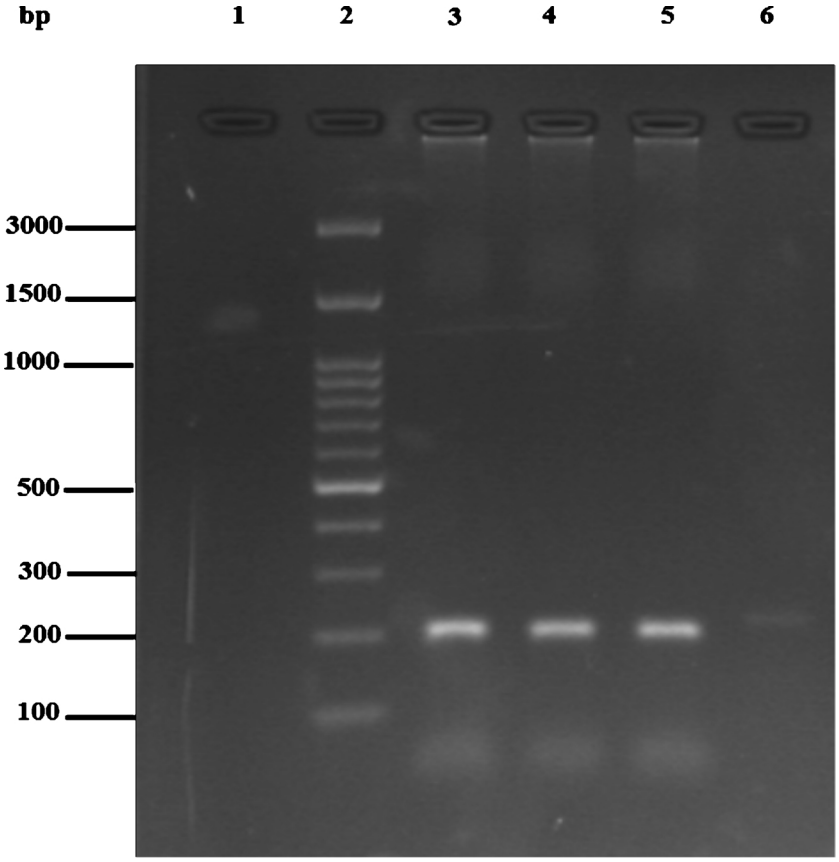

Fig. 2. Agarose gel image of amplified fragment of *C. burnetii* IS1111 gene (203 bp) using nested-PCR. Lane 1, Negative control, Lane 2, 100- bp molecular ladder (Smobio Technology Inc., Taiwan); lanes 3, Positive control (*C. burnetii* standard Nine Mile strain RSA 493), 4, 5, 6 positive samples for *C. burnetii*.

impact of *C. burnetii* on ruminants is pivotal [25].

Few studies have examined possible horses' role in transmitting Q fever to a human being [26]. *C. burnetii* may be widely prevalent but undiagnosed in livestock in Iran. It is noteworthy that the control of Q fever in animals and humans is important for the management of disease, as well as early and accurate detection of *C. burnetii,* is required [27]. Q fever is widely distributed in Iran [22,28]. *C. burnetii* has been isolated from arthropods, animals, and humans [1]. In the current study, among 200 collected sera samples, after amplifying a fragment of 203 bp of the IS1111 gene using nested-PCR, 15 samples (7.5 %) were positive for *C. burnetii*.

In some countries, studies performed on abortion rate in horses showed relatively low positive rates; (0%; 0/122) by using PCR in Italy [14], blood (0%; 0/105) by complement fixation test in Denmark [5], aborted fetuses (1.5 %; 6/407) by real-time PCR in France [29], and aborted fetuses (4.3 %; 1/23) by real-time PCR in Germany [30]. On the other hand, failed pregnancies were high (42.2 %; 19/45) in Croatia [31], blood (22.2 %; 4/18) by loop-mediated isothermal amplification in China [32], aborted or non-aborted placenta (7.7 %; 3/39) by real-time PCR in the Netherlands [33], and blood (12.5 %; 14/112) and urine (7.1 %; 1/14) by real-time PCR in Australia [34].

The pooled mean sera-prevalence evaluated in horses was not markedly distinct from the mean sera -prevalence in ruminants obtained in other systematic reviews: 20 % for cattle, and 15 % for goats and sheep [35] and 15.0–21.0 % for cattle, 2.5–88.1 % for goats and 3.5–56.9 % for sheep at animal level [36]. Recent studies were based on the characterization of considering heterogeneity in aims, geographical areas, specimens, sampling methods and results. With respect to *C. burnetii* infection, horses received only marginal attention compared to ruminants resulting in underscoring the important role of horses as a source of *C. burnetii* infection [36–38], the majority of studies could be partially biased because of special attention paid to ruminants in case of coxiellosis. In support of the aforesaid, a reporting bias was found in the Netherlands when addressing the role of goats, where Q fever was called "goat flu" [39].

Interestingly, in this study there was significant difference between horse $<$ Year $5-1$(3%) (p-value $<$ 0.05, 95 % CI: 1 %–8.5 %) and $>$ 6 years old (14.15 %) (p-value $<$ 0.05, 95 %, CI: 8.5 %–23 %) age group for *C. burnetii* infection. this result revealed that age may be a risk factor for *C. burnetii* in horses. The difference may be explained as a consequence of a higher probability of contact [40,41].

Other factors apart from species, such as farm densities of animals, seasonality of births or management, could explain the higher frequency of *C. burnetii* infection in ruminants. The risk factors identified for *C. burnetii* infection in ruminants should also be examined when studying the infection in horses. Moreover, different *C. burnetii* infections may have differing epidemiologic characteristics depending on the nature of specific outbreaks [36].

The most important diagnostic problems of *C. burnetii* is collection and storage of specimens.

C. burnetii is a very infectious intracellular bacterium. Thus, only biosafety level 3 laboratories and experienced personnel should be allowed to manipulate contaminated specimens and cultivate this microorganism from clinical samples [42,43]. There were many limitations in this research that we point as: no any history about abortion in horse herds, no accessible facilities for bacterial cell culturing and many limitations related with occurrence of Covid-19 pandemic.

Finally, because Q fever is not a notifiable disease and many human cases could have been misdiagnosed as some other infection, the public health impact of *C. burnetii* infection will be not understood until suitable epidemiologic surveillance is effectively employed in Iran.

8. Conclusion

To conclude, horses can play an important role in the epidemiology of Q fever as reservoir for *C. burnetii*. Attained data show that PCR can be used as an easy and reliable approach for detecting Q fever causative agent. Therefore, decreasing the risk of Q fever transmission from horse to other animals and humans is followed. Our study shows that the impact of Q fever disease on horses' health status in the studied area appears to be low, but quite important. One of the limitations of our study was little number of positive cases that made it impossible for us to properly analyze the risk factors Statistical and epidemiologically. The authors suggest application of PCR based method to reliable diagnosis of Q fever in animal with all kind of samples such as serum sample.

Declaration of Competing Interest

The authors have not conflicts of interest to disclose.

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