Genomic detection of *Coxiella burnetii* based on plasmid genes in horses

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Abstract

Q fever is a worldwide zoonosis caused by an obligate intra-cellular pathogen called *Coxiella burnetii* affecting a broad range of animal hosts including horses. Most of the isolates found carry plasmids which genetic studies of *C. burnetii* strains suggest a critical role in *C. burnetii* survival. The correlation between an isolated plasmid type and the chronic or acute nature of the disease has always been controversial. This study was conducted to investigate the prevalence of *C. burnetii QpH1* and *QpDG* plasmids in horses and assess the potential role of these species as reservoirs of infection and transmission. Nested-polymerase chain reaction (PCR) assays were performed on 320 blood serum samples drawn from horses in West Azerbaijan province, Iran, in 2020. In total, 26 (8.13%) Q fever-positive samples based on containing the *IS1111* gene were tested by nested-PCR approach to amplify *QpH1* and *QpDG* plasmid segments. The *QpH1* and *QpRS* plasmid-specific sequences were identified in 19 (73.07%) and none in the serum samples, respectively. According to the present study, the age of the animal can be considered as an important risk factor for the prevalence of *C. burnetii*; but, the season, sex, and breed of the horse had no effect on the prevalence of disease. The results indicate that nested-PCR method could be suitable for routine diagnosis, to gather new information about the shedding of *C. burnetii*, and to improve the knowledge of contamination routes.

Introduction

The obligate, Gram-negative bacterium *Coxiella burnetii* causes globally distributed zoonotic Q fever.[1] Although Q fever is not a reportable disease in many places, human and animal infections of Q fever is known to be endemic in Iran.[2] Acute form of Q fever is usually a mild disease with flu-like symptoms. Chronic form may vary from endocarditis to chronic hepatitis.[3] The role of horses in the natural cycle of *C. burnetii* remains unknown;[4] however, experimental infections have shown that all the infected horses can develop depression, fever, intestinal inflammation, and lobular pneumonia.[5,6]

On the other hand, serological evidence of *C. burnetii* infections in horses has long been reported. Study results, ranging from no evidence of infection to a seroprevalence of 52.50%, support the hypothesis that horses may occasionally be sensitive to *C. burnetii* infections;[7] but, their potential role as a source of further infection remains unclear. Several polymerase chain reaction (PCR)-based diagnostic tests have recently been developed to detect *C. burnetii* DNA in clinical samples; *C. burnetii* DNA has occasionally been reported in equine aborted fetuses, urine, placentas, whole blood, and blood serum samples.[5,7-10]

Presence of mobile genetic elements and virulence related pseudo-genes is predicted to be specific genome manifestations of the obligate intra-cellular lifestyle of this pathogen.[11] All isolates of *C. burnetii* carry a large, auto-replicating plasmid.[12]

Unique sequences for each plasmid type have been described,[6,13] being used to design primers for the differentiation of *C. burnetii* plasmid types by PCR. The plasmids range from 32 to 54 kb in size and share a 25-kb core region;[13] the sequences of all five representative plasmid types in *C. burnetii* have been determined. The *C. burnetii* can harbor five different plasmids of different sizes and compositions, namely *QpH1*, *QpRS*, *QpDG*, *QpDV*, and plasmidless.[14]

Plasmids function in the biology of *C. burnetii* supports the critical role for extra-chromosomal elements in *C. burnetii* pathogenesis.[15] A potential influence of the different *C. burnetii* plasmids on animal and human

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diseases has been discussed.[16] Early studies have suggested that the *QpH1* and *QpRS* plasmids are markers for acute or chronic diseases, respectively.[17] However, this assumption could not be verified in a later study.[18] The third plasmid, *QpDG*, was found in *C. burnetii* isolates from wild rodents and these isolates were shown to be virulent for guinea pigs;[19] their pathogenicity for humans is unknown.[17]

Nested-PCR is a modification of PCR being designed to improve sensitivity and specificity. Nested-PCR involves the use of two primer sets and two successive PCR reactions. The first set of primers is designed to anneal sequences upstream from the second set of primers and used in an initial PCR reaction. Amplicons resulting from the first PCR reaction are used as template for a second set of primers and second amplification step. Sensitivity and specificity of DNA amplification may be significantly enhanced with this technique. However, the potential for carryover contamination of the reaction is typically increased due to the additional manipulation of amplicon products. To minimize carryover, different parts of the process should be physically separated from each other, preferably in entirely separate rooms. Amplicons from nested-PCR assays are detected in the same manner as in PCR.

A recent study testing the hypothesis that obstetric complications in *C. burnetii* infections were associated with a particular genotype and the presence of *QpDV*, found that this plasmid type was detected more frequently in isolates associated with abortions.[20] Thus, identification of *C. burnetii* plasmids may provide important information for the differential diagnosis of Q fever and epidemiological investigations.

Materials and Methods

Data collection. In 2019 - 2020, 320 horse blood samples (Mare 155, Stallion 165) were collected from four geographical regions of West Azerbaijan province (including Urmia, Khoy, Piranshahr and Shahin Dezh) being located in the northwest of Iran (Fig. 1). The samples were labeled with identification numbers. Besides, information regarding the potential risk factors such as age, breed, and sex was recorded. Blood samples were centrifuged at 14,000 *g* for 10 min at 4.00 °C and then, the supernatant was frozen at – 80.00 °C.

Preparation of samples for PCR. The DNA was extracted using commercially available kit (Bioneer Corp., Seoul, Korea), following the manufacturer's protocol. The DNA concentration and purity were determined by measuring the optical density at both 260 and 280 nm with a DNA calculator (GeneQuant II; Pharmacia Biotech, Burladingen, Germany). The DNA solution was kept at – 20.00 °C.

Detection of *C. burnetii* in samples. Detection of *C. burnetii* in horse blood samples was performed in the Nested-PCR procedure targeting *IS1111* gene. The Trans-

PCR assay was performed as described formerly.[21] Amplifying a fragment of 203 bp of the *IS1111* insertion sequence used in this study is previously described by Parisi *et al.*[22] Primer sequences for detection of *C. burnetii IS1111* gene, plasmid genes and PCR conditions are used according to Parisi *et al.*, and Zhang *et al.*[14,22]
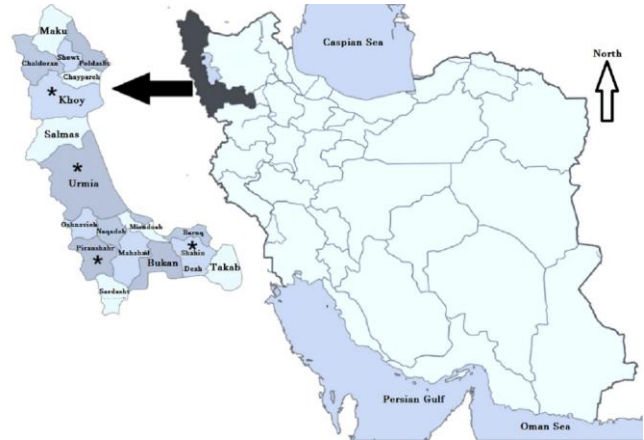

Fig. 1. Schematic map of the study areas (*) in west Azerbaijan province, Iran.

Primers used for detection of *C. burnetii* plasmids. The sequences of primers (Pishgam, Iran, Tehran) used in the study and PCR conditions are presented in Table 1. The modified primers were used to detect *C. burnetii* plasmid-specific sequences. The first set of primers was designed from a specific gene of the *QpH1* plasmid, *cbhE*.[21,23] The second set of primers, QpRS1-QpRS2 and QpRS3-QpRS4, was designed from a unique gene of the *QpRS* plasmid, *cbbE*.[19,24]

DNA amplification. The nested-PCR reaction (Trans-PCR stage) was prepared in 25.00 μL volume comprising 5.00 μL of extracted DNA, 50.00 pM of each primer (Trans 1 and Trans 2), and 12.50 μL of master mix. The Trans-PCR was applied and 2.50 μL of a 10^{-2} dilution of the reaction mixture was used for the nested-PCR. The encapsulation of two PCRs increased specificity and decreased the detection limit.

Detection of PCR products. The PCR products of both stages were electrophoresed on a 1.50% agarose gel containing safe stain and ethidium bromide (0.50 μg mL^{-1}), visualized using Ingenious Gel Documentation (Syngene Bio Imaging, Cambridge, UK) and then, photographed.

Statistical analyses. The obtained data were statistically analyzed by the Chi-square test using SPSS software (version 24.0; IBM Corp., Armonk, USA). The $p < 0.05$ was considered significant.

Results

A total of 320 equines were sampled for detection of *C. burnetii* specific gene. Twenty-six (8.13%) of these samples were positive by *IS1111* insertion (Fig. 2).

Table 1. The amplification protocol names, thermal program for both PCR and primer names and sequences and the size of PCR products.

Protocol	Primer Name	Sequence 5------3	Product size (bp)	PCR condition (Cycle No.)	Ref.
PCR	Trans 1 Trans 2	TATGTATCCACCGTAGCCAGTC CCCAACAACACCTCCTTATTC	687	95.00 ℃ for 3 min, 94.00 ℃ for 30 sec, 62.00 - 66.00 ℃ for 30 sec (5); 72.00 ℃ for 1 min, 72.00 ℃ for 10 min (35)	22
Nested-PCR	261F 463R	GAGCGAACCATTGGTATCG CTTTAACAGCGCTTGAACGT	203	95.00 ℃ for 3 min, 94.00 ℃ for 30 sec, 54 for 20 sec, 72.00 ℃ for 1 min, 72.00 ℃ for 10 min (35)	22
PCR	CB5 CB6	ATAATGAGATTAGAACAACCAAGA TCTTTCTTGTTCATTTTCTGAGTC	977	94.00 ℃ for 4 min, 94.00 ℃ for 2 min, 53.00 ℃ for 1 min, 72.00 ℃ for 2 min, (35)	14
Nested-PCR	QpH1F QpH1R	CTCGCTGACGGAAGAGGATCTTTT TAACACTGCCCGTCGCTTTACT	602	94.00 ℃ for 3 min, 94 for 45 sec, 50.00 ℃ for 45 sec, 72 for 45 sec, 72.00 ℃ for 5 min. (35)	14
PCR	QpRS1 QpRS2	CTCGTACCCAAAGACTATGAATATATCC AACACCGATCAATGCGACTAGCCC	693	94.00 ℃ for 4 min, 94.00 ℃ for 1 min, 54.00 ℃ for 1 min, 72.00 ℃ for 2 min, 72.00 ℃ for 7 min (36)	14
Nested-PCR	QpRS3 QpRS4	ACTTTACGTCGTTTAATTCGC CACATTGGGTATCGTACTGTCCCT	309	94.00 ℃ for 3 min, 94.00 ℃ for 30 sec, 51.00 ℃ for 20 sec, 72 for 90 sec, 72.00 ℃ for 7 min (35)	14

The results of this study showed a significant difference between age and contamination levels and no significant differences between study areas. By gender, a total of 10 (38.46%) males and 16 (61.54%) females were observed, respectively. However, there was no significant difference between stallions and mares infected with *C. burentii* (p > 0.05). The difference between breeds was not statistically significant (p > 0.05). Our results indicated that the incidence differed significantly by age group (p < 0.50). The higher incidence rate was identified in the ≤10 years old with most of the cases (17.39%). The C*. burnetii* was reported from all four regions in west Azerbaijan, Iran; the highest reported incidence rate was primarily observed in the Urmia; but the difference was not statistically significant among different districts (p > 0.05; Table 2). The C*. burnetii* plasmid type was identified directly in horse serum by nested-PCR using primers targeting the *QpH1* and *QpRS* plasmids from 26 horses. Nineteen (73.07%) serum samples were positive for the *QpH1* plasmid and negative for the *QpRS* plasmid (Fig. 3). Of 320 horse blood serum samples tested by nested-PCR,[25,26] serum samples were diagnosed with Q fever with molecular prevalence of (8.13%).

Table 2. Statistical analysis of the research results (sex, Age, Region and breed).

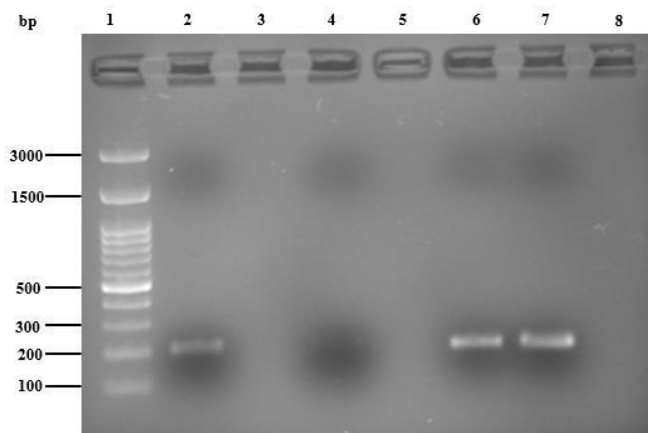| Parameter	Category	Sample	PCR-positive (%)
Sex	Mare (n = 155)	260	10 (3.84)
	Stallion (n = 165)	260	16 (6.15)
Age	< 1-3 years	100	4 (4.00)
	3-10 years	105	6 (5.71)
	> 10 years	115	16 (13.91)
Region	Urmia	80	9 (11. 25)
	Khoy	80	6 (7.50)
	Piranshahr	80	4 (5.00)
	Shahin Dezh	80	5 (6.25)
Breed	Kurd	70	10 (14.28)
	Turkmen	30	5 (16.66)
	Dareshuri	45	6 (13.33)
	Arab	50	2 (4.00)
	Foreign breeds	25	3 (12.00)

Fig. 2. An agarose gel electrophoretogram of the 203-bp amplification products after the nested-polymerase chain reaction and ethidium bromide staining. Lane 1: Molecular size markers (100 bp DNA ladder); Lanes 2, 6 and 7: Positive serum samples; Lanes 3, 4 and 5: Negative serum samples; Lanes 8: Negative control.
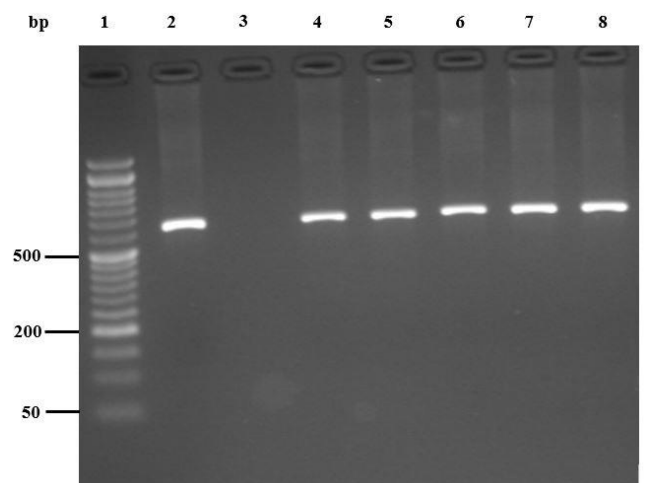

Fig. 3. Agarose gel image of amplified fragment of *Coxiella burnetii QpH1* plasmid. Lane 1: Molecular size markers (100 bp DNA ladder; Lane 2, Positive control: Lane 3, Negative control: Lanes 4, 5, 6, 7 and 8, Positive sample.

Discussion

The Q fever is a common zoonotic disease caused by *C. burnetii* that can infect a wide range of animal species and humans. Although the organism is widespread throughout the world, serological studies in New Zealand have shown no evidence of the disease.[25] The zoonotic role of horses as a reservoir for *C. burnetii* infection has not been clearly established. This role has not been adequately investigated and has long been reported with variable results, from no detection of any evidence of infection[26] to about 52.50% seroprevalence.[27] In fact, available serological examinations show that horses can be exposed to *C. burnetii* naturally; but, serological assays have low sensitivity and specificity.[4]

In general, seroprevalence in equines based on published studies is not significantly different from the average seroprevalence in ruminants. Data from sub-groups or individual studies of *C. burnetii* infection in horse showed a seroprevalence of 15.80% (95.00% confidence interval: 9.60 - 23.00%). This pattern is close to those previously reported in other species, especially ruminants. The average seroprevalence is reported to be 15.00 - 21.00% for cows, 2.50 - 88.00% for goats, and 3.50 - 56.90% for sheep.[7] The most important factors with respect to prevalence of *C. burnetii* in different parts of the world are geographical variations, type of survey, type and number of samples, and the season in which the sampling was done. Blood serum sampling in the early stages of systemic diseases makes it a suitable specimen for molecular diagnosis due to the possible presence of the pathogen's genome. Due to the presence of inhibitors in the blood and the small amount of microorganisms DNA in the serum, conventional PCR is not suitable for diagnosis.[28] The sensitivity of Trans-PCR is 100 times higher than conventional PCR.[21] In addition, an effective, simple, and rapid method of removing PCR inhibitors from the sample by diluting the DNA pattern to 10^{-2} has been described, making it possible to detect one microorganism per milligram of the sample.[21] Nested-PCR is therefore a very sensitive method for laboratory diagnosis of *C. burnetii* infection. Positive cases of *C. burnetii* infection in horses provided an important opportunity to identify the horse as a carrier of the organism and raise the possibility of transmitting the infection to humans and animals.[7] Comparison with other studies in horses with the aim of investigating *C. burnetii* is difficult due to the differences in the methods used in terms of specificity and sensitivity. Although, *C. burnetii* has been isolated from arthropods, animals, and humans.[29-31] The latest molecular study investigating the prevalence of *C. burnetii* in horse blood sera samples in northern Iran (Golestan province) showed 7.50% positivity for *C. burnetii*.[9] In the present study, the *IS1111* sequences were detected in 26 horses (8.13%). This result indicates that *C. burnetii* has spread in horses in west Azerbaijan, Iran.

The *C. burnetii* strains carry one of four large, conserved, and autonomously replicating plasmids (*QpH1*, *QpRS*, *QpDV*, or *QpDG*). The *QpH1* plasmid was first obtained from a tick isolate and found in most isolates derived from ticks, and patients with acute Q fever.[32] The *QpRS* plasmid was detected in a wild rodent isolate and then, found in most chronic Q fever patients isolates.[32] The *QpDG* plasmid has only been found in a few wild rodent isolates.[17,19] The *QpDV* plasmid was detected in a cow milk isolate and humans with pneumonia, acute Q fever, aortic aneurysm, and chronic endocarditis.[33] Samuel *et al.*,[17] demonstrated that the isolates originating from patients with acute Q fever contain the *QpH1* plasmid; while, the isolates originating from patients with chronic Q fever possess the *QpRS* plasmid or plasmid sequences integrated into the chromosome.

In the present study, the sera were taken from 26 horses being diagnosed with Q fever by nested-PCR targeting *IS1111* gene. It was found that all of the positive serum samples possessed the *QpH1* plasmid-specific gene; but, no *QpRS* plasmid. This is the first report of *QpH1* and *QpDG* plasmids of *C. burnetii* isolate occurring in horses in Iran. However, Q fever is not categorized in Iran as a notifiable disease and detailed clinical information is not available. Although some studies have shown higher seroprevalence in mares,[34] the reasons for this have not been clear. The difference is probably due to the differences in the management and maintenance of the animal; but, other studies have shown that the higher seroprevalence in females can be attributed to the high tendency of *C. burnetii* to mammary glands and uterus because the frequency of *C.burnetii* is higher in these tissues.[35] Although the number of reported cases of Q fever may vary from region to region, this may not reflect the reality of the disease.

It is noteworthy to mention that in Europe, cases of acute Q fever are more common in spring and early summer. Apparently, the highest incidence of Q fever in spring is related to the calving season of sheep, which is the time of severe environmental contamination with *C. burnetii*.[6]

The present study is the first study based on the plasmid pattern of *C. burnetii* causing Q fever in horses. While the relationship between horses in the epidemiology of Q fever is unclear, studies have shown that horses can be naturally exposed to *C. burnetii*. According to the present study, the age of the animal can be considered as an important risk factor for the prevalence of *C. burnetii*; but, the season, sex, and breed of the horse had no effect on the prevalence of the disease. Due to the effect of geographical location and differences in pollution in different regions, the extensive epidemiological study in different parts of the country is highly suggested. Given that plasmids can have host specificity, it is suggested that other studies be performed to investigate the prevalence of *C. burnetii* based on plasmids in other equines, including donkey and mule.

Acknowledgments

Conflict of interest

The authors declare no conflict of interest.

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