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Molecular diagnosis of *Coxiella burnetii* in milk based on Plasmid and Transposon Genes

Zahra Javadi, Abdolghaffar Ownagh*

Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, West Azerbaijan, Iran

ABSTRACT

Background and Aim: All *Coxiella burnetii* isolates carry one of four large, conserved, autonomously replicating plasmids or a plasmid-like chromosomally integrated sequence.

Materials and Methods: In this study, from 2020 to 2021, 400 milk samples were collected from domestic ruminants (cows, sheep, goats, and buffaloes) in West Azerbaijan province. DNA extraction (Forgen, Taiwan) was used to take the bacteria genome. Nested-PCR method was used to perform PCR to the amplification of *IS1111* genes and plasmids (*QPH1*, *QpRS*, *QpDV*, and *QpDG*) using specific primers for each gene.

Results: In total, out of 400 milk samples collected from cow, buffalo, sheep, and goats based on the *IS1111* gene, 62 (15.5%), (95% CI: 12.3%–19.4%) samples were positive for *C. burnetii*. Out of 62 positive samples, 16 (25.8%), (95% CI: 16.6%–37.9%) samples contained *QpH1* plasmid gene and 5 (8%), (95% CI: 3.5%–17.5%) samples contained *QpRS* plasmid gene. Also, there were 7 (11.3%), (95% CI: 5.6%–21.5%) positive samples for *QpDG* and 5 (11.3%), (95% CI: 3.5%–17.5%) positive to *QpDV* gene. The Phylogenetic analysis of plasmid sequences showed that all obtained sequences have 100% similarity. A phylogenetic tree constructed based on neighbor-joining analysis of partial genes revealed that 20 sequenced isolates were closely clustered together showing 99.9% similarity which can be considered identical and also revealed the 100% similarly of these sequences with more sequences in the gene bank from different sources.

Conclusion: Our results indicated that nested PCR has high sensitivity in detecting plasmids.

Keywords: Milk, Q fever, *Coxiella burnetii*, Plasmid, Transposon genes

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Corresponding Information: **Abdolghaffar Ownagh**, *Department of Microbiology, Faculty of Veterinary Medicine, Urmia University, Urmia, West Azerbaijan, Iran* Email: a.ownagh@urmia.ac.ir , ownagh@yahoo.com

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1. Introduction

Coxiella burnetii is a gram-negative intracellular bacterium that causes Q fever. Moreover, it is widely distributed as a zoonosis disease (1). This etiological agent is known as an important micro-organism in causing infection in human beings and animals (2). Clinical finding of Q fever is asymptomatic, but in acute form, it can cause self-limiting febrile illness, pneumonia, or hepatitis. In chronic form, the main sign is endocarditis in patients with previous valvulopathy (3). *C. burnetii* have integrated sequences and plasmids (4). These sequences involve the surveillance of *C. burnetii* and also help in designing new vaccines for preventing and controlling Q fever (5).

The *IS1111* -insertion sequence, coding for a transposase, is seen in up to 56 copies in *C. burnetii* genomes. Consequently, this element is often used as a specific target providing sensitive diagnostic PCRs (6). In 2007, Denison *et al.* established a genotyping system based on four of these insertion sequence regions, which were analyzed by PCR, using an antisense primer binding inside of the *IS1111* -elements in combination with an upstream sense

primer specific for each element **(7)**. The algorithm proposed by Denison *et al.* allowed a classification into genomic groups I–V, according to the six clusters derived by different authors described above using plasmid profiles, PCR, or restriction-endonuclease digested DNA. One advantage of this method is its ease of performing inside and between laboratories when comparing up to five different PCR results embedded in the above-mentioned decision tree **(8)**.

Coxiella burnetii strains normally possess one of four autonomously replicating plasmids termed QpH1, QpRs, QpDV, and QpDG, or a chromosomally integrated QpH1-like plasmid. QpH1 plasmids are closely related and likely identical to QpDG **(9)**.

All *C. burnetii* isolates examined to date maintain a related autonomously replicating plasmid or have chromosomally integrated plasmid-like sequences (IPS). Nucleotide sequences have been determined for QpH1, QpRS, QpDG, QpDV, and IPS of the G and S isolates **(4)**.

Five different plasmid types of *C. burnetii* including: four plasmids (*QpH1*, *QpRS*, *QpDV*, and *QpDG*) and one type of *QpRS*-like chromosomally integrated sequence **(9)**. The plasmids range from 32 to 54-kb in size and share a 25-kb core region **(10)**. According to these plasmid types, there are five associated genomic groups **(5)**. the genomic group-specific virulence was examined in mice and guinea pigs by experimental studies **(11, 12)**. According to the studies on *C. burnetii* mostly from Europe and North America, this bacterium is considered to have a clonal population structure with low genetic diversity. Further research on *C. burnetii* isolates has a considerable impact on investigating global genetic diversity (particularly from different eras) **(13)**.

The effectors play an important role in *C. burnetii's* pathogenicity. However, regarding the plasmid effectors, reports on their mutants are scarce. By using transposon mutagenesis, eight mutants of *QpH1* genes and strong phenotypes were observed. Martinez, Cantet **(13)**.

Detection of the *C. burnetii* using PCR amplification of chromosomal *IS1111* repetitive elements has revealed the extent to which zoonotic reservoirs have dispersed *C. burnetii* into the USA, with 23.8% of over 1600 random environmental samples containing *C. burnetii* DNA [7]. Q fever outbreaks can have a substantial financial impact as illustrated by the 2007–2011 Netherlands outbreak (>4000 cases) where human disease burden and infection control measures were estimated to cost 307 million Euro **(14)**. This study aimed to analyze the phylogeny of isolated *C. burnetii* isolates based on plasmid genes in milk samples (cow, sheep, goat, and buffalo) using the nested PCR method.

2. Materials and Methods

Sampling

Four hundred milk samples were collected randomly. The number of samples based on the type of livestock, geographic region, and seasons is given in Table 1. Also, the number of herds of cows, sheep, goats, and buffaloes was 10 each. The collected milk samples were placed on ice and immediately transferred to the microbiology laboratory at the Faculty of Veterinary Medicine.

DNA extraction

Ten ml of each milk sample was centrifuged at 2191g, and then the precipitate obtained from rinsing with DEPC water was used for DNA extraction with the Blood Genomic DNA Extraction Mini Kit (50 preps) (Favorgen, Taiwan). Furthermore, all extracted samples were examined with NanoDrop 2000c (Termo Scientific, USA) to investigate the quality and concentration of DNA.

Touchdown and Nested PCR

Used Primers

The primers used for amplifying the IS1111 gene were used according to a method by Khademi and Ownagh **(18)**. Furthermore, Zhang and Hotta **(19)** *utilized a method* to amplify plasmid genes in this study. Table (1) presents the sequence of primers, temperature programs, and cycles.

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

Protocol	Primer Name	Sequence 5'----3'	PCR product size (bp)	PCR condition (Cycle)	Reference
Trans-PCR	Trans 1	TATGTATCCACCGTAGCCAGTC	687	95c for 3m, 94 c for 30s, 62-66 (5) for 30s, 72c for 1m, 72c for 10m. (35)	(13, 15)
	Trans 2	CCCAACAACACCTCCTTATTC			
nested-PCR	261F	GAGCGAACCATTGGTATCG	203	95c for 3m, 94 c for 30s, 54 for 20s, 72c for 1m, 72c for 10m. (35)	
	463R	CTTTAACAGCGCTTGAACGT			
PCR	CB5	ATAATGAGATTAGAACAACCAAGA	977	94 for 4m, 94 for2m, 53 for1m, 72 for2m, 72/5. (35)	(14)
	CB6	TCTTTCTTGTTCATTTTCTGAGTC			
nested-PCR	QpH1-F	CTCGCTGACGGAAGAGGATCTTTT	602	94 for3m, 94 for45s, 50 for45s, 72 for45s, 72 for5m. (35)	This study
	QpH1-R	TAACACTGCCCGTCGCTTTACT			
PCR	QpRS1	CTCGTACCCAAAGACTATGAATATATCC	693	94 for4m, 94 for1m, 54 for1m, 72 for2m, 72 for7m. (36)	(14, 16)
	QpRS2	AACACCGATCAATGCGACTAGCCC			
nested-PCR	QpRS3	ACTTTACGTCGTTTAATTCGC	309	94 for 3m, 94 for 30s, 51 for 20s, 72 for 90s, 72 for7m. (35)	
	QpRS4	CACATTGGGTATCGTACTGTCCCT			
PCR	QpDG1	TGAAGCGGCGATTAAGCTAT	513	95 for 3m, 95 for 30s, 55 for 20s, 72 for 60s, 72 for10m. (35)	(17)
	QpDG2	GATGGCGGTGAGTACGGTTTT			
Nested-PCR	QpDG3	GGTTGCGCTATTTGAAGAGG	265	95 for 3m, 95 for 30s, 55 for 20s, 72 for 60s, 72 for10m. (35)	
	QpDG4	ATGTCCTTCTGCCACGACTT			
PCR	QpDV1	TTCCGCTACGTTTTTCAAGG	548	95 for 3m, 95 for 30s, 55 for 20s, 72 for 60s, 72 for10m. (35)	(17)
	QpDV2	CCAAGGTTTGGAAAAGCAAA			
Nested-PCR	QpDV3	ACTATCGTTCCCTGCCCTCT	288	95 for 3m, 95 for 30s, 55 for 20s, 72 for 60s, 72 for10m. (35)	
	QpDV4	AGCCACCGGTAAATACACGA			

Electrophoresis

Detection of PCR Products

Gel electrophoresis examined the PCR products using concentrations of 1 to 2.5 of agarose gel, and the device (Syngene Bio Imaging, UK) visualized the results.

Sequencing

The PCR products of twenty *C. burnetti* isolates, with the amplified fragment of *C. burnetii IS1111* (n=4), *QpH1* (n=4), *QpRS* (n=4), *QpDV* (n=4) and *QpDG* (n=4) genes for twenty samples, were sent to SinaClon Company (Tehran, Iran) for sequencing. Obtained nucleotide sequences were searched against GenBank (National Centre for Biotechnology Information, Rockville Pike, and Bethesda, USA) using the advanced BLAST similarity search option and compared to the same sequences of *C. burnetii* isolates from GenBank. Nucleotide sequences were aligned and compared to other nucleotide sequences from GenBank using Clustal W and the phylogenetic tree was generated using the neighbor-joining method in MEGA software version X.

Statistical Analysis

Data were analyzed using the Chi-square test in SPSS version 22 (IBM Corp. Armonk, NY, USA). Differences with a p-value <0.05 were considered significant

3. Results

Specificity and Sensitivity of the Nested PCR Assays

The primers IS1111 gene and QpH1F-QpH1R amplified expected products of 203 bp and 606 bp, respectively, in the second PCRs from the Nine Mile a strain containing the *QpH1* pla*smid* (Figure 1, 2). *T*he

primers QpRS3-QpRS4, QpDV3-QpDV4, and QpDG3-QpDG4 also yielded predicted products of 309, 288, and 265 bp in the second PCRs containing QpRS,QpDV and QpDG plasmids respectively (Figure 3, 4 and 5) .

Identification of *C. burnetii* Plasmids in Milk

The usefulness of the nested PCR was first evaluated for the direct identification of *C. burnetii* plasmids in animal milk. Initially, all of the samples were PCR-positive when primers *IS1111* were used. The genomic and plasmid sequences were detected in 62 (15.5%), (95% CI: 12.3%–19.4%) of the samples. In addition, the plasmid types of *C. burnetii* were directly identified in the milk by the nested PCR with primers targeted to the *QpH1* and *QpRS*, *QpDV,* and *QpDG* plasmids. Among the 62 milk samples tested, 16 (25.8%), (95% CI: 16.6%–37.9%) were positive for the *QpH1* plasmid, 5 (8%), (95% CI: 3.5%–17.5%) were positive for the *QpRS* plasmid were plasmids, respectively (Table 2). In addition, the contamination rate based on *QpDV* 5 (11.3%), (95% CI: 3.5%–17.5%) and *QpDG* plasmid genes was 6 (9.7%) and 7 (11.3%), (95% CI: 5.6%–21.5%), respectively. Out of 62 positive samples, 28 (45.1%) samples had no plasmid.

Table 2. The prevalence of *Coxiella burnetiid* DNA in milk samples based on the animal, season, and geographical regions

	Animal				Total
	Buffalo	Cattle	Sheep	Goat	
Age group (Years old)					
< 4	2/33 (6%)	1/30 (3.3%)	0/35 (0%)	3/40 (7.5%)	6/138 (4.3%)
5-10	5/32 (15.6%)	6/30 (20%)	2/30 (6.6%)	4/31 (12.9%)	17/123 (13.8%)
>10	8/35 (22.9%)	15/40 (37.5%)	4/35 (11.4%)	7/29 (24.1%)	34/139 (24.5%)
Season					
Spring	4/25 (16%)	3/25 (12%)	1/25 (4%)	4/25 (16%)	12/100 (12%)
Summer	8/25 (32%)	11/25 (44%)	3/25 (12%)	6/25 (24%)	28/100 (28%)
Autumn	3/25 (12%)	6/25 (24%)	1/25 (4%)	3/25 (12%)	13/100 (13%)
Winter	0/25 (0%)	2/25 (8%)	1/25 (4%)	1/25 (4%)	4/100 (4%)
Region					
North	8/33 (24.2%)	10/29 (34.5%)	3/30 (10%)	6/28 (21.4%)	27/120 (22.5%)
Center	1/30 (3.3%)	8/39 (20.5%)	2/34 (5.9%)	5/31 (16.1%)	16/134 (11.9%)
South	6/37 (16.2%)	4/32 (12.5%)	1/36 (2.8%	3/41 (7.3%)	14/146 (9.6%)

Table 3. The rate of positive samples based on *C. burnetii* plasmids

Plasmid	Animal				Total
	Buffalo	Cattle	Sheep	Goat	
QpH1	5	7	4	-	16 (25.8%)
QpRS	-	-	-	5	5 (8%)
QpDV	2	1	1	1	5 (8%)
QpDG	2	2	2	1	7 (11.3%)

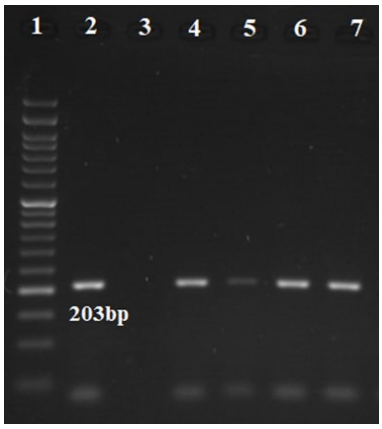

Figure 1. Agarose gel electrophoresis of amplified fragment of *C. burnetii IS1111* gene (203 bp) using nested-PCR. ; Lane 1; 50-bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 2, positive control; lanes 3, negative samples for *C. burnetiid*Lane 4 and 7, Positive sample.

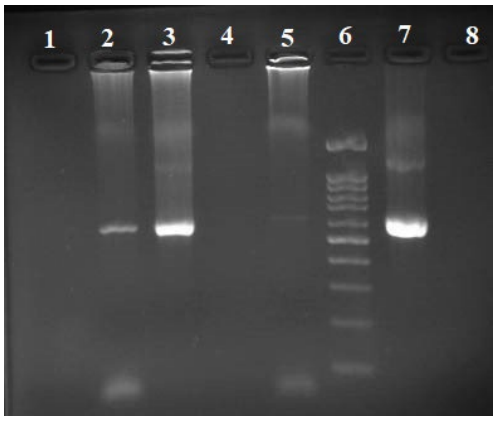

Figure 2. Agarose gel electrophoresis of amplified fragment of *C. burnetii QpH1* gene (606 bp) by using nested-PCR. lanes 2, 3 and 5; positive samples for *C. burnetii*, Lane 6, 100-bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 7, Positive control; Lane 8, negative control

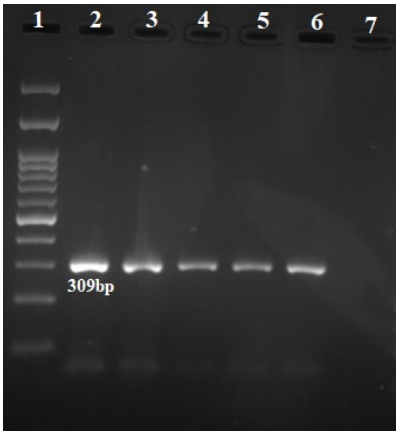

Figure 3. Agarose gel electrophoresis of an amplified fragment of *C. burnetii QpRS* gene (309 bp) using nested-PCR. Lane 1, 100-bp molecular ladder (Smobio Technology Inc., Taiwan); Lane 2, Positive control (Nine Mile strain), lanes 3, 4, 5, and 6; positive samples for *C. burnetii*, Lane 7, negative control.

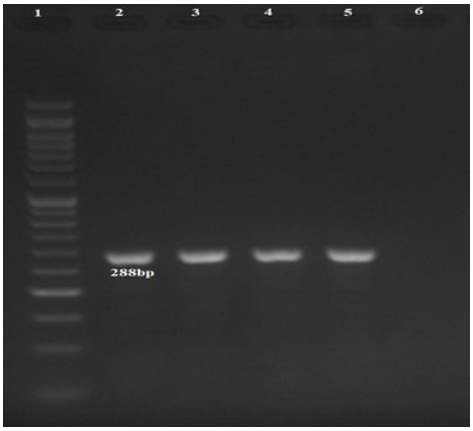

Figure 4. Agarose gel electrophoresis of an amplified fragment of *C. burnetii QpDV* gene (288 bp) using nested PCR. Lane 1, 100-bp molecular ladder (Smobio Technology Inc., Taiwan); lanes 2, 4, 5 positive samples for *C. burnetii*, Lane 6, negative control.

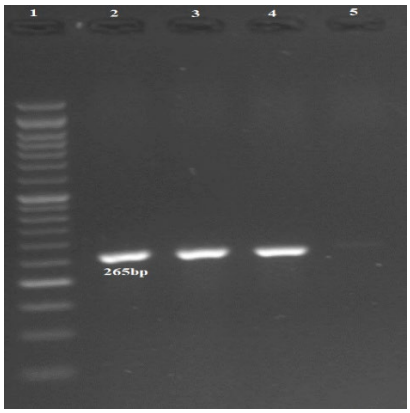

Figure 5. Agarose gel electrophoresis of an amplified fragment of *C. burnetii QpDG* gene (265 bp) using nested PCR. Lane 1, 100-bp molecular ladder (Smobio Technology Inc., Taiwan); lanes 2, 3, 4 positive samples for *C. burnetii*, Lane 5, negative control.

Phylogenetic Analysis

A phylogenetic tree constructed based on neighbor-joining analysis of *QpH1* and *QpDG* partial gene revealed that twenty isolates were closely clustered together showing 99.9% similarity that can be considered identical. *QpRS*, *QpDV,* and *QpDG* gene sequences are registered as accession numbers in the NCBI. Considering the 100% similarity between *QpDG* and *QpH1* genes in different sources and regions, there was no need to plot a phylogenetic tree (Figures 6 and 7). The submitted genes with accession numbers are as follows: (*QpDV*; OP677863, OP712504, *QpDG*; OP712505, *QpRS*; OP718633).

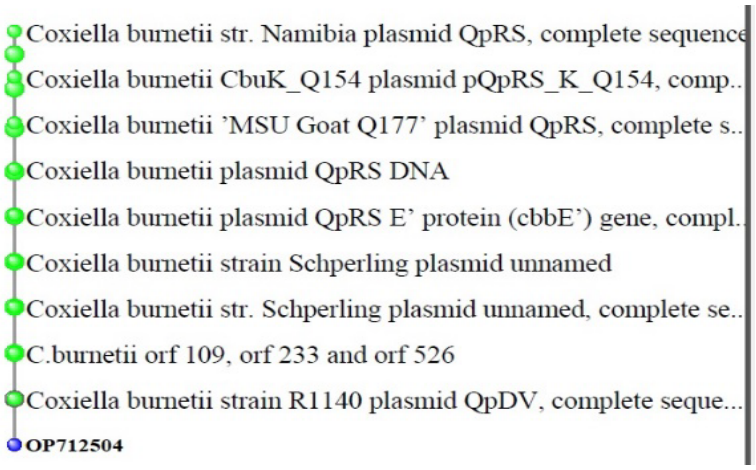

Figure 6. Phylogeny tree based on *QpDV* gene

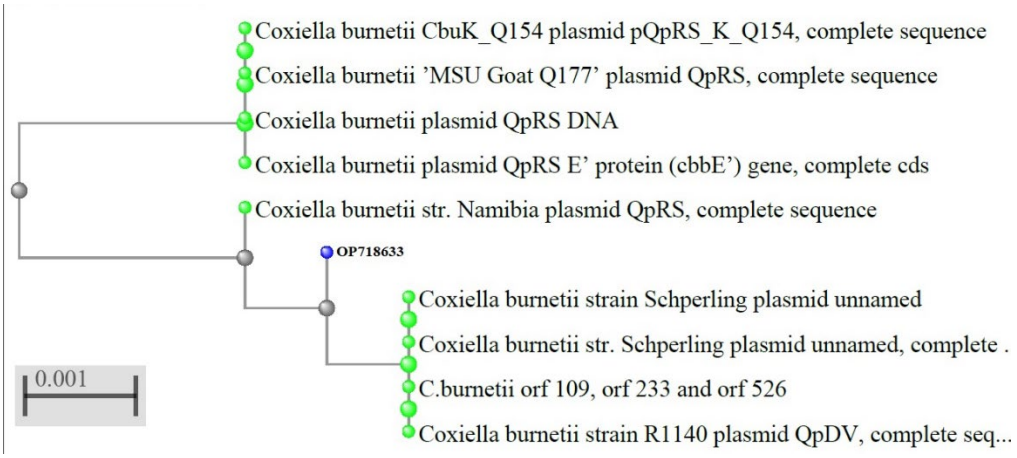

Figure 7. Phylogeny tree based on *QpRS* gene

4. Discussion

Coxiella burnetii was detected in a proportion of available raw milk, confirming that individuals who purchase and drink raw milk in Iran may be exposed to this pathogen. *Coxiella burnetii* strains carry one of four large, conserved, autonomously replicating plasmids (*QpH1*, *QpRS*, *QpDV*, or *QpDG*) and a *QpRS*-like chromosomally integrated sequence of unknown function. All *C. burnetii* strains have one of the four different types of plasmids or one plasmid-like chromosomally integrated sequence. The plasmids' role in *C. burnetii* biology has been implicated by identifying type IV secretion effectors among its genes **(4, 20)**.

The *QpH1* plasmid, first isolated from a tick, has been regularly found in isolates obtained from cattle, sheep, and goats **(9)**. Several serological and Molecular studies have suggested that Q fever is distributed widely in Iran **(15, 16, 18)**. In the present study, the *QpH1*, *QpRS*, *QpDV,* and *QpDG* specific sequences were detected in 16, 5, 5, and 7 samples with Q fever, respectively. This result indicates that different strains of *C. burnetii* have spread in cattle, goat, sheep, and buffalo milk in Iran. We also demonstrated that only 5 isolates originating from goats possessed the *QpRS* plasmid.

These data suggest that *C. burnetii* strains possessing the *QpH1* plasmid are the most prevalent strain in Iran. Samuel *et al.* **(6, 10)** demonstrated that the isolates originating from patients with acute Q fever contained the *QpH1* plasmid, while the isolates originating from patients with chronic Q fever possessed the *QpRS* plasmid or plasmid sequences integrated into the chromosome. However, because Q fever is still not diagnosed routinely in Iran, we have been unable to obtain detailed clinical data for these animals.

A study showed that fetal morbidity may be linked to the genotype of the infecting strain, as the plasmid *QpDV* was more common in isolates associated with abortions **(21)**. As already reported in the literature **(16)**, we found that the clinical manifestations of Q fever depended, at least in part, on the *C. burnetii* genotype, with strains carrying the *QpDV* plasmid

being more frequently associated with acute Q fever **(21)**. In addition, *C. burnetii* isolates associated with the *QpH1* plasmid have been shown to have fewer deleted genes than the isolates harboring the *QpRS* and *QpDV* plasmids **(21-27)**. Finally, *the QpDV plasmid harbors sequence* coding for four proteins not found in QpH1, which could explain the differences in clinical expression. However, as the plasmid type is associated with the genetic chromosome content **(21)**, only the ongoing pangenome analysis of *C. burnetii* will determine the comprehensive genomic basis for the difference in virulence between strains.

The present findings were consistent with similar studies in Iran and other countries. Based on the findings of prevalence reported by many researchers, *C. burnetii* is more prevalent in cow's milk compared to the milk of other animals. The reason for the high prevalence of *C. burnetii* in cow's milk than in other animals, such as sheep is related to the fact that the vaginal discharge of *C. burnetii* is commonly very short in cows (less than 14 days), while it is discharged in milk. It persists for much longer periods, and the bacterium is mainly excreted via feces and vaginal mucosa in sheep **(28, 29)**. Therefore, cow's milk can play an important role in the epidemiology of Q fever and greatly affects public health.

It was found that there was a significant relationship between age and discharge of *C. burnetii* in cow's milk. The finding was consistent with previous reports, indicating that age was a significant risk factor for the discharge of *C. burnetii* in cow's milk with a positive odds ratio of 1.67 times higher for each year of age **(30)**. This study's results indicated that there was significant regional diversity in the discharge of Q fever agents in raw milk. *C. burnetii* was the highest milk contamination in the province's south. It was reported that the regional distribution of Q fever in human cases was similar to the distribution and population density of sheep and cows. Therefore, it can be assumed that the population of buffaloes, cows, and sheep, which discharged the bacterium, increased the positive samples **(31)**.

Van der Hoek *et al.* and Khademi *et al.* reported a seasonal pattern of the onset of Q fever in humans in spring and early summer **(18, 32)**. It was found that the increase in the incidence of Q fever in animals was related to the lambing season, in other words, the highest number of cases was reported during the summer in terms of spring lambing season in many European countries. The highest prevalence of *C. burnetii* discharge in milk was in summer, and the result was consistent with previous reports **(5, 24)**.

If available, information on locally circulating strains may assist physicians in developing patient management strategies. The presented findings demonstrate *QpDG* and *QpH1* to be closely related and likely identical. Consequently, there is no need to further sequence *C. burnetii* plasmids at this time. Experiments carried out to analyze the function, especially of the conserved and unique plasmid regions, seem to be more important for the understanding of *C. burnetii* biology. All *C. burnetii* isolates contained plasmids or plasmid-homologous sequences integrated into the chromosome, suggesting that these sequences harbor essential factors and/or perform essential functions for the organism. Mutagenesis and transformation experiments may uncover the underlying functions of the conserved and unique genes.

5. Conclusion

The obtained results showed that the raw milk of buffalo, cow, goat, and sheep could be important sources of Q fever. Age can be considered an important risk factor in the prevalence of *C. burnetii* in raw milk. *C. burnetii* discharge in milk follows a seasonal and regional pattern. The buffalo could play an important role in the epidemiology of Q fever in West Azerbaijan; hence, it should be considered in terms of public health. This study's results indicated that nested PCR assays were useful for directly typing *C. burnetii* plasmids in animal milk. Plasmid typing by PCR seems to be a more promising and useful method for applying as a golden standard method to detect the microorganism and so, rapid differentiation of *C. burnetii* in clinical samples owing to its sensitivity and specificity. Therefore, there is a need for more studies to validate nested PCR methods for early differentiation of acute Q fever from chronic Q fever.

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Conflict of Interest

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

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