



Molecular Analysis of *Coxiella Burnetii* by Isocitrate Dehydrogenase Gene Sequence-Based Typing and PCR-RFLP in Isfahan, Iran

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ABSTRACT

In the recent years, considerable advances have been made in the detection and genotyping of *Coxiella burnetii*, the causative agent of Q fever. The selection of appropriate genotyping method has enabled description of the clonal diversity of *C. burnetii* around the world. Since, in the place of study, *C. burnetii* genotyping has not been done, the *icd* gene Restriction fragment length polymorphism (RFLP) and sequence-based typing for differentiation between the genomic detected *C. burnetii* from the various sources and compared the two methods is used. In an observational study, a total of 15 genomic positive cases of *C. burnetii* infection from different sources in Isfahan province (Central Iran) were enrolled and underwent two genotyping methods: the *icd* gene PCR-RFLP and *icd* gene sequence-based typing. The degree of similarity between the *icd* gene sequences was high (98.3-100%). In comparison with *C. burnetii* Nine Mile *icd* gene sequence, the nucleotide sequences were different at 11 positions, which resulted in 7 differences in the amino acid sequences. After digesting the 370 bp amplified *icd* gene fragments all the samples indicated only one band of 370bp, while amplified *C. burnetii* Nine Mile strain *icd* gene were digested into two bands with sizes of 221bp and 149bp. The results of two genotyping methods matched together. Used methods in present study were cheaper and easier than new methods and they can be used for detection of acute and chronic phases of infection.

Key words: *Coxiella burnetii*, Isocitrate dehydrogenase, Iran, Restriction fragment length polymorphism, Sequence-based typing

INTRODUCTION

Coxiella burnetii, the causative agent of Q fever in humans and animals, is a gram negative highly infectious coccobacillus, with an infectious dose of less than ten organisms (Massung et al., 2012). This bacterium has been found in all parts of the world except New Zealand and Antarctica (Sidi-Boumedine and Rousset, 2011; Prevention, 2013). The main reservoirs of human infection are ruminant such as cattle, goats and sheep (Capin et al., 2013). Humans are accidentally infected by *C. burnetii*. A common route of transmission of *C. burnetii* to humans occurs through inhalation of contaminated aerosols arising from the infected animal body fluids. However, human infection has also occurred via the following ways: placental transmission to the fetus (Raoult and Stein 1994), blood transfusions (Pantanowitz et al., 2002) and consumption of raw milk (Signs et al., 2012). *C. burnetii* is also a potential bioterrorism agent and belongs to the category B of CDC –list (Massung et al., 2012). In human Q fever presents in two forms: acute and chronic. Acute disease often manifests as self-limiting febrile flu-like illness, pneumonia and hepatitis (Prevention, 2013). Whereas, chronic Q fever is a serious condition that presents in forms of endocarditis, vascular infections and bone and joint infections (Million and Raoult, 2015). Even Q fever infection was seen as coinfection inside Scrub Typhus (Jeong et al., 2019).

Because Q fever is considered as a zoonotic disease, the human infection epidemiology relates to the circulation of the bacterium in animal reservoirs (Eldin et al., 2017). On the other hand, differentiation between the *C. burnetii* isolates is important in diagnostic and epidemiological research, due to the vast extent of Q fever infection and multiple hosts of *C. burnetii*. Genotyping can be a key tool for understanding and follow up the epidemiology of the Q fever and by using that it can be find the animal source of human infection (Eldin et al., 2017). Furthermore, in a study by Van Nguyen et al.

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(1999) was suggested that the differences at the molecular level between the strains of *C. burnetii* may be responsible for acute or chronic forms of Q fever (Van Nguyen and Hirai, 1999). The *C. burnetii icd* gene encoding isocitrate dehydrogenase is an acid-induced and housekeeping gene that may be associated with the ability of the bacterium to replicate in the acidic environment of the phagolysosomes (Van Nguyen and Hirai, 1999; Van Nguyen et al., 1999).

Several genotyping methods have been developed for differentiating *C. burnetii* isolates. One of these techniques is Restriction fragment length polymorphism (RFLP) analysis of genomic DNA and PCR-RFLP of specific genes (Eldin, et al., 2017). In the present study, we used the *icd* gene PCR-RFLP and sequence-based typing for differentiation between the genomic detected *C. burnetii* from the various sources. In addition, in this survey we compared the obtained *icd* gene sequences with some *C. burnetii* strains *icd* gene sequences submitted in GenBank and we found the relationship between the isolates based on the *icd* gene sequence.

MATERIALS AND METHODS

Ethical approval

Written informed consent was obtained from all individuals and farm owners and the study protocol was approved by the ethics committee of Isfahan University of medical sciences (No. 194033).

Bacteria

In a series of cross-sectional studies conducted by Isfahan Infectious Diseases and Tropical Medicine Research Center (Grant No. 293390 to 293393) on May to June 2015 in Isfahan province, Iran, 34 genomic positive cases of *C. burnetii* infection from different sources (Human whole blood, animal whole blood including: Sheep and Cow and Bulk Tank Milk (BTM) from dairy Cows) were detected. The present study was performed in the following of the mentioned studies with grant no. 194033. We randomly selected 15 cases from 34 positive cases that the original sources and other characteristics of them are shown in table 1.

C. burnetii icd gene nested PCR

Bacterial DNA was extracted from human and animal (Sheep and Cow) whole blood and BTM from dairy cows samples using the YTA Genomic DNA Extraction mini kit (Yekta Tajhiz Azma Co., Tehran., Iran) according to the manufacturer's instructions. Oligonucleotide primers used in this survey are presented in Table 2. First of all, we amplified a 400 bp fragment using *icd1*-F and *icd2*-R primers and then a 370 bp fragment using *icdN*-F and *icdN*-R primers (Van Nguyen and Hirai, 1999). PCR reaction was performed in a 25 µl mixture containing: 1X PCR buffer, 1.5 mM MgCl₂, 200 mM of each deoxynucleotide triphosphate (dNTP), 1U of Taq DNA polymerase (SinaClon Bioscience Co., Tehran., Iran), 0.2 mM of each primer (Bioneer Co., Daejeon., Korea) and 5 µl genomic DNA for first step of PCR and 1 µl PCR product for second step of PCR. PCR conditions were programmed in T100™ Thermal Cycler (Bio-Rad, USA) for both steps as follows: Initial denaturation at 94°C for 3 min; followed by 35 cycles at 94°C for 60 s, 58°C for 45 s and 72°C for 45s and final extension at 72°C for 10 min. PCR products of second step were separated with electrophoresis on 2% agarose gel (SinaClon Bioscience Co., Tehran., Iran) and after staining with ethidium bromide, visualized under UV gel documentation system. Genomic DNA of *C. burnetii* Nine Mile strain was used as a positive control.

Sequencing of the *icd* gene fragments

For DNA sequencing, amplified products of second step of PCR underwent bidirectional Sanger sequencing using the ABI 3730 XL DNA analyzer (Applied Biosystems, USA) by Bioneer Co., Korea. The obtained sequences were blasted against the nucleotide database of the National Center for Biotechnology Information (NCBI, 2019). Then, the obtained sequences were aligned against the *icd* gene sequences of *C. burnetii* Nine Mile and 5 other *C. burnetii* strains using the Clustal W v2.0 software. The *icd* gene sequences accession numbers of used *C. burnetii* strains are as follows: AF069035 (Nine Mile, Type strain for acute Q fever), AF146291 (Bangui strain isolated from acute Q fever), AF146285 (TK-1 strain isolated from acute Q fever), AF146294 (Priscilla, Type strain for chronic Q fever), NC_011527.1 (CbuG_Q212 strain isolated from chronic Q fever) and CP001020.1 (CbuK_Q154 strain isolated from chronic Q fever). Phylogenetic tree was constructed by MEGA Version 6.0 (Koichiro Tamura, 2013) and Neighbor-Joining method (Saitou and Nei, 1987).

PCR-restriction fragment length polymorphism (PCR-RFLP) analysis

The *icd* gene nested-PCR products were digested with the FastDigest *Bsh1236* I restriction enzyme (ThermoFisher Scientific., USA) as described by the manufactures. This enzyme recognizes CG↓CG site. Briefly, in a 30 µl reaction containing: 2 µL of appropriate 10X buffer, 1 µL of *Bsh1236* I enzyme and 17 µL distilled water, 10 µL of PCR products was added and incubated at 37°C for 5 minutes. Then, microtubes were incubated at 80°C for 10 minutes to deactivate

the enzyme. Digested products were separated by electrophoresis on 2% agarose gel containing 0.1 µl/ml ethidium bromide and were visualized using an UV gel documentation system. Genomic DNA of *C. burnetii* Nine Mile strain was used as the control.

Table 1. Characteristics of the evaluated genomic positive cases of *C. burnetii* in this study

Samples	Characteristics					
Origin: Human	Age (years)	Sex	Occupation	Length of employment (years)	Consumption of unpasteurized milk	
CH3	30	Male	Butcher	9	No	
DH4	43	Male	Farmer	15	Yes	
EH5	48	Male	Slaughterer	30	Yes	
Origin: BTM	Reproductive disorders in the herd	Types of cattle herds	Contact With other ruminant species	Contamination with ticks in the herd		
AM1	No	Traditional	No	No		
IM9	Yes	Traditional	No	No		
TM20	Yes	Traditional	Yes	No		
VM22	No	Traditional	No	No		
WM23	No	Commercial	No	No		
XM24	No	Traditional	No	No		
Origin: Ruminants	Type	Age (mounts)	Sex	Strain	Reproductive disorders	Contamination with ticks
FA6	Sheep	36	Female	Mixed	No	Yes
GA7	Sheep	40	Male	Afshari	No	No
HA8	Cow	54	Female	Holstein Friesians	No	No
JA10	Cow	50	Female	Holstein Friesians	No	No
RA18	Sheep	40	Female	Afshari	No	No

Table 2. Primers sequences used in the study

Name of primers	Sequences (5'-3')	Amplicon size	References
<i>icd1-F</i>	CGGAGTCTCTTAGTGATGACGGA	400 bp	(Van Nguyen and Hirai, 1999)
<i>icd2-R</i>	GCCTTCTTTAGAAACCGGTTTAA		
<i>icdN-F</i>	GGAGTTAACCGGAGTATCCA	370 bp	
<i>icdN-R</i>	ATTGAGCGAACGTATGCCAC		

RESULTS

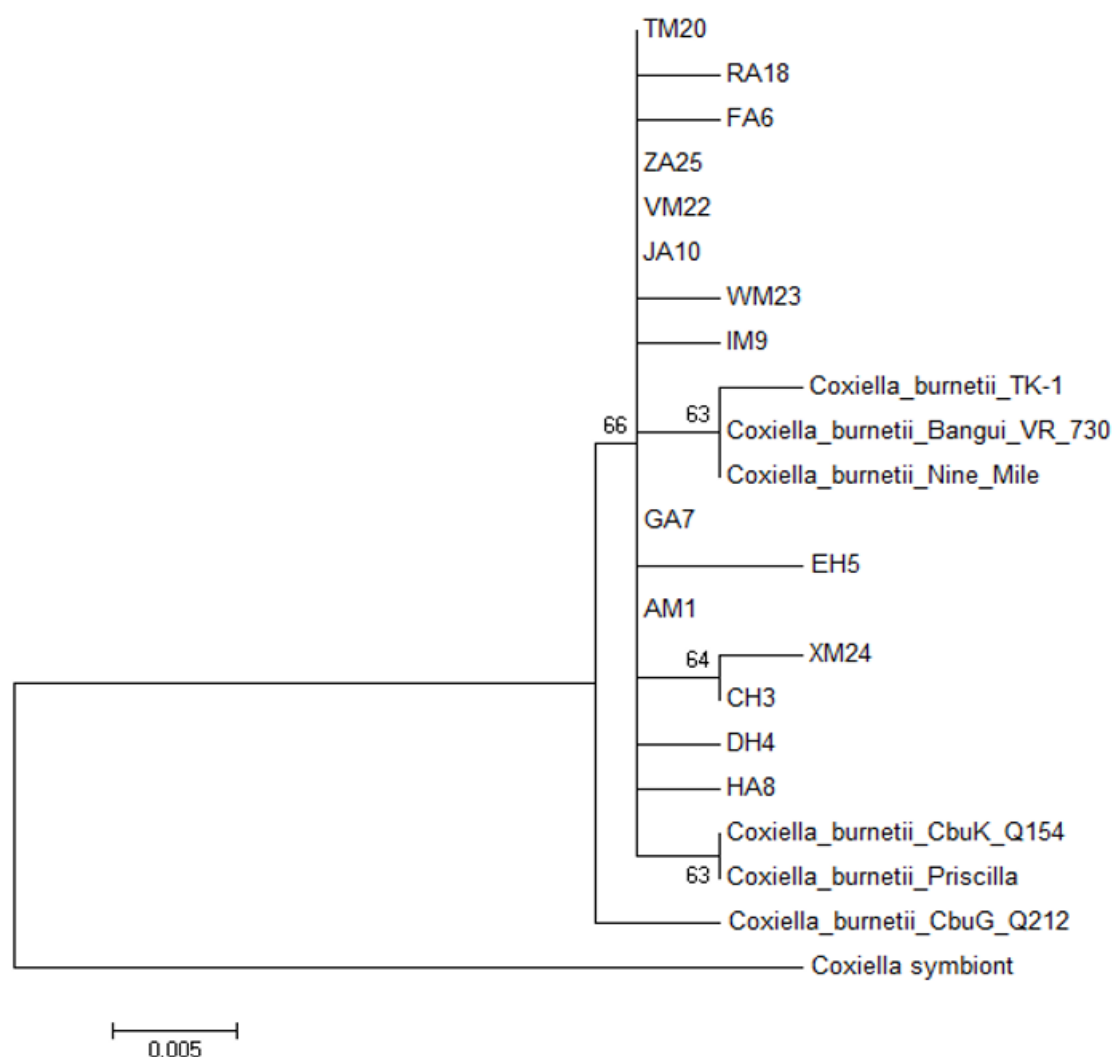
Fifteen obtained partial *icd* gene sequences were aligned and compared with six *C. burnetii icd* gene sequences derived from GeneBank. The degree of similarity between the nucleotide sequences of present study was high (98.3-100%). In compare with *C. burnetii* Nine Mile *icd* gene sequence (GenBank accession No.: AF069035.1), present nucleotide sequences were different at 11 positions, which resulted in seven differences in the amino acid sequences (Table 3). Point mutation was the cause of all changes and other type of mutations were not seen. Among our sequences there was a common mutation at position 745 according to the *C. burnetii* Nine Mile *icd* gene sequence. In six samples just one point mutation (745 G→A) were seen. However, in seven samples were seen two different point mutations and in other two samples were observed three other point mutations. Figure 1 showed the phylogenetic tree constructed based on the *icd* gene sequences obtained in this study and *C. burnetii icd* gene sequences from six mentioned strains.

After digesting the 370 bp *C. burnetii* Nine Mile strain *icd* gene amplified with primers *icd* N-F and *icd* N-R, two bands were produced with size of 221bp and 149bp on the agarose gel. In contrast, amplified fragments from all the samples indicated only one band of 370bp. Numbers of the PCR-RFLP patterns of *C. burnetii* samples are presented in figure 2.

Table 3. observed mutations in the samples compared with *C. burnetii* Nine Mile strain

Sample No.	Mutations		Gene Bank accession no.
	Nucleotide changes	Amino acid changes	
AM1	745 G→A	Ala53→Thr	KY962668.1
CH3	745 G→A/ 866 A→G	Ala53→Thr /Asp93→Ser	KY962669.1
DH4	745 G→A/ 800 A→G	Ala53→Thr /Lys71→ Arg	KY962670.1
EH5	745 G→A/ 690 T→A/ 861 A→G	Ala53→Thr	KY962671.1
FA6	745 G→A/ 883 A→T	Ala53→Thr/ Thr99→ Ser	KY962672.1
GA7	745 G→A	Ala53→Thr	KY962673.1
HA8	745 G→A/ 811 G→A	Ala53→Thr /Glu75→ Lys	KY962674.1
IM9	745 G→A/ 849 A→T	Ala53→Thr	KY962675.1
JA10	745 G→A	Ala53→Thr /Lys87→Asp	KY962680.1
RA18	745 G→A/ 934 A→C	Ala53→Thr	KY962681.1
VM22	745 G→A	Ala53→Thr	KY962676.1
WM23	745 G→A/ 750 C→T	Ala53→Thr	KY962677.1
XM24	745 G→A/ 866 A→G/902A→G	Ala53→Thr /Asp93→Ser/Glu105→Gly	KY962678.1
ZA25	745 G→A	Ala53→Thr	KY962679.1
TM20	745 G→A	Ala53→Thr	KY962682.1

No: Number

**Figure 1.** Phylogenetic tree constructed based on the *icd* gene sequences. The phylogenetic tree was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the p-distance method and were in the units of the number of base differences per site. The tree was rooted using *C. symbiont* as the out group. The support of each branch, as determined from 1000 bootstrap samples, is indicated by percentages at each nodes.

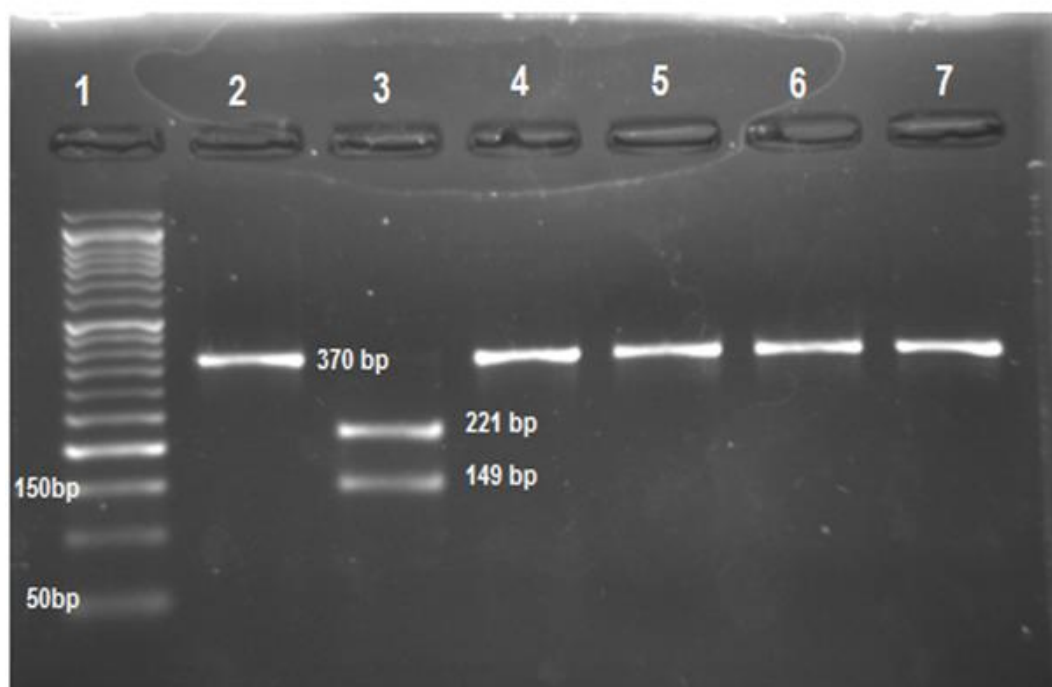


Figure 2. PCR-RFLP patterns of the *icd* gene from numbers of *C. burnetii* samples. Lanes: 1, 50bp DNA ladder; 2, undigested nested-PCR product from *C. burnetii* Nine Mile strain; 3, Bsh1236 I –digested nested-PCR products of *C. burnetii* Nine Mile strain; 4-7, Bsh1236 I –digested nested-PCR products of samples

DISCUSSION

In the recent years, the different epidemiological profile of *C. burnetii* infections was reported in Iran. In the seroprevalence studies Q fever infection rates varied from 7.8% to 68% in the different sources (animals and high-risk population) (Khalili and Sakhaee, 2009; Aflatoonian et al., 2014; Azizzadeh et al., 2014; Esmaeili et al., 2014; Esmaeili et al., 2017, Khalili et al., 2014; Nokhodian et al., 2017). In the molecular studies the prevalence rate of *C. burnetii* DNA was reported from 0% to 48.15% in various samples (animals, human and ticks) by amplification of different genes included *IS1111*, *16S rRNA* and *ComI* (Rahimi et al., 2010; Dehkordi 2011; Jamshidi et al., 2014; Khademi et al., 2014; Nokhodian et al., 2016). Unfortunately, until now, genotyping studies have not been done in Iran and according to the relative high prevalence of *C. burnetii* infection in this area, because of tremendous importance, to find the relationship between the isolates and the sources of the infection. In this study, for the first time in Iran, the number of isolates with the type strains of *C. burnetii* using the two simple and fast genotyping methods were compared. Based on the sequencing, it found that all the isolates had a common point mutation at nucleotide position 745 in the *icd* gene fragments. This point mutation also was seen in *icd* gene sequences of Priscilla (Type strain for chronic Q fever), CbuG_Q212 and CbuK_Q154 strains that isolated from chronic Q fever. However, this point mutation was not seen in *icd* gene sequences of Nine Mile strain (Type strain for acute Q fever), Bangui and TK-1 strains that isolated from acute Q fever.

On the other hand, in the Nine Mile strain *icd* gene amplified sequence there is one restriction site for *Bsh1236 I* restriction enzyme (position 744 to 747) and as it revealed in figure 2; Nine Mile strain nested-PCR product digested and two band were observed in agarose gel. However, none of samples were digested by restriction enzyme, so only one band was observed. Since the common point mutation in the isolates located at nucleotide position 745 and this site is located in restriction site, the results of both methods are consistent. Van Nguyen and Hirai (1999) studied the *icd* gene profile of 19 *C. burnetii* isolates. Based on the gene sequences, they divided the isolates into three groups included one group originated from acute Q fever and two groups originated from chronic Q fever. Similar to the results they found a common point mutation at position 745 in isolates originated from chronic Q fever (Van Nguyen and Hirai, 1999). In another study by Ando et al., using *icd* gene PCR-RFLP and sequencing analysis, 49 of 72 isolates had a completely identical nucleotide sequences and these isolates, same as 6 the isolates, had only one point mutation in position 745 (G→A). They called these isolates “Japanese-specific” isolates and they claimed that the *icd* sequences of these isolates were not similar to other chronic *C. burnetii* strains (Andoh et al., 2004). However, in this study, all 6 identical nucleotide sequences were same as the chronic isolates submitted in GeneBank. Unfortunately, in the present study, no records about the clinical manifestation have seen (acute or chronic) of sources of isolates. However, according to these

results, the isolates could be originated from the chronic or persistent focalized infections. Evaluating more *C. burnetii* isolates from different geographical area of Iran along with complete clinical information using these two methods is recommended in resent study. According to the primary studies, the initial steps of Citric Acid Cycle (CAC) pathway revealed the least conservation and changes in the genes encoding initial enzymes can effective on bacterial adaptations to different environments (Huynen et al., 1999). Since the isocitrate dehydrogenase enzyme is a member of the initial steps of CAC enzymes, mutations in the *icd* gene may be related to the *C. burnetii* pathogenicity and virulence. Therefore, it is suggested more attention and study on the role of the isocitrate dehydrogenase enzyme in the different *C. burnetii* strains in the various environments.

Currently, the new genotyping methods were developed such as multiple-locus variable-number tandem repeat (VNTR) analysis (MLVA), Multi-spacer Sequence Typing (MST), and Single Nucleotide Polymorphism (SNP) genotyping (Eldin et al., 2017). Since it is better to determine the phase of the disease (Acute or chronic infection) and type of the genotype of the bacterium in the epidemiological studies and to decide for treatment of the infection, so use of the RFLP method, that is cheaper and easier than sequenced-based methods, can be helpful.

DECLARATION

Acknowledgements

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Competing interests

All authors have no conflict of interest.

Consent to publish

All authors gave their informed consent prior to their inclusion in the study.

Authors' contributions

ZN, MK, BA and AF conceived and designed the project. ZN, MK and SR Managed activities to annotate (produce metadata), scrub data and maintain research data for initial use and later re-use. MY Verified whether as a part of the activity or separate, of the overall replication of results and other research outputs. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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