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Molecular characterization of *Coxiella burnetii* in the slaughtered animals of Southern Iran

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ABSTRACT

Objective: To investigate the presence of *Coxiella burnetii* and define its genetic characterization in the carcasses of sheep and cattle in Shiraz, Southern Iran.
Methods: Kidneys, mesenteric lymph nodes and supra mammary glands from 60 sheep and 70 cattle were randomly collected from the slaughtered animals. Nested PCR assay was used to identify *Coxiella burnetii* in tissues.
Results: The results showed that 6.66% of the sheep and 5.71% of the cattle carcasses were positive, respectively. Further details of the genomic characterization of the isolates were then analyzed.
Conclusions: This study denotes the presence of Q fever disease in the slaughtered animals and defines the genetic characteristics of the isolates which might be used to implement a proper preventive program among slaughterhouse workers.

1. Introduction

A worldwide zoonotic infection caused by *Coxiella burnetii* (*C. burnetii*), has been considered as imperative neglected public health issue for the last decades[1]. The main causative agent of Q fever, *C. burnetii*, is an obligate intracellular Gram-negative bacterium which may infect a variety rang of vertebrate and invertebrate populations and is also assumed as a biological terrorist organism[2-4]. The reports have implied that farm animals including cattle, sheep, and goats, play a major role as reservoir of the infection, with high risk to the vulnerable populations including ranchers, veterinarian, butchers and personnel of slaughterhouses[5-7]. Transmission routes of the infection to human are including infected ticks, aerosols, direct contact with the infected animal fluids such as milk, urine, seminal fluid, contaminated tissues such as kidneys, mammary glands and lymph nodes[2,8-12].

As most cases of the disease in human and animals show asymptomatic features, employing the molecular based techniques to recognize and identify the virulence factors and also further genome sequencing analysis of the microorganism to explore the details of its pathogenicity in mammals, are extremely recommended[13,14]. Furthermore, because of the potent risk of infection throughout the difficult isolation techniques, their time consuming and high biosafety standards requirements, recently the molecular techniques such as nested PCR is prevalent[13,15-18].

There is little information about the molecular identification and genetic characterization of *C. burnetii* in slaughtered animals in Fars Province, Southern Iran. The present study was therefore aimed to further investigate the molecular identification and genetic characterization of the microorganism in the associated tissues in the carcasses of food animals.

2. Materials and methods

2.1. Sampling

Sample collection was conducted during 2016–2017 from the

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Shiraz industrial slaughterhouse, Shiraz, Southern Iran. The age of animals were ranged from 12 to 24 months. The mesenteric lymph nodes, kidneys, and supra mammary lymph nodes were carefully collected and transported to the laboratory on ice which was later stored at –20 °C until further use.

2.2. DNA extraction

DNA from 45 samples was purified, using a DNA extraction kit (Bioneer, South Korea) as was described by the manufacturer. Two hundred milligram of each tissue was processed. ANG 100 spectrophotometer (Nanodrop Technologies, USA) was employed to assess the quantity of DNA.

2.3. Nested PCR assay

PCR was performed on the extracted DNAs of all the samples (n = 130)[19]; two pair of species-specific primers were used to amplify a region (438 bp) of the 27 kDa outer membrane protein (*com1*) gene, partial cds. Each 25 µL reaction consisted of 12.5 µL master mix (Amplicon, Fermentas, Tehran, Iran), 1 µmol/L of each primer, 2 µL of DNA and 8.5 µL of PCR grade water. Amplification was performed in a Thermal Cycler-Life Pro (Bioer Technology, China) using the following cycling conditions: denaturation at 94 °C for 3 min and then 35 cycles of 94 °C for 45 s, 65 °C for 45 s, and 72 °C for 45 s, followed by a final extension at 72 °C for 5 min. Furthermore, a positive control (Nine Mile strain, ATCC VR-615) and a negative control (Ultra Pure Water DNase and RNase-free, Cinnagen, Tehran, Iran) were incorporated in each assay. For each sample a volume of 8 µL of PCR product was run on a 1.2% agarose gel in Tris-acetate-EDTA (TAE) running buffer stained with Safe Mode DNA stain (SinaClon, Tehran, Iran) and visualized by a UV transilluminator.

A 27 kDa outer membrane protein (*com1*) gene was amplified, using the previously described method[19], from the mitochondrial genome, part of *com1* was amplified using two pair of primers as shown in Table 1.

Table 1
Primers used in the present study.

	Primer	Sequence (5-'3')	Amplicon size (bp)	Ref.
1st PCR	Cox1	AGTAGAAGCATCCCAAGCATTG	501	[19]
	Cox2	TGCCTGCTAGCTGTAACGATTG		
Nested	Cox3	GAAGCGCAACAAGAAGAACAC	438	
PCR	Cox4	TTGGAAGTTATCACGCAGTTG		

2.4. DNA sequencing and data analysis

The sequencing of 16s rRNA PCR product was performed on the amplified genes, using QIAquick gel extraction kit (Bioneer, USA), as described by the manufacturer. The pure products were subjected to a sequencing reaction (Macrogen, South Korea). After sequencing it was analyzed, using Mega4 software, the *C. burnetii* polymorphic VNTR sequences of the selected samples were analyzed, using GeneMapper 4.0 software and compared to the reference Nine Mile strain and the Q fever in the GenBank.

3. Results

Out of 130 animals (6–18 months cattle, 1–2 years sheep), 8 (6.15%) were found positive. Out of 70 samples taken from cattle, 4 (2 mesenteric lymph nodes and 2 kidney samples) (5.71%) were positive for the genome of *C. burnetii*. Additionally, 4 samples (1 kidney; 1 mesenteric lymph node and 2 supra-mammary lymph nodes) out of 60 carcasses of sheep (6.6%) were found positive (Table 2).

All the *C. burnetii* DNA samples examined in our study were 100% identical to each other at *com1* gene. This was confirmed by observation of a 438 bp fragment in the PCR assay (Figure 1).

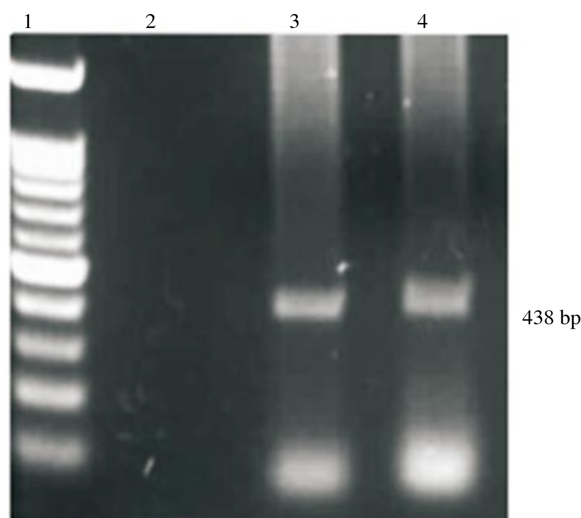

Figure 1. Gel electrophoresis of products of nested PCR on DNA template of some isolates.
Lane 1: 100 kb DNA ladder, Lane 2: Negative control, Lane 3: Positive control (pure DNA of the bacterium), Lane 4: Positive samples.

Table 2
Frequency of *C. burnetii* in various tissues of slaughtered animals.

Animal	Total No.	Kidney (%)	Mesenteric lymph node (%)	Supra mammary lymph nodes (%)
Sheep	60	1 (1.66)	1 (1.66)	2 (3.33)
Cattle	70	2 (2.85)	2 (2.85)	0

BLASTn comparison of the sequences of the samples, against the nucleotide database, did return a significant result to *C. burnetii*. The sequences of samples were submitted at NCBI with accession number KY433366 for *com1* gene. Comparison of the extracted 27 kDa outer membrane protein of *C. burnetii* to the published sequences for other *Coxiella* showed that the most genetic homology (99%) belonged to the previously reported sequence for *C. burnetii* (NZ_AKYP01000099.1).

The result of the phylogenetic analysis of the isolates is shown in Figure 2. Our analysis represented the most homology to one of the previously reported Iranian isolates from Tehran, however, it was similar to those reported from China, South Korea and Australia (99%). Finally it was clearly different from the American isolates (96%), in which, nine distinct mutation points were found.

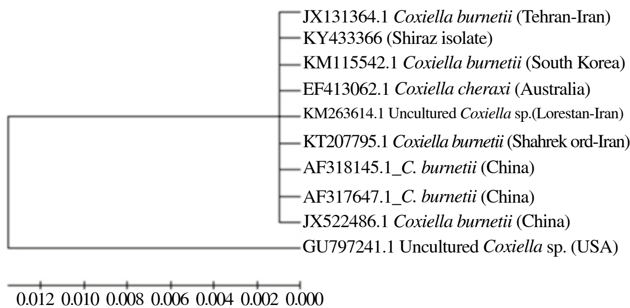

Figure 2. Phylogenetic tree based on ENO gene sequence data, constructed according to the Neighbor-Joining (NJ) algorithms, showing the position of *C. burnetii*, compared to other related species in the GenBank.
Numbers above the branches indicate bootstrap values (%) from 1 000 replicates.

4. Discussion

Serious limitations occur in detecting the etiological agent of Q fever in food animals. In general, not much information regarding to the infection of different tissues in food animals have been released, yet. However, in an experimental infection in sheep the identification of organism was confirmed in kidneys, udder and placenta[20]. Presence of *C. burnetii* genetic material in any part of carcasses is indicative of the past or ongoing infection[21]. In spite of the occurrence of infection in all seasons, the involvement of multiple tissues has been intermittently reported in spring, each year. In domestic ruminants, the clinical form of Q fever more likely occurs in late gestation and around the time of parturition[2,5]. In animals, no obvious clinical and/or gross lesions has been reported in both ante- and post mortem routine meat inspection program, however, acute and chronic severe clinical manifestations have been frequently reported[22]. In animals, the typical clinical signs of Q fever are abortion, stillbirth, weak calves, and repeat breeding[2,3].

Our study revealed the presence of *Coxiella* and its potent risk of infection in both livestock and the person in contact with the infected animals in Southern Iran. In human, both of acute and chronic forms of the disease have been reported, worldwide. A febrile flu like illness is the main symptom in the immunocompetent people; however, in the severe forms, pneumonia, hepatitis and CNS involvement have also been reported. In the chronic forms of infection, endocarditis and serious complications in the pregnant women are among the major symptoms. Moreover, in the pathological examinations tuberculoid lesions and endocarditis with valve lesions have been frequently reported[2,3,23-26].

Although, the PCR positive samples are not always indicative of presence of the live microorganisms[21], due to the low infection rate and potent risk of *Coxiella* to the staffs, our findings support the necessity of the preventive measures to the public.

The phylogenetic analysis of our isolates showed a homology to the strains identified from East Asian countries. This shows that the etiologic agent of the disease might have been imported to our country through the transporting of animal products and thus need more considerations.

To date, there is no evidence on the occurrence of the outbreaks of this disease in our country, both in humans and animals which might be associated with the lack of information about the potential risk of infection, problems to differentiate the clinical symptoms and insufficient laboratory supports in the accurate detection of the disease. Using an ELISA assay targeting IgGI and IgGII of *C. burnetii* in the serum samples of butchers and slaughterhouse workers in South-East of Iran revealed 18.1% and 14.4% seroprevalence rate of infection to the phase I and II of Q fever, respectively[27]. Moreover, in a systematically search from 1937 to 2012, on the published data regarding Q fever from Iran, the country was categorized as an endemic region and thus as a public health concern[28]. Considering the highly resistant nature of the bacterium and repeated reports on isolation of the microorganism from soil after six months removal of an infected animal, frequent shedding of the microorganism in the milk of infected animals and difficulties in identification of the disease in a routine meat inspection program make it essential to pay more attention to the disease as a neglected zoonotic disease[29]. In a report recently published by World Health Organization, an aggregate presence of Q fever in an Australian abattoir has also confirmed the importance of this zoonotic disease as a neglected occupational hazard to the slaughterhouse workers and to set a preventive program by using a potent vaccine[6].

As the final conclusion, the data presented here provided some details on the presence of the disease in the slaughtered animals. This study also assessed the genetic characterization of the microorganism which might be used to implement a proper preventive program among slaughterhouse workers.

Conflict of interest statement

We declare that we have no conflict of interest.

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