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The potential utility of nested PCR for investigation of *Coxiella burnetii* in Iranian snails

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ABSTRACT

Objective: To detect the prevalence of *Coxiella burnetii* (*C. burnetii*) in two species of snails consisted of *Lymnaea palustris* (*L. palustris*) and *Pomacea canaliculata* (*P. canaliculata*) by using nested PCR method in Chaharmahel Va Bakhtiari Province which is located in the southwest of Iran.

Methods: A total of 160 snail samples consisted of 100 *L. palustris* and 60 *P. canaliculata* were collected from 4 rice paddy fields in the southwest of Iran between June and August 2014. Snails' DNA was extracted by a genomic DNA purification kit according to the manufacturer's instructions. Detection of the presence of *C. burnetii*'s DNA was carried out by using a nested PCR assay with [specific primers outer membrane protein 1 (OMP1)-OMP2 and OMP3-OMP4] targeting the com1 gene.

Results: In this study, a total of 160 snail samples were tested and 15 (9.37%) samples were found positive for *C. burnetii*, 15 samples were positive from the *L. palustris* and there were no positive samples from *P. canaliculata*.

Conclusions: Snails are kind of gastropods which seem to be harmless in life, but these small gastropods can be very dangerous for farmers, especially in humid climates. Also, *C. burnetii* in snails showed that this bacterium can be a factor of transmission of contamination to human beings and animals.

1. Introduction

Coxiella burnetii (*C. burnetii*) is an obligate intercellular, Gram-negative bacterium[1]. The organism is ubiquitous in the environment where it can persist in a spore-like form for years. Goats, sheep, cattle are the species most clinically affected by infection and are often as the source of human infection called Q fever[2]. This bacterium can transmit to human via the inhalation of pathogen-contaminated dust or aerosols[3,4]. *C. burnetii* is very hardy and it can travel on winds current for over miles. So, the exposure to *C. burnetii* can occur without direct contact with infected animals[5,6]. Q fever is a common zoonotic disease with acute and chronic clinical manifestations including hepatitis, pneumonia and endocarditis but in animal is mostly asymptomatic[7]. The first identified Q fever was in 1935 in Queensland, Australia after an outbreak of febrile disease among slaughterhouse workers[8]. The disease was named "Query (Q)" fever, because its etiopathogenesis was not known[9]. The investigations which are done on Q fever are complex because of the aerosol transmission of organism, the ubiquitously and environmentally persistent nature of the organism, clinic diseases in most effected herds, shedding in animals, difficulties in test result interpretation in all species and so forth[2]. *C. burnetii* can show different morphological forms in the cycle of its development. Some forms can survive extracellularly and even accumulate in the environment[10]. In different countries, different species of animals are infected with *C. burnetii*. *C. burnetii* has been identified in marsupials, arthropods, fish, birds, rodents, and livestock[11]. Snails also can be infected by *C. burnetii*, so in this study we examined *Lymnaea palustris* (*L. palustris*), a species of air-breathing freshwater snail and an aquatic-pulmonate gastropod mollusk in the family of Lymnaeidaeand, and *Pomacea canaliculata* (*P. canaliculata*), a species of large fresh water snail with gills and operculum in the family of Ampullariidae[12,13]. No previous study on the prevalence of *C. burnetii* on snails existed in

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the nearby region. Many PCR diagnostic assays were developed to detect DNA of *C. burnetii* including conventional PCR, nested PCR and real-time PCR[14]. Nested PCR is a highly effective variant of the PCR technique, differentiated from the standard PCR protocol by the conceptual primer design, which involves two sets of external and internal primers in two successive PCR amplification runs, for amplification of extreme low quantities of template DNA. It is useful to identify serotypes of virus, parasites and bacteria. Many target genes which are used for specific *C. burnetii*[15]. Several target genes used for specific *C. burnetii* identification include the superoxide dismutase (Sod B) gene, *com*1 encoding a 27 kDa outer membrane protein (OMP), the heat shock operon encoding two heat shock proteins (htpA and htpB), isocitrate dehydrogenase, the macrophage infectivity potentiator protein (cbmip) and a transposon-like repetitive region of the *C. burnetii* genome (Trans)[16].

The aim of this study was to determine the prevalence of *C. burnetii* in two species of snails (*L. palustris* and *P. canaliculata*) by nested PCR in Chaharmahel Va Bakhtiari Province of the southwest of Iran.

2. Materials and methods

2.1. Sample collection

One hundred and sixty snail samples were collected randomly during June and August 2014. The snail samples consisted of 100 *L. palustris* samples and 60 *P. canaliculata* samples. *L. palustris* samples were collected from 4 rice paddy fields in Chaharmahal Va Bakhtiari Province of the southwest of Iran and *P. canaliculata* was bought from different fish stores. The samples were taken by clean gloves and immediately transported in plastic containers to the laboratory. Before being removed from their shells, snails were narcotized in isotonic (7.2%) magnesium chloride solution in a tray for 30 min. The shells were broken very carefully with a hammer. The whole body of each snail was removed and washed thoroughly with normal saline solution and blotted.

2.2. DNA extraction and nested PCR

DNA was extracted by using genomic DNA purification kit according to the manufacturer's instructions (CinnaGen Co., Iran) and assayed on 2% agarose gel electrophoresis and measured at 260 nm optical density. DNA samples were stored at -20 ℃ until they were used. In this study, we used primers which were designed from the nucleotide sequence of the *com*1 gene, which was previously described by Ogawa *et al.*[17].

Two primer pairs for nested PCR were designed and obtained by using the method listed in Table 1. For nested PCR with primers OMP1-OMP2 and OMP3-OMP4, the first amplification was performed in a total volume of 25 μL in 0.2 tubes containing 3 μL of DNA sample, 1 μmol/L of each primer (OMP1-OMP2), 5 μL of 10× PCR buffer, 2 mmol/L of $MgCl_2$, 200 μmol/L DNTPs and 1 IU of *Taq* DNA polymerase. The mixtures were overlaid with 2 drops of mineral oil. PCR was performed at 95 ℃ for 5 min and then for 32 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s, 72 ℃ for 2 min and a final extension at 72 ℃ for 5 min, whit a final hold at 10 ℃ in a thermal cycler (mastercycler gradient, Eppendrof, Germany). In the second amplification, the reaction mixtures were the same as those in the first amplification except for the primers and DNA templates. Primers OMP3 and OMP4 were used at 1 μmol/L of each and 2 μL of the first amplification product were used as the template. In this study, *C. burnetii*'s DNA (serial number: 3154; Genekam Biotechnology AG, Duisburg, Germany) and sterile distilled water were used as the positive and negative controls, respectively.

Table 1
Primers for identification of *C. burnetii.*

Primer	Sequence	Amplicon size (bp)
OMP 1	5´-AGTAGAAGCATCCCAAGCATTG-3´	501
OMP 2	5´-TGCCTGCTAGCTGTAACGATTG-3´	
OMP 3	5´-GAAGCGCAACAAGAAGAACAC-3´	438
OMP 4	5´-TTGGAAGTTATCACGCAGTTG-3´	

2.3. Detection of PCR products

The PCR amplified products were detected in 1% agarose gel electrophoresis with ethidium bromide stained. Constant voltage of 90 V for 30 min was used for produced separation. The DNA molecular weight marker (100 bp, Fermentas, Germany) was used as the size marker. The viewed on UV transilluminator and photographerd were obtained in ultraviolet irradiation doc gel documentation system (UK). The length of reproduced DNA by nested PCR method is related to OMP1, OMP2, OMP3, OMP4 and they are 501 and 438 bp, respectively.

2.4. Statistical analysis

The data were then analyzed by using SPSS software (ver. 20, SPSS, Inc., Chicago, IL, USA) and *P* value was calculated by using χ^2. To bind in significant relationship, *P* value less than 0.05 was considered statistically significant.

3. Results

The quality of the extracted DNA from samples was examined by electrophoretic analysis through a 2% agarose gel. In this study, 160 snail samples were tested for *C. burnetii* by using a nested PCR assay (Figure 1). In total, 15 (9.37%) samples were found positive for *C. burnetii*. All positive samples were obtained from the *L. palustris* and there were no positive samples from *P. canaliculata*. The OMP1-OMP2 and OMP3-OMP4 primers amplified the predicted products 501 bp (Figure 1) in the first amplification and 438 bp (Figure 2) in the

second amplification of PCR with DNA templates from all 15 isolates used of *C. burnetii*.

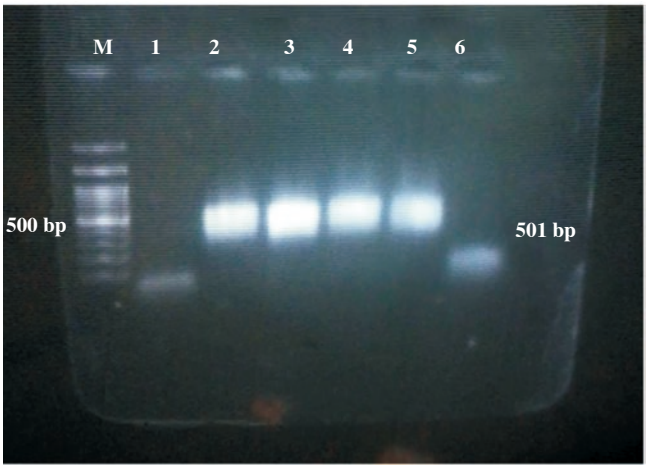

Figure 1. The PCR products amplified with used primers (OMP1-OMP2). Lane M: 100 bp DNA ladder; Lane 1: Negative control; Lanes 2, 3, 4 and 5: Positive samples of *C. burnetii*; Lanes 6; Negative sample.

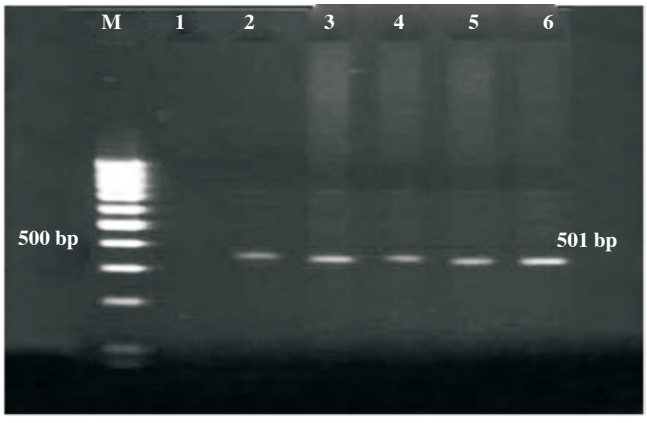

Figure 2. The PCR-amplified products.
Lane M: 100 bp DNA ladder; Lane 1: Negative control; Lanes 2, 3, 4 and 5: Positive samples of *C. burnetii*; Lane 6: Positive control.

4. Discussion

C. burnetii, a zoonotic parasite, belongs to the γ subdivision of the Proteobacteria phylum[18]. Those who are in contact with farm animals including farmers, abattoir workers, and veterinarians are at greatest risk[19]. By 1955, *C. burnetii* was found in 51 countries of five continents. In the 1990s, New Zealand was one of the few countries which was free of *C. burnetii* infection. However, major differences occur in the manifestations of Q fever from country to country. In Nova Scotia, Canada, and in the Basque region of Spain, pneumonia is the predominant manifestation of Q fever[20]. Clinical Q fever has two forms in human: acute and chronic. Acute Q fever manifests as a self-timing flue like illness characterized by high-grade fever, pre-orbital, headache, and myalgia. Chronic Q fever most commonly presents endocarditis occurring weeks to years after an acute infection. Other manifestations include vascular infections and infections of the bone, liver or reproductive organs. The rout of infection and the inoculum size are the factors that can affect the expression of *C. burnetii* infection[21]. *C. burnetii* can lead to reproductive disorders such as abortion, stillbirth, and delivery of weak and nonviable neonates in ruminant[22]. The purpose of this study was to determine the prevalence of *C. burnetii* in snails. This study showed that only 9.3% of samples via nested PCR tested and they were positive for *C. burnetii*. No previous study on the prevalence of *C. burnetii* on snails existing in this region. Most of the studies which were conducted on *C. burnetii* were on the animals such as cows, goats, sheep and so on. This disease can be seen in ticks, some wild birds such as bat and pigeon, mammals such as goat, sheep, *etc*. Also, this disease can be seen in dairy products such as milk[19,23].

Kargar *et al.* tested the sensitivity of PCR method for detection of *C. burnetii* in milk samples. The results of their study showed that tran-PCR is highly sensitive and useful for the direct detection of *C. burnetii* in milk samples[24]. Fard and Khalili examined *C. burnetii* in ticks collected from sheep and goats by PCR in Southeast Iran in 2011. The results of their study showed three pools, each consisting of five females of *Hyalomma anatolicum* and one pool (6 ticks) of *Rhipicephalus Sanguineus* ticks collected from goats and sheep, were found to be positive by trans-PCR[25].

In another study, Rahimi *et al.* examined the prevalence rate of *C. burnetii* in bovine, ovine, and caprine bulk milk samples by PCR in Chaharrmahal va Bakhtiari Province in 2009. In their study, 62% of bulk milk samples was tested positively for *C. burnetii* while ovine bulk milk samples were negative for *C. burnetii*. Also, 1.8% of caprine bulk milk was tested positively. They reported that clinically healthy cattle are the main sources of *C. burnetii* in Iran[26].

Kim *et al.* examined *C. burnetii* in bulk tank milk samples in the United States in 2005. They reported 94% prevalence in samples of bulk tank milk from U.S. dairy herds during the past 3 years. This study was the first one which was conducted on the prevalence of *C. burnetii* on two species of snails[27].

Snails are potential hosts for many zoonotic diseases including liver and intestinal trematodes so snails' infection could be lead to these zoonotic infections in human or animals. The farmers who work in these areas can be a factor of the transmission of this disease. Also, the animals such as goats, sheep which live in this area and use water of this area are the factor of the transmission of this disease. Therefore, this bacterium is resistant against physical and chemical factors and has the direct relation with human life. The results of the study showed that the percentage of pollution in the animals and humans were fairly high. The uses of the safe masks, gloves, vaccines are suggested in order to prevent from this disease.

Cleaning of the polluted areas, separating and controlling polluted animals and rapid recognition of Q fever and its treatment can prevent from the prevalence of this disease. It is predicted that the prevalence of *C. burnetii* in winter season reduces because amount of food for this snail reduces in winter. As a result, this

study confirms that snails are important reservoirs for *C. burnetii* in southwest Iran and play an important role in the transmission of the disease. The detection of *C. burnetii* by nested PCR has a significant application for assessing the absence of Q fever disease ruminants and minimizing the potential risks of Q fever outbreaks. The large number of samples should be analyzed in subsequent studies in order to obtain reliable results. Also, it is better to compare different methods for the detection of *C. burnetii*.

Conflict of interest statement

We declare that we have no conflict of interest.

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