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MOLECULAR DETECTION AND IDENTIFICATION OF *COXIELLA BURNETII* IN ABORTED SHEEP AND GOATS IN SULAIMANI PROVINCE, KURDISTAN-IRAQ

FATTAH MARIF HARDI [1]; HANA SHERZAD RAUF [1]; SHAKHAWAN LATIF MAHMOOD [1]; REBWAR BAHIR AHMAD [1]; BASIM ABDULWAHID ALI [1] AND MOHAMED OMAR BABA SHEIKH [2]

[1] College of Veterinary Medicine, University of Sulaimani, New Sulaimani, Street 27, Sulaymaniyah, Kurdistan Region, Northern Iraq

[2] Directorate of Veterinary in Sulaimani, Salim Street, Sulaimaniyah, Kurdistan Region, Iraq

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ABSTRACT

Q fever is an almost ubiquitous zoonotic disease with a worldwide distribution, caused by *Coxiella burnetii* which can infect different types of animal species such as cattle, sheep and goats, as well as humans. Infection with *C. burnetii* in small ruminants, are mostly sub-clinical, although, abortions and stillbirths can occur. The aim of this study was to provide molecular evidence of *C. burnetii* in aborted small ruminants in different districts of Sulaimani province, Kurdistan-Iraq. Blood and faecal samples were collected from 180 aborted sheep and goats (90 samples each) and analyzed by conventional Polymerase Chain Reaction (PCR) for DNA detection of transposase gene (IS1111) of *C. burnetii* from February to June 2019. (Comparison sequence analysis exhibit that field isolates highest identities of (Iraq, Iran and China) strains with the rate of 99.84%). Shedding of *C. burnetii* by aborted sheep and goats was found only in (5.55%) faecal samples investigated. Only 10 out of 60 faecal samples (16.66%) were positive. Six out of 48 faecal samples (12.5%) and 5 out of 12 faecal samples (41.66%) were positive in aborted sheep and goats respectively. None of the blood samples revealed positive amplification for *C. burnetii* DNA. This paper documents the first molecular detection of *C. burnetii* in aborted small ruminants in Sulaimani province Kurdistan-Iraq.

Keywords: *Coxiella burnetii*, Q fever, Sulaimani province, PCR and zoonotic disease.

INTRODUCTION

Q fever is a zoonotic disease in humans and animals affecting a wide range of hosts. The causative agent, *Coxiella burnetii*, is a Gram-negative obligate intracellular bacterium and is known for its high tenacity and infectivity present worldwide (Heinzen, *et al.*, 1999), (McCaul & Williams, 1981), (Aitken *et al.*, 1987).

Reservoirs of *C. burnetii* include many wild and domesticated mammals, birds and ticks (Raoult, *et al.*, 2005). Ticks are considered to be the natural primary reservoirs of *C. burnetii* responsible for the spread of the infection in wild animals and for transmission to domestic animals (Norlander, 2000). In animals, *C. burnetii* infection does not usually provoke severe symptoms. However, in cattle it has been associated with infertility and in small ruminants (goats and sheep) the infection can result in late abortions. Increased abortion rates in infected caprine herds have been described, with up to 90% abortions in pregnant animals (Van den Brom, van Engelen, Roest, van der Hoek, & Vellema, 2015). Infected females shed a huge amount of bacteria in birth products and in urine, feces, and milk. This shedding can persist for several months in vaginal mucus, feces, and milk (Rodolakis, 2009). The massive shedding of *C. burnetii* during such abortions makes sheep and goats the main reservoirs responsible for infection of humans (Parisi *et al.*, 2006).

C. burnetii is most often transmitted to humans by inhalation of an aerosol that has been contaminated with parturient products, urine, and feces of the infected animals (Parisi *et al.*, 2006). Main clinical presentations may be as flu- like illness or a febrile pneumonia or hepatitis (Arricau-Bouvery & Rodolakis, 2005), (Kampschreur *et al.*, 2012), While, in few cases, the disease progresses to a chronic stage characterized by endocarditis or vascular infection (Kampschreur *et al.*, 2012), (Raoult *et al.*, 2005), (Anderson *et al.*, 2013). Polymerase Chain Reaction (PCR) assays are commonly used to directly detect *C.*

Corresponding author: Dr. FATTAH MARIF HARDI
E-mail address: hardi.marif@univsul.edu.iq
Present address: College of Veterinary Medicine, University of Sulaimani, New Sulaimani, Street 27, Sulaymaniyah, Kurdistan Region, Northern Iraq

burnetii in biological materials such as placentas, genital swabs, feces or milk samples, and reveal the existence of ongoing infections associated with bacterial shedding (Norlander, 2000). PCR assays provide a valuable approach that is sensitive, easy to perform, and safe for laboratory personnel and it holds the promise of timely diagnosis, since it should be positive before antibodies are detectable (Berri, *et al.*, 2003).

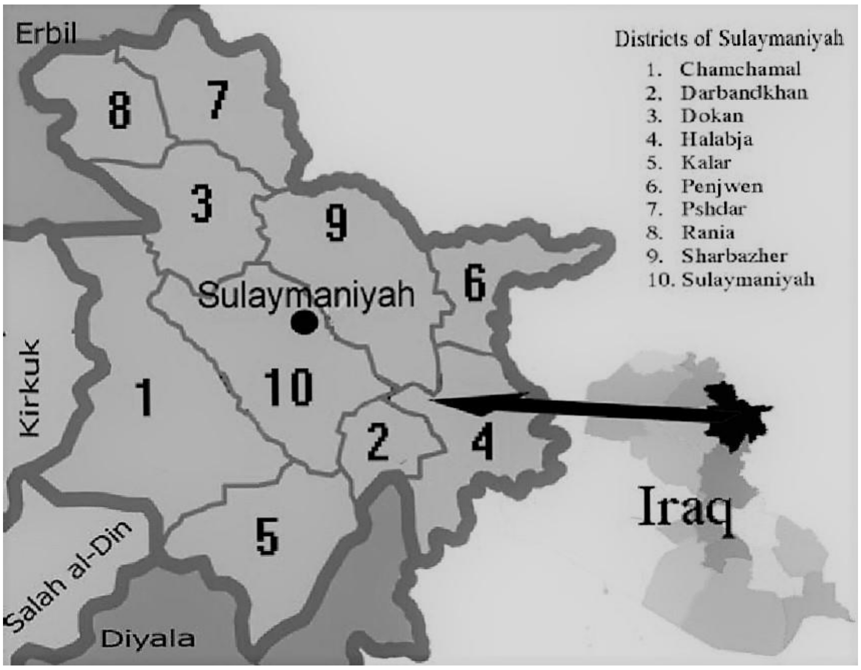

Figure 1: Area of the research, samples were taken from different districts of Sulaimani province, Kurdistan-Iraq.

MATERIALS AND METHODS

Sample collection

This study was carried out from February to June 2019. Blood and fecal samples were collected from 90 sheep and 90 goats. Blood (5 ml) was collected from the jugular veins of the aborted small ruminants using disposable needles (18 gauges) and 10ml syringes. Blood samples were then stored at room temperature for one hour to allow clotting. After centrifugation (1,500 x g, 10 minutes) the serum samples were ready for DNA extraction.

Fecal (3g) was obtained and placed in sterile plastic tubes, transported to the laboratory at 4°C and subsequently preserved at -20°C.

Sample preparation and DNA extraction

The fecal samples were vortexed in phosphate buffered saline (1 mL 0.1 M PBS of pH 7), then genomic DNA was extracted from fecal and serum specimens using a DNA extraction kit (Genaild, Co, Korea) according to the manufacturer's instructions.

Oligonucleotide primer

A PCR protocol targeting IS1111 repetitive transposon-like region of *C. burnetii* was used for the detection of *C. burnetii* DNA in feces and serum. For the PCR amplification primers Trans-1 (5’-TAT GTA TCC ACC GTA GCC AGT C-3’) and Trans-2 (5’-CCCAAC AAC ACC TCC TTA TTC-3’) were used (Hernychova et al., 2008). The length of the genome target for amplification was expected to be (687) bp (table 1).

Table 1: Sequences of primers and PCR conditions

Protocol	Primer	Sequence	Gene	Amplifier length (bp)	PCR conditions (°C/s)			
					Denaturation	Annealing	Extension	No. of PCR cycles
Uniplex Polymerase Chain Reaction	Trans-1	5’-TAT GTA TCC ACC GTA GCC AGT C-3’	IS1111	687	95/30	58/35	72/40	
	Trans-2	5’-CCCAAC AAC ACC TCC TTA TTC-3’	IS1111	687	95/30	58/35	72/40	40

Uniplex Polymerase Chain Reaction

C. burnetii was amplified by using PCR Premix (2X). This kit provides a complete system for fast, high yield and reliable single tube PCR (Genet-bio, Korea). The reactions were carried out in 0.2 ml PCR tube based on the following specifications: 10 µL supreme script PCR premix, 5 µL DNA, 1 µL forward (10 pmol), 1 µL reverse primers (10 pmol), and 3 µL ultra-pure water to make up a final volume of 20 µL. The conventional PCR machine (Hercuvan, USA) was programmed as followed: initial denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s; annealing at 58 °C for 35s, and extension at 72 °C for 40 s and a final extension at 72 °C for 10 min. Five microliters of each amplified DNA sample were loaded on to a 1% agarose gel stained with a safe dye (Eurx-poland) on preparation. Electrophoresis was performed at 100V for 50 minutes.

Sequencing the PCR products

In order to confirm the PCR results, sequencing method was depended, 25 µl of amplified PCR product of two positive samples with both direction primers were sent for sequencing using Sanger sequencing method (Macrogen, South Korea).The results were submitted in NCBI/ GenBank with accession number (MK994501 & MK994502).

Phylogenetic tree and sequence analysis

Phylogenetic trees were constructed based on partial sequence IS1111A transposase gene 26 strain of *C. burentii.* The sequence homology and multiple sequences alignment at the nucleotide and amino acid level was performed by the CLUSTALW program (Thompson, *et al.*, 1994), the phylogenetic tree was constructed by MEGA.X program employing the neighbor-joining (NJ) method (Kimura, 1980), (Kumar, *et al.*, 2018).

RESULTS

Positive amplification was obtained, using the primers which amplify the repetitive transposon-like regions of *C. burnetii*, from 10 out of 180 blood and faecal samples with 5.55% investigated in the present study. None of the blood samples collected from aborted sheep and goats revealed positive amplification for *C. burnetii* DNA. Shedding of *C. burnetii* by aborted small ruminants was found only in 10 out of 60 faecal samples (16.66%) investigated. Six out of 48 faecal samples (12.5%) and 5 out of 12 faecal samples (41.66%) were positive in aborted sheep and goats respectively (table 2).

Table 2: PCR results of examined blood and fecal samples in aborted sheep and goats according to places in Sulaimani province, Kurdistan-Iraq.

No.	Districts	Animals: Sheep			Animals: Goats		
		Blood samples	Fecal samples	PCR+ (%)	Blood samples	Fecal samples	PCR+ (%)
1.	Chamchamal	4	-	0 (0%)	6	-	0 (%)
2.	Darbandikhan	3	-	0 (0%)	2	-	0 (%)
3.	Dokan	3	-	0 (0%)	3	-	0 (%)
4.	Halabja 1	4	-	0 (0%)	4	-	0 (%)
5.	Kalar	5	-	0 (0%)	11	-	0 (%)
6.	Penjwen	1	-	0 (0%)	11	-	0 (%)
7.	Pshdar	3	-	0 (0%)	2	-	0 (%)
8.	Rania 1	5	-	0 (0%)	7	-	0 (%)
9.	Sharbazher 1	2	-	0 (0%)	9	-	0 (%)
10.	Sulaymaniyah	3	-	0 (0%)	5	-	0 (%)
11.	Halabja 2	2	-	0 (0%)	9	-	0 (%)
12.	Sharbazher 2	3	-	0 (0%)	3	-	0 (%)
13.	Rania 2	4	-	0 (%)	6	-	0 (%)
14.	Kalar		6	2 (33.33 %)	-	4	2 (50 %)
15.	Penjwen	-	10	1 (10 %)	-	6	1 (16.6 %)
16.	Pshdar	-	32	3 (9.375 %)	-	2	1 (50 %)
Total		42	48		78	12	180

Figure 2: lane 1: 100 bp DNA ladder, +ve: positive control, -ve: negative control, lanes (2-5): an example of positive samples**.**

Sequence and phylogenetic tree analysis

Nucleotide and amino acid identity of different countries of partial sequence IS1111A transposase gene were compared with other fourteen *C. burentii* strains. Field sequences strain exhibited identities ranging from 96.82 to 99.84%, and the highest similarity with (Iran, Brazil, Iraq and China) strain with identity rate of 99.84%, and lowest identity with Algeria strain with identity 96.82%. A comparative analysis of the two field isolates exhibited a 100% identity to each other. Interestingly, these isolates presented very limit amino acid diversity from other country strain except Nigeria and Namibia strain ranging from 1.51-20.0%. The partial sequence IS1111A transposase gene of both field sequences were aligned and compared with the reference strains for sequence analysis (Fig. 3), transposaese protein revealed limited variation between them (only one or two amino acid changed) except Namibia and Negeria strain due to different origin (Fig. 3). In phylogenic tree is shown in (Fig. 4), the two sequence of field isolate clustered together and make grouped with Brazil, Portugal and china strain.

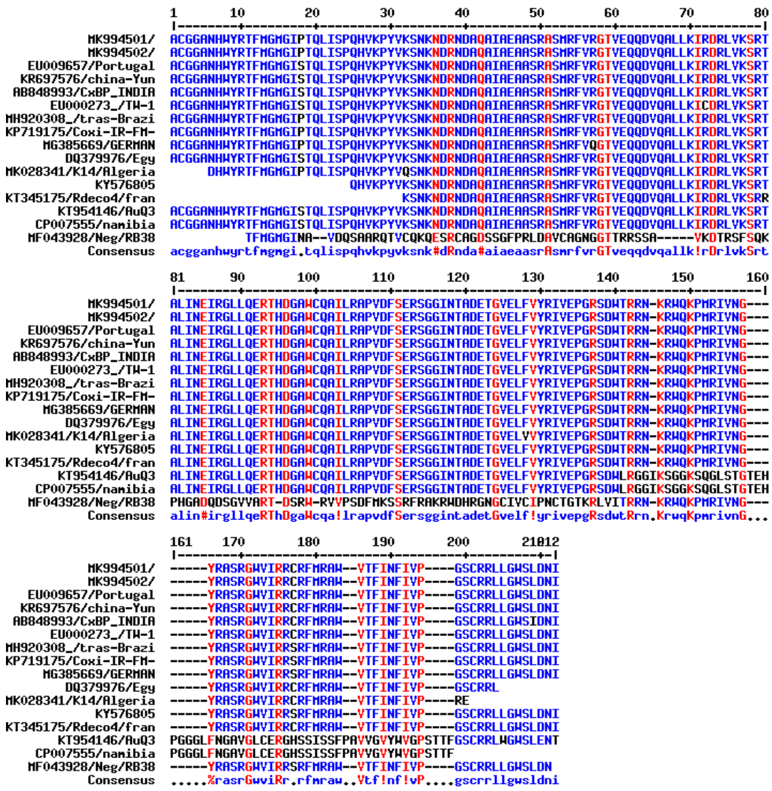

Figure 3: Two fields isolate sequences alignment with differences reference. Multiple sequences alignment of the amino acid of transpoanes gene region two fields isolates *Coxiella burentii* with fourteen reference strain in different countries.

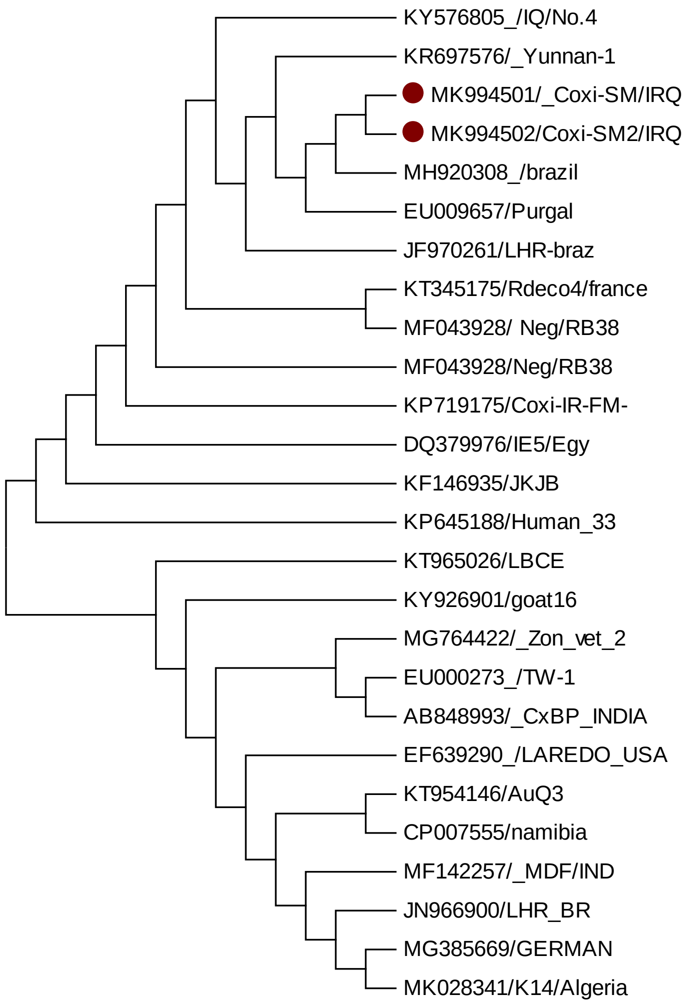

Figure 4: Neighbor-joining (NJ) phylogenetic tree generated using the nucleotide sequences for the partial sequence transpaneas gene of *coxiella burentii* spp.

DISCUSSION

The amplification of transposase gene (IS1111) allowed for the sensitivity of the assay to be increased, because this is a multi-copy gene (7-110 copies) (Klee *et al.*, 2006). DNA sequences generated in the present study confirm that *C. burnetii* is circulating in goats and sheep from some herds of Sulaimani province. A few studies were conducted on *C. burnetii* in Iraq. An outbreak of Q fever occurred with high morbidity in U.S. marines located in Iraq (Faix *et al.*, 2008).

The circulation of *C. burnetii* has been reported in AL- Diwaniayh city of Iraq in 2017. The current study constitutes the second attempt to genotype *C. burnetii* strains in Iraq. Certainly much more work needs to be done and many more samples need to be tested in order to record as many different genotypes as possible, as well as, to cover most of the country territory. Furthermore, the collection of such data and their comparison with data deposited in international databases will help towards both the continuing of the active surveillance and strain genotyping of the pathogen.

Q fever cases have been reported from some countries neighboring Iraq, such as Turkey and Iran. Results of a serosurvey undertaken on 42 sheep flocks in Turkey showed that 20% of sheep were seropositive (Kennerman, *et al.*, 2010). Serologic evidences indicate people and animals in Iran are exposed to *C. burnetii* (Khalili & Sakhaee, 2009), (Khalili, *et al.*, 2010).

C. burnetii has already been detected using different PCR methods in blood samples of infected camels in Iran (Schoffelen *et al.*, 2014).The sequence results analysis revealed 99% identity with Iranian, Brazilian and china strains. This indicates that the source of the bacteria in Iraq is Iran, Brazil and China which entered the country by importing meat and animal products from these countries.

Limited variation between field sequences, past Iraqi strain, and other countries is an indicator for stability of transposase gene (Fig. 3). In phylogenetic tree the sequences of current study make cluster with other Iraqi strain, this result epidemiologically exhibit that *Coxiella burnetii* has the same source in Iraq (fig. 4). The main route of shedding by ovine was found to be the faeces and vaginal mucus, while these routes were rare in bovine herds. Caprines were found to shed the organism via vaginal discharges, faeces and milk (Rodolakis *et al.*, 2007).

Shedding of *C. burnetii* in goats via faeces lasted for 2-5 weeks. In the present study the absence of *C. burnetii* DNA from the serum samples of sheep and goats could be attributed to the fact that the organism in these animal species is shed primarily via vaginal mucus and faeces, and this probably confirms that milk and blood are not the preferred routes of discharge for *C. burnetii* in sheep and goats (Rodolakis *et al.*, 2007).

CONCLUSION

The present study reports the first molecular detection of *C. burnetii* in sheep and goat in Sulaimani province. Further studies are necessary to characterize the genotype of *C. burnetii* and to identify the potential risk of transmission between human and animals regarding the public health issue of Q fever.

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CONFLICT OF INTEREST

The authors disclose no conflict of interest.

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الكشف الجزئي وتحديد الإصابة ب *Coxiella burnetii* في الاغنام والماعز المجهضة في محافظة السليمانية- كردستان- العراق

هردى فتاح مارف ، هانا شيرزاد رؤف ، شاخوان لتيف محمود ، ريبوار باهر أحمد ، باسم عبد الواحد على ، محمد عمر بابه شيخ

كلية الطب البيطري، جامعة السليمانية ، السليمانية ، العراق

E-mail: hardi.marif@univsul.edu.iq Assiut University web-site: www.aun.edu.eg

تعتبرحمى كيو من اكثر الامراض المشتركة انتشارا في جميع أنحاء العالم ، والمسببة من قبل جرثومة *Coxiella burnetii* التي يمكن أن تصيب أنواع مختلفة من الحيوانات كالماشية والأغنام والماعز اضافة الى الانسان. بالرغم من ان معظم اصابات العدوى بهذه الجرثومة هي تحت سريرية ، الا ان من الممكن حدوث حالات الإجهاض والإملاص في هذه في المجترات الصغيرة. هدفت الدراسة إلى تزويد شواهد جزيئية على وجود *C. burnetii* في المجترات الصغيرة المجهضة في مناطق مختلفة من محافظة السليمانية، كردستان - العراق. جمعت ١٨٠عينة دم وبراز من الاغنام والماعز المجهضة (٩٠ عينة لكل منهما) خلال الفترة الممتدة من شباط ولغاية حزيران (٢٠١٩) ومن ثم تحليلها باستخدام تفاعل البلمرة المتسلسل التقليدي (PCR) للكشف عن الحمض النووي للجين transposase (IS1111) في *C. burnetii*. تم الكشف عن طرح *C. burnetii* في (٥,٥٥ ٪) من عينات البراز التي تم فحصها للأغنام والماعز المجهضة. من اصل ٦٠ عينة براز، ١١ عينة فقط (16.66%) كانت موجبة للفحص. ومن أصل ٤٨ و ١٢ عينة براز، هناك ٦ عينات (12.5%) و ١٢ عينة (٤١,٦٦٪) موجبة للفحص في الأغنام والماعز المجهضة على التوالي. لم تظهر أي من عينات الدم نتيجة موجبة تجاه ايجاد و تضخيم الحمض النووي لجرثومة *C. burnetii*. يعتبرهذا البحث الاول في محافظة السليمانية، كردستان العراق في استخدام الكشف الجزيئي لـ *C. burnetii* في المجترات الصغيرة المجهضة.