Molecular detection and phylogenetic analysis of *Coxiella burnetii* in goats

Hayder N. Ayyez

Zoonotic research Unit, Veterinary Medicine College, University of Al-Qadisiyah, Iraq

email: Hayder.ayiz@qu.edu.iq

Abstract

Over the last years, the number *Coxiella burnetii* infections increased throughout the word. A molecular detection and phylogenetic analysis were performed in Al-Diwaniayh city in which the first phylogenetic documentation of *C. burnetii* reported in this area with accession numbers KY576802- KY576805. Molecular detection and phylogenetic analysis of *C. burnetii* targeting the transposase gene partial sequence was done on the milk samples from 50 apperently healthy goats . DNA sequence was amplified in 6 out of 50 (12%) milk samples collected from different regions of Al Diwaniya city. Four of these amplicons were submitted to sequences analysis and gave 99% nucleotides sequence similarity. Also the amplicon sequences of local strain were compared with global *C. burnetti* sequences in NCBI which revealed close related to NCBI-Blast *C. burnetii* transposase gene of Indian and Brazilian strains, whereas there was genetic variation to NCBI-Blast *C. burnetii* transposase gene for USA, Portugal and Taiwan. This work confirm goats infection and shedding the *C. burnetii* in Al Diwaniayh, Iraq and determined the phylogenetic tree of local strain of this bacterium.

Keywords: *Coxiella burnetii*, Query fever, goat, Molecular diagnosis ,Phylogenetic analysis

التحري الجزيئي وتحليل الشجرة الوراثية للكوكزلة بورنيتي في الماعز

حيدر ناجي عايز

وحدة بحوث الامراض المشتركة، كلية الطب البيطري، جامعة القادسية

email: Hayder.ayiz@qu.edu.iq

لوحظ في السنوات الاخيرة زيادة نسبة الاصابة بـ*C. burnetii* في انحاء العالم مما شجع على دراسة تلك البكتريا بالتحري عنها جزيئيا وتشخيص النمط الوراثي الموجود في مدينة الديوانية والذي تم تسجيلة بالجين بنك بالارقام KY576802- KY576805 .

استهدفت دراست التحري الجيني والتحليل الوراثي لجزء من الجين المسؤول عن التشفير لانزيم الترانسبوزيز (Transposase) لبكتريا *C. burnetii* في عينات حليب ماعز سليمة ظاهريا. اظهوت ستة عينات (١٢%) نتيجة موجبة من خلال تضخيم الدنا في عينات الحليب التي جمعت من مناطق مختلفة من مدينة الديوانية، تم تحليل تتابع القواعد النايتروجينية للمنطقة المضخمة من الدنا لاربعه منها واظهرت نسبة تطابق ٩٩% فيما

بينها، كذلك تم مقارنتها مع عتر عالمية في المركز العالمي لمعلومات التكنلوجية الحيوية (NCBI)حيث وجد تطابق مع العتر الهندية والبرازيلية بنسبة ١٠٠% بينما اظهرت نسبة اختلاف مع العتر الامريكية والبرتغالية والتايوانية. اكدت الدراسة الحالية الاصابة وطرح هذة البكتريا في الماعز في مدينة الديوانية كذلك تم تحديد الشجرة الوراثية للعتر المحلية.

الكلمات المفتاحية: الكوكسلة بورنيتي، حمى الاستعلام، الماعز، التحري الجزيئي، تحليل الشجرة الوراثية.

Introduction:

Coxiella burnetii, gram negative obligate intracellular bacteria, is the causative microbe of query (Q) fever which is a serious zoonotic disease spread everywhere ,except Newzealand (1 , 2). This bacterium is present fundamentally in goats, sheep and cattle which considered the main resrvoir for human infection (3). In animal the infection is often subclinical but has been combined with abortion and stillbirth. Clinical form in cattle may be sporadic while in goats and sheep the same symptoms may be appeared in epidmic form (4).

Q fever in human is typically an acut febrile disease with nonspecific signs and about 60% of infection are asymptomatic. Clinically acute infection in human appear as flu-like symptom often followed by pneumonia, while chronic type of infection showed endocarditis and cause death (5). High risk human groups to infection involve persons working with material of infected animals like slauterhouse worker, veterinarians, people living in or near to farm and laboratory personnel dealing with infected samples (6).

C. burnetii may persist in the enviroment for years and it is transmitted via inhalation of contaminated dust particals and through contact with carrier animal specially their reproductive fluids or other product such as wool (7).

The serological methods may be not useful to discovery of acute infection because the retard in antibodies development in addition it is diffecult to differentiate between current and previous infection due to the antibodies usually persist after the bacteria disappear from the body (Zhang *et al*., 1998). Molecular investigations are very useful for detection, specially to clarify and determine sources of infection and for evaluating control methods (9)

There are little information about *C. burnetti* infection in Iraq, so the aim of the this study was to investigate the presence of *C.burnetti* DNA in goat raw milk that may contribute to be source of transmission of this bacterium and identify genotypes occurring in Iraq to compare them with strains in other countries.

Material and methods: the study was conducted in Augat 2016 to March 2017 in the City of Al-Diwaniayh in Iraq .Milk samples from goats were collected from four deffernt areas. Specimens were evaluated by PCR assay using primers targeting the gene for transposase of *C. burnetii* F (GCAGCACGTCAAACCGTATG) and R

(TTCCCCCTCGAATGTTGTCG) which producing an amplification product of 549 bp .DNA was exrtacted from milk samples by genomic DNA purification kit supplemented byQIAamp DNA mini kit (Qiagen, Hilden, Germany) depending on the manufacturer's prescript. quantification of extracted DNA concentration and purity was done using a nanodrop to assessment the concentration and purity of the extracted DNA according to the manufacturer's informations.

The PCR reaction was done on 5 µl of tamplet DNA from each prepared specimen in atotal volume of 50 µl. The mixture of final reaction contained 10 pmol of each primer (Bioneer R& D center, Korea)

The amplification procedure consisted of an initial denaturationat 95°C for 120 sec, followed by30 cycles of DNA denaturation at 94 for 30s, primer annealing at 59 for 30s and strand extension at 72 for 60s. Finally one step at 72 for 5 min to complete DNA extension.

PCR product were visualized via electrophoresis, using 8 µm of it mixture and DNA ladder in 1.4% agarose gel for 45 min at 80 V, after stained with ethidium bromide and examind using ultraviolat transilluminator.

Four amplicons obtained from PCR were send to Bioneer Company in Korea for nucleotide sequencing by (AB DNA sequencing system). The results were analyzed with the multiple sequence alignment program. Phylogenetic analysis were performed by MEGA-6 and aligned sequences Coxiella tranposase partial sequence were compared to determined homology.

Results:

Products of anticipated size (549bp) for the transposase gene parcial sequence of *C. burnetii* were obtained in 12% (6 of 50) of goat milk samples collected from different region of Al-Diwaniyiah city(Figur .1)

Phylogenetic analysis via DNA sequencing

Only four among the six milk specimens selected according regions that give an amplicon of the anticipated base pair for *C. burnnetii* transposase gene from different region of Al-Diwaniyiah city were studied.

The results showed that these sequnces shared 99% similarity. The sequences of targeting gene (transposase partial gene) obtained from four goats were deposited in gene bank (accession numbers KY576802, KY576803, KY576804, KY576805).

Comparative analysis of transposae nucleotide sequences from Iraq samples with the number of *C. burnetii* strains present in the gene bank database shown in figure 2.

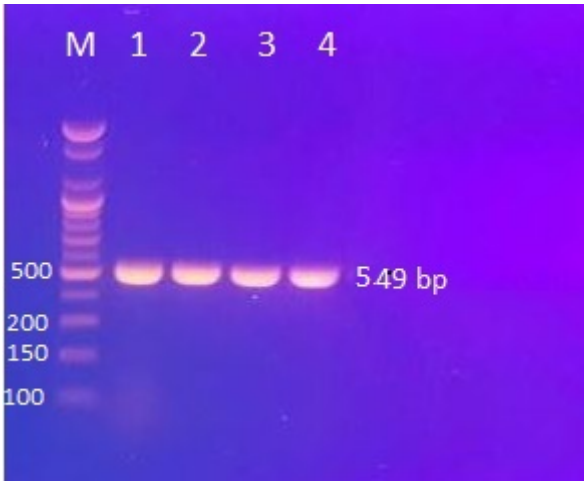

Figure 1 : Electrophoresis on agarose gel which revealed the PCR product of transposase partial sequences gene for diagnosis of *C. burnetii*. Where M: DNA ladder (1500-100bp), well (1-4)positive samples, at 549 bp PCR product size.

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Iraq-Seq1    ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCATCGACAACATTCGAGGGGG-A--A
Iraq-Seq2    ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCATCGACAACATTCGAGGGGG-A--A
Iraq-Seq3    ATTTCATCGTTCCCGGCAGT-GTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGTGGG-AATA
Iraq-Seq4    ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGTGGGAA--T
India        ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCATCGACAACATTCGAGGGGG-A--A
China        ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGGGGG-A--A
Taiwan       ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGGGGG-A--A
Portugal     ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGGGGG-A--A
Brazil       ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGTGGG-A--A
USA          ATTTCATCGTTCCCGGCAGTTGTCGGCGTTT---------------------------------A--T
France       ATTTCATCGTTCCCGGCAGTTGTCGGCGTTTATTGGGTTGGTCCCTCGACAACATTCGAGTGGG-A--A

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Figure (2): Multiple sequence alignment analysis of the partial transposase gene for local Iraqi *Coxiella burnetii* isolates (Seq1- Seq4 isolate) with NCBI-Blast *Coxiella burnetii* transposase gene by using (MEGA 6.0, multiple alignment analysis tools). The multiple alignment analysis similarity (*) in transposase gene nucleotide sequences.

The sequences of Coxiella partial transposase gene were compared to published sequnces (present in Gene Bank). The phylogenetic analysis revealed closed relatedof Seq-1 and Seq-2 to NCBI-Blast *C. burnetii* transposase gene of Indian strain AB848993.1 (100% identity), whereas Seq-3 and Seq-4 were close related to NCBI-Blast *C. burnetii* transposase gene of Brazilian strain JF970261.1 (Figure 3) & (Table

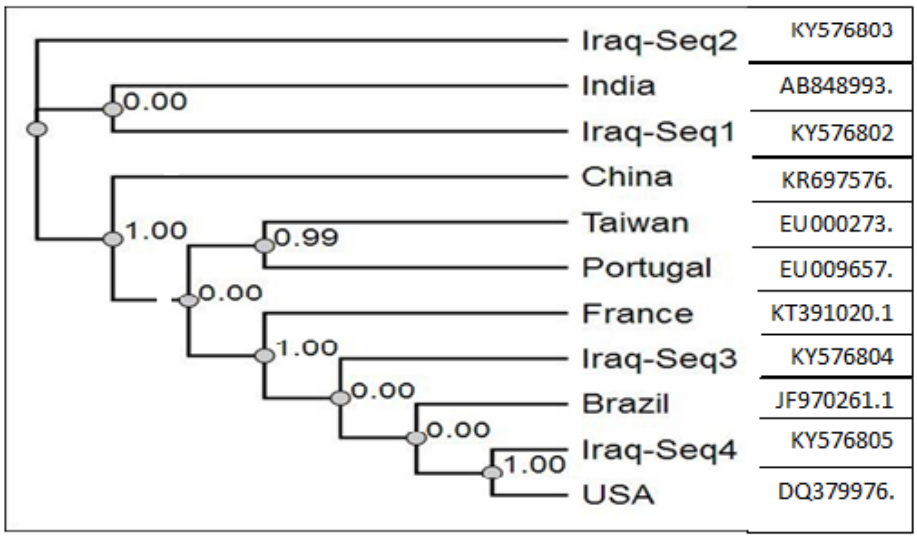

Figure (3): Genetic tree interpretation based on the transposase gene partial sequence that used for *Coxiella burnetii* genetic changes according regions. The genetic tree was constructed according Unweighted Pair Group method with Arithmetic Mean (UPGMA tree) in (MEGA 6.0 version). The local *Coxiella burnetii* isolates (Seq.1 and Seq.4) were showed closed related to NCBI-Blast *Coxiella burnetii* transposase gene of Indian strains, whereas Seq.3 and Seq.4 revealed identity with Brazilian strains.

Table 1 : Percentage of identity with some Global strains.

isolates	Iraq-Seq1	Iraq-Seq2	Iraq-Seq3	Iraq-Seq4
Iraq-Seq1		100.00	99.27	99.27
Iraq-Seq2	100.00		99.27	99.27
Iraq-Seq3	99.27	99.27		100.00
Iraq-Seq4	99.27	99.27	100.00	
Brazil	99.27	99.27	100.00	100.00
India	100.00	100.00	99.27	99.27
China	99.64	99.64	99.64	99.64
USA	99.81	99.81	99.81	99.81
Taiwan	99.45	99.45	99.45	99.45
Portugal	99.45	99.45	99.45	99.45

Discussion

This study used molecular tools which is specific and efficient for detection of *C. burnetii* infection compared with the use of serological diagnosis that often loss of sensitivity. Cross-reactions have been described with *Legionella* species and with *Bartonella* species and can make interpretation of serological results difficult (10).

Convential PCR technique targeting the transposase partial sequence gene in specimens collected from apparently healthy goats of different separate farms of Al-Diwaniyia showed the presence of this bacterium DNA in 12% (6 of 50) of the goat milk samples which tested in this study. This result revealed relatively high positive yield of Coxiella compared to study on native Korean goats in which infection was 9.5% (11) and 2% of healthy goat milk samples were positive in Iran(12), while low positive yield compared to other study on Egyptian goats that confirmed the found of *C. burnetii* DNA in 85.2% of goats raw milk (9). The variation in detection rates greatly dependes on sampling time after parturition due to shedding of this bacterium by infected goats occurs mainly during parturition (13)

Also Molecular study of *C. burnetii* is a important method to know the genetic diversity in a region and to explore relationships among variants of this organism. Genotyping methods revealed two genotype prevalance among samples under study. The first was identical to the Brazilian strain, while the other genotype showed a 100% match to the Indian strain. This indicates that the source of these bacteria in Iraq is Brazil and India which entered the country by importing meat and animal products from those countries.

The study results refer to the goats is the important reservoir of *C. burnetii* and it was the source of infection in the regoin and because of ability of this bacterium to survive in the enveronment and transmession by inhalation, these gave indicated the potential of an increase infections in humans specially persons whose work in contact with animals and to other animals in Al-Diwaniayh city and its surroundings.

The sequence results analysis showed the presence of 99% identity among sequenced strains and there are two genotype circulating in the different regoin and this result highly suggests a clonal spread of coxiella with this predominant genotype over the goat farms in the Al Diwaniya part of the Iraq.

Conclusions

This is the first work gives information about the genotypic similarity and diversity of *C. burnetii* that found in Iraq with other regions.

Refrences

1- Raoult D, Marrie T, Mege J: Natural history and pathophysiology of Q fever. Lancet Infect Dis 2005, 5:219–226.
2- Ming-Yang Y, Si-Xian Q, Qi-Dong T, Sheng- Yong F, Guang-Xue L, Dong-Hui Z, Xing-Quan Z. First reort of C.burnetti seroprevelance in Tibetan sheep in China. Vector-Borne and Zoonotic Diseases 2015; 15(7):419-422.
3- Domenico M, Curini V, Massis F, Provvido A, Scacchia M, and Camma C (2014) Coxiella burnetii incentral italy Novel genotype are circulating in cattle and goats. V 14, No. 10.

4- Agarholm JS. (2013) Coxiella burnetii associated reproductive disorder in domestic animals a critical review. Acta Vet Scand 55:13.
5- Setiyono A, Ogawa M, Cai Y, Shiga S, Kishimoto T, Kurane I. New criteria for immunofluorescence assay for Q fever diagnosis in Japan. J Clin Microbiol 2005; 43:5555–5559.
6- Borriello G, Iovane G, Galiero G. La febbre Q negli animali domestici. Large Animal Review 2010; 16: 273-283.
7- ECDC (Eurooean Centre for Disease Prevention and Control) . Panel with Representatives from the Netherlands, France, Germany, United Kingdom, United States of America. Risk assessment on Q fever. ECDC Technical Report 2010; 40 pp.
8- Zhang GQ, To H, Yamaguchi T, Fukushi H, *et al*. (1997) Differentiation of Coxiella burnetii by sequence analysis of the gene (com1) encoding a 27-kDa outer membrane protein. Microbiol Immunol. 41:871–877.
9- Khalifa N. o.; Elhofy F. I.; Fahmy H. A.; Sobhy M. M., and Agag M.A.(2016). Seropervelance and molecular detection of Coxiella burnetii infected in sheep, goats and human in Egypt.vol 2 No. 1-7.
10- Markey B., Leonard F., Archambault M., Cullinane A. and Maguire D. (2013). Clinical Veterinary Microbiology. Second edition
11- BY, Seo MG, Lee SH, Byun JW, Oem JK, Kwak D.(2014) Molecular and serologic detection of Coxiella burnetii in native Korean goats (Capra hircus coreanae). Vet Microbiol..
12- Abbasi S, Farzan R, and momtaz H, (2011) Molecular detection of Coxiella burnetii in goat bulk milk samples in some provinces of Iran. 10 (80): 18513-18515.
13- Guatteo R, Beaudeau F, Joly A, and Seegers H, (2007) Assessing the within-herd prevalence of Coxiella burnetii milk shedder cows using a real-time PCR applied to bulk tank milk. Zoonoses Puplic health, 54: 191-194.