

## Molecular detection of *Coxiella burnetii* in human and sheep in Al-Diwaniyah province by Real Time- PCR

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### Abstract

**Background:** Iraq is agricultural country, but the important aspect of livestock shown by most of the studies conducted in previous years, which dealt with Q fever infection in animals ( cattle, sheep and goat ) strikingly. Q fever it is a worldwide zoonotic disease caused by *Coxiella burnetii* .

**Material and methods:** One hundred and five (105), human samples used in the study were (50) (22 sputum/28 bronchial-alveolar lavage) collected from Teaching Hospital and Center Pulmonology and Respiratory Specialist in Al-Diwaniyah. Collected sheep samples were (55) Sample (28 nasopharyngeal swabs and 27 bronchial fluids) from the slaughterhouse of Al-Diwaniya during January – august 2016. All samples were collected from peoples & sheep that suffer from clear respiratory signs. By using Sybr Green Real Time- PCR technique the samples tested directly.

**Results:** showed that the infection incidence in humans (16%) and in sheep (38.181%), the study proved that there were a significant differences at the site of infection in the respiratory tract (URT& LRT) for humans and sheep alike, as well as in terms of age groups and gender.

**Conclusions:** Q fever is a serious disease circulating between peoples in Al-Diwaniyah city and clearly defined risk groups: people with respiratory infection, heart and vascular disease, immunosuppressed patients and distributed in sheep that have respiratory disorder. Real Time –PCR is a potential tool to diagnosis of *C. burnetii* in humans and animals, because of its rapid and sensitive detection.

**Keyword :** *C. burnetii*, human, sheep, real time-pcr SberGreen, respiratory samples, & Iraq

الكشف الجزيئي للالوكسيلا البيورنتية في الإنسان والأغنام في محافظة الديوانية باستخدام  
تقنية تفاعل سلسلة البلمرة في الوقت الحقيقي.

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### الخلاصة:

خلفية : العراق بلد زراعي، لكن جانب الثروة الحيوانية يعد مهما و هذا ما أبدته معظم الدراسات التي أجريت في السنوات السابقة و التي تعاملت مع عدوى حمى Q في الحيوانات (الأبقار والأغنام والماعز) , أن بكتيريا

*Coxiella burnetii* هي العامل المسبب لحمى Q، وهو مرض حيواني المنشأ ينتقل بين البشر والحيوانات و ذو انتشار عالمي.

**المواد وطرق العمل :** تم جمع عينات من الأشخاص والأغنام التي تعاني من علامات واضحة في الجهاز التنفسي. وكانت مائة وخمسة (105) العينات البشرية التي استخدمت في الدراسة (50) (22 بلغم / 28 سوائل الغسل القصبي) التي تم جمعها من المستشفى التعليمي ومركز الامراض الصدرية والتنفسية التخصصي في الديوانية وكانت العينات التي تم جمعها من الأغنام (55) عينة (28 مسحات البلعوم و 27 السوائل الشعب الهوائية) في مجزرة الديوانية خلال يناير - أغسطس 2016.

**النتائج :** أظهرت أن حالات عدوى بين البشر (16%) و في الأغنام (38.181%)، وقد أثبتت الدراسة أن هناك اختلافات كبيرة في موقع الإصابة في الجهاز التنفسي للبشر والغنم على حد سواء، كما اوجدت الدراسة فروق معنوية من حيث الفئات العمرية والجنس.

**الاستنتاجات :** حمى Q هو مرض خطير لا يزال دائرا بين الاشخاص في مدينة الديوانية، الفئات المعرضة للخطر واضح المعالم هم : الأشخاص الذين يعانون من التهابات الجهاز التنفسي وأمراض القلب والأوعية الدموية، والمرضى الذين يعانون كبت المناعة و في الأغنام التي لديها اضطراب في الجهاز التنفسي. وتعد تقنية ال real time pcr تقنية معتمدة في التشخيص لسرعتها وحساسيتها لبكتريا *C. burnetii*.

## Introduction

Coxiellosis is a zoonotic widespread disease with profile risk the causative agent bacterium *Coxiella burnetii*. It's an emerging disease that can cause considerable morbidity and serious long-term complications in humans, circulating in cattle, sheep and goats and in accordance with Centers for Disease Control and Prevention (CDC) in the United States, the disease classified as a bioterrorism agent (1,2). *C. burnetii*, is an obligate intracellular bacterium similar to rickettsia, that relies exclusively on a eukaryotic cell for growth (3), and it can survive in a dry and harsh environment (4). Ruminants (Cattle, sheep and goats) are the primary reservoirs of animals (5). Inhalation of contaminated aerosols, it is the main route of infection, oral transmission by contaminated raw milk or dairy products is also possible (6). As well as tick bites, and human-to-human transmission considered other rare modes of transmission in human (7). From every world region Q fever infections have been reported in humans and animals except Antarctica (8). There is a difficulties in distinguished Q fever in human & animals from other febrile diseases because symptoms frequently are unspecialized, making diagnosis be provoked (7).

The disease has many forms including without clinical symptoms or moderately symptomatic to fatal disease, in humans Q

fever can manifest as an acute disease (mainly as a self-limited febrile illness, pneumonia, or hepatitis) or as a chronic disease (endocarditis), patients with former valvulopathy and less in immunocompromised hosts and in pregnant women. In comparison to human, Q fever in animals most cases, strikingly asymptomatic (9). In ruminants (sheep and goat), infections are mostly asymptomatic, however, abortions and stillbirths can occur, mainly in late pregnancy (5). But there is no prop proof to support a supposition of *C. burnetii* causing disorders such as subfertility, endometritis / metritis, or retained fetal membranes in any kind of domestic animal species (10).

The information supplied from Iraq most of which revolved about US military personnel returning after the war in 2003 (11). Investigations of these cases related satisfactory to tick bites, sleeping in stockade, and living near helicopter zones with environmental exposure resulting from helicopter-generated aerosols (12,13). In western Iraq, during 2005 the infection recorded and the potential risk factors included dust and exposure to animals and ticks (14,15). Q fever infection between Iraqi individuals particular detected firstly in Nassiriya city during 2010, in patient women hospitals suffering from abortions and unknown

fever (16). Then It appeared again in same city during 2015 (17). In Thi-Qar province the infection by Q fever recorded in cows & sheep during 2010 (18). In the mid-Euphrates region exactly in AL-Qassim city during 2011 the infection identified between small ruminants (sheep & goat) (19). After all past, the disease appeared once more in the Nassiriya city during 2015, where *Coxiella burnetii* responsible of abortion cases in small ruminants (17). In Basra southern of Iraq, announced the prevalent of Coxiellosis across the province on the reality in 2015, where the infection incidence between local sheep breeds. (20) With all these data, it is an indication for remaining Q fever an important concern in the current conflicts in Iraq. With high levels of sensitivity and specificity, Real-time PCR assays have been developed already (21), and it is an excellent way for the detection and measurement of *C. burnetii* cells in body fluids (22).

#### **Aim of the study :**

The study aimed to highlights the spreading of *Coxiella burnetii* infection in patients & in sheep across the province, and evaluation of some risk factors (site of infection, age and sex )

#### **Materials and Method :**

**Samples collection :** fifty (50) human respiratory samples included 22 Sputum swab (upper respiratory tract)(URT) and 28 bronchial-alveolar lavage (BAL) (lower respiratory tract)(LRT). were collected from patients with different respiratory infection from Teaching Hospital and Center Pulmonology and Respiratory Specialist in Al-Diwaniyah city. Most patients suffering from fever (temperature  $\geq 38^{\circ}\text{C}$ ), respiratory disease (hard breathing, expectoration, cough, chest pain-associated by abnormal results on the chest radiograph), hepatitis (< twofold elevation in serum levels of aspartate aminotransferase). In addition to

55 sheep samples 28 nasopharyngeal swab and 27 bronchial fluids collected from same animals except one sample taken from upper part of respiratory tract only, they pull together in AL- Diwaniyah abattoir, all sampling occur during January to august 2016, the animals suffering from respiratory signs ( nasal discharge, lacrimation and dyspnea ), and abortion. Each sample collected in 25ml sterile containers processed immediately, 1mL of sputum /BAL/ bronchial fluids were homogenized and diluted in 5mL of sterile distilled water, transported into laboratory then centrifuged at 12000r for 10 minutes, 0.5mL of supernatant had been taken and re-suspend centrifuged deposit in residual fluid, stored at  $-20^{\circ}\text{C}$  in a refrigerator until use for genomic extraction.

**Genomic extraction :** Bacterial genomic DNA was extracted from respiratory swabs by using (AccuPrep® DNA Extraction Kit. Bioneer. Korea). DNA was examined by Nanodrop spectrophotometer, and stored in  $-20^{\circ}\text{C}$  until outright PCR assay.

**Real Time PCR Technique :** was carried out for direct detection of *Coxiella burnetii* based amplification of transposase gene (*IS1111a*). **The Oligonucleotides were designed by employing NCBI-GenBank recorded sequence to amplify a 147bp fragment, forward primer / TAAAAGCGGTGGCAAAAGCC, reverse primer /ACCCAATAACGCCGACAAC,** were equipped by (Bioneer company. Korea). The Real-Time PCR amplification reaction was done by using (AccuPower® GreenStar™ qPCR PreMix kit, Bioneer. Korea) and the qPCR master mix it has been prepared to each sample depending on company directive. These qPCR master mix component convey into green star qPCR premix standard plate tubes which contain the Syber Green dye and

other PCR amplicon, the plate mixed by exispin vortex centrifuge for 3 minutes and placed in MiniOpticon RealTime PCR system and setting the thermocycler conditions as the following: initial denaturation, at 95 °C for 3 minutes, one repeated cycle / denaturation at 95 °C for 10 sec/ annealing\ extension 10sec and detection (scan) at 60 °C for 30sec during 45 repeat cycle and melting 60-95°C, 0.5sec through 1 repeat cycle.

**Results and discussion :** To the best of our attention, there is no notify from Iraq confirms that *C. burnetii* being responsible of atypical pneumonia between Iraqi individuals; this could possibly be traced to the fact that atypical organisms have rarely been isolated from patients with community-acquired pneumonia. Culture of *C. burnetii* is difficult, hazardous and technically is hard and not routinely ready in most laboratories. Molecular detection of bacterial DNA is more used (23). The present study which done in Al-Diwaniyah consider the first detection of *C. burentii* in the city in human and animals (sheep), by using Sybr Green Real-Time PCR, globally it is first detection of Q fever infection depending on respiratory samples by same primer in molecular assay. The study showed that *C. burnetii* infection is fairly widespread in our society, out of 50 human samples 8 (16%) were positive for *C. burnetii* this finding closed to results of (16) who detected coxiellosis in blood and breast milk of aborted women but lower than the results which recorded by (17) who was confirmed that the infection was 31.5% in

serum of women depending on Elisa technique.

Human respiratory samples showed amplification threshold at 28 to 34 cycle, while sheep samples amplified at 22 to 34 cycle, the primers expressed specificity at approximately 83<sup>0</sup>C melt peak in human & sheep samples ( figure 1,1-1,1-2,1-3 and 2,2-1, 2-2, 2-3), This results of Sybr Green based real-time PCR targeting the *IS1111a* (transposase gene), the test show highly reproducible results for the detection and quantification of the pathogen and agreed with (24, 39, 40) were they found that real-time PCR consider better rapid and reliable diagnosis of Q fever more than isolation of pathogen, and an excellent test for the detection and/or quantification of the pathogen in clinical samples.

The highest rate according to the site of infection recorded in LRT 25 % than URT 4.545% with a significant difference ( $p<0.05$ ) table (1). The study results agreed with occurrence nature of disease which was characterized by two common manifestation, [flu-like symptoms](#) and typical pneumonia caused by variety of microorganisms (25). Epidemiological studies represent a reminder for confirmation of viruses are an important etiologic agent of lower respiratory tract infections, as well as essential assistant factor in the development of severe bacterial pneumonia (26,27), also viruses alter the bacterial community in the URT and promote bacterial colonization of the LRT (28). Smoking also have an importance in compromising the pulmonary clearance mechanisms and the host immune response by increasing the risk of pneumonia (29).

**Table (1): human Q fever infection rate according to the infection site.**

Sit of sample in R.T	No. of respiratory samples	No. of positive samples	Positivity percentage %
Sputum swab	22	1	4.545 <sup>a</sup>
BAL	28	7	25 <sup>b</sup>
Total	50	8	16

\*different letters referred to the significant difference at (p<0.05)

The study demonstrated that the high significant difference at (p<0.05) was seen among the age groups, 40-60yrs and 15-30yrs compared with 1-12 years old in table (2) this results meet with the high ratios of infection with Q fever occur among the adult ages more than younger persons (30,31,16). Whereas children most probably have milder illness and symptoms than adults (32). Kids have good immunity during the first years of their lives more than adults (32).

**Table (2): human Q fever infection rate according to the age groups**

Age groups	No. of respiratory samples	No. of positive samples	Positivity percentage %
1-12 y	19	1	5.263 <sup>b</sup>
15-30 y	10	2	20 <sup>a</sup>
40-60 y	21	5	23.809 <sup>a</sup>
Total	50	8	16

\*different letters referred to the significant difference at (p<0.05)

Regarding to the gender, the results showed that the males had more infections with Q fever 22.727 % than females 10.714 % with significant differences (p<0.05) table (3), this results was agreement with many previous studies ensured that the symptomatic infection is more prevalent in men than women (33). The predisposition for infection in men may be explained by the protective role of female sex hormones (34).

**Table (3): human Q fever infection rate according to the gender.**

Gender	No. of respiratory samples	No. of positive samples	Positivity percentage %
Male	22	5	22.727 <sup>a</sup>
Female	28	3	10.714 <sup>b</sup>
Total	50	8	16 %

\*different letters referred to the significant difference at (p<0.05)

**In sheep** Out of 55 samples 21 (38.181%) were appeared infection with coxiellosis and this ratio agreed with the studies of (17-20), where they explained and confirmed the zoonotic and worldwide distribution of this microorganism. This results consider the first one which confirms the present of infection in animal (sheep) in Al-Diwaniya city. In Iraq the infection with *C. burnetii* expected due to cumulative exposure and multiple of the reservoirs in the nature which include different species of mammals, birds and ticks in addition to the infections of *C. burnetii* is more often inherent in animals with continued shedding of bacteria to the environment.

The infection rate according to the infection site of RT was higher 60.714% in URT than LRT 14.814% with a significant differences (p<0.05) table (4). The interpretation of this results was related to the fact of various etiologies cause respiratory diseases and described in different domestic animals. However, the problem is more common in sheep due to the verity ratio of the alveolar surface to metabolic weight is very low in sheep compared to other species (35). Also, *C. burentii* spore like form can stay alive for years in the environment and journey widespread as an aerosol, windy, dry

conditions may cofactors to exposure the animals and disease transmission (36). Also poor feeding, bad ventilation and alien new animals to the herd with unknown case history play an role important in distribution of infection in flocks.

**Table (4): sheep Q fever infection rate according to the infection site.**

Sit of sample in R.T	No. of respiratory samples	No. of positive samples	Positivity percentage %
Nasopharyngeal swab	28	17	60.714 <sup>a</sup>
Bronchial fluids	27	4	14.814 <sup>b</sup>
Total	55	21	38.181

- Different letters referred to the significant difference at ( $p < 0.05$ ).

Q fever prevalence between sheep according to the age groups showed that lower percentage rate was found among 1-2 years age group 23.529 % whereas the highest rate recorded at < 2 yrs age group 61.904 % with a significant differences ( $p < 0.05$ ) table (5), in *C. burnetii* infection the differences related to ages expected because older animals are exposed to tick bite and *C. burnetii* for longer periods (37).

**Table (5): sheep Q fever infection rate according to the age groups.**

Age groups	No. of respiratory samples	No. of positive samples	Positivity percentage %
1-2 y	34	8	23.529 <sup>a</sup>
2 y	21	13	61.904 <sup>b</sup>
Total	55	21	38.181

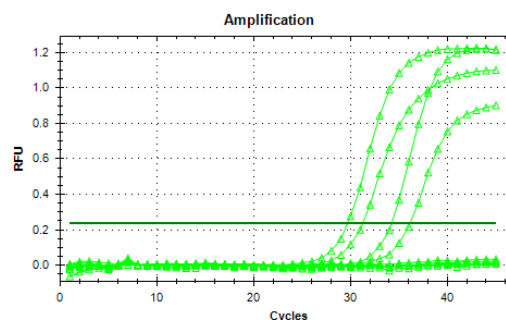
\*different letters referred to the significant difference at ( $p < 0.05$ ).

The high occurrence of disease in females 45.945% than males 22.222 % with a significant differences ( $p < 0.05$ ) table (6) and this may be related to the organism nature, which has a high affinity for the placenta, fetal membranes and mammary glands, and is found in large numbers in these tissues (38).

**Table (6): sheep Q fever infection rate according to the sex.**

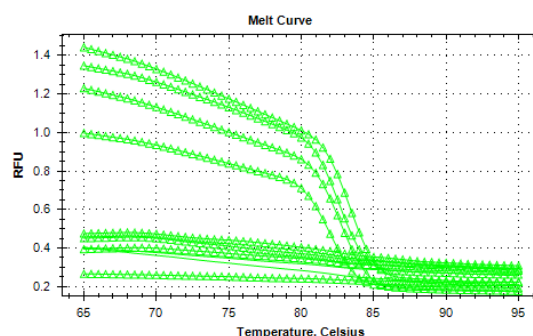
Sex	No. of respiratory samples	No. of positive samples	Positivity percentage %
Male	18	4	22.222 <sup>a</sup>
Female	37	17	45.945 <sup>b</sup>
Total	55	21	38.181 %

\*different letters referred to the significant difference at ( $p < 0.05$ ).

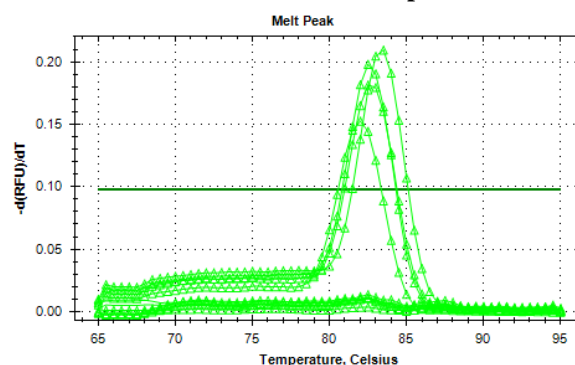




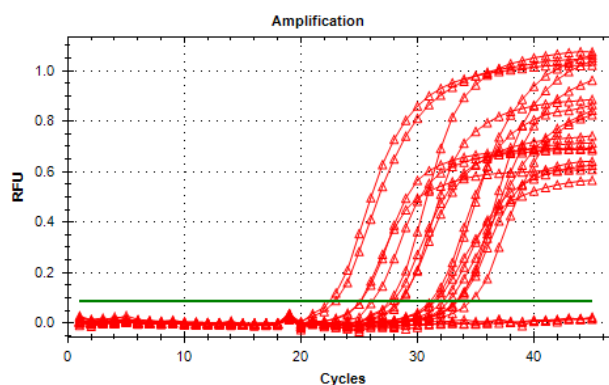
(Fig.1): Real-Time PCR amplification plots for transposase gene in *Coxiella burnetii* positive in human respiratory samples that show threshold amplification cycle at 28 to 34 cycle.



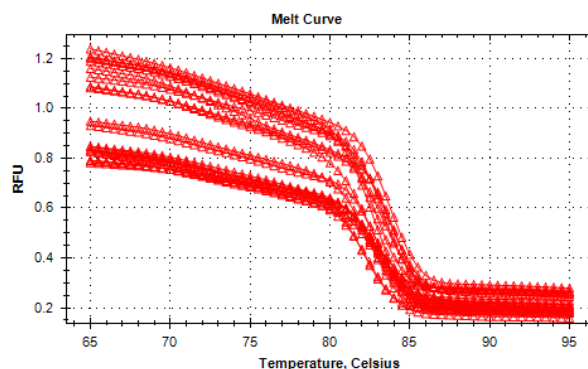
(Fig. 1-2): Real-Time PCR melt curve of *Coxiella burnetii* positive in human bronchial samples.



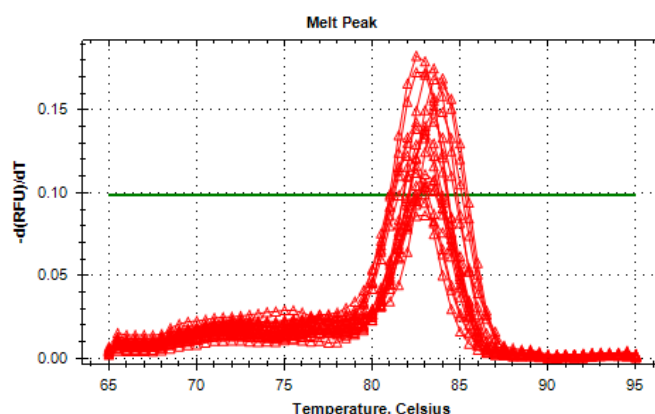
(Fig.1-3): Real-Time PCR melt peak of *Coxiella burnetii* positive in human bronchial samples that show primers specificity at approximately 83°C melt peak.



(Fig. 2): Real-Time PCR amplification plots for transposase gene in *Coxiella burnetii* positive in sheep respiratory samples that show threshold amplification cycle at 22 to 34 cycles.



(Fig.2-2): Real-Time PCR melt curve of *Coxiella burnetii* positive in sheep bronchial samples



(Fig.2-3): Real-Time PCR melt peak of *Coxiella burnetii* positive in sheep bronchial samples that show primers specificity at approximately 83°C melt peak.

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