



RESEARCH

Seroprevalence and Molecular Detection of *Coxiella burnetii* Infection in Sheep, Goats and Human in Egypt.

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ABSTRACT

Query (Q) fever is an ubiquitous zoonosis caused by *Coxiella burnetii*. The present study was carried out to determine the prevalence of *C. burnetii* in apparently healthy sheep, goats and farm workers. Raw milk and serum samples were randomly collected from 200 sheep, goats (100 of each) and 30 farm workers from Qalubia governorate, Egypt during 2014/2015. The milk and serum samples were investigated for IgG antibodies against *C. burnetii* phase II antigen by indirect immunofluorescent antibody test (IFAT). The seropositive samples were confirmed by touch-down PCR with specific primers which amplify transposon-like region of *C. burnetii*. The results showed that antibodies against *C. burnetii* in sheep raw milk and sera were 17% and 23% respectively, in goat raw milk and sera were 19% and 27% respectively and in human was 23.3%. PCR targeting IS1111 gene confirmed the presence of *C. burnetii* DNA in sheep and goats raw milk and sera were 82.4%, 89.5%, 91.3% and **85.2%** respectively and in farm workers was 57.1%. **These** results proved that the apparently healthy sheep and goats are an important reservoir of *C. burnetii* infection. The farm workers constitute an occupational risk group for *C. burnetii* infection, for their contact with infected livestock.

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INTRODUCTION

Q fever is a zoonotic disease caused by *Coxiella burnetii*, a small obligate intracellular Gram-negative pathogen worldwide spread, except New Zealand (Maurin and Raoult, 1999 ; YinMing-Yang et al., 2015). Sheep and goats are considered as the main reservoir of infection for man, like cattle. The most common pathological manifestations of chronic Q fever are abortions and stillbirths. In sheep and goat flocks with reproductive disorders, animals contemporarily shed the bacterium through vaginal mucus, feces and milk. In a recent work, goats have been shown to eliminate *C. burnetii* mostly through milk while sheep mainly through vaginal mucous or feces (Rodolakis, 2009). *C. burnetii* was a cause of abortion waves at 28 dairy goat farms and 2 dairy sheep farms in the Netherlands (Van den Brom et

al., 2015). It persists for several years, and is probably lifelong. Sheep, goats and cows are mainly subclinical carriers, but can shed bacteria in various secretions and excreta. Humans generally acquired the infection through air-borne transmission from animal reservoirs, especially from domestic ruminants (OIE, 2010). Epidemics still occur in other slaughterhouse workers as well, recent cases have been reported in New South Wales and in Scotland (Gilroy et al., 2001; Wilson et al., 2010). High risk groups include people working with possible infected material (slaughterhouse workers, veterinarians, meat-processing workers), persons living in or next to farms, and laboratory personnel processing eventually infected organs and tissues (Borriello et al., 2010). Infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals,

their reproductive tissues or other animal products, like wool (ECDC, 2010). *C. burnetii* is secreted in the milk, therefore the ingestion of contaminated food such as raw milk and dairy products, represent a possible source of infection for humans (Maurin and Raoult, 1999). Clinically, acute Q fever in human showed flu-like symptom often followed by pneumonia, whereas chronic type of the disease showed endocarditis and caused death (Setiyono et al., 2005).

C. burnetii undergoes phase variation in which antigenic transition occurs from the wild type phase I to a virulent phase II during successive passages in embryonated eggs or in cell cultures (Kovacova and Kazar, 2002). Serologic assay may detect antibodies to phase II and phase I of *C. burnetii*. Phase II antibodies are more prevalent during acute infection, while chronic infection is characterized by a predominantly phase I antibody response (Fornier et al., 1998). A micro-immunofluorescence technique (IFAT) is the current method for the serodiagnosis of Q fever (Tissot-Dupont et al., 1994). IFAT is suitable technique for diagnosing Q fever and its therapeutic follow-up and is a good candidate for screening sera in large numbers (Slabá et al., 2005). Seroprevalence studies did not provide information on the incidence of current infection in humans or animals. Several typing methods have been used for the identification of *C. burnetii* strains, such as nested PCR assay (Mazyad and Hafez, 2007, Rahimi et al., 2010), real-time PCR (Szymańska-Czerwińska et al., 2015) and touch-down PCR (Khanzadi et al., 2014), trans-PCR targeting IS1111, the repetitive transposon-like region of *C. burnetii* (Vaidya et al., 2008). These tools are very useful for epidemiological investigation, particularly to clarify links regarding sources of infection, for better understanding of the epidemiological emerging factors, and to a lesser extent, for evaluating control measures.

Little information is available about *C. burnetii* in sheep and goat infection in Egypt, so the objective of the present study was to investigate the seroprevalence of *C. burnetii* by IFA and to determine the presence of *C. burnetii* DNA in seropositive raw milk and sera that may contribute to be source of transmission of the organism.

Materials and Methods

Sampling

Raw milk, and blood samples were collected from apparently healthy 200 sheep and goats (100 of each) 1 to 3 years old. Samples were collected under aseptic conditions from randomly distributed farms of sheep and goats in different localities in Toukh city, Qalubia governorate, Egypt during 2014/2015. Blood samples were collected from brachial veins of farm workers (n=30) from the same farms. Milk and sera samples

were transferred in sterile screw capped tubes and stored at -20°C until processed.

Identification of *Coxiella burnetii* by Indirect Immunofluorescence Assay (IFA)

The reagents were commercially provided from bioMerieux SA, Marcy-l'Etoile, (France). Milk and sera were tested for IgG antibodies against *C. burnetii* phase II by using slides coated with *C. burnetii* phase II. The IFA was performed as described by the manufacture. Serial twofold dilutions of milk and sera were prepared in a phosphate buffered saline (PBS) with a goat anti-human IgG-fluorescein isothiocyanate (FITC) immunoconjugate, *C. burnetii*-positive rabbit serum and *C. burnetii*-negative rabbit serum. The slides are examined under ultraviolet light at a magnification of 400x by fluorescence microscope. Samples that showed green fluorescent color at a titre $\geq 1:64$ are considered positive (Vaidya et al., 2008).

DNA Extraction

A volume of 1 ml milk was centrifuged at 8000 g for 60 min, the cream and milk layers were removed and the pellet was washed and resuspended twice in 50 μ l distilled water (Mohammed et al., 2014). Then DNA extraction was applied by using QIAamp kit (Qiagen, Hilden, Germany). according to the manufacturer's instructions for milk and serum samples.

Oligonucleotide Primers

The oligonucleotide primers were designed according to published DNA sequences of the transposon-like repetitive region of the *Coxiella burnetii* genome (IS1111 gene). Trans-1 and trans-2 primers with the following sequence were used (Metabion, Germany). Trans-1 (5'-TAT GTA TCC ACC GTA GCCAGT C-3') and trans-2 (5'-CCC AAC AACACC TCC TTATTC-3') (Hoover et al., 1992).

DNA amplification by Touch Down PCR (trans-PCR)

The trans- PCR was carried out as described by Vaidya et al., (2008). The PCR mixture (25 μ L) included 3 μ L of template DNA, 2 μ M of each primers and 2.5 μ L PCR Master Mix (Jena Bioscience Co. Jena, Germany), in an automated DNA thermal cycler (T- Biometra, Gottingen, Germany). The cycling denaturation of DNA at 95°C for 2 min, followed by five cycles at 94°C for 30s, 66 to 61°C (the temperature was decreased by 1°C between consecutive steps) for 1 min, and 72°C for 1 min. These cycles were followed by 35 cycles consisting of 94°C for 30s, 61°C for 30s, and 72°C for 1 min and then a final extension step of 10 min at 72°C. Following amplification, 15 μ l from each PCR containing amplified products were loaded onto 1.5% agarose gel and stained with ethidium bromide in gel electrophoresis and visualized under UV rays against GeneRuler 100 bp plus DNA ladder (molecular weight marker) ready to

use (Fermentas, Canada). Every PCR run included deionized distilled water as negative control. The positive result is indicative at 687- bp.

Results

The presence of *C. burnetii* was evaluated in sheep and goats raw milk and serum samples. A percentage of 17% (17) sheep raw milk, 23% (23) sheep sera, 19% (19) goat raw milk and 27% (27) goat sera were seropositive by IFA test at a titre $\geq 1:64$ for *C. burnetii*. For farm workers, 23.3% was seropositive for IgG

antibodies against *C. burnetii* phase II (Table, 1 and Figure, 1).

Touch-down PCR assay targeting the IS1111 gene of the organism by trans-1 and trans-2 primers resulted in 14 out of 17 sheep raw milk samples (82.4%), 21 out of 23 sheep sera (91.3%), 17 out of 19 goat milk samples (89.5%), and 23 out of 27 (74.1%) goat serum were positive for *C. burnetii*, whereas 4 out of 7 human serum samples (57.1%) were detected (Table, 1 and Figure, 1).

Table 1: Prevalence of *Coxiella burnetii* in sheep, goats and farm workers.

samples	No. of samples	IFA		PCR	
		Positive samples		Positive samples	
		number	%	number	%
Sheep raw milk	100	17	17.0	14	82.4
Sheep serum	100	23	23.0	21	91.3
Goat raw milk	100	19	19.0	17	89.5
Goat serum	100	27	27.0	23	85.2
Farm workers	30	7	23.3	4	57.1
Total	430	93	21.62	79	84.94

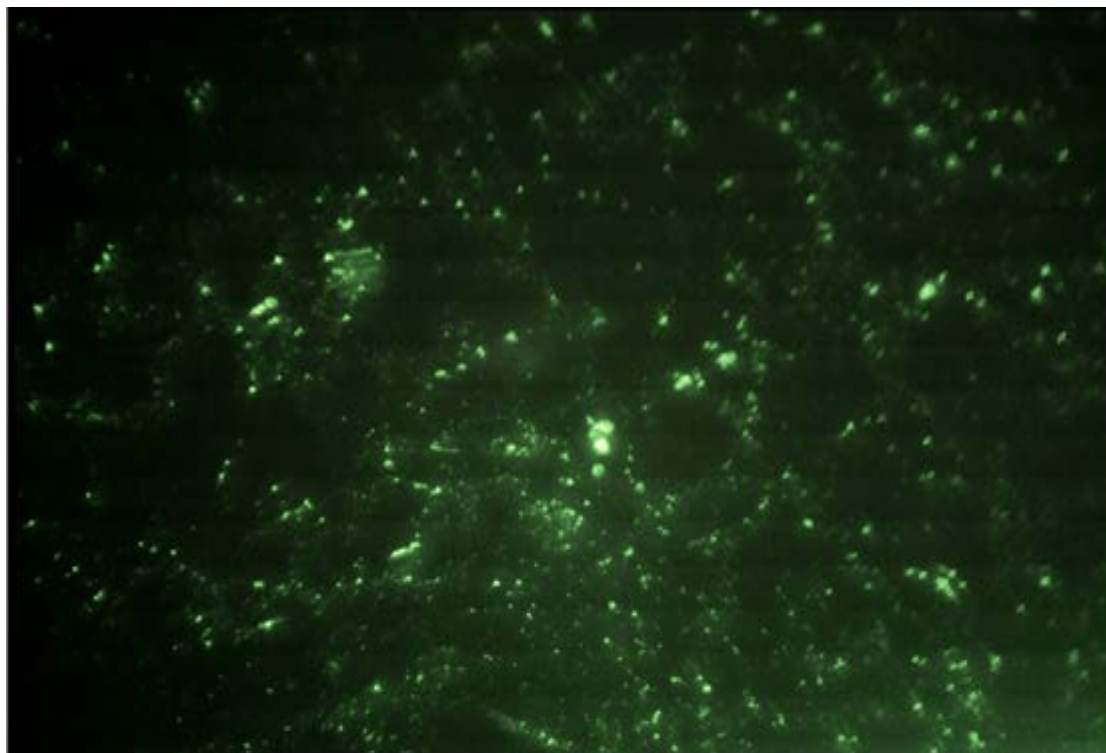


Figure 1: *C. burnetii* phase II antibodies IgG by IFA under fluorescence microscope 400x

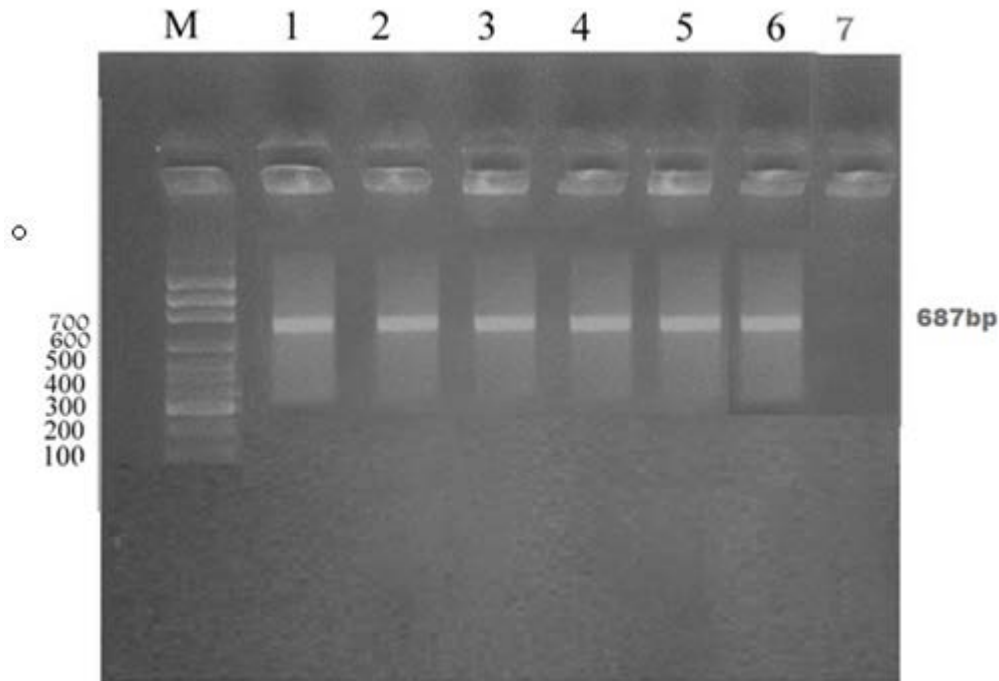


Figure 2: Agarose gel electrophoresis of PCR amplification of the 687 bp products of DNA extracted from positive *C. burnettii* phase II antibodies IgG. Lane M: 100bp molecular size DNA marker. Lanes 1 & 2: Positive sheep milk and serum samples, lanes 3 & 4: Positive goat milk and serum samples, lanes 5 & 6: Positive Human serum samples and lane 7: negative control

Discussion

The first report of Q-fever outbreak was recorded in Australia in 1935, and then spread to almost worldwide up to date (Setiyono et al., 2005). The prevalence of Q fever was higher in females than in males, for her susceptibility. In addition, pregnancy is an important parameter in occurrence of Q fever and the higher seroprevalence rate of disease has been reported in sheep during pregnancy (Berri et al., 2001).

In the current study, IFAT revealed the presence of antibodies for *C. burnettii* where the seroprevalence of *C. burnettii* were 17% of sheep raw milk, 23% of sheep sera, 19% of goat raw milk and 27% of goat sera.

Previously in North Sinai, Egypt, the prevalence *C. burnettii* antibodies were detected in 22.5% and 16.8% of sheep and goats respectively (Mazyad & Hafez, 2007). In Sudan seropositivity to *C. burnettii* in healthy sheep and goats had been reported to be 62 % and 24.22% respectively (Reinthalder et al., 1988 ; Hussien et al., 2012). A total of 3 % analyzed sera had antibodies to *C. burnettii* in apparently healthy sheep in the region of Aydin, Turkey (Kilic et al., 2005). The prevalence of antibodies in sheep was 0.6% while antibodies were not detected in Swedish goats (Ohlson et al., 2014). The prevalence of *C. burnettii* infection in animals were varied widely with species tested, year, geographic location, assay type, and criteria used to define positive results (McQuiston et al., 2002).

In Japan, a survey on the presence of Q fever in domestic ruminants reported values of 25.4% in healthy

cattle, 28.1% in sheep and 23.5% in goat. In Japanese bovine herds with reproductive disorders; the seroprevalence of Q fever was 84.3% (Htwe et al., 1992). In the European states to the moment it is not clear yet the exact entity of Q fever in domestic ruminants, since rules or recommendations are not harmonized for monitoring and report of the disease. Epidemiological data on Q fever in animals pointed out that the general prevalence of the disease in domestic ruminants is increased from the 7.4% to 10.0% within the period 2007 -2008. Particularly, the greatest increase had been recorded for goats, with values of 9.7% and 15.7% in 2007 and 2008, respectively. Member States mostly affected are Bulgaria, France, Germany and, in particular way, the Netherlands (EFSA Panel on Animal Health and Welfare, 2010). In Southern Italy the presence of *C. burnettii* in ovicaprine flocks had been reported (Parisi et al., 2006). In Iran, all 110 ovine bulk milk samples from 31 sheep breeding farms were negative and only 1.8% (1/56) caprine bulk milk samples from 20 goat breeding farms was positive for *C. burnettii* (Rahimi et al., 2010). Gyuranecz et al., 2012 reported a seropositivity in sheep by CFT and ELISA as 0% (0/100) and 6.0% (3/50) respectively in Hungary. Also in China, the *C. burnettii* seroprevalence in Tibetan sheep serums which examined by ELISA was 14.39% (Yin Ming –Yang et al., 2015) and in Northeastern China and inner Mangolia Autonomus region was 13.5% (Cong Wei et al., 2015) . In the present work, farm workers were positive for IgG antibodies against *C. burnettii* phase II 23.33% (7/30). In Egypt previous researchers recorded seroprevalence of Q fever (*C.*

burnetii) among man as 5% and 32% in North Sinai and Nile Delta village respectively (Mazyad and Hafez ,2007 ; Corwin et al.,1993).

The present work revealed that the presence of *C. burnetii* antibodies for human in contact with sheep and goats (23.3%). While *C. burnetii* seropositivity was 16% for humans in close contact with *C. burnetii* seropositivity in 13% of cattle, 23% of goats, 33% of sheep, 0% of buffalo (Ghoniem and Abdel-moein, 2012) and higher than 1% of humans in close contact with seropositive 80% of camels, 4% of cattle, 13% of goats, 11% of sheep in Chad (Schelling et al.,2003). While the prevalence of *C. burnetii* infection in humans occupationally exposed to animals in Poland was 31.12% (Szymańska-Czerwińska et al.,2015). In the USA, 132 cases of Q fever with onset in 2008 have been reported; of these, 117 were acute Q fever and 15 were chronic Q fever (Nasphv and Cdc, 2011). A serological survey in Japanese healthy humans revealed a prevalence of 22.2% (Htwe et al., 1992). Moreover it has been observed that in Japanese children Q fever is often characterized by a clinical expression, mostly represented by atypical pneumonia (Hirai and To, 1998). A recent outbreak in the Netherlands started in 2007 and made the European Commission concerned about this zoonosis. The Dutch epidemic counted more than 2,200 confirmed human cases of the disease and more than 20 people died. In an effort to prevent the disease from spreading further, over 50,000 dairy goats were slaughtered and the government launched a mandatory animal vaccination campaign at the start of 2009. The main cause of infection has been attributed to infected goat and sheep farms located in the southern Brabant province (EFSA Panel on Animal Health and Welfare, 2010). Although the main route of infection in human is inhalation of contaminated aerosols, oral transmission by contaminated raw milk or unpasteurized dairy products is also a possible route of infection. Raw milk or dairy products produced from unpasteurized milk may contain virulent *C. burnetii* (Khanzadi et al., 2014).

Serologic assay may detect antibodies to phase II and phase I *C. burnetii*. Phase II antibodies are more prevalent during acute infection, while chronic infection is characterized by a predominantly phase I antibody response (Fornier et al., 1998). The IFA results are the most specific and sensitive for phase II and phase I IgG antibodies and, to a lesser extent, also for the phase II and phase I IgM antibodies (Setiyono et al.,2005). Serologic techniques are not useful tools in order to determine which animal represents a current risk for transmission, as animals may seroconvert without shedding or remain seropositive long after the acute infection has resolved. Conversely, some animals may pose a risk for infection prior to the development of

antibodies by shedding the bacteria and some of the infected animals never seroconvert (Berri et al., 2001). In the current study, samples from the animals and humans that showed positivity IFA were further screened by the touch-down PCR (trans-PCR) for detection of *C. burnetii* in raw milk and blood. Trans-PCR assay was used for targeting the repetitive transposon-like region of *C. burnetii*. Touch-down PCR assay targeting the IS1111 gene of the organism by Trans-1 and trans-2 primers resulted in 14 of 17 sheep raw milk samples (82.4%), 21 of 23 sheep sera samples (91.3%), 17 of 19 goat milk samples (89.5%), and 23 of 27 (74.1%) goat serum samples were positive for *C. burnetii*, whereas 3 of 5 human serum samples (60%) were detected as positive at 687bp.

Previous studies found trans-PCR to be highly specific and sensitive for the direct detection of *C. burnetii* in genital swabs, milk and fecal samples from ewes (Berri et al., 2000), unpasteurized milk and dairy products in North – East of Iran (Khanzadi, et al.2014) and clinical samples of women with spontaneous abortions (Vaidya et al., 2008). Moreover PCR has the efficiency to detect one *C. burnetii*-cell in 1 ml of milk (Berri et al., 2000).

Positive amplification was detected in the blood (5.3%) obtained from goats but not from sheep in Saudi Arabia (Mohammed et al.,2014). in North – East of Iran, 34.78% (8/23) sheep milk samples were found to be positive for *C. burnetii* DNA. However, 10 goat milk samples were found to be negative (Khanzadi et al., 2014). Prevalence of *C. burnetii* (28% antibody positive herds) in clinically healthy German sheep flocks, whereas real-time PCR revealed the presence of *C. burnetii* DNA in 5% of the flocks (Hilbert et al., 2012). *C. burnetii* was identified by real-time PCR in 69.0% (113/163) and 75% (72/96) sheep and goat abortion submissions, respectively (Hazlett et al., 2013).

Conclusion

The current results indicated that the apparently healthy sheep and goats are an important reservoir of *C. burnetii* infection. Investigations on *C. burnetii* using PCR as well as serological surveys of animals are important methods for diagnosis and control of Q-fever. Although the isolation of the pathogen is the reliable method for diagnosis, but it is a time consuming and requires biosafety 3 practices. The extreme persistence of *C. burnetii* in the environment and its diffusion in domestic ruminants and wild animals indicated therefore that good management practices played an important critical role for the control of Q fever, not only in animals but also in man .

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