MOLECULAR DETECTION OF *COXIELLA BURNETII* IN RAW COW'S MILK

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ABSTRACT

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This study aimed to detect *Coxiella burnetii* in raw cow's milk by using Polymerase Chain Reaction (PCR). A total of 50 raw milk samples collected from dairy farms were tested. The obtained result showed that, 7 (14%) of the tested samples were positive for *Coxiella burnetii*. The public heath importance of the disease and control measures was also discussed in this study.

Key Words: *Molecular detection, Coxiella burnetii, cow's milk, Assiut*

INTRODUCTION

Coxiella burnetii (*C. burnetii*) is a strict fastidious obligate intracellular Gram-negative bacterium similar to rickettsia, which is a causative agent of important ubiquitous worldwide zoonotic infectious disease named coxiellosis or query fever (Q fever) (Raoult *et al.,* 2005). It has a wide range of hosts including mammals such as ruminants, dogs, cats, primates, wild rodents, small mammals and non-mammals such as reptiles, amphibians, birds, fish and ticks (Parker *et al*., 2006; Fretz *et al*., 2007). Cattle, sheep and goats are the main sources of infection in humans (Angelakis and Raoult, 2010).

Coxiella burnetii is the causative agent of Q fever in human and animals. The ticks are considered to be the natural primary reservoirs of *Coxiella burnetii* and are responsible for the spread of the infection in wild animals and for transmission to domestic animals (Norlander, 2000 and Pluta *et al.,* 2010).

The Q fever, a zoonosis that occurs worldwide (Marrie, 1990). Infected animals, especially livestock, are considered the most important source of transmission to humans (Lang, 1990). Whereas, animals in general show no clinical signs of infection except occasional abortions and other problems with reproduction such as premature birth, dead or weak offspring, and endometritis and infertility in domestic ruminants (Aitken, 1989; Maurin and Raoult, 1999 and Kazar, 2005). The infected animals usually shed the agent intermittently in milk, feces, and urine, with no outward signs of disease, and should be regarded as possible sources of human infection (Guatteo *et al.*, 2011).

Coxiella burnetii can cause serious illness in humans. This agent is very resistant to environmental influences, and even a single infective particle can initiate an infection in the animal model (Ormsbee *et al*., 1978). It also, highly infectious and one to ten organisms can cause Q fever in humans (Kersh *et al*., 2010). *Coxiella burnetii* can be present in milk, urine, faeces, vaginal mucus and semen. In milk, it can be secreted for 8 days in ewes and up to 13 months in cattle (Roest *et al*., 2011).

Infection via inhalation of aerosolized organisms or ingestion of raw milk or fresh dairy products has been reported in humans and animals (Tissot-Dupont and Raoult, 1992). Q fever has two major manifestations in man, acute and chronic infection. Acute Q fever has a variety of clinical presentations including self-limited febrile illness, pneumonia, hepatitis, meningoencephalitis and pericarditis. Rarely, it is a cause of constrictive pericarditis (Bautista-Hernandez *et al.,* 2004). Chronic Q fever is a much more serious illness and almost always means endocarditis, although infection of an aortic prosthesis or aneurysm is another manifestation of chronic Q fever (Raoult and Marrie, 1995). While still not adequately described in man, it is likely that Q fever during pregnancy results in chronic uterine infection with relapse during subsequent pregnancies as it does in other female mammals (Marrie, 1993 and Raoult and Stein, 1994).

The raw milk of infected cows is an important material for detecting *Coxiella burnetii* and diagnosing bovine coxiellosis (Stoenner, 1951). Routine diagnosis of Q fever is usually based on the detection of specific antibodies by complement fixation and immunofluorescence and enzyme-linked

immunosorbent assay (ELISA) tests. Isolation of *Coxiella burnetii* is hazardous, difficult and time-consuming, and requires confined biosafety level 3 laboratories due to the zoonotic nature of the microorganism (Stein and Raoult, 1992 and Field *et al.,* 2000).

In contrast, PCR assay is a safe and useful method for detection and diagnosis of *Coxiella burnetii*. Moreover, at present, the polymerase chain reaction (PCR) technique has become a useful tool to detect *Coxiella burnetii* in biological samples (Berri *et al.,* 2000). Several PCR-based methods have been developed targeting the isocitrate dehydrogenase gene (Nguyen and Hirai, 1999), the superoxide dismutase gene (Heinzen *et al.,* 1999) and a transposon-like repetitive region (Hoover *et al.*, 1992).

PCR is a highly sensitive and specific detection method that has been used previously to trace *Coxiella burnetii* in clinical samples (Muramatsu *et al*., 1996; Muramatsu *et al*., 1997 and Yuasa *et al*., 1996). A PCR performed with primers based on a repetitive, transposon like element (Trans-PCR) proved to be highly specific and sensitive, but extraction of DNA from milk samples took considerable effort and there was a high risk of contamination due to the numerous preparation steps (Willems *et al*., 1994).

Due to the dangerous of *Coxiella burnetii* for human being, the aim of this study is to detect *Coxiella burnetii* in raw cow's milk consumed in Assiut City, Egypt, by using PCR assay.

MATERIALS and METHODS

A total of 50 raw milk samples were collected from dairy farms in Assiut city, Egypt, under aseptic conditions. The samples were stored at 4^0C during transportation without delay to the laboratory to be examined by Polymerase Chain Reaction (PCR) according to Berri *et al.* (2002); Vicari *et al.* (2013) and Kargar *et al.* (2014).

1. Materials used for PCR:

1.1. Reagents used for agrarose gel electrophoresis:

1.1.1. Agarose powder, Biotechnology grade (BioshopR, Candainc. lot No: OE16323).
It prepared in concentration 2% in 1× TAE buffer.

1.1.2. Tris acetate EDTA (TAE) electrophoresis buffer (50×liquid concentration) (Bioshop R, Candainc. lot No: 9E11854).
The solution diluted to 1× by adding 1 ml stock solution to 49 ml double dist. Water to be used in the preparation of the gel or as a running buffer.

1.1.3. Ethedium bromide solution (stock solution) biotechnology grade (Bioshop ® CandaInc, Lot No: 0A14667):
The stock solution was diluted by 25µl /200ml double distilled water and stored covered at 4ºC. It was used for staining of PCR products that electrophoreses on agarose gel to be visualized by UV light.

1.2. Gel loading buffer (6×stock solution) (Fermentas, lot No: ooo56239).
The components were dissolved in sterile double distilled water and stored covered with aluminum foil at room temperature.

1.3. DNA ladder (molecular marker):
100 bp (Fermentas, lot No: 00052518).

1.4. 5X Taq master (Fermentas):
Containing polymerase enzyme, Magnesium chloride (Mg Cl2), Deoxy nucleotide triphosphate (dNTP) and PCR grade water.

1.5. Primer sequences of *Coxiella burnetii* used for PCR identification system:
Application of PCR for identification of gene specified for *Coxiella burnetii* was performed essentially by using Trans Primers (Invitrogen, Life Technologies, Thermo Fisher Scientific Inc., UK) as shown in the following table.

Primer	Oligonucleotide sequence (5′ → 3′)	Amplicon length (bp)	Reference
Trans 1 (F)	5′TGGTATTCTTGCCGATGAC′3	687	Kirkan *et al*. (2008)
Trans 2 (R)	5′GATCGTAACTGCTTAATAAACCG′3		

2. DNA extraction:
One ml of raw milk was centrifuged at 2000 rpm for 10 minutes. This procedure was performed to isolate the microbial cells in pellet of the milk samples (Berri *et al*., 2002). DNA was extracted from the pellet by a genomic DNA purification kit (Fermentas) according to the manufacturer's protocol. Therefore, DNA extracts were stored at -20ºC until they were used.

3. DNA amplification reaction:
The amplification was performed according to Kargar *et al*. (2014) in a thermocycler (Eppendorf –Master

cycler personal). A total reaction volume of 50 µl, containing 5 µl of 10 PCR buffer (10 mMTris-HCl, pH 9·0, 50 mM potassium chloride, 0.1% Triton X-100), 5 µl 25 mM magnesium chloride, 250 µM of each deoxynucleotide triphosphate, 2 U of TaqDNA polymerase (MBI Fermentas), 1 µM of each primer and 5 µl of template DNA.

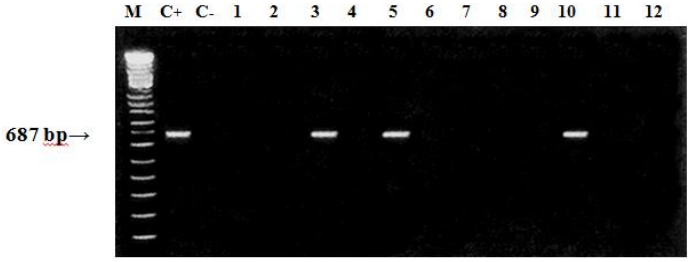
4. The reaction conditions of PCR:
The reaction conditions consisted of 5 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 66°C (the temperature was decreased by 1°C between consecutive steps) for 1 minute and the extension at 72°C for 1 minute. Accordingly, 40 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72 °C for 1 minute was carried out (Vicari *et al*., 2013).

The 10 µl amplified products were detected by 1.5% of agarose gel electrophoresis stained with ethidiumbromide at 100 volts for 1 hour then visualized and captured on UV transilluminator. Thus, PCR products of 687 base pairs were considered indicative for identification of *Coxiella burnetii.*

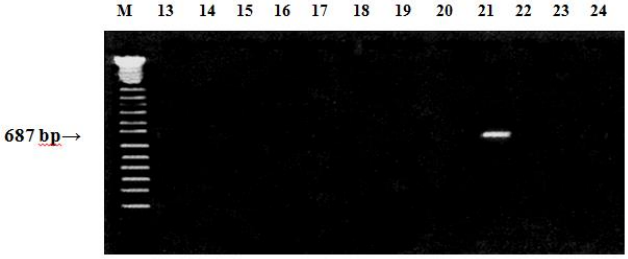
RESULTS

Table1: Incidence of *Coxiella burnetii* in raw milk samples by using PCR assay.

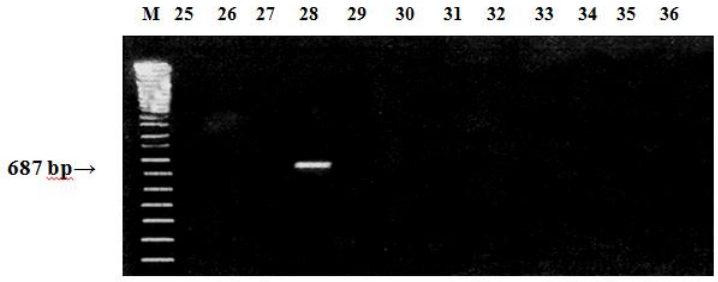
Number of Samples	Positive Samples		Negative Samples	
50	No.	%	No.	%
	7	14	43	86



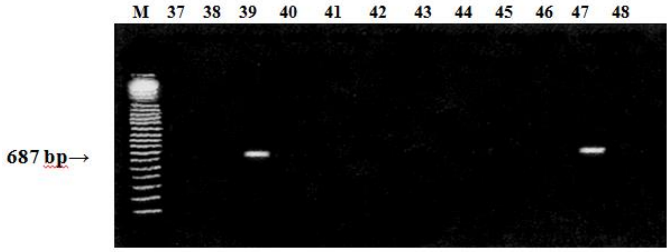
Photograph (1): Agarose gel electrophoresis of PCR of specific (Trans) primer for characterization of *Coxiella burnetii* in the examined milk samples.
Lane M: 100 bp ladder as molecular size DNA marker.
Lane C+: Control positive for *Coxiella burnetii.*
Lane C-: Control negative for *Coxiella burnetii.*
Lanes 3, 5 and 10: Positive milk samples for *Coxiella burnetii.*
Lanes 1, 2,4, 6, 7, 8, 9, 11 and 12: Negative milk samples for *Coxiella burnetii.*



Photograph (2): Agarose gel electrophoresis of PCR of specific (Trans) primer for characterization of *Coxiella burnetii* in the examined milk samples.
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 21: Positive milk sample for *Coxiella burnetii.*
Lanes 13, 14, 15, 16, 17, 18, 19, 20, 22, 23 and 24: Negative milk samples for *Coxiella burnetii.*



Photograph (3): Agarose gel electrophoresis of PCR of specific (Trans) primer for characterization of *Coxiella burnetii* in the examined milk samples.
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 28: Positive milk sample for *Coxiella burnetii.*
Lanes 25, 26, 27, 29, 30, 31, 32, 33, 34, 35 and 36: Negative milk samples for *Coxiella burnetii.*



Photograph (4): Agarose gel electrophoresis of PCR of specific (Trans) primer for characterization of *Coxiella burnetii* in the examined milk samples.
Lane M: 100 bp ladder as molecular size DNA marker.
Lane 39 and 47: Positive milk sample for *Coxiella burnetii.*
Lanes 37, 38, 40, 41, 42, 43, 44, 45, 46, 48,49 and 50: Negative milk samples for *Coxiella burnetii.*

DISCUSSION

Routine isolation of *Coxiella burnetii* is very tedious and highly dangerous as presence of these bacteria in the environment, even in small numbers, poses a serious health risk to the population and its isolation required biosafety level-3 facilities (Loftis *et al*., 2010 and Brooke *et al*., 2013). Therefore, PCR assay for detection of the bacterial genome especially in milk samples is reliable but its disadvantages are due to the high cost.

The result determined in this study revealed that, out of the 50 raw cow's milk samples examined by PCR assay, 7 samples (14%) were positive for *Coxiella burnetii* genome (Table 1). Nearly similar result (16.8%) was detected by Ho *et al*. (1995). While, lower result (4.7%) was estimated by Fretz *et al.* (2007). In contrary, higher results (33.1, 94.3, 24.4, 22, 51.1, 32, 50, 51.7 and 27%) were determined by (Muramatsu *et al.*, 1997; Kim *et al*., 2005; Guatteo *et al*., 2006; Amin and Ahmed, 2009; Loftis *et al.*, 2010; Angen *et al.*, 2011; Muskens *et al.*, 2011; Astobiza *et al.*, 2012 and Petruzzelli *et al.*, 2013), respectively.

The variance in the recovery rate may be attributed to difference in geographic distribution and to the different methods of detection. Also, variation to different seasons of the year may have an effect (Amin and Ahmed, 2009).

Adequate heat treatment of raw milk must be done before consumption to safeguard the human from being from infection as *Coxiella burnetii* is more heat-resistant than *Mycobacterium tuberculosis*, and could be isolated from pasteurized milk processed according to minimum standards (Huebner *et al.*, 1949). Moreover, if large numbers of the Q fever organism were present in raw milk, some would survive pasteurization at 143°F (61.7°C) for 30 min (Enright *et al.*, 1957). High temperature, short-time pasteurization standards (71.7°C for 15 seconds) in the United States have been developed to inactivate *Coxiella burnetii* in milk (Cerf and Condron, 2006). Furthermore, UHT milk is safe for consumption as it is sterilized milk.

CONCLUSION

The result obtained in this study showed that, raw milk consumed in Assiut city, Egypt, could be representing a potential source for *Coxiella burnetii* infection for human being consumed such infected raw milk. Therefore, adequate heat treatment of raw

milk before consumption must be done to safeguard the consumers from being infected by Q fever.

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الفحص الجزيئي للكوكزيلا بيرنيتي في اللبن البقري الخام

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يعد اللبن الخام مصدراً لكثير من الأمراض الخطيرة التي تؤثر علي صحة المستهلك ومن ضمن هذه الأمراض مرض الحمي المجهولة الذي يسببه ميكروب كوكزيلا برنيتي. لذلك تهدف هذه الدراسة لمعرفة مدي تواجد هذا الميكروب في لبن الأبقارالخام. فقد تم تجميع عدد ٥٠ عينة لبن خام من بعض المزارع وتم فحص هذه العينات باستخدام تفاعل البلمرة التسلسلي. وقد أظهرت نتيجة الفحص أن٧ عينات بنسبة (١٤٪) كانت ايجابية لوجود كوكزيلا برنيتي. وقد تم ايضاً مناقشة أهمية الميكروب من الناحية الصحية وكيفه القضاء علية.