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Risk of *Coxiella burnetii* infection in Dairy Farms

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ABSTRACT

Coxiella burnetii is a strict fastidious obligate intracellular Gram-negative bacterium, causative agent of an important zoonotic disease called coxillosis or query fever (Q-fever). Due to the dangerous of *C. burnetii* for human being, the aim of this study is to detect the incidence of such microorganism in farm milk in Assiut governorate in Egypt, by using polymerase chain reaction (PCR). A total of 150 raw milk samples from dairy cattle, ewes and goats (50 samples each) were collected from 5 farms in Assiut governorate, in Egypt. The sampling priority from the animals was upon those having the reproductive problems or mastitis in addition to the presence of ticks which are responsible for transmission of the infection among animals. All the collected milk samples were examined for the incidence of *C. burnetii* for PCR products of 687 base pairs (*IS1111* gene). The obtained results revealed that 9 cattle milk samples (18%), 4 ewes milk samples (8%) and 3 goat milk samples (6%) were positive. In total, 16 out of the 150 milk samples (10.67%) were contaminated with *C. burnetii*. The revealed results concluded that the raw milk produced in Assiut governorate could be considered a potential source for *C. burnetii* infection for human being and represented a public health hazard. Consequently, application of food safety management systems and adequate heat treatment of raw milk must be applied to safe the consumer from being infected by coxillosis.

INTRODUCTION

Nowadays, there is increased awareness of bovine coxiellosis as an economically and public health important disease. It has historically been described as an occupational disease primarily noted in abattoir workers, veterinarians, shearers, tanners, and farmers. Query fever is a potential risk factor for some reproductive problems in dairy farms and a possible risk factor for human infection **(Dobos et al. 2022)**. This agent has high transmissibility and can spread over long distances via wind, in which a small number of aerosolized particles are needed to infect susceptible host **(Souza et al. 2022)**. Humans are primarily infected by inhalation of aerosols containing spores, as well as by ingesting cottage cheese or unpasteurized

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milk **(Lencastre Monteiro et al. 2021)**. Clinical presentation in humans varies from asymptomatic to flu-like illness and severe sequelae may be seen.

Although the main route of infection in human is inhalation of contaminated aerosols or at transmission by contaminated caw milk, or unpasteurized dairy products produced from unpasteurized milk ay contain virulent C. burnetii **(Khan zadi et al. 2014)** and **(Khalifa et al. 2016).**

Although this agent can replicate in various animal hosts, such as wild mammals, domestic mammals, birds, and arthropods **(Eldin et al. 2017)**, ruminants have been implicated as the main reservoirs for human infection **(Pexara et al. 2018)**. Ruminants are often sub-clinically infected or show reproductive disorders such as abortions **(Klemmer et al. 2018)**. Infected animals shed the infectious agent predominantly through feces, urine, saliva, vaginal discharge, the placenta, and amniotic fluid **(Abiri et al. 2019)**.

According to the **World Organization for Animal Health - WOAH (2018)**, there is no gold standard for the techniques used for diagnosis of *Coxiella. burnetii*. which is classified as a category B biological agent and its isolation is hazardous, difficult and time consuming, and requires confined biosafety level 3 laboratories due to the zoonotic nature of the microorganism. Polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA) have been considered methods of choice. Most studies have chosen the insertion sequence of the *IS1111* gene as the target for detection of *C. burnetii* by PCR because it is a repeating element of multiple copies with 7–110 copies per isolate, allowing a greater sensitivity of the technique **(Sahu et al. 2020)**.

According to the aforementioned, the present investigation was aimed to study the incidence of *Coxiella burnetii* in raw farm milk in Assiut governorate, Egypt by using PCR to detect *IS1111* gene.

MATERIALS and METHODS

Sampling:

A total of 150 raw milk samples were directly taken from some dairy farms at different localities in Assiut Governorate as 50 samples from each of dairy cows, dairy ewes and dairy goats. The fore milk was discarded then the sample was taken in a sterile container and transported directly without delay to the laboratory to be examined by PCR.

Not all the dairy animals were apparently healthy, in which the animals showed any clinical signs were taken in the considerations. The clinical signs were varied from anaestrum, repeat breeder, retained placenta, weak offsprings, stillbirth, cough, mastitis, lowered milk production, emaciation, lameness, ticks & flea infestations and also whose of case history of abortion.

PCR:

The samples were examined for the detection of the gene specific for *Coxiella burnetii* by using of conventional polymerase chain reaction (PCR). This part was done in animal health research institute. Application of PCR for the identification of gene specified for *C. burnetii* was performed essentially by using Trans Primers (Invitrogen, Life Technologies, Thermo Fisher Scientific Inc., UK) as following:

Target gene	Primer	Oligonucleotide sequence (5′ → 3′)	Product size (bp)	Reference
IS1111	Trans 1 (F)	5′TGGTATTCTTGCCGATGAC′3	687	Kirkan et al. (2008)
IS1111	Trans 2 (R)	5′GATCGTAACTGCTTAATAAACCG′		

DNA extraction (Berri et al. 2002):

One ml of the raw milk sample was centrifuged at 2000 rpm for 10 min. This procedure was performed to seperate the microbial cells in pellet of the milk samples. DNA was extracted from the pellet by a genomic DNA purification kit (Fermentas) according to the manufacturer's protocol. The DNA

DNA amplification reaction (Kargar et al. 2014):

The amplification was performed on a Thermal Cycler (Master cycler, Eppendorf, Hamburg, Germany). A total reaction volume of 50 µl, containing five µl of 10 PCR buffer (10 mMTris HCl, pH 9.0, 50 mM potassium chloride, 0.1% Triton X-100), five µl 25 mM magnesium chloride, 250 µM of each deoxynucleotide triphosphate, 2 U of TaqDNA polymerase (MBI Fermentas), 1 µM of each primer and five µl of template DNA. The reaction conditions consisted of five cycles consisting of denaturation at 94° C for 30 s, annealing at 66° C (the temperature was decreased by 1° C between consecutive steps) for 1 min and the extension at 72° C for 1 min. Accordingly, 40 cycles consisting of denaturation at 94° C for 30 s, annealing at 61° C for 30 s and extension at 72 °C for 1 min was carried out **(Vicari et al. 2013)**. The 10 µl amplified products were detected by electrophoresis at 1.5% of agarose gel (Applichem, Germany, GmbH) stained with ethidium bromide at 100 volts for 1 h then visualized and captured on UV trans illuminator. A 100 bp plus DNA Ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes, as PCR products of 687 base pairs were considered indicative for identification of *C. burnetii*.

Agrarose gel electrophoresis:

Agarose powder, Biotechnology grade (BioshopR, Candainc.lot No: OE16323): was prepared by concentration 2% in 1× TAE buffer. Tris acetate EDTA (TAE) electrophoresis buffer (50×liquid concentration) (BioshopR, Candainc. lot No: 9E11854): The solution was diluted to 1× by adding 1 ml stock solution to 49 ml double distilled water to be used in the preparation of the gel or as a running buffer. Ethedium bromide solution (stock solution) biotechnology grade (Bioshop ® CandaInc, Lot No: 0A14667): The stock solution was diluted by 25 µl /200 ml double distilled water and stored covered at 4º C. It was used for staining of PCR products that electrophoreses on agarose gel to be visualized by UV light.

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. DNA ladder (molecular marker): 100 bp (Fermentas, lot No: 00052518). 5X Taq master (Fermentas) containing polymerase enzyme, magnesium chloride ($Mg\ Cl_2$), deoxy nucleotide triphosphate (dNTP) and PCR-grade water.

RESULTS

Table1. prevalence of *C. burnetii* in the examined raw milk samples in some dairy farms

Farms	Cow's milk			Ewe's milk			Goat's milk			Total No.	Total +ve	Total%
	No.	+ve	%	No.	+ve	%	No.	+ve	%			
Farm A	6	2	33.33	8	1	12.5	17	2	11.76	31	5	16.13
Farm B	16	3	18.75	16	1	6.25	10	0	0	42	4	9.52
Farm C	17	3	17.65	5	0	0	15	1	6.67	37	4	10.81
Farm D	11	1	9.09	0	0	0	8	0	0	19	1	5.26
Farm E	0	0	0	21	2	9.52	0	0	0	21	2	9.52
Total	50	9	18	50	4	8	50	3	6	150	16	10.67

Photo 1. Agarose gel electrophoresis of PCR of *IS1111*gene (687 bp) specific for characterization of *C. burnetii* in cow's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lane C+: Control positive for *C. burnetii*
Lane C-: Control negative
Lanes 6, 11, 12 & 14: Positive milk samples for *C. burnetii*
Lanes 1, 2, 3, 4, 5, 7, 8, 9, 10, 13, 15, 16, 17 & 18: Negative milk samples for *C. burnetii*

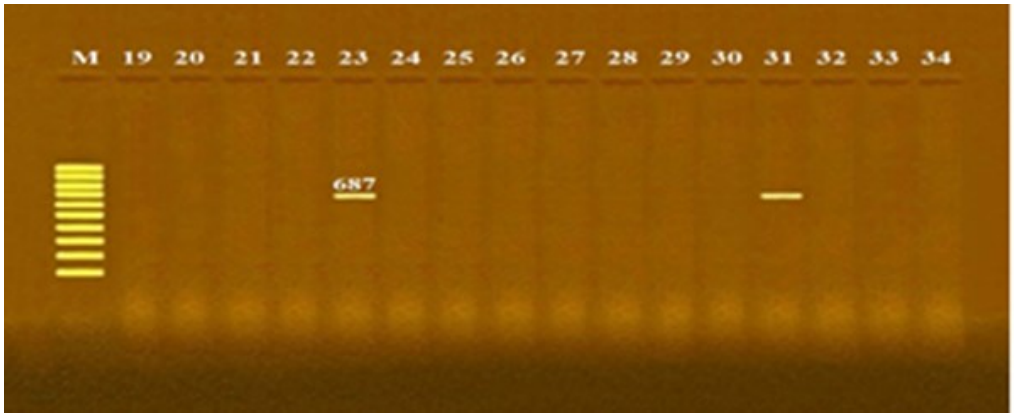

Photo 2. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in cow's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lanes 23 & 31: Positive milk samples for *C. burnetii*
Lanes 19, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 32, 33 & 34: Negative milk samples for *C. burnetii*

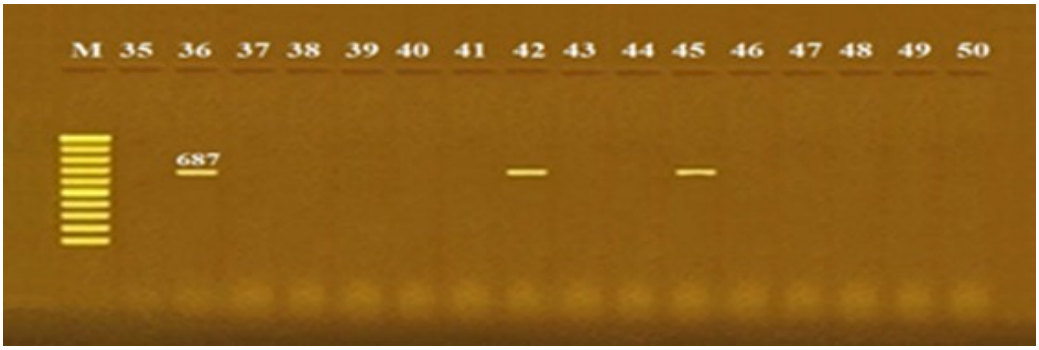

Photo 3. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in cow's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lanes 36, 42 & 45: Positive milk samples for *C. burnetii*
Lanes 35, 37, 38, 39, 40, 41, 43, 44, 46, 47, 48, 49 & 50: Negative milk samples for *C. burnetii*

Photo 4. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in ewe's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lanes from 1 to 18: Negative milk samples for *C. burnetii*

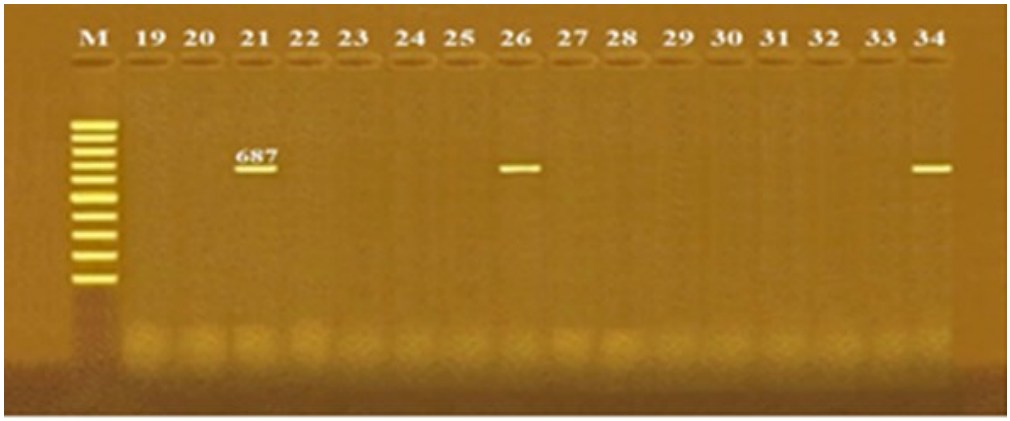

Photo 5. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in ewe's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lanes 21, 26 & 34: Positive milk samples for *C. burnetii*
Lanes 19, 20, 22, 23, 24, 25, 27, 28, 29, 30, 31, 32 & 33: Negative milk samples for *C. burnetii*

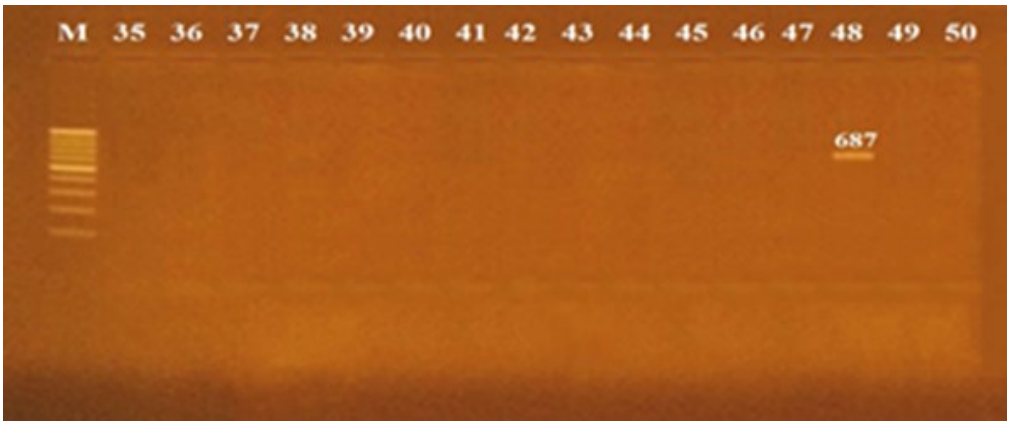

Photo 6. Agarose gel electrophoresis of PCR of (687 bp) specific for characterization in ewe's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lane 48: Positive milk sample for C. burnetii
Lanes from 35 to 47, 49 & 50: Negative milk samples for *C. burnetii*

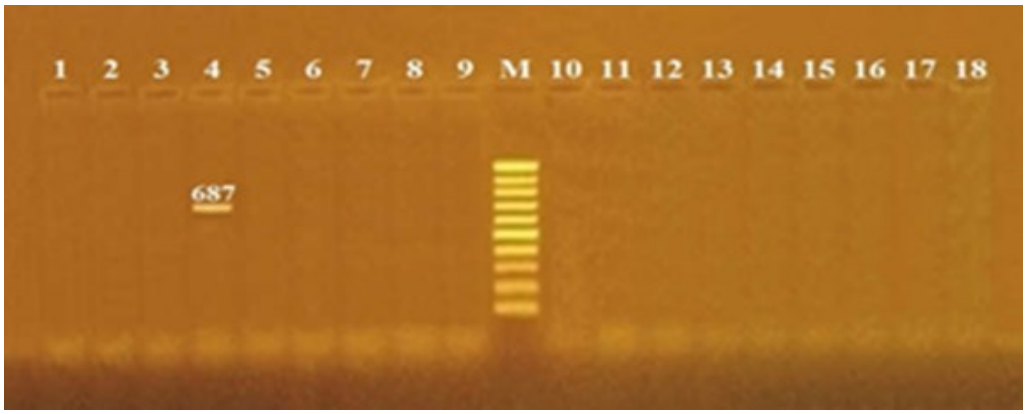

Photo 7. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in goat's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lane 4: Positive milk sample for *C. burnetii*
Lanes 1, 2, 3 & from 5 to 18: Negative milk samples for *C. burnetii*

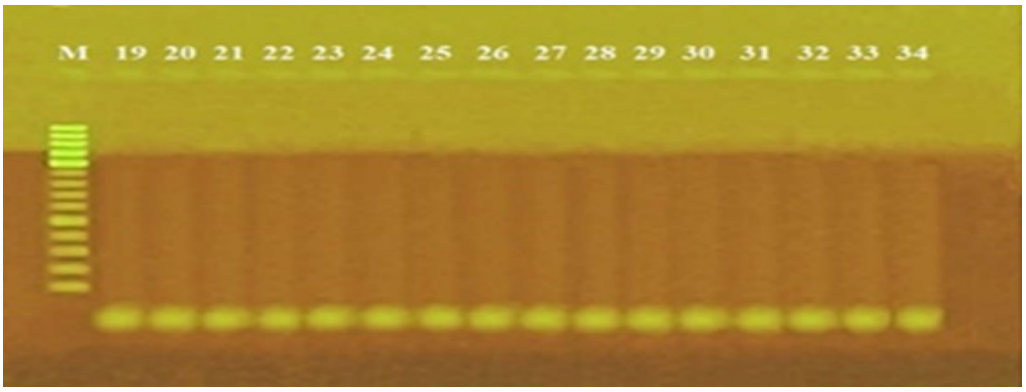

Photo 8. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in goat's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lanes from 19 to 34: Negative milk samples for *C. burnetii*

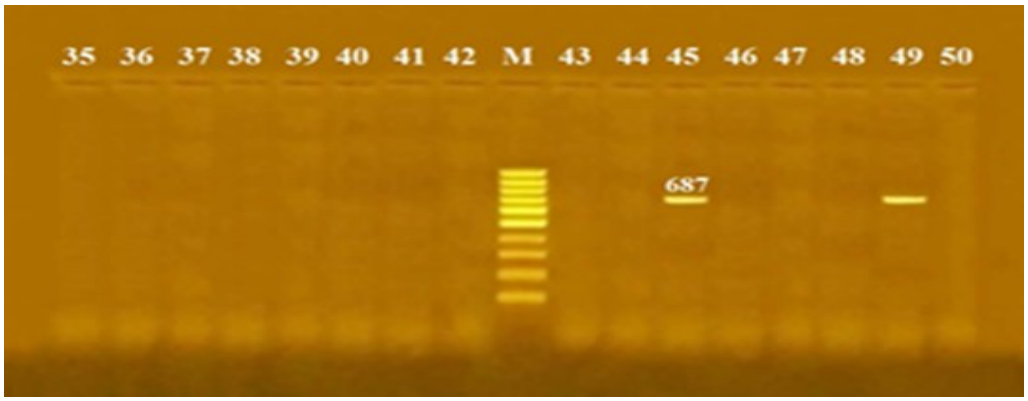

Photo 9. Agarose gel electrophoresis of PCR of *IS1111* gene (687 bp) specific for characterization of *C. burnetii* in goat's milk samples
Lane M: 100 bp ladder as molecular size DNA marker
Lanes 45 & 49: Positive milk sample for *C. burnetii*
Lanes from 35 to 44, 46, 47, 48 & 50: Negative milk samples for *C. burnetii*

DISCUSSION

The ingestion of contaminated food such as row milk and dairy products perform a possible source of infection of humans as Coxiella burnetii secreted in milk **(Maurin and Raoult, 1999), (Khalifa 1. N.O. et al. 2016).**

The examined milk samples in the current study were taken from different localities with special attention to the animals with reproductive disorders and mastitis, whereas, animals in general show no clinical signs of *Coxiella burnetii* infection except occasional abortions and other problems with reproduction such as premature birth, dead or weak offspring, and endometritis.

The obtained results in **Table 1 and Photos 1, 2 & 3** revealed that, out of the examined 50 raw cow's milk samples, 9 samples (18%) were positive for *C. burnetii*. In another study, **Dobos et al. (2022)** examined cow milk samples and found that *IS1111* element of *C. burnetii* was detected in 10 out of the 24 samples (41.67%) by real-time PCR.

Table 1 and Photos 4, 5 & 6 presented the incidence of *C. burnetii* in the examined ewe's milk samples, in which, 4 samples (8%) were positive. Lower results were detected by **Rahimi et al. (2011)**; **Gyuranecz et al. (2012); Can et al. (2015)** where they revealed incidences of 5.7, 4 and 4%, respectively. In contrast, **Fretz et al. (2007)**; **Rahimi et al. (2009)** failed to detect of *C. burnetii* genome in ovine milk samples.

Concerning the examined goat's milk samples, 3 out of the 50 samples (6%) were positive **(Table 1 and Photos 7, 8 & 9)**. Lower incidences were revealed in bulk goat's milk samples by **Rahimi et al. (2011)**; **Can et al. (2015)**. On the other hand, **Fretz et al. (2007)** failed to detect *C. burnetii* genome in caprine milk samples.

The obtained results showed higher incidence of *C. burnetii* in the examined milk samples in the farm A (16.13%) than the other farms. While, the farm D showed the lowest incidence (5.26%). On the other hand, *C. burnetii* could not be detected in the ewe's milk of the Farm C and in the goat's milk of the Farms B & D/E **(Table 1)**. For instance, geographical areas with low vegetation and low soil moisture levels have higher levels of *C. burnetii* transmission **(Van Leuken et al. 2016)**. Infection can reach up to 18 km from the point of origin via wind **(Clark and Soares Magalhães, 2018)**.

Regarding the species, the examined cow's milk samples revealed higher incidence (18%) in comparison with the other species of ewe's milk (8%) and goat's milk (6%). **Guidia et al. (2017)** determined the prevalence of *C. burnetii* in cattle and sheep raw milk farms in central Italy using commercial real-time PCR; by 17 out of a totally 66 dairy farms had at least 1 positive milk sample; and the cattle farms showed higher positive results than sheep ones as 50% and 21%, respectively. A serological survey found that *C. burnetii* specific antibodies were detected in 19.29 %of cattle (162/840), 8.94% of sheep (64/716) and 6.75% of goats (21/311).

Although infected animals often do not show clinical signs of disease, many reproductive disorders have been associated with the presence of the pathogenic bacteria **(Agerholm, 2013)**. The economic impact of Q -fever in industrial dairy cattle farms can be attributed to impaired reproductive performance due to the abortion, premature delivery, stillbirth and weak offspring complex, early pregnancy loss, metritis and retained placenta **(Agerholm, 2013; Dobos et al. 2020)**.

CONCLUSION

The present study is reflecting the public health hazard of consumption of raw milk toward the *Coxiella burnetii*. Therefore, the application of food safety management systems and adequate heat treatment of raw milk must be adopted.

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