

Molecular and serological studies on *Coxiella burnetii* in camels at Marsa Matrouh governorate in EgyptSamah F. Ali¹, Ibrahim E. M¹ and Jakeen El Jakee²¹ Animal Health Research Institute, Nadi El-said, Dokki, Egypt² Microbiology Department, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
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Abstract: *Coxiella burnetii*, the bacterium causing Q fever, is an obligate intracellular biosafety level 3 agent. In animals, the organism is mainly found in the reproductive system and may primarily cause abortion or infertility. Antibodies to this organism have been reported in a wide range of animals including mammals, reptiles, amphibians, and birds. In the present study, a total of 125 serum, bulk tank milk, vaginal swab, placenta and internal organ of aborted fetus samples were collected from aborted she camels and apparently healthy in Marsa Matrouh governorate, Egypt to detect the prevalence of *C. burnetii* at flock level by ELISA and Real-Time PCR as well as the pathogen amplification in Embryonated Chicken Egg (ECE). This study proves that camels in Marsa Matrouh governorate may play an important role as a reservoir of *C. burnetii* and they could be a significant source for the transmission of Q-Fever to other animal species and to humans. The preferred route of shedding of *C. burnetii* in camel appeared to be the milk followed by placenta.

[Samah F. Ali, Ibrahim E.M and Jakeen El Jakee. **Molecular and serological studies on *Coxeillaburnetii* in camels at Marsa Matrouh governorate in Egypt.** *J Am Sci* 2016;12(1):77-82]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). <http://www.jofamericanscience.org>. 10. doi:[10.7537/marsjas12011610](https://doi.org/10.7537/marsjas12011610).

Key words: *Coxeilla burnetii*, Camels, Real-Time PCR, ECE

1. Introduction

Q-Fever (or Coxiellosis) is widely distributed throughout the world. The causal agent, *Coxiella burnetii*, is present in virtually all animal kingdoms, including arthropods, but the disease affects mostly humans, cattle, sheep and goats (OIE, 2015).

Coxiella burnetii is a short, pleomorphic rod (0.2 to 0.4 μm wide, 0.4 to 1 μm long) that possesses a membrane similar to that of Gram-negative bacteria (Angelakis and Raoult, 2010). This organism is an obligate intracellular bacterium adapted to thrive within the phagolysosome of the phagocyte. *C. burnetii* belongs now to the family Coxiellaceae together with the genus Rickettsiella. Typical for this family is the intracellular intravascular lifestyle in cells of invertebrate or vertebrate hosts (de Bruin *et al.*, 2012).

Domestic ruminants are mainly subclinical carriers, but can shed bacteria in various secretions and excreta. Animals constantly shed *C. burnetii* in their feces, urine and milk with substantial shedding occurring during parturition (EFSA, 2010).

Diagnosis of Q- Fever in ruminants, including differentiating it from other abortive diseases, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results (Lang, 1990). Direct detection and quantification by PCR and serological ELISA should be considered as methods of choice for clinical diagnosis (Sidi-Boumedine *et al.*, 2010).

Detection of *C. burnetii* DNA in animals can be achieved by the PCR in a wide range of clinical

materials including; vaginal discharge, abortion products, feces, milk, urine and blood. It has become increasingly common in diagnostic laboratories with PCR capabilities.

The aim of recent study was to estimate the sero- and antigen prevalence of *C. burnetii* on flock level among apparently healthy and aborted she camels in Marsa Matrouh governorate as a model example.

Materials and methods**Samples**

A total of 125 serum, bulk tank milk, vaginal swabs, placenta and internal organ of aborted fetus samples were collected from two groups of camels, 83 samples from aborted she camels and 42 samples from apparently healthy one at Marsa Matrouh governorate, the samples design are shown in Table 1. The samples were collected under sterile conditions and were immediately transported to the laboratory on ice box.

Serological assay

All serum and bulk milk samples were tested using the CHEKIT Q-Fever Antibody ELISA Test Kit (IDEXX laboratories, Westbrook, WE, USA) following the manufacturer's instructions. The results were interpreted using the cutoffs recommended by the manufacturer, where the optical densities (OD) of the sample and the positive control were corrected by subtracting the OD value of the negative control, and the ratio between the sample and the positive control (S/P ratio) was calculated. The manufacturer considers an S/P ratio of <30% as negative, 30% - 40% as inconclusive and >40% as positive.

2. Samples preparation:

Serum:

The serum samples were allowed to come to 18-26°C before use and mixed by gentle swirling or vortex. Serum samples were diluted to 1:400 in a tube using the wash buffer.

Milk Samples:

Whole-milk samples were used after overnight refrigeration at 2–8°C. The sample should be drawn from below the cream layer, and then the milk samples were diluted to 1:5 in a tube using the wash buffer.

Real – Time PCR assay

The milk, vaginal swabs, placenta, Internal organ of aborted calves and inoculated yolk sacs were tested for *Coxiella burnetii* DNA using RT-PCR which targeting the IS1111 gene of *C. burnetii* (Natale *et al.*, 2012) and the test performed following the manufacturer's instructions of the commercial kit (using an ADIAVET® COX REALTIME, Adiaçène). Pre-treatment and sample processing were performed according to the manufacturer's instructions.

DNA extraction:

Coxiella DNA was extracted from the pellet by a genomic DNA purification kit (Gene JET Genomic DNA purification Kit (#K0722), 50 preps, according to the manufacturer's protocol.

Samples preparation:

From milk: 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 twice in distilled water; DNA was extracted using DNA extraction kit.

From placental tissue as follows: collection of some cotyledons and their intercotyledonary membranes were placed in a sterile watertight container rinsed them by BPS and dried with filter paper. The mixture was centrifuge for 30 min. at 3000g at 4°C. The supernatant was treated with extracted Kit.

From vaginal swabs: 1 ml of sterile physiological saline was added to the vaginal swab, the sample was vortexed for 30 s, and 2 ml of the supernatant was transferred to a 2 ml microtube.

From yolk sacs as follow: The collected yolk sac was washed twice in physiological saline. DNA was extracted from 200 µl of this homogenate.

All reactions are done in applied biosystem stepone (USA) apparatus with the following thermal profile: Initial denaturation 95°C for 5min. followed 40 cycle of denaturation at 95°C for 30sec. and annealing at 60°C for 1min. The primer set was described in Table (2).

Isolation of *C. burnetii*

Isolation of the coxiellosis from positive PCR samples was amplified through infection of 6-7 days old specific free Embryonated chicken egg in the yolk sac. The inoculations were carried out in sterile conditions, through the opening in the center of an air chamber. The embryos were incubated at 37°C with the ovoscopy being carried out on a daily basis during the period of 15 days. In order to detect the coxiellae by light microscope we prepared smears of yolk sacs of CE, The preparations were stained by the classical methodology of Gimenez stain and displayed by light microscopy[9].

3. Results:**Serology**

The serological results obtained by ELISA illustrated that the sero-positivity for *C. burnetii* in the examined serum and milk samples were 16 and 10% respectively as shown in Table 3.

Molecular analyses

Table 4 and Figure 1. Shows That 13 out of 75 samples were positive for the presence of *C. burnetii* DNA using real-time PCR.

Isolation results

The infection with the *Coxiella* pathogen lead to very typical pathological changes in the chicken embryos and especially, in their yolk sacs, which became a useful indicator of the coxiellosis infection. In diligently stained preparations of yolk sacs, dot-like oval and spherical coxiellae, colored in different shades of red or in violet, were observed and located in the cytoplasm of the endodermal cells in the shape of inclusions or with a diffusion distribution. The results of inoculated yolk sac by positive PCR samples from aborting and nonaborting camels for the presence of *C. burnetii* pathogen are shown in Table 5 and Fig (2): shows the light microscopic preparation Gimenez of yolk sacs of CE infected *C. burnetii* pathogen.

Table (1): samples design for *C. burnetii* detection in camels at MarsaMatrouh

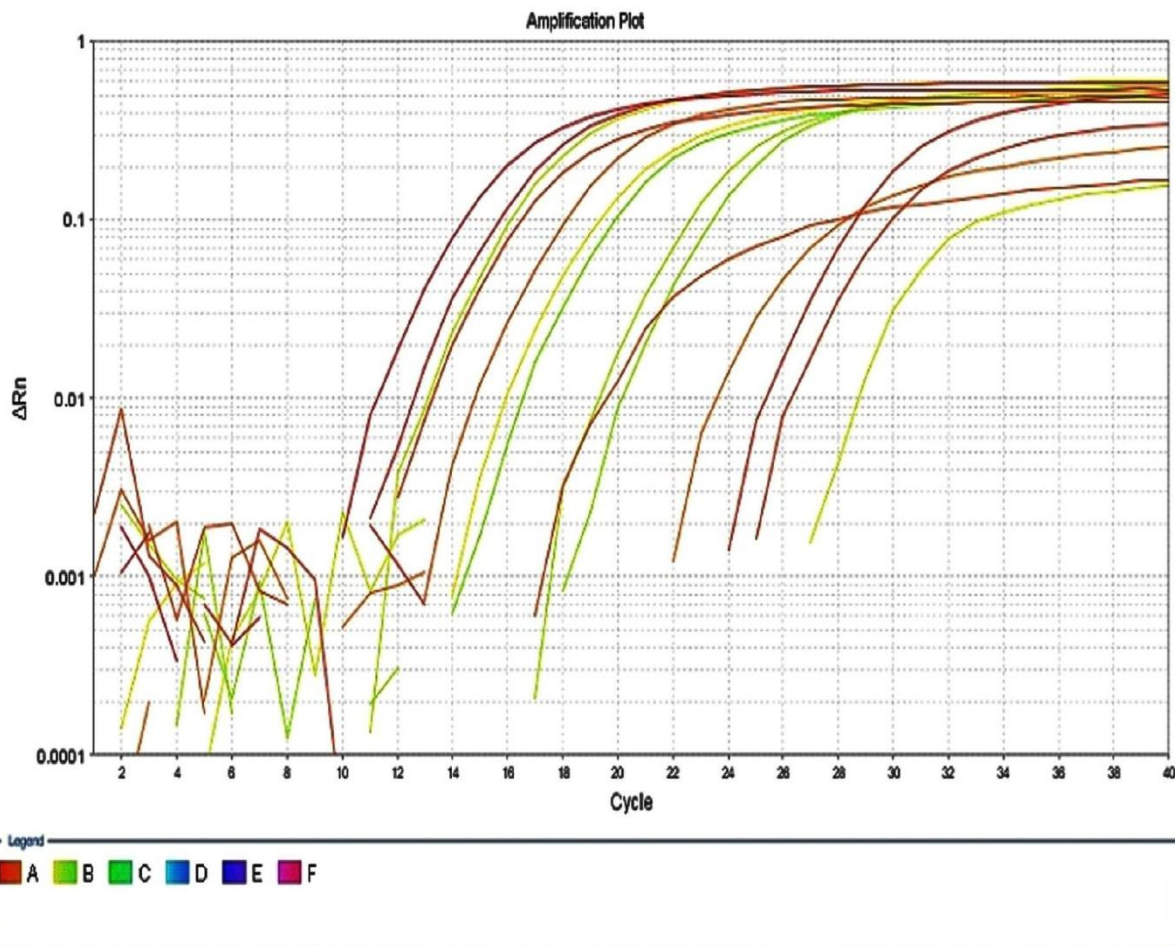
Samples	Animal status		Total number of samples
	Aborted she camels	Apparently healthy camels	
serum	30	20	50
milk	18	22	40
placenta	4	-	4
Vaginal swabs	26	-	26
Internal organ of aborted calves	5	-	5
Total	83	42	125

Table(2):Nucleotide sequences and the internal probe trans-p used for Real-Time PCR.

Primer name	Description	Nucleotide sequence(5'-3')	Reference
Trans 1	Universal forward primer	TATGTATCCACCGTAGCCAGTC	Hoover <i>et al.</i> (1992)
Trans 2	Reverse primer	CCCAACAACACCTCCTTATTC	
The internal probe trans-p:	(5'AACGATCGCGTATCTTTAACAGCGCTTG-3') was labeled with the reporter dye 5-carboxyfluorescein (FAM) on the 5' end and the quencher dye N', N', N', N'-tetramethyl-6 carboxyrhodamine (TAMRA) at the 3' end.		

Table (3): The prevalence and percentage of positive specific antibodies to *C. burnetii* in camel sera and milk samples.

Animal status	Sera samples		BTM samples	
	Total number	positive	Total number	positive
Aborted she camels	30	6 (20%)	18	2(11%)
Apparently healthy camels	20	2 (10%)	22	2(9%)

**Fig (1): The amplification plot of *trans* gene in *C. burnetii* directly detected from BTM, 13 samples out of 75 were positive samples and the control positive Nine Mile RSA493 *C. burnetii* strain where supplied by Giessen University, Germany**

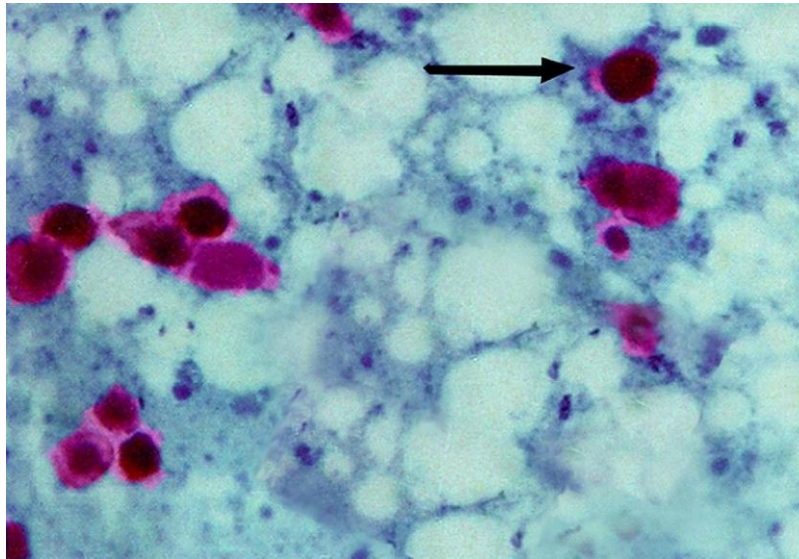


Fig. (2): The inclusion bodies in the infected yolk sac membrane stained with Gimenez stain. X1200

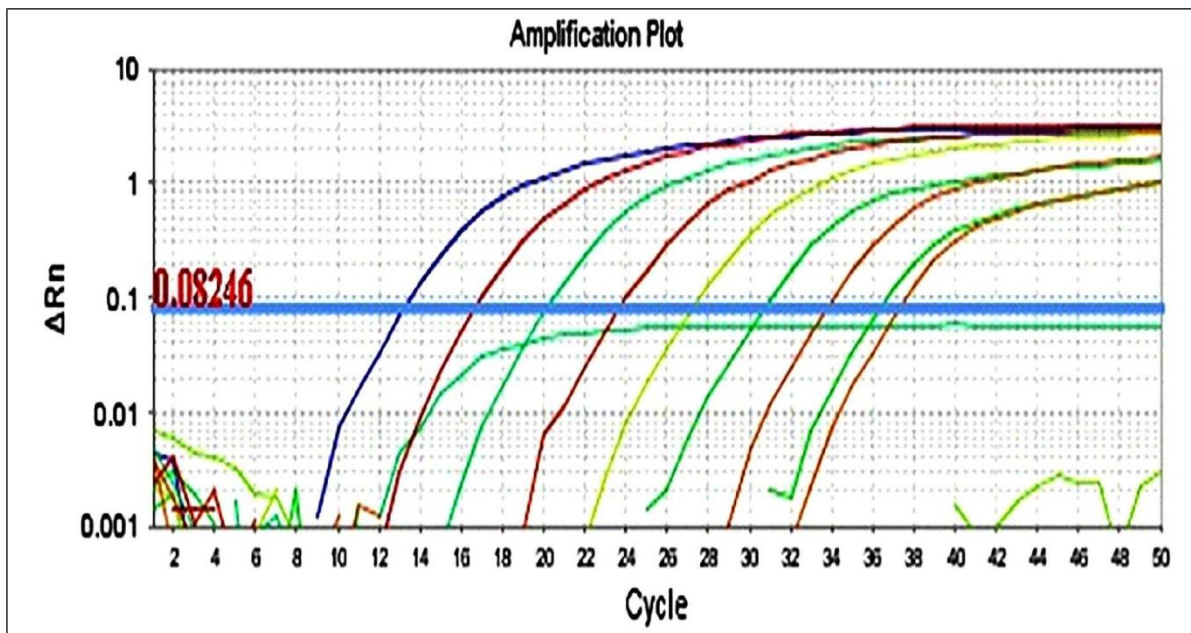


Fig (3) The amplification plot of Trans gene in *C. burnetii* directly detected after propagation of positive PCR Samples from aborting and nonaborting in embryonated chicken eggs showing 8 positive samples and the control positivethe control positive Nine Mile RSA493 *C. burnetii* strain where supplied by Giessen University, Germany

Table 4: real-time PCR results from aborted she camels and apparently healthy one for the presence of *C. burnetii* DNA.

Animal status	BTM samples		Vaginal swabs		Placenta samples		Internal organs of foeti	
	Total number of	Positives samples	Total number of	Positives samples	Total number of samples	Positives samples	Total number of samples	Positives samples
aborted she camels	18	4 (22.2%)	26	3 (11.5%)	4	3 (75) %	5	0%
apparently healthy camels	22	3 (13.6%)	-	-	-	-	-	-

Table 5: The results of inoculated yolk sac by positive PCR samples from aborting and nonaborting camels for the presence of *C. burnetii* pathogen

Animal status	BTM samples		Vaginal swabs		Placenta samples		Internal organs of foeti	
	Total number of samples	Positives samples	Total number of samples	Positives samples	Total number of samples	Positives samples	Total number of samples	Positives samples
aborted she camels	4	3 (75%)	3	2 (66.6)	3	3(100)%	-	-
apparently healthy camels	3	-	-	-	-	-	-	-

Table 6: The results of inoculated harvested yolk sac.

Animal status	BTM samples		Vaginal swabs		Placenta samples		Internal organs of foeti	
	Total number of samples	Positives samples	Total number of samples	Positives samples	Total number of samples	Positives samples	Total number of samples	Positives samples
aborted she camels	4	3 (75%)	3	2 (66.6)	3	3(100)%	-	-
apparently healthy camels	3	-	-	-	-	-	-	-

Results of inoculated yolk sac samples by (RT-PCR)

The results of harvested inoculated yolk sac by positive PCR samples from aborting and non aborting for presence the transposing-like repetitive region of the *C. burnetii* genome by RT-PCR and are given in Table (6) and Fig. (3).

4. Discussion

Q-Fever, caused by the organism *C. burnetii*, is an emerging or reemerging rickettsial zoonotic disease worldwide (Vaidya *et al.*, 2008). Q-Fever in camel has been reported from many countries, including the Sudan, Egypt, Tunisia, India, Kenya, Central Africa, Ethiopia, Nigeria, United Arab Emirates, and Chad (Mansour *et al.*, 2008). The epidemiology of Q-fever in Egypt is essentially unknown, but there are some studies that reported detection of *C. burnetii* in various clinical samples in Egypt (Mazyad and Hafez, 2007; Nahed *et al.*, 2012 and Mayada *et al.*, 2014).

In present study, the overall seroprevalence of *C. burnetii* in aborted she camels and apparently healthy camels were 20 % and 10 %, respectively. These results in agreement to those obtained in Egypt by Mazyad and Hafez 2007; Osama *et al.*, 2014. In another study, Soliman *et al.*, 1992 reported results which showed that the competitive enzyme immunoassay (CEIA) detected 71 % (n=34) of the camel serum samples positive for *C. burnetii* antibody, while 65 % (n=31) were detected by the enzyme immunoassay with protein A conjugate (EIA-PA), with 60 % agreement. Of 40 % with discordant results, 11 camel serum specimens were positive by CEIA and negative by EIA-PA. They also detected *C. burnetii* antibody in sheep (n=40) and goat (n=96) serum samples. Also the prevalence *C. burnetii* in BTM samples in aborted she camels and apparently healthy camels were 11 % and 9 %, respectively.

This indicate that camel in Egypt have exposed to *Coxiella burnetii* infection and this may be responsible for some of the reproductive disorder among infected camel and neighboring camel. In the majority of cases, *C. burnetii*'s abortion occurs at the end of gestation without specific clinical signs until abortion is imminent, as observed with brucellosis or chlamydiosis. Aborted fetuses appear normal but infected placentas exhibit intercotyledonary fibrous thickening and discolored exudates, which are not specific to Q-Fever.

In the study, we tested the presence of *C. burnetii* DNA in different clinical samples, because *C. burnetii* may be shed by other routes such as vaginal mucus, urine, feces or birth fluids, hence testing animals on only blood samples can lead to misclassifying the status of the animal and misidentifying the route of excretion or discharge. Out of 75 camel samples, 13 (17.3%) were found real-time PCR positive for *C. burnetii* DNA. The higher positive percent were found in placenta (75%) follow by BTM samples (22.2%) then in Vaginal swabs (11.5%) the results are in agreement with (Osama *et al.*, 201 and Mansour *et al.*, 2013) whom identify *C. burnetii* by PCR in the milk, feces and urine of naturally infected camels.

The trans-PCR assay targeting the IS1111a gene of *C. burnetii* proved to be highly specific and sensitive for the detection of *C. burnetii* in clinical samples. Earlier workers found trans-PCR to be highly specific and sensitive for the direct detection of *C. burnetii* in genital swabs and milk and fecal samples (Berri *et al.*, 2000). The high degree of efficacy of the trans-PCR can be attributed to the fact that the targeted region exists in at least 19 copies in the *C. burnetii* Nine Mile, phase I, genome, which gives the trans-PCR a level of sensitivity 100 times higher than that of the PCR assay (Henning and Sting, 2002).

While PCR is most useful in establishing a microbial diagnosis for samples that may include other bacteria, PCR cannot distinguish between living and dead bacteria. The isolation of *C. burnetii* definitively demonstrates a current infection with viable bacteria. In the present study, Coxiella isolation attempts were performed on the PCR positive samples to confirm the presence of the involved bacteria as well as amplification through embryonated egg inoculation, the study results were 8 (80 %) from aborted she camels.

The study results indicate clearly that camels in Egypt may play an important role as a reservoir of *C. burnetii* and that they could be a significant source for the transmission of Q-Fever to other animal species and to humans.

Acknowledgments

The authors are grateful to Animal Health Research Institute and all of the staff members for supporting this work.

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