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First serodetection and molecular phylogenetic documentation of *Coxiella burnetii* isolates from female camels in Wasit governorate, Iraq

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Abstract

This study aims to detect *Coxiella burnetii* in one-humped female camels (*Camelus dromedarius*) using ELISA and confirmation of infection by PCR with the phylogenetic analysis of local isolates. The 91 adult female camels were selected for clinical examination and blood sampling from different areas in Badra and Al-Numaniyah districts in Wasit governorate, Iraq, from February to April 2019. The prevalence of *Coxiella (C.) burnetii* was 19.8% and 4.4% by ELISA and PCR, respectively. Targeting 16S rRNA genes from three positive samples were documented in the Genbank-NCBI under accession numbers of MN900579.1, MN900580.1, and MN900581.1. Clinical evaluation revealed insignificant variation in temperature, pulse, respiratory rates, and lymph node enlargement among the positive and negative animals. The findings also showed that camels of the Badra regions have positive sign*s. burnetii* compared to other regions, and the infection was increased significantly in April and March. In conclusion, our findings confirmed the prevalence of *C. burnet*h among Iraqi female camels, suggesting that these animals might be a source of the pathogen for humans and other animal species. Therefore, further studies are necessary to provide more detailed data about the prevalence of *C. burnetii*to to improve effective control measures.

Introduction

The *Coxiella (C.) burnetii* is an obligate intracellular, Gram-negative, non-motile bacterium belonging to the Coxiellaceae family of Legionellales order, which causes a highly contagious neglected zoonotic disease known as Q (Query) fever (1). Many studies reported that this pathogen is considered a potential agent for bioterrorism due to its highly high infectivity (one bacterium may produce disease) in humans and a wide range of domestic and wild animals and its survival under harsh environmental conditions (2). *C. burnetii* is an occupational pathogen of farmers, slaughterhouse workers, and veterinarians and can be transmitted by inhaling aerosolized contaminated dust, direct contact with infected tissues and fluids, as well as by arthropod bites (3). Multiple hosts can serve as the main

reservoir for infection, particularly the infected females that shed vast numbers of highly stable bacteria into their birth products (amniotic fluids and placenta) and smaller amounts in milk, feces, and urine which may continue over several months (4-6). Worldwide, numerous studies investigate the prevalence of *Coxiella* in camels such as the United Arab Emirates (7), Egypt (8), Algeria (9), Saudi Arabia (10), and Iran (11). These studies showed that the prevalence of *C. burnetii* varies widely by geographical location, type of management, and flock numbers. ELISA is the most recommended and preferred diagnostic technique for Q fever and other diseases for screening assays and epidemiological studies because of its high specificity and sensitivity, relatively low cost, and easier to use (11,12). For confirmation, the isolation of *C. burnetii* by inoculation of the yolk sac of 5-7 old chick embryos or in vitro tissue

culture has been difficult, time-consuming, hazardous, and requires biosafety level 3 laboratories (1). Molecular assays are beneficial and safe methods with high specificity and sensitivity and decrease the time for detecting targeted *C. burnetii* DNA in suspected samples (4). Serological assays and direct detection by PCR must be used as combinatory methods to diagnose infection (5). In Iraq, the dromedary camel (*Camelusdromedarius*) is one of the most important domesticated animals in arid and semi-arid areas. It is a source of milk, meat, wool, and leather, mainly for Bedouin communities (13).

Nonetheless, there are no data or studies about the detection of *C. burnetii* in camels. Furthermore, the biological management and veterinary care for camels in Iraq lag behind those for other animals such as sheep, goats, and cattle; this may decrease productivity due to disease-related morbidity and mortality. Hence, the present study was designed to investigate the seroprevalence of *C. burnetii* in adult female camels using the ELISA to detect the bacterium by PCR for subsequence phylogenetic analysis.

Materials and methods

Ethical approval

Approval for the present study was obtained from the College of Veterinary Medicine, University of Baghdad, Baghdad, and the College of Veterinary Medicine, Wasit University, Wasit, Iraq.

Samples

Ninety-one adult female camels were selected randomly from different areas in Badra and Al-Numaniyah districts in Wasit governorate, Iraq, from February to April 2019. Each animal was examined clinically, and the data regarding vital signs (temperature, pulse, and respiratory rates), examination of scapular and supra-mammary lymph nodes, tick's infestation, and reproductive status (number of pregnancies, abortions, stillbirths, and milk production) were reported. Additionally, 10 ml of jugular venous blood was drained from each camel under aseptic conditions and divided equally into without- and with-anticoagulant (EDTA) tubes to be used for serology and molecular assay, respectively. After centrifugation (3000 rpm / 10 minutes), the serum sample of each animal was kept in a labeled Eppendorf tube. Both sera and whole blood samples were stored frozen at -20°C until analysis.

Serology

Camel *C. burnetii* ELISA kit (SunLong, China) was used to detect the anti -*C. burnetii* IgG antibodies according to manufacturer's instructions and protocols. The serum samples were analyzed and read at 450 nm optical density (OD) using an automatic plate reader (BioTek, USA). The cut-off got 0.471, and it was at the mean of the negative OD +0.15.

Molecular testing

The genomic DNA of *C. burnetii* was extracted from whole blood samples according to the manufacturer's instruction of G-spin total DNA extraction mini kit (iNtRON, Biotechnology, South Korea). The purity and concentration of extracted DNA were measured using a Nanodrop spectrophotometer (Thermo-Scientific, UK). The PCR premix (Bioneer, South Korea) and the primer (4) [(F: 5′-AGTACGGCCGCAAGGTTAAA-3′) and (R: 5′-CTCCAATCCGGACTACGAGC-3′)] of 16S rRNA gene at 20 µl final volume were carried out to amplify *C. burnetii* DNA at 425bp. The PCR reaction was performed by Thermal-Cycler (Bio-Rad, USA) utilizing the following optimized conditions: 1 cycle (95ºC/5 min.) for initial denaturation, 30 cycles comprised (95ºC/40 sec.) denaturation, (56ºC/40 sec.) annealing, and (72ºC/1 min.) extension for each one, and followed by one cycle (72ºC/7 minutes) for the final extension. Electrophoresis in 1.5% agarose gel was examined the PCR products using 100-1500 bp of DNA ladder (Qiagen, Germany). The agarose was stained with ethidium bromide (Biotech, Canada), and electrophoresis was done at 100 V, 80 mA for one h. The DNA bands were visualized by a UV trans illuminator (Clinx Science, China).

Phylogenetic analysis

The amplified 16S rRNA gene of three positive samples by the conventional PCR assay was sent for sequencing (Macrogen, Korea) and analyzed using the MEGA-X program (14) Based on the NCBI-BLAST data, Multiple Sequence Alignment Analysis of 16S rRNA gene, phylogenetic tree, and homology sequence identity were made with the Genbank-NCBI strain/isolate.

Statistical analysis

All data were documented and analyzed using Microsoft Office Excel and SPSS. Chi-square and t-test were applied to detect significant differences in clinical, serological, and molecular results at $P<0.05$ (15).

Results

The serological analysis showed that out of 91 serum samples tested by ELISA, 18 (19.8%) (95% Confidence limits 11.6-28.0%) were positive for *C. burnetii*. In addition, OD values of seropositive camels were ranged 0.481-1.174 and having 0.782±0.042.

The molecular assay of whole blood samples showed that 4.4% (95.6% Confidence limits 0.08-10.2%) of 91 samples were positive for the 16S rRNA gene of *C. burnetii* at 425 bp (Table 1, Figure 1).

The phylogenic analysis of the three positive samples is based on the 16S rRNA gene. It showed that the local *C. burnetii* IRAQ/Camel No.1 reported a close relationship to NCBI-BLAST *C. burnetii* ATCC: VR-615 strain-16s ribosomal RNA gene (NR_104916.1) of USA strains. On the

other hand, the local *C. burnetii* IRAQ/Camel No.2 and IRAQ/Camel No.3 were showed a close relationship to NCBI- BLAST CoxiellaburnetiiCB-30 strains (LC464975.1) of Indonesia strains, at total genetic changes (0.005%), the sequences findings were recorded in NCBI under the accession numbers of MN900579.1, MN900580.1, and MN900581.1, respectively (Table 2). Based on the phylogenetic tree, the local *C. Brunetti* strains (IRAQ/Camel-No.1, 2, and 3) showed a significant genetic association to NCBI-BLAST strains of *C. Brunetti* strains at total genetic changes of 0.005% (Figure 2).

Table 1: Prevalence of *C. burnetii* in female camels by ELISA and PCR

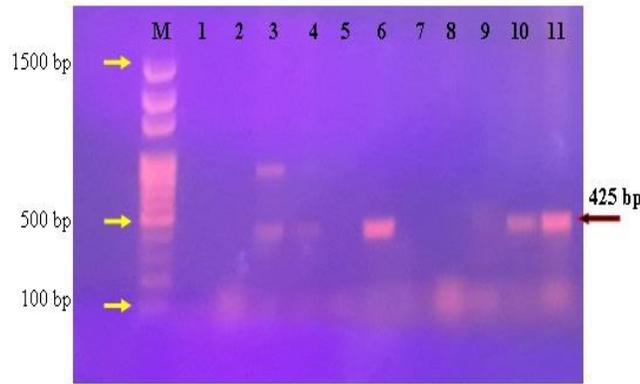| Total No.	Test	Positives	Negatives	Confidence level
91	ELISA	18 (19.8%)	73 (80.2%)	11.6-28%
	PCR	4 (4.4%)	87 (95.6%)	0.08-10.2%

Figure 1: PCR product analysis of 16S rRNA gene (425bp) of *C. burnetii* on agarose-gel electrophoresis. M: Ladder marker (100bp); Lane 1 represents negative control; Lanes 2, 5, 7, 8, and 9 represent negative samples; Lanes 3, 4, 6, and 10; Lane 11 represents positive control.
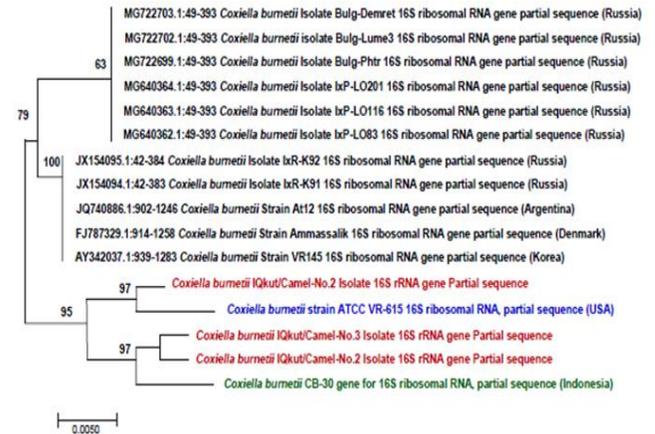

Figure 2: Phylogenetic tree (UPGMA tree) of local isolates and their association with some Genbank-NCBI isolates.

Table 2: Homology Sequence identity between local and NCBI-BLAST *C. burnetii* strains

Local *C. burnetii* isolate		NCBI-BLAST Homology Sequence identity (%)			
Isolate	Accession No.	Isolate	Accession No.	Country	Identity (%)
IRAQ/Camel-No.1	MN900579.1	*ATCC: VR-615*	NR_104916.1	USA	96.52%
IRAQ/Camel-No.2	MN900580.1	CB-30	LC464975.1	Indonesia	96.93%
IRAQ/Camel-No.3	MN900581.1	CB-30	LC464975.1	Indonesia	96.94%

The clinical examination of camels showed no significant differences in positive camels' temperature, pulse, and respiratory rates using the ELISA and PCR methods compared to negatives (Table 3).

Pre-scapular and supra-mammary lymph nodes of all camels were clinically normal at the time of sampling, and no ticks were found in the study. According to the region of the study's camels, significant increases in positive results were reported in areas of Badra by ELISA 42.9% and PCR 10.71% (Table 4).

An association of positive findings to the period factor showed significant increases in the positive results by ELISA and PCR methods respectively in April 23.3and 6.7% and March 20.9 and 4.7% (Table 5).

Table 3: Results of vital signs of study camels

Factor	ELISA		PCR	
	Positive	Negative	Positive	Negative
Temperature	37.54 ± 1.02	37.18 ± 0.69	37.91 ± 0.83	37.33 ± 0.45
Pulse rate	46.21 ± 2.05	45.82 ± 1.56	45.93 ± 1.22	45.61 ± 1.04
Respiratory	9.25 ± 0.88	9.64 ± 0.26	9.37 ± 0.51	9.79 ± 0.34

Table 4: Total positive results according to the region of study's animals

Region	No.	ELISA		PCR	
		Positive	Negative	Positive	Negative
Al-Numaniyah	63	6 (9.52%)	57 (90.48%)	1 (1.59%)	62 (98.41%)
Badra	28	12 (42.86%) *	16 (57.14%)	3 (10.71%) *	25 (89.29%)

* Significance at P<0.05.

Table 5: Total positive results according to the period of samples collection

Period	No.	ELISA		PCR	
		Positive	Negative	Positive	Negative
February	18	2 (11.1%)	16 (88.9%)	0 (0.0%)	18 (100%)
March	43	9 (20.9%) *	34 (79.1%)	2 (4.7%)	41 (95.4%)
April	30	7 (23.3%) *	23 (76.8%)	2 (6.7%)	28 (93.%)

* Significance at P<0.05.

Discussion

Camels are considered multi-purpose animals of high economic importance due to their milk, meat, leather, and soft wool, especially in dry and hot areas (13). Formerly, it was assumed that camels are resistant to many pathogens, usually affecting other animals. However, numerous studies reported that they are susceptible to other livestock populations and act as reservoirs or carriers for several pathogens (11).

In the present study, the anti-*C. burnetii* antibodies were detected by ELISA in 19.8% of camels. The seroprevalence of anti-*C. burnetii* in humans and many different animals have been detected in Iraq's neighboring countries such as Saudi Arabia (10), Iran (11), Turkey (16), and Kuwait (17). Our findings are primarily in line with many studies reported in other countries such as Selimand Ali (8), who detected that the seroprevalence rate was 22% in camel in Egypt; however, our results were lower than detected 71.2% in Algeria (18), 44% in Tunisia (19) and 40.7% in Egypt (20).

The molecular assay showed that the 16S rRNA gene of *C. burnetii* was detected in whole blood samples of 4 female camels with 4.4%. These results were lower than the molecular detection in the camels recorded 15.9% in Saudi Arabia (10) and (17.5%) in Egypt (21). The results of the serological and molecular tests confirmed wide variations in the prevalence of *C. burnetii* among the camel population worldwide. The quarantine measures could influence these variations, type, and sensitivity of diagnostic methods, type of studied sample, age and sex of animals, poor hygienic and sanitary conditions. The current study reveals significant variations between the seropositive rate 19.8% of ELISA and PCR 4.4%, this shows that only a fraction of exposed animal carries the pathogen at a given time might be due to continuous immunological responses of the infected host, which leads to decrease and/or interrupted shedding of this pathogen (5).

The sequencing of the 16S rRNA gene of our isolates revealed a significant genetic association to NCBI-BLAST strains of *C. burnetii* strains at total genetic changes of 0.005%. Whereas the local *C. burnetii* IRAQ/Camel No.1 showed a close relationship to the USA (NR_104916.1) strain, the local *C. burnetii* IRAQ/Camel No.2 and IRAQ/Camel No.3 were showed a close relationship to Indonesia (LC464975.1) strains. The 16S rRNA gene sequencing analysis can be used to identify and differentiate phenotypically aberrant, uncultivable, or poorly described microorganisms such as *C. burnetii* (22-24).

In the clinical examination of the study's camels, there are no statistical differences in values of vital signs (temperature, pulse, and respiratory rates) and enlargement in superficial lymph nodes of positive and negative camels. This may be since the infected animals with *C. burnetii* neither revealed the visible clinical signs nor apparent pathological alterations in the tissues, milk, and meat. Consequently, it is unlikely to diagnose this pathogen according to the clinical or postmortem examination (25). Consequently, definitive diagnosis of this disease is based on a significant rise in antibody and molecular detection (25-27). The decrease in pregnancy and/or milk production might be attributed to the high susceptibility of *C burnetii* to the placenta, amniotic fluids, and udder (8,18).

The season and geographical location are the main factors that participated in a high prevalence of *C. burnetii* infection. The significant increases in *C. burnetii* positive camels at Badra compared to the Al-Numaniyah region may be partly correlated with the local ecological factors, such as type of management and practice, flock size, and drought-related to low rainfall. Concurrently, this area has been suffering from the dust arising from the regional countries, which is named Middle Eastern Dust. The dust can be carried and transport various pathogens for long distances (28). The windy and dry environments play an essential role in the transport of *C. burnetii* in numerous outbreaks. Worthwhile, Badra is a town in Wasitgovernorate, near the Iraqi-

Iranian border. In Iran, *C. burnetii* has been detected in camels and other animal species, as mentioned above (11). A comparison with Iranian strains could demonstrate cross-border transmission.

Finally, the apparent high seroprevalence of *C. burnetii* during the April and March months 23.3 and 20.9%, respectively, might be attributed to the climatic changes and the high wind activity during these months. These winds commonly facilitate the transmission and inhalation of contaminated dust with this pathogen (28). Furthermore, increasing the activity of the ticks and other arthropods may increase, which plays an essential role in transmitting this bacterium during these months (29).

Conclusion

Our findings indicate that *C. burnetii* is prevalent among female camels in different areas in Badra and Al-Numaniyah districts in Wasit province, Iraq. Additional molecular studies targeting other genes must be performed to improve the control measures of this highly contagious zoonotic pathogen.

Acknowledgment

Conflict of interest

The authors declare that the current study was carried out without conflict of interest.

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الكشف المصلي الأول وتوثيق النشوء الجزيئي عن جراثيم الكوكسيلا البورنتية المعزولة من إناث الإبل في محافظة واسط في العراق

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الخلاصة

إن هذه الدراسة تهدف الكشف عن جراثيم الكوكسيلا البورنتية في إناث الإبل وحيدة السنام باستخدام اختبار الاليزا وتأكيد الإصابة بواسطة تفاعل البلمرة المتسلسل مع تحليل النشوء والتطور للعزلات المحلية. تم اختيار ٩١ من إناث الإبل البالغة لإخضاعها للفحص الكلينيكي وجمع نماذج الدم بشكل عشوائي من مناطق مختلفة في مدينتي بدرة والنعمانية في محافظة واسط (العراق) خلال الفترة من شباط إلى نيسان ٢٠١٩. كان معدل انتشار الكوكسيلا البورنتية ١٩,٨ و ٤,٤٪ بواسطة الاليزا وتفاعل البلمرة المتسلسل على التوالي باستهداف جين *16S rRNA* وتم توثيق الحمض النووي الجيني لثلاث عينات إيجابية في -Genbank NCBI تحت أرقام الانضمام MN900579.1 و MN900580.1 و , MN900581.1. أظهر التقييم السريري لجمال الدراسة تباينًا طفيفًا في قيم درجة الحرارة والنبض ومعدلات التنفس وتضخم العقدة الليمفاوية بين الحيوانات الإيجابية والسلبية. وكذلك وأظهرت النتائج أن الإبل في منطقة بدرة كانت إيجابية بشكل معنوي للكوكسيلا البورنتية مقارنة بالمناطق الأخرى، كما زادت الإصابات بشكل ملحوظ في شهري نيسان وأيار، أكدت النتائج التي توصلنا إليها انتشار جراثيم الكوكسيلا البورنتية بين الإبل العراقية، مما يشير إلى أن هذه الحيوانات قد تعمل كمصدر للأمراض في الإنسان وأنواع حيوانية أخرى، علاوة على ذلك، فإن الدراسات ضرورية لتوفير بيانات أكثر تفصيلاً حول انتشار الكوكسيلا البورنتية لتحسين خطط السيطرة.