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Serological and Molecular Phylogenetic Detection of *Coxiella burnetii* in Lactating Cows, Iraq

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A B S T R A C T

This study is carried out to investigate the prevalence of *Coxiella burnetii* (*C. burnetii*) infections in cattle using an enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) assay targeting IS1111A transposase gene. A total of 130 lactating cows were randomly selected from different areas in Wasit province, Iraq and subjected to blood and milk sampling during the period extended between November 2018 and May 2019. ELISA and PCR tests revealed that 16.15% and 10% of the animals studied were respectively positive. Significant correlations ($P<0.05$) were detected between the positive results and clinical data. Two positive PCR products were analyzed phylogenetically, named as *C. burnetii* IQ-No.5 and *C. burnetii* IQ-No.6; and then recorded in the National Center for Biotechnology Information (NCBI) under an accession numbers of MN473204.1 and MN473205.1. Comparative identity of the local strains with NCBI-BLAST strains/isolates revealed 97% similarity and 0.1-0.6% of total genetic mutations/changes. NCBI-BLAST Homology Sequence reported high significant identity ($P<0.05$) between the local, *C. burnetii* IQ-No.5 and *C. burnetii* IQ-No.6; strains and *C. burnetii* 3345937 (CP014354.1) Netherlands isolate at 99.10% and 99.06%, respectively. The current study concluded that the percentage of infected cows with coxiellosis is relatively high, and *Coxiella* should be listed as abortive pathogen. Therefore, additional studies should be performed including different animals, samples, and regions.

Keywords: *Coxiella burnetii*, cattle, Iraq, *IS1111A transposase* gene, ELISA, PCR

INTRODUCTION

Bovine coxiellosis is a common zoonotic infection caused by *Coxiella burnetii*, an obligate intracellular gamma proteobacterial organism (1). The disease is worldwide distribution except New Zealand and Antarctica; however, the incidence rate appears to vary considerably throughout all countries (2). In recent years, changes in social-economical, environmental, and ecological factors may be in contributing of emergence a large number of human cases that are attributed mostly to livestock sheep, goats and cattle (3). In cattle, *Coxiella burnetii* (*C. burnetii*) infection is commonly subclinical (asymptomatic) but it can result in reduced reproductive performance (4).

Various quantities of bacteria can be shed by infected animals through different routes (5). Intra-herd infection dynamic of a dairy herd is mainly influenced by the heterogeneity of the shedding routes. One of the important uncertainties concerning dynamics of infection lies in the contributions of the different routes in transmitting *C. burnetii* between livestock herds (6). In pregnant cows, the outcomes of infection involve an abortion, weak offspring, stillbirth, and premature delivery (7). Airborne transmission of *Coxiella* is well documented phenomenon (8). For humans, inhalation of contaminated aerosols generated during parturition, and ingestion of raw milk/unpasteurized dairy products of infected cows consider as the principal sources of infection (9, 10). Hence, intensive cattle farming with high prevalence could become a concern for public health, and investigation of infection dynamics in cattle herds is essential in the emergence to control continuum (11).

For the diagnosis, there are many laboratory tools that available for detecting *C. burnetii* using many biological samples (12). Even though the direct detection of *Coxiella* is more useful in veterinary pathology, cultivating of this fastidious microorganism is difficult and restricts to reference centers as it requires biosafety-level 3 laboratories and relies on cell culture which is performed by expert technicians (13). Serological screening of humans and animals for the detection of the organism specific antibodies by enzyme-linked immunosorbent assay (ELISA) has been found to be more sensitive and easier to be performed (14). Using only this method is not sufficient to demonstrate the infection so additional testing focused on confirmation the presence of the organism or its DNA is necessary (15). The development of highly sensitive and specific molecular assays has prompted the routine use of polymerase chain reaction (PCR) based analysis to assess the health status of the herds towards many pathogens including *C. burnetii* and ensure the safety (16). Hence, this study aimed to use serological ELISA and molecular PCR in detecting *C. burnetii* in both serum and milk samples of lactating cow, and documentation some positive PCR strains in the National Centers for Biotechnology Information (GenBank-NCBI) for the first time in Iraq. This study also targeted to correlate their positivity associated with the clinical data of reproductive system.

MATERIALS AND METHODS

Animals and Sample Collection

The present study is approved and performed under the Council of College of Veterinary Medicine, and the authority of the Department of Internal and Preventive Veterinary Medicine, College of Veterinary Medicine, University of Baghdad.

A total of 130 lactating cows from many areas in Wasit province, and of different ages and breeds were subjected for this study during the period extended from November 2018 to May 2019. Under aseptic conditions, approximately 50 mL of milk was collected directly from udder quarters of each cow into a plastic container that was labeled, noted, and kept into a cooled icebox. Furthermore, 10 mL of jugular venous blood was drained by a disposable syringe into free-anticoagulant glass gel tube. At laboratory, blood samples were centrifuged (4000 rpm, 5min), and sera were preserved in 1.5 mL labeled Eppendorf tubes. Both sera and milk tubes were frozen at -20 °C until be tested by ELISA and PCR, respectively. Case history data concerning to age, breed, tick infestation, and other animals' data at same herds/pasture, herd size, reproductive performance, and milk production were detected.

Serology by Indirect ELISA

Using a commercially available indirect ELISA (Sunlong Biotech, China) Kit, serum samples and buffer diluents were prepared and diluted. Following the manufacturer's instruction, a total 10 µL of each serum sample from each sample was used for detecting specific IgG antibodies against Phase I and Phase II antigen of *C. burnetii*. After the final step of ELISA procedure and adding of Stop Solution, absorbance (OD) was measured at a wavelength of 450nm using an ELISA microplate reader (BioTek, USA). Critical value (CUT OFF) was calculated as the following formula: CUT OFF= Average value of Negative Control + 0.15. Sample is considered positive when OD value ≥ CUT OFF; and negative when OD value < CUT OFF.

Molecular Conventional PCR Assay

Using 200 µL of each milk sample, DNAs were extracted following the Protocol A of G-spin™ Total DNA extraction (Intron, Biotechnology, South Korea) Kit. Concentration and purification of extracted DNAs were checked by Nanodrop spectrophotometer (Thermo-Scientific, UK) at an absorbance of A260/A280nm; and estimated as approximately as 6 µg for concentration and 1.6-1.9 nm for purification.

For PCR amplification, one set of primers [(F: 5´-TAT GTA TCC ACC GTA GCC AGT C-3´) and (R: 5´-CCC AAC AAC ACC TCC TTA TTC-3´)] targeting *IS1111A transposase* gene was designed as previously described (17, 18), and provided by the Macrogen Company (South Korea). A ready AccuPower PCR-PreMix (Bioneer, South Korea) Kit was used to prepare the PCR-Master mix at a final volume of 20 µL (5 µL DNA template, 1 µL F-Primer, 1 µL R-Primer, and 13 µL free-nuclease water) for each sample. For PCR reaction and Thermal Cycler (Bio-Rad, USA) conditions were as follows: initial denaturation (98 °C, 30 sec) 1 cycle; denaturation (98 °C, 7 sec), annealing (60 °C, 20 sec), extension (72 °C, 20 sec) 30 cycles, final extension (72 °C, 7 min) 1 cycle, and hold (4 °C, Forever). At 100 volt and 80 mA for 1 hour, 1.5% agarose gel-electrophoresis stained

with ethidium bromide to analyze the PCR-products that were visualized under UV illuminator (Clinx Science, China). According to standard size of the band of Ladder Marker (100-1500 bp), the samples of PCR product were considered positives at an amplicon size of 687 bp.

To confirm local *C. burnetii* strains, two positive PCR products were sent for phylogenetic analysis at Macrogen Company (South Korea). Sequencing results were received by private mail were analyzed by the Multiple Sequence Alignment Program, and phylogenetic tree was constructed using of MEGA-6 software; and homology identity between the local strains *C. burnetti* and GenBank-NCBI strains/isolates was detected.

Statistical Analysis

All collected data were documented and tabulated using the Microsoft Office Excel (version 2016) and analyzed statistically by IBM/SPSS (version 23). Chi-square (x^2) test and one-way analysis of variance (ANOVA) were applied for the finding of ELISA and PCR, and for the risk factors, respectively. Differences were considered significant at P<0.05 (19, 20).

RESULTS

Of 130 tested sera using indirect ELISA, 21 (16.15%) cows were positive to IgG antibodies against *C. burnetii*. Whilst the results of PCR assay showed that among 130 milk samples tested, 13 (10%) samples were positive for *IS1111A transposase* gene (Table 1, Figure 1). Statistically, ELISA positivity was higher than that detected in PCR assay (P<0.05). In addition, ODs' levels of seropositive study cows were showed significant differences in their values (Figure 2).

Table 1. Total positive results of ELISA and PCR among 130 study cows

Test	Total No.	Positives	Negatives
ELISA	130	21 (16.15%) *	109
PCR	130	13 (10%)	117

*Significant (P<0.05)

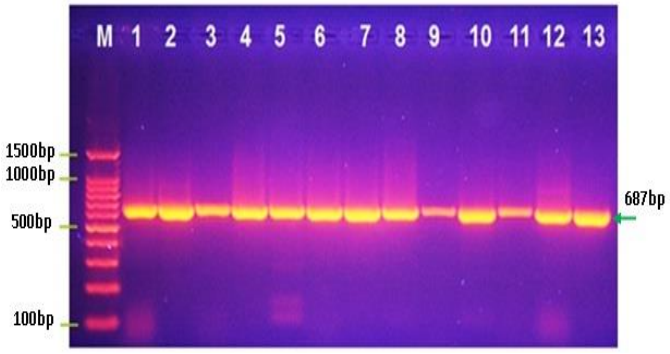

Figure 1. Agarose-gel electrophoresis at 100 Volt and 80 mA for 1 hour

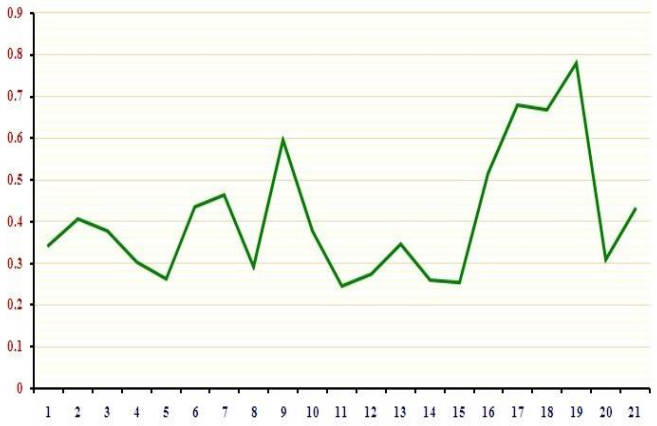

Figure 2. ODs' levels of seropositive study cows

Association between the positive results of ELISA and PCR was showed to be 11 (8.46%) of cows were positives by both assays in comparison with 10 (7.69%) were positive by ELISA only and 2 (1.54%) were positive cows by PCR only (Table 2).

Table 2. Relationship between positive results of ELISA and PCR

Test	PCR		
ELISA	Positives	Negatives	**Total**
Positives	11 (8.46%)	10 (7.69%)	21 (16.15%)
Negatives	2 (1.54%)	107	109 (83.85%)
Total	13 (10%)	117	130

Regarding to clinical data, significant increases (P<0.05) in positive cows by both assays were reported in cows of >5 years of age, crossbred, infested with ticks, lived with sheep, and raised in a herd of >25 cattle. As well as a significant elevation (P<0.05) in positivity was detected in cows suffering from low milk production and abortion (Table 3).

Genomic DNAs for two PCR-positive samples of *IS1111A transposase* gene were analyzed phylogenetically, named as *C. burnetii* IQ-No.5 and *C. burnetii* IQ-No.6, and recorded in NCBI under an accession numbers of MN473204.1 and MN473205.1, respectively. Comparative analysis of *IS1111A transposase* nucleotides sequence of study samples with number of *C. burnetii* strains present in the GenBank database was constructed using the ClustalW Alignment of MEGA software. The results showed that there were nucleotide alignment similarities (*) and substitution mutations in *IS1111A transposase* gene (Figure 3).

Comparative identity for genetic variations between the local strains and NCBI-BLAST strains/isolates was showed similarity of 97%; whereas, the total genetic mutations/changes were 0.1-0.6% (Figure 4, Table 4).

Table 3. Association of clinical data to positive results of ELISA and PCR assay

Factor	ELISA		PCR	
Age (year)				
≤ 3-4	0/21 (0%)		0/13 (0%)	
> 4-5	4/21 (19.05%)	*P = 0.035*	4/13 (30.77%)	*P=0.037*
> 5	17/21 (80.95%) *		9/13 (69.23%) *	
Breed				
Local	2 (9.52%)		0/13 (0%)	
Crossbred	19 (90.48%) *	*P = 0.021*	13/13 (100%) *	*P=0.013*
Pure	0 (%)		0/13 (0%)	
Tick infestation				
Infested	21 (100%) *	*P = 0.019*	13/13 (100%) *	*P=0.015*
Non-infested	0 (%)		0 (0%)	
Other animals at same pasture				
Sheep	17 (80.95%) *	*P = 0.048*	13 (100%) *	*P=0.046*
Goats	14 (66.67%)		11 (84.62%)	
Herd size				
<10	2 (9.52%)		0 (0%)	
10-25	4 (19.05%)	*P=0.039*	2 (15.38%)	*P=0.030*
>25	15 (71.43%) *		11 (84.62%) *	
Reproductive and milk				
Abortion	8 (38.10%)	*P=0.072*	4 (30.77%)	*P=0.043*
Low milk	9 (42.86%)		8 (61.54%) *	

*Significant (P<0.05)

Figure 3. Multiple sequence alignment analysis similarity

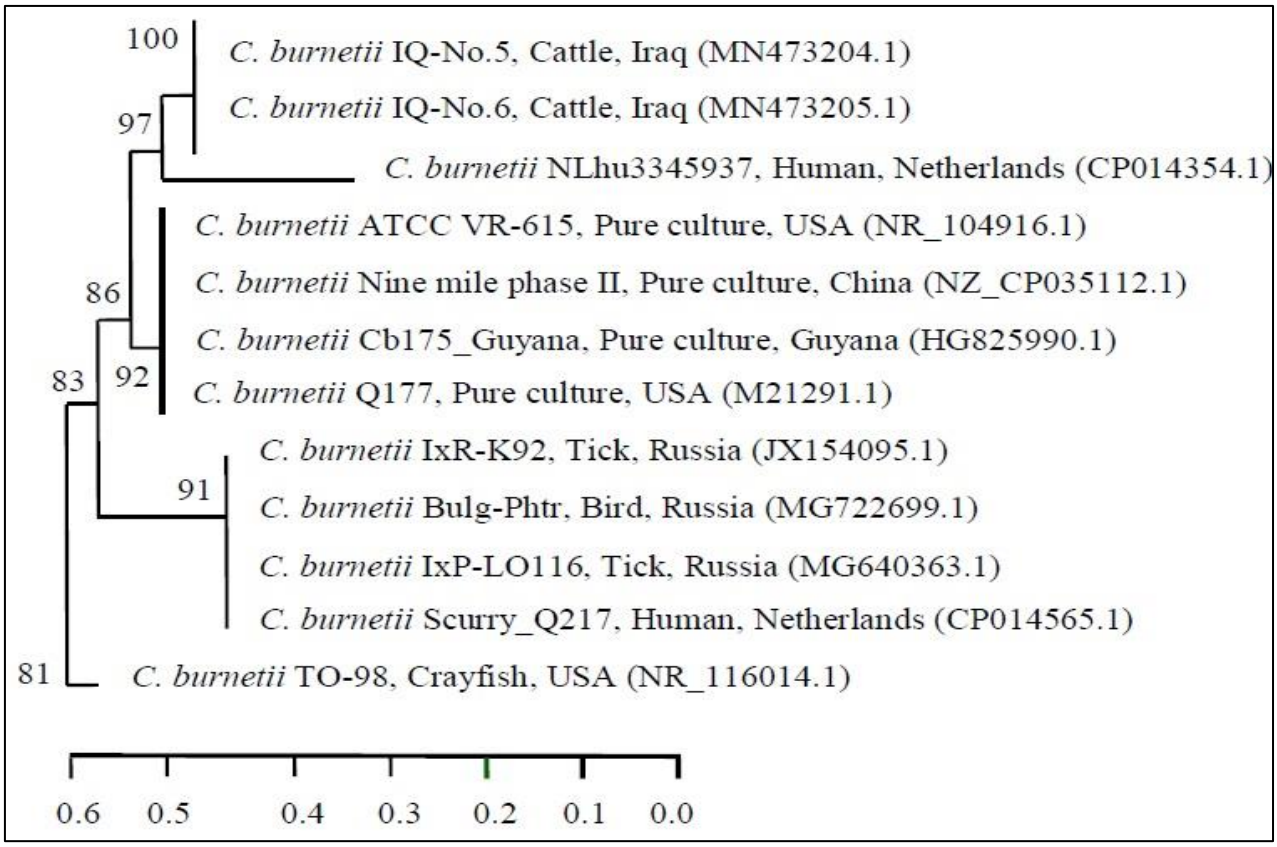

Figure 4. Phylogenetic tree analysis based on *IS1111A transposase* gene

Table 4. Phylogenetic tree analysis based on *IS1111A transposase* gene

Numbers	Country	Identity (%)	
		IQ-5	IQ-6
Coxiella burnetii strain IQ-No.5 16S ribosomal RNA gene, partial sequence	Iraq	-	100
Coxiella burnetii strain IQ-No.6 16S ribosomal RNA gene, partial sequence	Iraq	100	-
Coxiella burnetii strain 3345937 sequence	Netherlands	97.10	97.06
C. burnetii strain ATCC VR-615 16S ribosomal RNA, partial sequence	USA	96.74	96.67
Coxiella burnetii strain nine mile phase II chromosome, complete genome	China	96.74	96.63
Coxiella burnetii Cb175-Guyana, complete genome	Guyana	96.74	96.64
Coxiella burnetii 16S ribosomal RNA	USA	96.74	96.68
Coxiella burnetii isolate IxR-K92 16S ribosomal RNA gene, partial sequence	Russia	96.72	96.69
Coxiella burnetii isolate Bulg-Phtr 16S ribosomal RNA gene, partial sequence	Russia	96.44	96.43
Coxiella burnetii isolate IxP-LO116 16S ribosomal RNA gene, partial sequence	Russia	96.44	96.43
Coxiella burnetii strain Scurry-Q217 chromosome	Netherlands	96.44	96.42
Coxiella burnetii strain TO-98 16S ribosomal RNA, partial sequence	USA	94.33	94.32

Table 5. NCBI-BLAST Homology Sequence identity (%)

Strain	GenBank Accession No.	NCBI-BLAST Homology Sequence		
		Isolate name	Accession No.	identity
C. burnetii IQ-No.5	MN473204.1	*C. burnetii* 3345937	CP014354.1	99.10%
C. burnetii IQ-No.6	MN473205.1	*C. burnetii* 3345937	CP014354.1	99.06%

NCBI-BLAST Homology Sequence recorded that there was a high significant identity ($P<0.05$) between the local, *C. burnetii* IQ-No.5 and *C. burnetii* IQ-No.6 strains and *C. burnetii* 3345937 (CP014354.1) of Netherlands isolates at 99.10% and 99.06% respectively (Table 5).

DISCUSSION

Coxiellosis in cattle herds is known to be widespread and enzootic (3). The diagnosis of *C. burnetii* infection in animals is of great importance not only to identify the infected herds/flocks but also to determine the risk of disease transmission to humans (21, 22). In Iraq, there is limited information about this organism in humans and livestock populations. In cattle, only one serological report was carried out previously (23). Serologically, ELISA finding of our study was higher than what was reported in Iraq (7.37%), (23); and Albania (7.9%), (24); compatible with that was recorded in Germany (14.8%), (25); Australia (16.8%), (26); and Egypt (19.3%), (27); and lower than was observed in Iran (27.83%), (28); and Turkey (29%), (18). To the best of our knowledge, this is the first Iraqi molecular study performed to investigate the prevalence of bovine coxiellosis in lactating cows. In comparison to other studies, the finding of present study was higher than what it was reported in Turkey (1.42%), (18); and Iran (6.2%), (29); compatible with that was reported in Italy (11.9%), (30); and lower than what it was confirmed in France (21.1%), (11); and Netherland (56.6%), (31).

Significant increases in positive findings of ELISA comparing to PCR were reported; and this could be because of presence of more chronic infections than acute cases due to a host or seasonal factors. Astobiza et al. (2012) detected that serological results should be further analyzed with caution, and that other complementary analysis needed to be done (32). Therefore, our results were in expectance since PCR was more sensitive and greatly specific than ELISA in detecting of *C. burnetii* (33, 34). There was a high possibility of false positive or negative ELISA can be resulted due to insufficient blocking of immobilized antigen, antibody instability, seroconversion, and cross-reaction of the secondary antibody (35). For PCR, false negative results might be caused by many factors correlated with the type of tested sample, method for collection, preservation, and DNA extraction, type of PCR assay, targeted gene, and length of designed primers (36).

Among seropositive cows, a significant variation in levels of ODs, showed in this study, may represent different phases of infection among the studied animals. High values of OD indicated a recent chronic phase of disease; whereas lower values referred to either early acute phase of infection where there was limit development for IgG antibodies, or the terminal period of chronic phase of *C. burnetii* infection. Combination of ELISA and PCR in current study showed that a number of study cows were positives by both assays. It is suggested in this study that these animals are of great importance as they represent the source of potential risk to other animals, as well as, for their owners. In addition, combination of ELISA and PCR can be used as a gold standard method for more reliable detection of infections, and for increasing the sensitivity and specificity of diagnosis.

As shown in present study, positive percentage of ELISA and PCR were revealed a significant elevation in their values with increasing the age of a cow, crossbreed animals, tick infestation, presence of sheep at the same pasture, and cows within a herd size of >25 ones. Although several studies have identified the risk factors of infection in cattle, their respective quantitative contributions to the transmission or spreading of infection are still unknown (37, 38). Regarding to age, Seo et al. (2017) referred to that the early exposure and continues infection in addition to occupational or environmental influences, breeding may play a role in development of infection (39). In this study, it is suggested that diminishing immunity can occur along with increasing the age as a result concurrent different infection, and high level of stress particularly in cows due to large milk production and pregnancies. However, all these reasons might act together for increasing of susceptibility. Crossbreed study cows were showed a high positivity rather than local and pure breeds; and this might belong to that different breeds might have variation in genetic map which was reflected on their sensitivity or resistance to infection in addition to management factors that played a great role in controlling of a disease.

Although, ticks were not essential in the natural cycle of *C. burnetii* in livestock, they formed part of the transmission cycle of the organism from animals to animals and from animals to humans (40, 41). Mediannikov et al. (2010) reported that at least viable *Coxiella* might survive for a long time in ticks, particularly soft tick, and transmit of organism transovarially and transstadially, and may secret via feces, saliva, and coxal fluid (42). Pandit et al. (2016) found that among all the new herd infection, 925 were attributed to an airborne transmission and the rest to cattle trade (3).

Even though most of the recent human outbreaks were known to originate from small ruminants (sheep and goats), the considered role of these animals in transmission of infection to other domestic animals remained to be elucidated. In this study, the finding showed that there was a high positive rate of infected cows that lived and fed at the same pasture. Intensive cattle farming with high prevalence of infection could become a concern for public health. In this study, a large herd size was associated with *C. burnetii* positivity, consistent with other studies (22, 43).

Although there was no evidence for *C. burnetii* being associated with herd outbreaks of abortion in cattle, number of studies concluded that this organism was an infrequent cause of abortion in cattle (44, 45). In Iraq, brucellosis has been recognized as the main causes of

decreased reproductive efficiency and abortions in cattle as well as in sheep (46, 47). For this reason, many abortion cases attributed to brucellosis might be largely correlated with coxiellosis. In a recent study, the authors showed that the prevalence of brucellosis in cattle was 0.97%, while 3.23% of which were positives to *Coxiella* (49). Other study detected no positive results for *B. Abortus*, but a high prevalence for *C. burnetii* (52.9%) was found in group of cattle (49). Low milk production detected in positive study cows was one of the most clinical signs that reported in cattle infected by brucellosis (50). These findings indicated that the clinical signs of reproductive pathogens might be shared leading to misdiagnosis of the real pathogenic cause.

In this study, phylogenetic analysis of local *C. burnetii* strains was revealed on a high relative identity with GenBank-NCBI Netherland strains/isolates NL3262 which originated from the largest Global human outbreak (51). These findings indicated that the local strains might be descended from the ancestor of Dutch strains/isolates and that certain factors might be played a role in distribution of the ancestor around the world such as moving of animals and humans in addition to importation and exportation processes.

The positive findings of present study demonstrated that the percentage of infected cows in Iraq is relatively high. ELISA and PCR can provide more reliable findings. Age, breed, ticks, sheep, and herd size as risk factors showed a significant association with bovine coxiellosis. Further assessment needs to be performed to ensure the correct identification and areas subjected to the increased risk of *C. burnetii* infections. *Coxiella* should also be listed as abortive pathogen. There is a high level of identity detected between the local *C. burnetii* strains reported in this study and GenBank-NCBI Dutch strains/isolates. Additional studies in other areas/herds are required to establish epidemiological database.

CONTRIBUTIONS

This work was part of PhD dissertation in the Department of Internal and Preventive Veterinary Medicine, University of Baghdad (Baghdad, Iraq). Hasanain A.J. Gharban was responsible on the work as a PhD student and Prof. Dr. Afaf Abdulrahman Yousif as the supervisor.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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التحري المصلي والجزيئي الجيني للكوكسيلا بورنيتي في الأبقار المُدرة للحليب، العراق

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الخلاصة

أجريت الدراسة الحالية للتحري عن انتشار اصابات الكوكسيلا بورنيتي في الابقار باستخدام تقنية المقايسة الامتصاصية المناعية للأنزيم المرتبط (الاليزا)؛ وتقنية تفاعل البلمرة المتسلسل للكشف عن الجين IS111A transposase المسؤول عن الكوكسيلا بورنيتي. اختير لهذا الغرض اجماليا 130 بقرة من مناطق مختلفة في محافظة واسط والتي خضعت الى جمع عينات الدم والحليب خلال الفترة من تشرين الثاني 2018 الى ايار 2019. سجلت النتائج الكلية ان 16.15 % و10 % من حيوانات الدراسة كانت موجبة للاليزا وتفاعل البلمرة المتسلسل على التوالي. لوحظ وجود علاقة معنوية ($P < 0.05$) بين النتائج الموجبة والبيانات السريرية. تم تحليل ناتج تفاعل البلمرة المتسلسل لأثنين من العينات الموجبة عرقياً، والتي تم تسميتهما بالكوكسيلا بورنيتي IQ-No.5 والكوكسيلا بورنيتي IQ-No.6 وتسجيلهما في المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI) تحت الرقمين التسلسلين MN473204.1 و MN473205.1. كشف التماثل المقارن للعتر المحلية مع عتر/ عزلات بنك جينات المركز الوطني لمعلومات التقنية الحيوية عن نسبة التشابه 97 %؛ ونسبة طفرات / تغيرات جينية 0.1-0.6 %. سجل تتابع التطابق للمركز الوطني لمعلومات التكنولوجيا الحيوية بأن هناك نسبة تطابق معنوي عالية ($P < 0.05$) بين العترتين المحليتين؛ الكوكسيلا بورنيتي Q-No.5والكوكسيلا بورنيتي IQ-No.6؛ وعزلة هولندا للكوكسيلا بورنيتي 3345937 (CP014354.1) بلغت 99.10% و99.06% على التوالي. استنتجت الدراسة الحالية ان نسبة الابقار المصابة بداء الكوكسيلا عالية نسبيا، وان جرثومة الكوكسيلا يجب ان تدرج ضمن قائمة الممرضات المجهضة. لذلك، يجب اجراء دراسات اضافية متضمنة حيوانات، عينات، ومناطق مختلفة.

الكلمات المفتاحية: كوكسيلا بورنيتي، ابقار، عراق، جين *IS1111A transposase* ، الاليزا ، تفاعل البلمرة المتسلسل