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RESEARCH ARTICLE

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Molecular identification and phylogenetic analysis of *Chlamydophila abortus* isolated from sheep and goats

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ABSTRACT

Chlamydophila abortus is one of the major causes of pregnancy failure (abortion) in sheep and goats in many countries. In the present study, milk samples from sheep and goat herds of West Azerbaijan, Iran were examined for C. *abortus* using PCR and nucleotide sequencing. A total number of 360 milk samples were randomly collected from sheep (n=180) and goats (n=180) of three different regions of West Azerbaijan province during 2018. DNA was isolated from samples and the nested-PCR was employed targeting the *16S rRNA* gene for detection of *Chlamydia spp.* The *omp* gene was amplified and sequenced for the characterization of detected *C. abortus.*The results showed that 8.61% (95% CI: 6.13%–11.96%) of the examined samples (11.67% sheep and 5.56% goat milk samples) were positive for *C. abortus.* The frequency of positive samples in the central region was significantly higher than in other regions. Positive samples for *C. abortus* from animals with a history of abortion were significantly higher than those without a history of abortion. Positive samples in autumn were significantly higher than the other seasons and also, in animals more than four years old were significantly higher than other age groups. Sheep infection was significantly higher than the goats. Phylogenetic analysis based on the helicase gene showed that two sequenced isolates clustered closely with the other *C. abortus* isolates reported in the GenBank. In conclusion, small ruminants in West Azerbaijan province were contaminated with C. *abortus* and they could shed this organism into the milk.

Keywords

Chlamydophila abortus, omp gene, nested-PCR, helicase gene

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Abbreviations

C. abortus: Chlamydophila abortus
PCR: polymerase chain reaction
PZ: plasticity zone
OMP: outer membrane protein

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Introduction

The *Chlamydia psittaci* serotype 1 or *Chlamydophila abortus* is a non-motile, coccoid, obligate intracellular parasite from the family *Chlamydiaceae*. It has been recently given a new classification with 11 separate species. It is important as a causative agent of the reproductive system in a small ruminant [1]. *C. abortus* causes enzootic abortion in sheep, which is an infectious disease featured with placentitis and abortion. The complication decreases the breeding rate of sheep in different countries and it is not limited to sheep as it also affects goats and cattle [2-4]. The infected animals demonstrate no clinical signs until abortion or delivery of very weak lambs [2-4]. Normally, abortion happens during the last 2-3 weeks of pregnancy. There are reports that the abortion rate in one year age is low and increased to thirty percent at the age of two, followed by a 5-10% increase in the third year [5]. There are reports of latent infections for more than three years [6]. Pathological findings in this infection can begin in the third month of pregnancy. The finding in the placenta is exudate with yellow color, thickness in the membrane of cotyledons, and cotyledon color change to red [7].

To have a direct diagnosis, pathogen isolation, direct microscopic examination, serological tests, and DNA-based methods are used [8, 9]. Isolation of *chlamydia* in cell culture is not easy and time-consuming. In the CFT test, cross-reactions between *C. abortus* and other bacteria like *Acinetobacter* can be observed [4]. There has been a surge in the conventional and real-time PCR use to identify *C. abortus* in clinical samples. To this end, the PCR methods are used with amplification on the chlamydial outer membrane protein (*omp1, ompA,* and *omp2* genes), genes encoding 16S rRNA and helicase, the polymorphic membrane gene *pmp*, and the 16S-23S rRNA intergenic interval [10-12]. It is important to use rapid and reliable diagnostic tests for fast disease control. A highly sensitive method to find highly low copy number target DNA is nested-PCR. The nested-PCR method can find a variety of fastidious microorganisms with a significant increase in sensitivity and specificity [13].

While there are serological studies on *C. abortus* infection, our knowledge of the prevalence of *C. abortus* infections in sheep and goats' milk in Iran is very limited. To date, seven *C. abortus* genome sequences have been published [14, 15]. The UK strain S26/3 was the first reference genome, comprised of a 1.1 Mb chromosome and, unlike other *Chlamydia*, lacked any virulence-associated plasmid [16]. Two Greek isolates, LLG and POS, originating from the aborted fetuses of a goat and sheep respectively, represent the most diverse variant strains identified to date, with a further closely related strain recently described [17]. Two molecular typing schemes exist, using either multiple-locus variable-number tandem repeat analysis (MLVA), which has only identified seven MLVA sequence types (MTs) [18], or multiple locus sequence typing (MLST) [18], where only six MLST sequence types (STs) have been defined. This is in sharp contrast to greater diversity in other species of *Chlamydia* [19]. Studies on limited numbers of samples suggest that *C. abortus* isolates in livestock appear to be largely monomorphic: low diversity is observed throughout the genome, even within the plasticity zone [PZ], a region of high genomic variation in other chlamydial species [15, 16]. Infections of *C. abortus* in sheep and goats have generally been documented serologically in West Azerbaijan province, in the North West of Iran. However, studies on pathogen isolation and detection by PCR are rare in this region. Therefore, we conducted this study to isolate *C. abortus* in sheep and goats in West Azerbaijan province, Iran, using the nested-PCR technique. We also conducted a phylogenetic analysis to compare our isolates with other *Chlamydia* species that were deposited in GenBank based on partial helicase gene sequence.

Results

Amplification of 16SrRNA gene

Among 360 milk samples collected from sheep and goats, 31 samples (8.61%, 95% CI = 6.13–11.96) were positive for *chlamydia spp.* amplifying a fragment of 127 bp of the *16S rRNA* gene using nested-PCR. The prevalence of *Chlamydia spp.* in the milk of two examined species were statistically significant. The prevalence of *C. abortus* infection was significantly different in terms of regions with the highest frequency in the central region (Table 1). Animals with abortion history showed higher number of positive milk samples for C. abortus. In terms of seasonal prevalence, the highest number of positive samples for *C. abortus* was recorded in autumn. *C. abortus* was found in 17.24% of milk samples from animals more than 4-year-old, which was significantly higher than the other age groups (Table 1).

Amplification of ompA gene

Among 31 positive samples (*16S-rRNA* gene), all samples were positive with *C. abortus* amplifying a fragment of 479-bp of the *ompA* gene using PCR. In this study, only 31 cases (8.61%, 95% CI = 6.13–11.96) were identified as *C. abortus* in sheep and goats by producing a 479-bp fragment using PCR. (Figure 1).

Table 1.
The epidemiological characteristics associated with *C. abortus* prevalence in sheep and goats in West Azerbaijan province, Iran

Variable	Category	No. of examined milk samples (No. of herd)	No. of positive sheep's milk samples (No. of herd)	No. of positive goat's milk samples (No. of herd)	Frequency of positive milk samples (%95 CI)	*p* value
Region	South	120 (6)	3 (2)	1 (1)	3. 33 (2.6.-7.98)	0.00068
	North	106 (5)	4 (2)	2 (1)	5.66 (3.01-10.41)	
	Center	134 (7)	14 (5)	7 (4)	15.67 (10.48-22.77)	
History of abortion	With history	160	15	8	14.38 (9.78-20.65)	0.00048
	Without history	200	6	2	4.00 (2.04-7.69)	
Season	Spring	99	5	1	6.06 (1.68-19.61)	0.000022
	Summer	88	1	1	2.22 (0.87-5.57)	
	Autumn	87	9	7	18.18 (12.38-25.71)	
	Winter	86	6	1	8.13 (2.79-3.01)	
Age	< 2 years	109	2	1	2.75 (0.94-7.78)	0.015
	2 – 4 years	222	15	8	10.36 (7.00-15.07)	
	> 4 years	29	4	1	17.24 (7.60-34.55)	
Species	Sheep	180	21	-	11.66	0.038
	Goat	180	-	10	5.55	
Total		**360 (18)**	**31 (9)**		**% 8.611 (%50)**	

Gel Extraction:

To identify *C. abortus*, PCR products containing 343-bp gene fragments were sent to (Pishgam Biotechnology Co.) for sequencing after gel purification (Expin Combo GP-mini, Co Gene All, South Korea). (Figure 2).

Phylogenetic inferences

The phylogenetic tree was constructed based on the neighbor-joining analysis of helicase partial gene. This analysis revealed that two isolates of *C. abortus* were closely clustered with other *C. abortus* isolates from GenBank with more than 99.0% similarity (Figure 3).

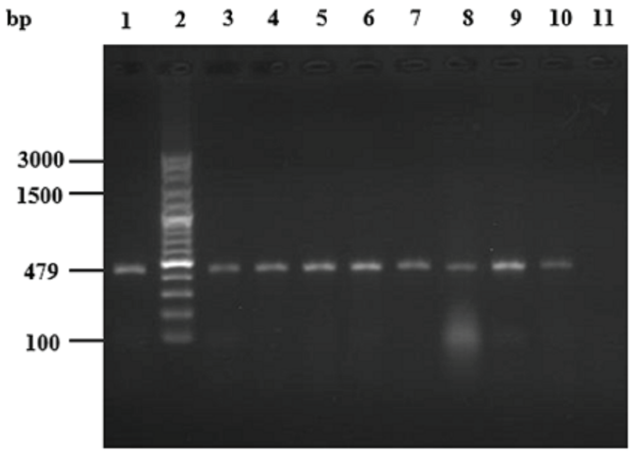

Figure 1.
Agarose gel image of an amplified fragment of the *C. abortus ompA* gene (479 bp) using PCR. Lane 1, Positive control; Lane M, 100 bp DNA size marker (SMOBIO Technology INC., Taiwan); Lanes 2, 3, 4, 5, 6, 7, 8, and 9, positive samples for *C. abortus*; Lane 10, negative control.

Discussion

Chlamydia is the cause of a variety of pathological syndromes in small ruminants. Among many, the most common clinical expression of infection is abortion that brings notable financial losses and risks to human health, particularly in pregnant women [3]. The present study was the first-ever epidemiological study on *C. abortus* in sheep and goats in West Azerbaijan province. Results of the present study revealed

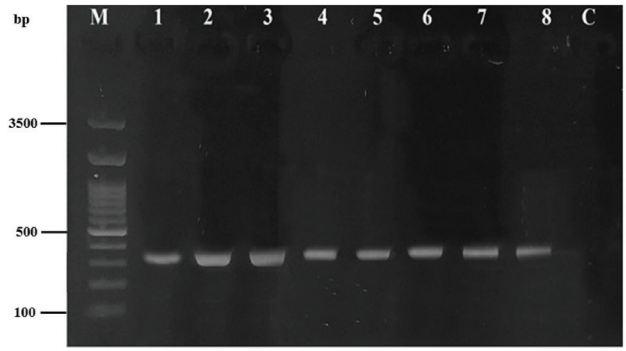

Figure 2.
PCR amplification of partial helicase gene from *C. abortus* bacteria. M: 100-bp DNA size marker; Positive samples: 1, 2, 3, 4, 5, 6, 7, and 8; C: Negative control

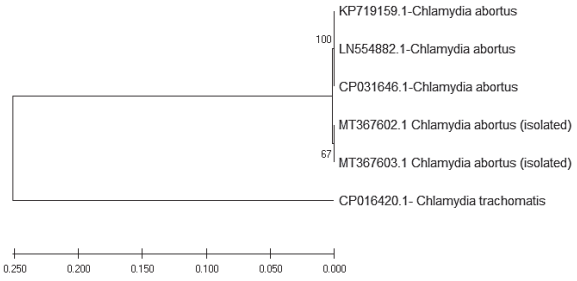

Figure 3.
Evolutionary analyses were conducted in MEGA X blast to show phylogenetic positioning of MT367602.1 and MT367603- *C. abortus* based on partial helicase gene, employing maximum likelihood available in GenBank sequences. Numbers on nodes indicate the bootstrap values.

that 8.1% of all examined raw milk samples were positive for *Chlamydia spp.* The prevalence of *C. abortus* in sheep and goats' raw milk has been reported by many researchers from Iran and other countries [24-26]. In a study in Mexico, the prevalence of *C. abortus* in goat milk was reported at 4.87% using the ELISA test [27]. Serological studies showed that 9% and 25% of sheep flocks of Khuzestan and Shahre-Kord provinces in Iran had antibodies against *C. abortus,* respectively [26, 28]. Pinheiro Junior et al. showed that 21.5% of sheep in Alagoas-Brasilia had antibodies against *C. abortus* and 77.7% of the population had at least one seropositive animal [29]. Huang et al. reported that 20.9% of Tibetan sheep had chlamydial antibodies [30].

The results of the current study showed that the prevalence of the *C. abortus* in sheep's milk (11.67%) was significantly more than goats' milk (5.56%). A study in Mexico showed that the prevalence of *C. abortus* in sheep and goats was 22.6% and 4.9% respectively [27]. The prevalence of *C. abortus* in sheep and goats with an abortion history was 14.38% which was significantly higher than of those without abortion history [4%]. This finding is consistent with other studies reported from Iran and other countries [24, 28]. The reason for this finding is that protective immunity does not develop when non-pregnant sheep are infected, and it can result in abortion [31, 32].

In a study in Iran, the prevalence of *C. abortus* in milk and other samples conducted by the molecular method in Tehran, Lorestan, Qom, Fars, Bushehr, East Azerbaijan, Khuzestan, and Chaharmahal va Bakhtiari provinces and results were 37.7%, 32.9%, 30.3%, 30.3%, 19.6%, 17.5%, 15.6%, and 52%, respectively [26]. The last reported finding is in accordance with those of Zaibet et al. [33] who reported that the risk of chlamydial infection in sheep is multiplied by 4 and 1.08 in the presence of cattle and goats on the same farm. We also found that flocks exposed to Chlamydiae showed not only risks of abortions, but also high sheep mortality rates. Indeed, chlamydial infection induces high animal mortality that finally reduces the financial capital of breeders and increases the costs of production [34]. Although the proportion of the positive sample in female sheep and goats was higher than male animals, sex was not significantly associated with the chance of seropositivity. Also, the seroprevalence of antibodies against *C. abortus* was not statistically different among age categories. Similar results were reported by McCauley et al. [35] who studied on seroprevalence of *C. abortus* in Australian sheep and Cubero-Pablo et al. [36] who reported seroepidemiology of chlamydial infection of wild ruminants in Spain. In a study in Turkey, the prevalence of *C. abortus* in ovine was 2.1% [37]. In other countries, the prevalence of chlamydia in milk was reported in the range of 3.70–61.0% so that the maximum and minimum prevalence of chlamydia in milk belonged to Sweden and Germany, respectively [27, 38-43].

It was shown that the geographical location may be a risk factor for *C. abortus* infection in sheep and goats. In the present study, the results showed that in the central region, the frequency of positive milk samples for *C. abortus* was 15.67%, nearly five times higher than in the south region (3.33%). This finding indicates that the sheep and goats populations in the south region are at lower risk of infection. The highest prevalence of *C. abortus* was in the region surrounded by mountains and Plateau [24, 26, 28]. The reasons for these differences in the prevalence of *C. abortus* might be the high density of livestock population in the central region than south of West Azerbaijan province.

The results of the present study showed that animals in the age group >4-year-old had the highest

prevalence of *C. abortus* (17.24%), which is in agreement with other studies [2, 44-46]. Qin et al. reported a significant correlation between age and infection, a by the increase of the age, the seroprevalence of *C. abortus* infection went up all the time, indicating that there may be a cumulative likelihood for exposure to *C. abortus* infection with age [47]. In a study by Esmaeili et al., the prevalence of *C. abortus* infection in small ruminant flocks according to age was 23.91% in the age group of 5-6 years [45].

The seasonal prevalence of *C. abortus* infection ranged from 2.22% to 18.11%. The highest prevalence (18.11%) was in autumn, and the lowest was in summer (2.22%). This finding of the current study was in agreement with similar studies from Iran and other countries [48]. In a study by Shi-Feng Hu et al. [48] in a seasonal survey of the *C. abortus*, the higher prevalence was in the autumn season.

In the present study phylogenetic analysis based on helicase gene revealed that the two sequenced isolates were almost identical with more than 99% similarity with the other *C. abortus* isolates from GenBank. In a study by Seth-Smith et al., phylogenetic analysis of a total of 64 genomes shows a deep structural division within the *C. abortus* species with a major clade displaying limited diversity. Also, the number of variable nucleotide positions across the sampled isolates is significantly lower than those published for *C. trachomatis* and *C. psittaci* [49]. Finally, regarding the public health issue of chlamydiosis, we suggest serological and molecular surveys on other species of livestock in West Azerbaijan province and other provinces of Iran will be necessary to clarify the picture of *C. abortus* infection in the country.

The prevalence of *C. abortus* infection in sheep and goats' milk was determined for the first time in West Azerbaijan, Iran. It was concluded that sheep and goats can play an important role in the epidemiology of Chlamydiosis as the reservoir for *C. abortus*. Our study showed that genetic diversity appears to be very stable. The molecular detection of *C. abortus* using the nested-PCR method in milk samples showed that PCR can be used as an easy and reliable approach for detecting *C. abortus*. The prevalence of *C. abortus* was higher in sheep's milk than in goat. Therefore, the consumption of sheep milk exposes humans to a higher risk of Chlamydial infection.

Materials & Methods

Study areas

West Azerbaijan province is in the northwest of Iran with over 3.5 m population. Urmia city is the center of the province. This province has diverse climates and geographical areas (e.g. relatively flat terrains, mountains, and the coasts of Urmia Lake). The climate is mostly featured with rainy winds of the Mediterranean and the Atlantic Ocean (https://www.britannica.com/place/Azerbaijan-region-Iran) (Figure 4). This province is very important in agricultural and animal production in Iran.

Milk sampling

A total number of 360 milk samples were randomly collected from sheep (n=180) and goats (n=180) belong to 36 different flocks in West Azerbaijan Province. The flocks were selected from three different geographical regions including the north, center, and south of the province. A total of 160 milk samples were from flocks with abortion history, and the other 200 samples were taken from flocks without abortion history throughout the year 2018. The sampled animals were classified into three age groups (<2 years old, 2-4 years old, and >4 years old). We avoided sampling from pregnant, early lactating dairy animals (<100 DIM) because the metabolic stress of early lactation may lead to immunosup-

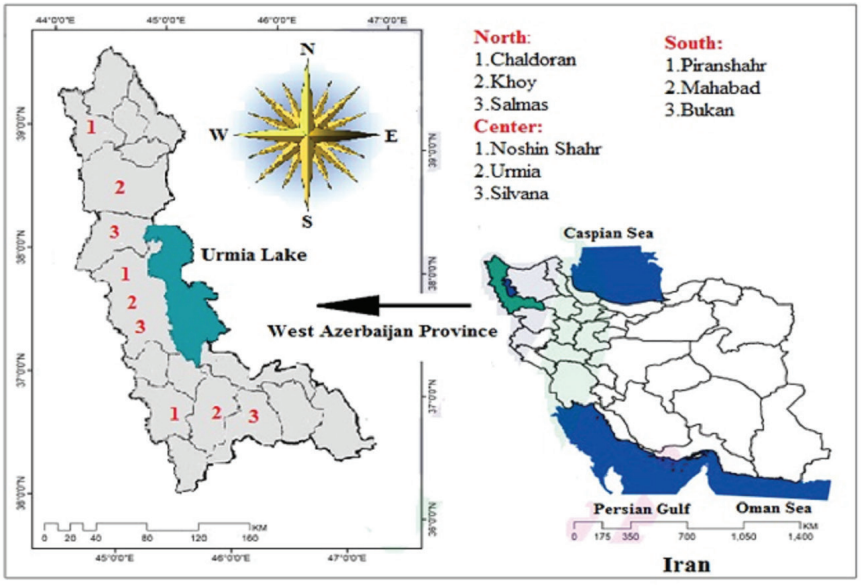

Figure 4.
The schematic map of the study areas, West Azerbaijan, Iran.

pression and therefore, an increase in disease susceptibility [20]. There has been no attempt to determine the cause of abortion in flocks with abortion. The milk samples were placed next to ice and transferred to the microbiology laboratory of the faculty of veterinary medicine.

DNA Extraction

Initially, the samples processed following a protocol described by White et al. [21]. Genomic DNA Extraction Kit (Favorgen, Taiwan) was used to extract DNA from milk samples according to kit's manufacturer instructions. The extracted DNA was quantified using NanoDrop 2000c (Thermo Scientific, USA) and kept at -20˚C until later use in PCR.

Amplification of 16s rRNA gene using Nested-PCR

Nested-PCR targeting the *16S rRNA* gene was used for molecular detection of *Chlamydia spp.* Using primers described by Messmer et al. [21] and modified by Longbottom et al. [8, 22] (Table 2). The first stage of the nested-PCR carried out by using Taq DNA Polymerase Master Mix RED (Amplicon, Denmark). The PCR reaction was prepared in 25 µl volume consisting of 5 µl of DNA template, 50 picomole of each primer (16SIGF, 16SIGR), 12.5µl of the master mix, and 6.5 µl of distilled water. For the second stage, PCR reaction was prepared as described above, except for the DNA template, for which 2.5 µl of 1:100 diluted PCR product from the first stage was used. The thermal cycling condition was described according to Messmer et al. [21]. The PCR products were electrophoresed on a 1.5% and 2% agarose gel stained with safe stain (Quanta. England) for stages 1 and 2, respectively. The gels were visualized through Ingenius Gel Documentation (Syngene Bio-Imaging, UK) (Figure 5).

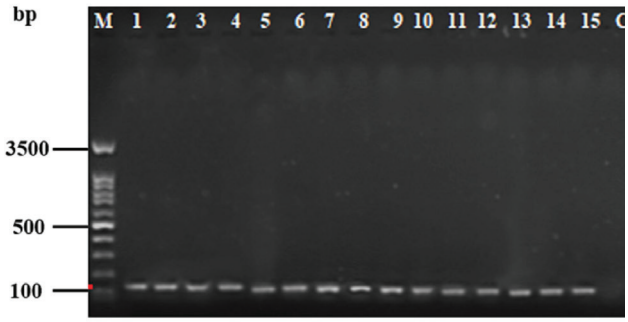

Figure 5.
Agarose gel image of an amplified fragment of the *C. abortus 16S rRNA* gene (127 bp) using nested-PCR. Lane 1, Positive control; Lane M, 100-bp DNA size marker (SMOBIO Technology Inc., Taiwan); Lanes 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, and 15, positive samples for *C. abortus*; Lane C, negative control.

Amplification of the ompA gene in C. abortus

For the identification of *C. abortus ompA* gene was amplified using primers previously described by Creelan et al. (Table 2). The PCR reaction was performed in 25 µl volume comprising 5 µl of extracted DNA, 50 pmol of each primer (ompA 1, ompA 2), and 12.5µl of master mix. Amplification conditions were described according to Creelan et al. Amplified products were electrophoresed on 1% (w/v) agarose gel containing safe stain and then visualized using Ingenius Gel Documentation (Syngene Bio-Imaging, UK) (Figure 1).

Table 2.
The list of primers used for detection of *C. abortus* based on 16S *rRNA*, *ompA* and helicase genes.

Application	Primer Name	Sequence (5' to 3')	Amplicon length (bp)	PCR step (°C/seconds)				cycles
				pre denaturation	Denaturation	Annealing	Extension	
PCR	16SIGF	ACGGAATAATGACTTCGG	436	95/180	94/30	70/30	72/45	45
	16SIGR	TACCTGGTACGCTCAATT						
Nested PCR	F	ATAATGACTTCGGTTGTTATT	127	95/120	94/60	55/30	72/60	35
	R	TGTTTTAGATGCCTAAACAT						
PCR	OMPA-1	TGGTATTCTTGCCGATGAC	479	95/180	94/30	70/30	72/45	45
	OMPA-2	GATCGTAACTGCTTAATAAACCG						
PCR	12SM-FW	CTAGAGGAGCCTGTTCTATAATCGATAA	343	93/120	93/30	63/30	72/45	40
	12SBT-REV2	AAATAGGGTTAGATGCACTGAATCCAT						

Amplification of the Helicase gene in C. abortus

In order to amplify the helicase gene was used the PCR primers and conditions described by Cantekin, et al. [23] (Table 2).

Nucleotide sequencing

The PCR products of the helicase gene of *C. abortus* isolates were sent to SinaClon Company (Tehran, Iran) for sequencing. The obtained nucleotide sequences of the helicase gene were searched against GenBank (National Centre for Biotechnology Information, Rockville Pike, and Bethesda, USA) using the advanced BLAST similarity search option and compared to the helicase sequences of *Chlamydia spp.* from GenBank. Nucleotide sequences were aligned and compared to other nucleotide sequences from GenBank using Clustal-W and phylogenetic tree was generated using the neighbor-joining method in MEGA software (version X; Biodesign Institute, Tempe, USA).

Statistical analysis of data

The epidemiological data were analyzed using the *Chi*-square test in SPSS version 22 (IBM Corp. Armonk, NY, USA). Differences with a p value <0.05 were considered significant.

Authors' Contributions

FT and AO conceived and planned the experiments. FT, AO, and KM carried out the experiments. FT and AO contributed to sample preparation. AO and KM contributed to the interpretation of the results. All authors took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analyses, and the manuscript.

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Competing Interests

The authors declare that there is no conflict of interest.

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