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Phylogenetic Analysis and Certain Risk Factors of *Chlamydia Abortus* in Ewes in Nineveh Governorate, Iraq

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Abstract

Chlamydia is an obligate intracellular Gram-negative organism that causes abortion, or "enzootic abortion of ewes", which is characterized by causing economic loss in sheep breeding. *Chlamydia abortus* spread could be stopped, and monitoring efforts could be more successful by gathering data on the condition of the disease. This ground-breaking study focused on the phylogenetic analysis and certain risk factors of *Chlamydia abortus* in ewes in Nineveh governorate, Iraq. 52 placenta samples were collected, representing 52 flocks (8660 ewes) who suffered from abortion in various Nineveh provincial locations between August 2022 and January 2023. Two local sequences were submitted to GenBank and assigned the accession numbers OR334580 and OR334581. The *Chlamydia abortus* 16S rRNA sequences were analyzed using the GenomeNet multiple sequence alignment tool (CLUSTALW), an online resource. After that, the sequences were compared to other *Chlamydia abortus* sequences that were accessible in the GenBank using NCBI BLAST (BLASTn) of NCBI. The Tamura-Nei model in the MEGA11 software program was used to create the phylogenetic trees. Results indicate that 15.38% (8/52) of the ewes had positive results for *Chlamydia abortus,* which was significantly affected by the increase in herd size and interspecies farming. Two sequences were put through individual sequence analysis, which revealed new *Chlamydia abortus* sequences, which were recorded for the first time in the governorate of Nineveh. We concluded that a higher risk of infection occurs in farms that contain more than 100 ewes and are mixed with other species of animals.

Keywards: Chlamydia abortus, phylogenetic, Risk factors, sheep, Nineveh-Iraq

التحليل الجيني وبعض عوامل الخطورة للكلاميديا المجهضة في النعاج في محافظة نينوى، العراق

خلاصة

الكلاميديا كائن حي سالب الجرام داخل الخلايا تسبب إجهاض المتدثرة "الإجهاض المتوطن في النعاج"، والذي يؤدي الى خسائر اقتصادية في تربية الأغنام. يمكن إيقاف انتشار الكلاميديا المجهضة، ويمكن أن تكون جهود المراقبة أكثر نجاحًا من خلال جمع البيانات عن حالة المرض. تضمنت الدراسة على تحليل النشوء والتطور وعدد من عوامل الخطورة ذات العلاقة بإجهاض الكلاميديا في النعاج في محافظة نينوى في العراق، تم جمع 52 عينة مشيمة تمثل 52 قطيعًا (8660 نعجة) عانت من الإجهاض في مناطق مختلفة من محافظة نينوى من في الفترة بين اب 2022 وكانون الثاني 2023، تم تقديم تسلسلين محليين إلى البنك الجيني وتعيين أرقام الانضمام OR334580 و OR334581 . تم إجراء تحليل تسلسل للجين 16S rRNA للكلاميديا المجهضة باستعمال أداة محاذاة التسلسل المتعددة GenomeNet (CLUSTALW). ثم تمت مطابقة التسلسلات الجينية مع السلالات الأخرى للكلاميديا المجهضة والموجودة في بنك جينات المركز الوطني لمعلومات التكنولوجيا الحيوية GenBank باستخدام NCBI BLAST (BLASTn). تم استخدام نموذج Tamura-Nei في برنامج MEGA11 لإنشاء شجرة النشوء والتطور. بينت النتائج ان 15.38٪ (52/8) من النعاج موجبة للكلاميديا المجهضة، وقد تأثرت هذه النسبة بشكل معنوي بحجم القطيع والتربية المختلطة، كما تم اخضاع تسلسلين من الجينات المحلية لتحليل التسلسل الفردي، والذي كشف عن تسلسل جديد للكلاميديا المجهضة وسجل لأول مرة في محافظة نينوى. خلص البحث إلى أن اعلى خطر للإصابة يحدث في المزارع التي تحتوي على أكثر من 100 من النعاج وتختلط بأنواع أخرى من الحيوانات.

Introduction:

Abortion is one of the most prevalent issues facing sheep breeders, which is simply the ending of a pregnancy at any time because of an infection and, less frequently, non-infectious diseases [1]. The most common infectious causes are Brucella [2], Toxoplasma [3], and Chlamydia, which I have dealt with in this study. Chlamydia is a Gram-negative, obligate intracellular, organism that has a unique biphasic life cycle; they have two morphological structures called: elementary body (EB), considered the infective form, and reticulate body (RB), an active form of the bacteria [4]. Greig first identified chlamydial abortion in 1936 under the name "enzootic abortion of ewes" (EAE) [5] and the first detection in Iraq was done by [6]. Chlamydial abortion results in a significant reproductive loss in numerous sheep-farming regions around the world, with New Zealand and Australia standing out as notable exceptions [7], Goats are also affected, as are less frequently cattle and deer. It is especially common in herds that are intensively managed throughout the lambing season, and it continues to be the most frequent reason for ewe abortions in several countries worldwide [8].

In scientific reports, more than 60 chlamydial diseases are known in mammals, in addition to infecting 465 species of domestic and wild birds, as well as causing 30 diseases in humans, this is because they closely resemble in their biological characteristics that they are only represented by a single Chlamydial genus, which includes all presently defined species [9]

Chlamydia is classified as a biological paradox. One paradoxical trait is the group's unified antigenic structure; this antigenic unit includes a wide range of pathogenicity, which explains the importance of these organisms should not be overlooked [10]. The OIE has listed Chlamydia as a notifiable disease [11]. Among the most important of these types are *Chlamydia abortus*, which causes Chlamydial abortion, a disease of high importance that is also referred to as Enzootic abortion in ewes (EAE), and Ovine enzootic abortion (OEA) which is endemic in sheep.

Chlamydia can be diagnosed by many techniques and confirmed using a polymerase chain reaction (PCR) which allows direct identification of the causal agent and species differentiation based on DNA from clinical samples [12]. Many different PCR protocols have been proposed. It has been established that PCR is highly durable for regular use and further confirmation assays and has proven to be the most delicate among several protocols [13], but the fact is that primers have been created. based on the traditional classification of species according to the latter classification [14; 15], and some tests that could be pertinent to veterinary diagnostic laboratories often rely on published conventional PCR techniques on specific genetic targets such as the 16S rRNA gene, OMP2, and the MOMP gene, each specific to an antigen region, between a specific genus and species [16].

Middle Eastern countries have reported chlamydia. Therefore, numerous studies have been accomplished in Turkey, Chlamydia was identified by PCR technique in 6% of the vaginal swab samples of ewes and goats [17], and in Iran [18] found that (24.1%) of sheep have *Chlamydia abortus* by real-time PCR technique, furthermore, in Saudi Arabia, Chlamydia was isolated from tissue samples taken from goat and sheep, like aborted fetuses and vaginal swabs. [19].

This study used conventional polymerase chain reaction for the first time in the governorate of Nineveh to find *Chlamydia abortus*. (c-PCR), even though there have been numerous studies

to detect chlamydia by various other diagnostic methods in sheep in Iraq in general [20; 21; 22], and in Nineveh in particular [23;24], and that as mentioned [25] *Chlamydia abortus* is among the major causes of abortion in Nineveh.

Although there is another study for the molecular detection of Chlamydia in sheep in Sulaimani Province, Northern Iraq [26], we conducted our study to detect *Chlamydia abortus* using conventional polymerase chain reaction (c-PCR) targeting 16S rRNA gene

Previously, in the Nineveh governorate no phylogenetic study of *Chlamydia abortus* has been published. The current study attempts to validate the results of the phylogenetic analysis, furthermore, herd size and interspecies mixing as risk factors associated with *Chlamydia abortus* in ewes.

Materials and methods

Ethical approval

The Institutional Animal Care and Use Committee of the University of Mosul College of Veterinary Medicine granted ethical permission for this work. (UM.VET.2022.069) on 15 Sep 2022.

Samples collection

Our study was conducted on 52 placenta samples representing 52 flocks (8660 ewes) who suffered from abortion. Samples were attended from ewes 1-3 years of age 1-2 weeks after abortion of local breed from various areas of Nineveh governorate, Iraq. The study was conducted from August 2022 to January 2023. Intensive or Semi-intensive breeding strategy was used in most of the included farms. Data about management were recorded including herd size and interspecies farming. The herd size was ≤ 100 and >100 and, some of these farms raised and bred mixed with other breeds. Biopsies were taken from the placenta, which was carried in specialized containers and kept between 4 and 8 °C in a refrigerator with a buffer solution (Sucrose-Phosphate-Glutamate Buffer, SPG Buffer) was added to them [27], It was kept at a temperature of (-20°C) until the diagnosis was made by PCR.

Extraction and Amplification of DNA

The samples' DNA was extracted using the (ADDBIO, South Korea), DNA extraction kit. The process was followed according to the manufacturer's guidelines. Primers prepared from (Macrogen, South Korea) were used to amplify specific parts of DNA (16S rRNA) during the polymerase chain reaction, forward primer 16SIGF (5'-GATGAGGCATGCAAGTCGAACG -3') and reverse primer 16SIGR (5'-CCAGTGTTGGCGGTCAATCTCTC - 3') with the amplification size 278 base pair [28].

Sequencing of DNA

For sequencing and purification, Two PCR amplicons from ewes were sent to Macrogen Company (South Korea) after they tested positive for PCR. Using multiple sequence alignment and the online program GenomeNet (CLUSTALW), NCBI BLAST (BLASTn) from NCBI (http://www.ncbi.nlm.nih.gov) evaluated the 16S rRNA sequences. The results were compared to other *Chlamydia abortus* sequences that were accessible in GenBank. Using bootstrap analysis and the Likelihood method on the Tamura-Nei model in MEGA11 software (1000 replicates), [29]. Moreover, phylogenetic trees were constructed using the Chlamydia psittaci (16S rRNA gene sequences, MK788308) as an outgroup.

Statistical analysis

Data were analyzed by IBM-SPSS Version 22 (Inc., Chicago, USA), using the X^2-test and Fischer's exact tests to evaluate the variation in prevalence across the main risk factors for *Chlamydia abortus*. Data was considered

statistically significant when the P value was ≤ 0.05.

Results and discussion

Eight positive samples were identified to have two Chlamydia abortus sequences in Nineveh governorate using individual sequence analysis (BLASTn). Following their submission to GenBank, these two sequences (n = 2) were assigned the accession codes OR334580 and OR334581. These sequences were 100% identical. Table 1

It was feasible to show how closely connected the local sequence was to those of many other nations by comparing the sequences. DNA sequences with numbers registered in the Genome Banks of various nations, such as (CP024084, U76710, and NR_111993) in the United States [30, 31, 32] and (CP021996) in China [36], and (LN554883, LN554882, CP070224, CR848038, Z49871) in the United Kingdom [26, 33, 34, 35], and (EF486854 and EF486853) in Greece [37], are present and are highly similar to those local sequences by 100%.

Furthermore, a phylogenetic tree analysis using the Tamura-Nei model in the MEGA11 software program revealed that the local Chlamydia abortus sequence was closely related (100% identity) to the other Chlamydia abortus sequences that were available in the GenBank database. The tree was rooted using Chlamydia psittaci (MK788308), which functioned as an outgroup (Figure 1). Following the reconfiguration of the 1000-nucleotide sequences, the phylogenetic tree of the local Chlamydia abortus genetic sequences shows a strong evolutionary relationship with the genetic sequences and shares common phylogenetic characteristics with the genetic sequences of Chlamydia abortus registered in the gene bank from all over the world under the aforementioned serial numbers and countries at a rate of 100%. Once Bootstrap MEGA11-analysis was used [29].

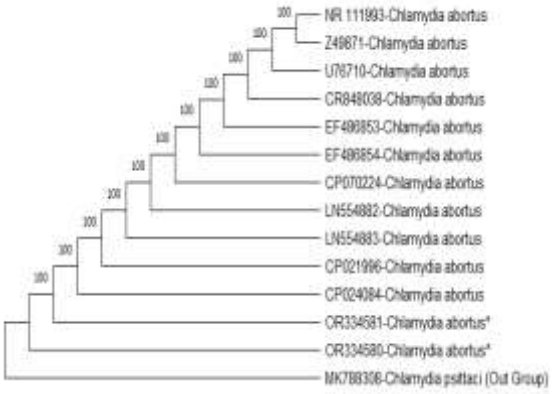

Figure 1 demonstrates the 16S rRNA partial sequences that were used to construct the *Chlamydia abortus* evolutionary tree. The local *Chlamydia abortus* sequences are represented by the written code with (*), and the outgroup used was *Chlamydia psittaci* (MK788308).

Eight out of 52 samples (15.38%) had positive results from conventional polymerase chain reaction (c-PCR) using the 16S rRNA gene. Additionally, a significant difference (P <0.05) in Chlamydia abortus is found in connection to herd size, as indicated by Table 3, which shows that farms with more than 100 heads had a higher probability of infection (odds ratio =11.11, Cl: 1.255–9.849), P = 0.017. Furthermore, there was a higher incidence of *Chlamydia abortus* in mixed farms (P<0.05) (odds ratio = 5.66, cl: 1.149–27.944), P = 0.03.

Table 1: The nucleotide sequence of 16S rRNA gene for the local *Chlamydia abortus* in sheep *Nineveh governorate,* Iraq.

local strain	Gene	Sequence	Accession No.
Chlamydia abortus	16S rRNA	TCAGTCCCAGTGTTGGCGGTCAATCTCTCAATCCGCCTAGACGTCA AAACCTTGGTAGGCCATTACCCCACCAACAAGCTGATATCCCATAG ACTCTCCCTTAACCGAAAGGTCCGAAGATCCCCTTCTTTAATATGT TTTAGATGCCTAAACATACCACATTCGGTATTAGCGGTCGTTTCCAA CCGTTATTCCCAAGTTGAGGACAGATTATCTATGTATTACTAACCCT TCCGCCACTAAATAACAACCGAAGTCATTATTCCGT	OR334580
		TCAGTCCCAGTGTTGGCGGTCAATCTCTCAATCCGCCTAGACGTCA AAACCTTGGTAGGCCATTACCCCACCAACAAGCTGATATCCCATAG ACTCTCCCTTAACCGAAAGGTCCGAAGATCCCCTTCTTTAATATGT TTTAGATGCCTAAACATACCACATTCGGTATTAGCGGTCGTTTCCAA CCGTTATTCCCAAGTTGAGGACAGATTATCTATGTATTACTAACCCT TCCGCCACTAAATAACAACCGAAGTCATTATTCCGT	OR334581

Table 2: Homology by using BLASTn between the local *Chlamydia abortus* sequence and other sequences

Name of strains	NCBI No.	Query cover	Identity	Country
Chlamydia abortus strain GIMC 2006:CabB577, complete sequence.	CP024084	100%	100%	USA
Chlamydia abortus strain GN6 chromosome, complete genome.	CP021996	100%	100%	China
Chlamydophila abortus strain 1H genome assembly, chromosome: 1	LN554883	100%	100%	United Kingdom
Chlamydophila abortus genome assembly CAAB7, chromosome : 1	LN554882	100%	100%	United Kingdom
Chlamydia abortus strain MRI-10/19 chromosome, complete genome	CP070224	100%	100%	United Kingdom
Chlamydophila abortus strain FAG 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	EF486854	100%	100%	Greece
Chlamydophila abortus strain FAS 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence	EF486853	100%	100%	Greece
Chlamydophila abortus strain S26/3, a complete genome	CR848038	100%	100%	United Kingdom
Chlamydophila abortus strains EBA 16S and 23S ribosomal RNA operon, Complete sequence.	U76710	100%	100%	USA
Chlamydia abortus strain B577 16S ribosomal RNA, partial sequence.	NR_111993	100%	100%	USA
Chlamydophila abortus 16S rRNA gene for 16S ribosomal RNA.	Z49871	100%	100%	United Kingdom

Table 3: The risk variables of *Chlamydia abortus* in ewes based on c-PCR technique

Factors	No. of tested samples	No. of +ve (%)	CI	OR	P
Herd size	52	8 (15.38%)			
≤100	28	1 (3.57%) [a]		1	
>100	24	7 (29.16%) [b]	1.255-9.849	11.11	0.017
Interspecies					
Non-mixed	37	3 (8.10%) [a]		1	
Mixed	15	5 (33.33%) [b]	1.149-27.944	5.66	0.03

CI: Confidence of interval, OR: Odds ratio,P:P value

In ewes and goats, *chlamydia abortus* was discovered, which has a major negative influence on reproductive health. In this study, the incidence of abortion was 15.38%. The lack of control measures may be a major contributing cause to the illness prevalence in the governorate of Nineveh. Furthermore, bringing animals into Iraq from nations like Turkey and Iran, where *Chlamydia abortus* is common, is may be the major contributor to the spread of infected animals in the country. This outcome is consistent with the explanation provided by [18].

The prevalence of chlamydia infection in sheep varies according to the country or study area. Previous serological studies carried out in Iraq indicate that the infection rates of chlamydia in sheep were 32.22% and 29.44%, respectively [38, 39]. The transmission of diseases between sheep and other farm animals, such as goats, dairy, and beef cattle, is another factor that may greatly raise the incidence of disease. These results are in line with those of [26], who discovered that despite the widespread belief that *Chlamydia abortus* is the cause of sheep sickness, it may infect a wide range of domestic animals, people, and wild animals. Furthermore, fluid discharges from infected animals can transfer *Chlamydia abortus* through embryonic tissue.According to this study, large-size herds (>100) had a much higher frequency of the EAE than small-size herds. This outcome aligns with the findings of [18, 19]. The data discrepancy could be attributed to various factors, such as direct pasture contact, continuous animal introduction, housing space constraints, breeding, susceptibility, mortality, and the presence of multiple animal species in one collection, which could suggest a higher chance of infection. This study found that there was a substantial difference in the prevalence of *Chlamydia abortus* in sheep between Non-mixed and Mixed touched animals, with the contacted animals showing a higher risk (odds ratio = 5.66). This result was in line with the reports [18, 40].

Conclusion

The study's conclusions indicate that sheep in Iraq's Nineveh Governorate are particularly susceptible to *Chlamydia abortus*. Disease incidence is significantly influenced by herd size and interspecies management. There has never before been published phylogenetic data on *Chlamydia abortus* in the Nineveh province of Iraq. The current study suggests further thorough investigation of the Nineveh governorate's ruminant population's contamination with *Chlamydia abortus*.

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Conflict of interest

According to the authors, there are no conflicts of interest with this paper.

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