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Serological and molecular diagnosis of *Neospora caninum* from ewe milk in Al-Diwaniyah province, Iraq

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Abstract

Neospora caninum is a protozoan parasite with a global distribution, and there is increasing evidence of its association with abortion in infected ewes. Based on our knowledge, there is no clear information regarding the existence of this infectious agent in ewe milk in Iraq. According to this, the current study was conducted to identify the presence of *N. caninum* in ewe milk in Al-Diwaniyah Province. Ninety-six milk samples were collected from ewes in different study area sites. These samples were subjected to ELISA, PCR, and Nc5-partial gene sequencing. The sequencing was followed by a phylogenetic study to identify genetic evolution. The ELISA findings revealed the presence of the parasitic antigen in 23/96 (23.96%), while PCR revealed only 5/96 (5.2%). However, The PCR-positive samples were sequenced, and the phylogenetic results demonstrated that the *N. caninum* isolates were closely similar to those recovered from milk, brain, and milk samples from different countries, such as New Zealand and the United States. The present study indicates that *Neospora caninum* has existed in milk samples from ewes in Al-Diwaniyah Province. This presence could indicate an essential link to abortion in ewes. The genetic evolution may provide substantial information that the current study isolates may have descended from USA or New Zealand isolates due to different means of dissemination, such as traveling and importing animals.

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Introduction

Neospora caninum, a protozoan from the apicomplexan phylum, has a wide geographical distribution and exerts substantial financial burdens on farmers and the livestock sector (1-5). The life cycle of the parasitic organism encompasses many phases, including the tachyzoite, tissue cyst, and oocyst. Ruminants primarily serve as intermediate hosts, whereas canines function as final hosts (6). The transmission of this parasite occurs both laterally and vertically among herds. A placental infection during pregnancy may lead to outcomes such as abortion, stillbirth, or the delivery of an asymptomatic diseased animal. The longevity of this pathogen within farms and herds is notable, and its spread by congenital means, which is the primary cause of abortion attributed to *N. caninum*, is crucial in facilitating its persistence over extended periods (7-10). While cattle are considered the primary host for *N. caninum*, it is worth noting that spontaneous infections have also been documented in other ruminant species, such as sheep and goats. *N. caninum* infection in sheep and goats exhibits notable variations globally across continents and nations (11). The observed variations in seroprevalence could be attributed to distinct features inherent to each region, including climate variables, inequalities in animal nutrition and health handling practices, fluctuations in serological diagnostic methods employed, alterations in sheep and goat communities, and dissimilarities in study design (12). According to the results obtained from comprehensive reviews and meta-analyses, the zero incidence of *N. caninum* infection in sheep and goats throughout the globe was determined to be 12% and 6%, respectively. Sheep often

engage in grazing behavior, rendering them more susceptible to infections near the ground, unlike goats, who mostly exhibit browsing behavior. The induction of *N. caninum* infection in small ruminants during gestation leads to a physiological state similar to that described in bovines. Nevertheless, the comprehensive understanding of *Neospora*'s clinical, epidemiological, and economic significance in sheep and goats remains incomplete, mainly owing to the scarcity of research papers conducted so far (13,14). The precise etiology of abortion often remains unclear due to the involvement of a diverse array of variables. However, infectious etiologies seem more prevalent in sheep and goats. The accurate identification of abortions necessitates the use of a specialist veterinary laboratory, resulting in a significant proportion of abortions being misdiagnosed (15). The global economic impact of reproductive failure in ruminants resulting from *N. caninum* infection is believed to be around 1.3 billion dollars per year. Consequently, the significance of this infection concerning the occurrence of abortion in sheep and goats should not be disregarded. In order to identify *N. caninum* infection, experts have conducted investigations into several diagnostic techniques that exhibit varying degrees of sensitivity and specificity. These techniques include histopathological, immunohistochemistry, serological, and PCR (16-21). The existing research on *N. caninum* infection in sheep and goats is currently restricted. Nevertheless, there is a lack of extensive study that aims to gather and methodically evaluate this particular field (22).

Neospora caninum is a worldwide transmitted protozoan in sheep and reports increasingly showed its relation to abortion in infected ewes. Based on our knowledge, there is no clear information regarding the existence of this infectious agent in ewe milk in Al-Diwaniyah Province, Iraq. According to this, the current study was conducted to identify the presence of *N. caninum* in ewe milk in this region.

Materials and methods

Ethical approve

Write the name of scientific or institutional board that give the ethical approve to conduct this scientific work and give the approval issue number and date. The study protocol was approved for the animal care and use by the College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah city, Iraq. The study was approved in the 22nd of November, 2022, under the No. 1891.

Samples

Ninety-six milk samples were collected from ewes in Al-Diwaniyah province, Iraq. The samples were collected in sterilized containers. These milk-filled containers were transported in a cool box to the Laboratory of Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah city, Iraq.

Indirect ELISA

The indirect-Elisa test was performed using a kit and following its procedure steps, in which 100µl of milk was used. Ovine antibodies were used in the current test. The optical density (OD) used was at 450nm and employed an ELISA reader (BioTek, USA). The following equation was followed for the interpretation of the results. S/P%=(OD sample-OD NC/OD PC-OD NC)*100. S/P%≤20% means negative. S/P%≥20% means positive.

Polymerase chain reaction

The milk samples were subjected to ADDBio Kit (South Korea), using the kit protocol to perform the extraction. The DNA extracted was NanoDrop-estimated for its quality and quantity. The DNA was stored in a -20°C-freezer for later work. The PCR was performed utilizing an ADDBio Kit (South Korea) for the master mix. The reaction of 20µl of total volume included the master mix at 10µl (20mM pH=8.8 tris-HCl, 100mM KCl, 4mM $MgCl_2$, loading dye, 0.5mM dNTPs, and 2x Taq polymerase), 1.5µl for each direction of the primer (0.5 pmol/20µl), 2µl DNA, and 5µl PCR water. The Nc5-gene-primer set was F: CAGTCAACCTACGTCTTC and R: GTGCGTCCAATCCTGTAA (23). The agarose 1.5% gel electrophoresis was done at 100 volts and 80Amp. The PCR products were explored under a UV-equipped machine.

PCR-positive product-based sequencing

The PCR products were sent to sequencing at Macrogen Company (Korea). The obtained files were processed, and the phylogenetic study was conducted using the NCBI-related websites and MEGA X software.

Results

The result of PCR revealed that only 5/96 (5.2%) (Figure 1). However, the ELISA findings revealed the presence of the parasitic antigen in 23/96 (23.96%).

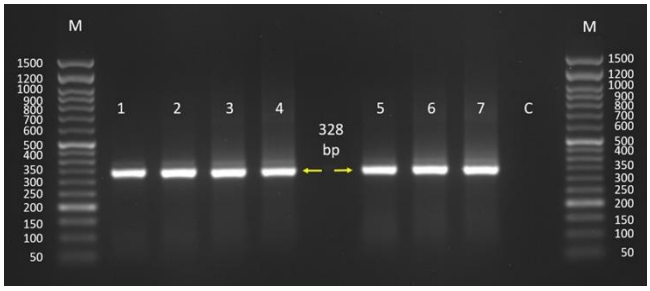

Figure 1: Image of 1.5% agarose gel electrophoresis of the NC5 gene of *Neospora caninum* from milk samples of ewes. Lanes, 1-7: Positive PCR at 328bp, C: Negative control (no DNA was added in the PCR reaction), and M: Ladder (50-1500bp).

The PCR-positive samples were sequenced, and the phylogenetic results demonstrated that the *N. caninum* isolates were closely similar to those recovered from milk, brain, and milk samples from different countries, such as New Zealand and the United States (Table 1 and Figure 2).

Table 1: The NCBI-BLAST Homology Sequence identity (%) in *Neospora caninum* isolates of local sheep based on the NC5 gene, and these isolates are compared with other world isolates.

	Obtained Accession number	Source	Bank Accession number	Country	Identity	Query	Host
1	OQ054165	Brain	X84238	Switzerland	99.32%	100%	Mice
2	OQ054166	Brain	AY459289	New Zealand	100%	95%	Cattle
3	OQ054167	Brain	LN714488	UK	98.29%	100%	no
4	OQ054168	Brain	JF827721	USA	98.63%	99%	Wolf
5	OQ054169	Blood	KF649847	USA	97.95%	100%	Wolf
6	OQ054170	Blood	MT709295	Iran	96.9%	98%	Cattle
7	OQ054171	Blood	KP715560	Italy	97.67%	100%	Deer
8	OR125081	Milk	KF649847	USA	97.59%	99%	Wolf
9	OR125082	Milk	KU253799	Australia	98.29%	100%	Dog
10	OR125083	Milk	LN714488	UK	98.29%	100%	no
11	OR125084	Milk	AY459289	New Zealand	98.49%	100%	Cattle
12	OR125085	Milk	MT709295	Iran	96.21%	98%	Cattle

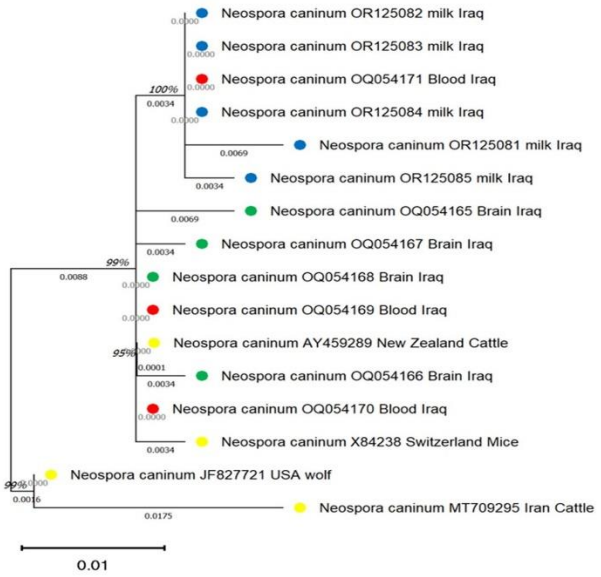

Figure 2: Phylogenetic tree of the partial *Nc5* gene sequencing of *Neospora caninum* from the milk of ewes based on the Maximum Likelihood method (500 replicates).

Discussion

Neospora caninum is a pathogenic intracellular parasite that leads to abortion or neonatal death in several animal species, with a specific impact on cattle. Although there have only been a few reports in individual sheep or flocks, neosporosis has sometimes been linked to abortions in sheep. The serological studies conducted in Iraq have provided evidence supporting the potential infections of sheep, goats, and cattle by *N. caninum*; however, evidence is scarce about the occurrence of *N. caninum* in spontaneously infected ovine, specifically in terms of DNA detection. Most available material pertains to experimentally and spontaneously infected cattle (15,24,25).

The ELISA findings demonstrated that the parasite antigen was detected in about 24% of the milk samples. Al-Gharban *et al*. (26) reported that the indirect ELISA and PCR results were 27.22 and 12.36%, respectively. Our results agree with Al-Gharban *et al*. (26), who showed a lower positive rate for the PCR than that from the ELISA. This could be because DNA extraction from milk requires much milk (27). Moreover, it could be because cattle milk has a unique proteolytic system that could destroy DNA but not proteins (28).

The current investigation conducted an in-depth evaluation of the precise number of cases of *N. caninum* in sheep in Al-Diwaniyah Province, Iraq, using DNA PCR-based screening techniques, which showed around 5% occurrence. The obtained results revealed a lower rate than that reported by Al-Shaeli (29), who revealed a prevalence rate of 13.73%. The authors (29) indicated the noteworthy involvement of neosporosis in inducing abortion in sheep, hence emphasizing the criticality of the placenta as a potential reservoir of infection. The findings indicate that molecular analysis of the placenta may be used to identify aborted fetuses affected by ovine neosporosis. This is supported by the observation that the placenta transmits the infection to the fetal tissues (30).

The pathogenesis of neosporosis is initiated with the transmission of the parasite across the placenta to the fetal tissues, resulting in the simultaneous damage of these tissues alongside the immune responses of both the fetus and the mother (31). Nevertheless, it is essential to note that several additional illnesses may contribute to a decrease in

reproductive performance and induce abortion in flocks, which were not involved in the scope of this particular research. The DNA of *N. caninum* can be detected in several tissues, including the brain, heart, kidney, liver, and umbilical cord of aborted fetuses. The DNA may also be detected in the dam's blood. This is particularly important since obtaining fresh placenta within a precise timeframe is sometimes challenging (32-40).

Conclusion

The present study indicates that *Neospora caninum* has existed in milk samples from ewes in Al-Diwaniyah Province. This presence could indicate an essential link to abortion in ewes.

Conflict of interest

This is a declaration that no conflict of interest is found in the current study.

Acknowledgment

The authors would like to thank the owners of the animals for their help in collecting the milk samples from their ewes.

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التحديد المصلي والجزيئي للنيوسبورا الكلبية والمعزولة من حليب النعاج في محافظة الديوانية، العراق

أزهار جفات كروان و منصور جدعان علي خالد

فرع الأحياء المجهرية، كلية الطب البيطري، جامعة القادسية، مدينة الديوانية، العراق

الخلاصة

إن النيوسبورا الكلبية هو من الأوالي المنتشرة في جميع أنحاء العالم في الضأن وأظهرت التقارير بشكل متزايد علاقته بالإجهاض في النعاج المصابة. وبناء على معلوماتنا لا توجد معلومات واضحة عن وجود هذا العامل المعدي في حليب النعاج في العراق. وبناء على ذلك فقد أجريت الدراسة الحالية للتعرف على وجود النيوسبورا الكلبية في حليب النعاج في محافظة الديوانية. تم جمع ستة وتسعين عينة حليب من النعاج في مواقع مختلفة من منطقة الدراسة. تم إخضاع هذه العينات لفحص التسلسل الجيني الجزئي لجين الـ *Nc5* وفحص الادمصاص الإنزيمي المناعي واختبار تفاعل إنزيم البلمرة المتعدد. وأعقب التسلسل دراسة النشوء والتطور لتحديد التطور الجيني. أظهرت نتائج فحص الادمصاص الإنزيمي المناعي وجود المستضد الطفيلي في ٩٦/٢٣ (٢٣,٩٦%) بينما أظهر اختبار تفاعل إنزيم البلمرة المتعدد وجود الطفيلي في ٩٦/٥ (٥,٢%) فقط. وأظهرت النتائج التطورية أن عزلات النيوسبورا الكلبية كانت مشابهة إلى حد كبير لتلك المعزولة من عينات الحليب والمخ من بلدان مختلفة، مثل نيوزيلندا والولايات المتحدة. تشير الدراسة الحالية إلى وجود النيوسبورا الكلبية في عينات حليب النعاج في محافظة الديوانية. يمكن أن يشير هذا الوجود إلى وجود صلة مهمة بالإجهاض في النعاج. قد يوفر التطور الوراثي معلومات جوهرية تفيد بأن عزلات الدراسة الحالية قد تنحدر من عزلات الولايات المتحدة الأمريكية أو نيوزيلندا بسبب اختلاف وسائل الانتشار مثل السفر واستيراد الحيوانات من تلك الدول الى العراق.