Nc-5 gene-dependent molecular identification and phylogenetic investigation of *Neospora caninum* in sheep

A.C. Karawan and M.J. Alkhaled

Department of Microbiology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah, Iraq

**Article information**

**Article history:**
Received 13 September, 2023
Accepted 02 December, 2023
Published online 15 March, 2024

**Keywords:**
Coccidia
Enteroparasites
Protozoa

**Correspondence:**
A.C. Karawan
azhar.karawan@qu.edu.iq

**Abstract**

The current study was conducted to identify the occurrence of *Neospora caninum* in sheep using molecular techniques. The investigation included 200 sheep samples (20 heart and 80 brain tissues and 100 blood) collected from different Al-Qadisiyah Province, Iraq districts. After extracting the DNA, the samples were subjected to molecular techniques, polymerase chain reaction (PCR), and partial gene sequencing accompanied by estimating the phylogenetic status of the *N. caninum* coccidial microorganism. Both techniques relied on detecting the Nc-5 gene, a repetitive region of the organism’s DNA. The finding of the PCR revealed the genetic identification of 7 (3.5%) isolates of *N. caninum* in the samples of the examined sheep. For the sequencing, seven PCR products were detected as *N. caninum* with nucleotide close similarity to isolates from some regions, such as New Zealand and Switzerland. The findings demonstrated by the herein study that sheep can be an intermediate host for *Neospora caninum*; however, all these global isolates were reported only from species other than from sheep; the current study target animals.

**Introduction**

The apicomplexan protozoan, *N. caninum*, causes substantial economic losses for farmers and the animal production sector worldwide. Ruminants serve as intermediate hosts for the parasite throughout its two phases of development (tachyzoite and tissue cyst), whereas canines serve as final hosts. Herds may spread this pathogen both horizontally and vertically. Fetal abortion, stillbirth, or the delivery of an animal with no apparent signs of illness are all possible outcomes of placental infection (1-3). The major pathway of abortion produced by *N. caninum* is congenital transmission, which is why this parasite can live in farms and herds for years. There have been reports of spontaneous infections in other ruminants, such as sheep and goats, even though cattle are the most crucial host for *N. caninum*. Infection rates with *N. caninum* in sheep and goats vary widely between regions and nations (4-8), which have various seroprevalence rates, which may be due to climatic conditions, livestock nutrition, and health care practices (9-12). Published research findings and meta-analyses indicate the seroprevalence of *N. caninum* infections in sheep and goats to be 12.0 and 5.99%, respectively (13). On the other hand, goats are often browsers, whereas sheep spend most of their time grazing, making them more susceptible to infections from the ground (14). When small ruminants were experimentally inoculated with *N. caninum*, pregnant settings comparable to those in cows were generated. Due to a shortage of research, we still do not know the potential of neosporosis via its clinical, epidemiological, and economic impact on sheep and goats (15,16). Abortion causes are often unknown because of the complexity of the underlying mechanisms. However, it seems that infectious factors are the most common cause of mortality in the fetuses of sheep and goats. It is essential to recognize *N. caninum*’s contribution in causing abortion in sheep and goats (17). Multiple diagnostic strategies, including histopathological techniques, immunohistochemistry, serological tools, and PCR, have been developed by scientists to detect *N. caninum* infection in fetal abortions (18-21). Until recently,
neosporosis genetic identification has relied on various genes, including those for internal transcribed spacer (ITS) sequences, 18s rRNA, and the Nc-5 genes. Meanwhile, due to its repetition in the N. caninum sequence, the Nc-5 gene has been shown to have high sensitivity and specificity for identifying neosporosis (22).

The primary reason for conducting this research was to examine the feasibility of employing PCR with primers exclusive for the Nc-5 gene for the diagnosis and genetic identification of neosporosis in sheep in Al-Qadisiyah Province, Iraq.

Materials and methods

Ethical approval

The current study was designed and conducted under the permission provided by the Ethical Approval Committee at the College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq, and according to national and international criteria for the animal care and use.

Samples

The investigation included 200 asymptomatic sheep (2-10 years old) samples (20 heart and 80 brain tissues and 100 blood) collected from different districts of Al-Qadisiyah Province, Iraq, from June 2022 to January 2023. Whole sheep heads and whole hearts were collected and placed in ice-cooled containers for the organs. The collected samples were transported immediately to the Laboratory of Parasitology, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Diwaniyah City, Iraq, where the Lab work was performed directly.

DNA extraction

The DNA materials were extracted using the AddBio extraction kit (Korea) and following its protocol steps. The DNA obtained from the extraction process was evaluated for quality and quantity, utilizing a NanoDrop. The target DNA was placed in a -20°C-storage.

Nc-5 gene PCR

After extracting the DNA, the samples were subjected to PCR. Nc-5 gene-based primers (F: CAGTCAACCTACGTCTTTC and R: GTGCGTCCAATCCTGTAA), design obtained from Li et al. (23), were used in the PCR reaction solution. The PCR solution (20µl of total volume) included (10, 1.5 (0.5pmol/each), 5, and 2µl) of master mix for each of F or R primer, PCR water, and DNA template, respectively. The steps of the thermocycler were under the following conditions: 95°C-5mins, 95°C-30s, 53°C-35s, 72°C-40s, and 72°C-5mins of initial denaturation (one cycle), (denaturation, annealing, and extension) at 38 cycles, and final extension (one cycle). The PCR products of the target Nc-5 gene were 1.5%-agarose-gel-run for an electrophoresis process at 100 volts and 80A for 60 mins. Then, the gel with the PCR product bands was evaluated by employing a UV imager.

Nc-5 gene-specific region sequencing

The PCR-purified products were Nc-5 gene sequenced at the Macrogen company in Korea, recruiting a Sanger sequencing service. After that, the sequences were processed using NCBI-related websites and Mega X software to build a phylogenetic tree (24).

Results

Molecular findings

The finding of the PCR revealed the genetic identification of 7 (3.5%) isolates of N. caninum in the samples of the examined sheep (Figure 1). These positive samples were from the brain samples, with no detection from the heart samples. For the sequencing, seven PCR products were detected as N. caninum with nucleotide close similarity to isolates from some regions, such as New Zealand and Switzerland (Table 1 and Figure 2).

Figure 1: Image of 1.5%-agarose gel for the Nc-5 gene-dependent PCR of Neospora caninum from sheep brain, heart, and blood samples. M: (50-1500bp) scaled ladder and lanes (1 to 7): positive PCR Nc-5 products.

Figure 2: Phylogenetic tree for the Nc-5 gene-dependent sequencing of Neospora caninum from sheep brain, heart, and blood samples.
Discussion

There is an insufficient amount of data detailing the genetic-based detection of silent neosporosis in sheep despite reports of a correlation between ovine abortion and neosporosis (25-29). The current study found that the protozoan caused infection in 7 (3.5%) sheep. This data agrees with Arbabi et al. (30), who reported that the overall infection rate in their sheep samples (hearts and brains) was 3.9% in 13 out of 330 sheep. They found that the parasite was detected in 12 (6.7%) hearts and one (0.7%) brain. Previous research has shown that between 1.1 and 8.3% of sheep in western Iran test positive for antibodies to N. caninum (31,32). The DNA of N. caninum was also found in 8.5% and 0.9% of aborted fetuses in Iran (33,34).

Among 181 goat youngsters studied in Romania, the DNA of N. caninum was found only in 2 (1.1%) of the diaphragm tissues (35). In most investigations, aborted or spontaneously infected sheep brain samples were positive for N. caninum (33,34). For instance, in Brazil, Silva et al. (36) found the DNA of N. caninum in 2 per every 102 (1.9%) sacrificed goats. All heart and tongue specimens were negative for the parasite. However, both test results were extracted from brain tissues. In Brazil, Santos et al. (37) found the DNA of N. caninum in 5% of brain tissues from beef cattle but in 0% of heart tissues. Some research found that the majority of samples tested 6.7% were found in the heart specimens instead of the brain extracts 0.7% (37).

In this work, we employed the Nc-5 gene to identify N. caninum and conduct a phylogenetic analysis. This gene is described as a DNA region with high sensitivity and specificity for identifying neosporosis since it is a repeated DNA fragment of N. caninum (38). Yamage et al. (39) examined the sensitivity and specificity of several primers for diagnosing N. caninum. To detect neosporosis in mice infected experimentally, they examined the sensitivity and specificity of Np1, Np3, Np5, Np7, and Np21 forward primers and Np2, Np4, Np6, and Np8 reverse primers generated from the Nc-5 genes (39). The Np21-Np6, Np7-Np6, and Np21-Np4 primer sets were the only ones out of the 19 tested that could pinpoint a minimum of 10pg DNA with distinct separate bands (39). Similar apicomplexan pathogens, such as Toxoplasma gondii and Sarcocystis species, may be distinguished from N. caninum by its unique Nc-5 gene. This led to the Nc-5 gene being utilized as a susceptible and specific genetic material for identifying neosporosis. Because of its high sensitivity and specificity, the Nc-5 gene was chosen for this study neosporosis identification (30).

For the sequencing, seven PCR products were detected as N. caninum with nucleotide close similarity to isolates from some regions, such as New Zealand and Switzerland. These similarities could be because N. caninum can infect different animal species, which allows the protozoan to disseminate quickly to any country via any tool, such as importing animals (40-47).

Conclusion

The findings demonstrated by the herein-study that sheep can be a host for Neospora caninum, in which similar isolates are isolated from different countries; however, all these global isolates were reported only from species other than from sheep; the current study target animal.

Acknowledgments

The authors would like to thank the College of Veterinary Medicine, University of Al-Qadisiyah.

Conflict of Interest

There is no conflict of interest for the current work.

References


Investigation of *Neospora caninum*, *Hammondia* sp., and *Toxoplasma gondii* in tissues from slaughtered beef cattle in Bahia, Brazil. Parasitol Res. 2010;106(2):457-61. DOI: 10.1007/s00436-009-1686-4


