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Molecular Identification and Histopathological Findings of *Neospora caninum* as a Cause of Bovine Abortion in Some Egyptian Dairy Farms

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ABSTRACT

Neospora caninum infection is a major cause of abortion in dairy and beef cattle in many countries. Fast and accurate diagnosis of neosporosis is still the best disease control strategy. Thus, Np6 and Np21 primer sets were employed for the detection of *N. caninum* in the blood of 139 aborted cows and tissues of 25 foeti during a wave of abortion in five dairy farms in Egypt. The results revealed a 47.5% overall prevalence of *N. caninum* infection in aborted cows and a 64% overall prevalence in foeti. Two farms were PCR negative for the infection, and the other farms presented 45.7%, 87.0% and 90.9% molecular prevalence. The majority of PCR-positive foeti were aborted in the fifth month of pregnancy. The most common PCR-positive fetal tissue was the brain, followed by the heart, liver, stomach contents, and then the lung. The histopathological lesions in 5-month-old aborted foeti were microgliosis in the brain meninges and submeningially and myocarditis in heart tissue. At 7 months of abortion, multifocal necrosis, mononuclear cell infiltration, and neuron degeneration were observed in the brain. Heart tissue showed hemorrhage and necrotic changes. A tissue cyst was observed in the heart of 5-month aborted foeti and the brain of 7-month-old foeti. The sequenced amplicons from aborted cows and foeti were 100% identical to each other. As far as we are aware, our investigation is the first to sequence isolates of *N. caninum* from cattle hosts. The sequences were submitted to GenBank with the accession numbers OR939832.1 and PP708713.1. Multiple sequence alignments revealed variation between the study isolates and other published isolates from different regions and hosts. Phylogeny revealed clustering of our sequences with sequences of isolates from South Korea, China, and Italy. The sequences were distinct from sequences previously isolated from camels in Egypt.

Keywords: Bovine abortion, Histopathology, Nc5 phylogenetic analysis, *N. caninum* tissue cyst, Np6/Np21PCR.

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INTRODUCTION

Neospora caninum is an apicomplexan unicellular parasite. It belongs to the order Eucoccidiorida, phylum Apicomplexa, family Sarcocystidae, and genus *Neospora*. The definitive hosts for *N. caninum* are different species of canines. A variety of domestic and wild ruminants, equids, camels, rodents, and bird species are intermediate hosts **(Dubey and Schares, 2011).** They may acquire infection through the transplacental route (vertical infection) or by ingesting sporulated oocysts with food and water (horizontal infection). Other suggested routes of transmission are via milk and semen **(Dubey *et al.*, 2007).** Notably, dogs may act as both the final and intermediate hosts simultaneously **(Dubey *et al.*, 2005).**

Neospora caninum infection is considered one of the most common causes of reproductive failure in bovines worldwide **(Dubey 2003; Dubey and Schares, 2006).** The disease is latent and asymptomatic in nonpregnant cattle. This makes control of the disease more difficult. In pregnant cattle, the principal signs are abortion, the birth of a diseased calf, or the birth of a persistently infected calf **(Buxton *et al.*, 2002).**

Consequently, it is one of the main causes that affects the livestock industry and can lead to considerable financial losses due to calf loss, the possible loss of milk yield, and reduced male infertility **(Bahrami *et al.*, 2018).** Early detection of neosporosis remains the most effective disease control approach because of the unavailability of vaccines or efficient medications to combat the protozoan *N. caninum* **(Sinnott *et al.*, 2017).**

Since *N. caninum* was discovered in 1984 **(Bjerkas *et al.*, 1984)**, many diagnostic methods have been developed to support disease diagnosis. It is challenging to determine whether *N. caninum* is present in live animals via conventional procedures since the parasite is hard to isolate and typically resides in tissues that are not accessible for sampling **(Dubey *et al.*, 2007).** Seroprevalence provides the basis for epidemiological data on the prevalence of *N. caninum* infection in various regions of the world. While serologic investigation for *N. caninum* is a basic and noninvasive technique that can be used to detect and screen for *N. caninum* infection, the type of ELISA that is utilized can affect the results **(Dubey and Schares, 2006; Almeria, 2013).** Histopathology remains a valuable diagnostic method that aids in our comprehension of how infections proceed. However, it should be carried out in combination with additional verifying diagnostic tests **(Dubey and Schares, 2006).**

The immune system instability that occurs during pregnancy may reactivate a parasite's latent form, turning it into an active tachyzoite that can infect the fetus via transplacental transmission. Thus, it may be feasible to directly identify whole parasites or their DNA in pregnant animals. Compared with conventional approaches, polymerase chain reaction (PCR) currently plays a major role in the identification of *N. caninum* DNA in infected cows and tissues of aborted foeti, yielding greater sensitivity and specificity **(Van Maanen *et al.*, 2004; Dubey and Schares, 2006; Almeria, 2013).** In Egypt, the prevalence of *N. caninum* infection in dairy cattle has reached 24.6%–38.04% **(Gaber *et al.*, 2021; Metwally *et al.*, 2023; Selim *et al.*, 2023).**

However, assessments rely mainly on serological methods that could be affected by fluctuations in *N. caninum*-specific antibodies throughout pregnancy, which may drop below measurable levels. Furthermore, not all infected animals produce a significant antibody response **(Dubey and Schares, 2011**). Therefore, in this study, we aimed to utilize molecular techniques for accurate diagnosis and assessment of *Neospora caninum*-linked bovine abortion on some Egyptian dairy farms along with histopathological investigations.

MATERIALS AND METHODS

Ethical approval

The animal handling and sampling protocols used were approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Veterinary Medicine, Cairo University (Vet CU 25122023833).

Sample collection

One hundred thirty-nine blood samples were collected from Holstein cows subjected to recent abortion at 5 dairy farms located at Ismailia Desert Road, Ismailia governorate, Egypt, in 2021. Blood samples were collected in EDTA and transferred on ice to the laboratory. Furthermore, 25 aborted foeti were collected and subjected to investigation. The ages of the aborted foeti ranged from five to eight months. Portions of aborted fetal tissues (brain, heart, liver, lung, and stomach contents) were chilled on ice and delivered to the laboratory for molecular examination. Other parts (brain, heart) were preserved in 10% neutral-buffered formalin for histopathological investigation.

Genomic DNA extraction

For nucleic acid extraction from cow blood samples, 500 µL of 0.1 M ammonium chloride was mixed with 200 µL of whole blood for RBC lysis. The pellet was suspended in 100 µL of 5% Chelex-100® suspension in TE buffer and 100 µL of 0.002% SDS and then processed according to the methodology described by **Bereczky *et al.*, (2005**). Chelex-100® was also used for DNA extraction from 100 mg of different fetal tissues according to **Collantes-Fernández *et al.*, (2006)**. Finally, the extract was transferred to a clean sterile 1.5 ml Eppendorf tube and stored at -20 °C for *N. caninum* PCR. DNA extraction from the *N. caninum* positive control (mash of *N. caninum* positive brain sample, ID Gene™ *Neospora caninum* Duplex, IDvet Genetics, France) was performed via a GeneDirex Genomic DNA Isolation Kit (GeneDirex, Inc., Taiwan) following the manufacturer's instructions.

Polymerase Chain Reaction

A sensitive and specific *N. caninum* primer pair named Np6 (5′-CAGTCAACCTACGTCTTCT-3′) and Np21 (5′-GTGCGTCCAATCCTGTAAC-3′) was used in the PCR to detect the Nc-5 gene of *N. caninum* DNA **(Yamage *et al.*, 1996)**. PCR was carried out to amplify a fragment 329--336 bp in length in a reaction volume of 25 µl. Each reaction contained 2X Dream™ Green PCR Master Mix (Thermo Scientific, Lithuania), 15 pmol of each primer (forward & reverse), 2 µl of the DNA template, and finally, nuclease-free water up to 25 µl. Positive and negative controls were included in all PCRs. The PCR conditions were optimized as follows: initial denaturation for 4 min at 94 °C; 35 cycles of denaturation at 94 °C for 45 sec, annealing at 54 °C for 30 sec, and extension at 45 °C for 30 sec; and

termination with an extension cycle at 72 °C for 10 min. A SimpliAmp thermocycler (Applied Biosystems, Thermo Fisher Scientific, Singapore) was used for the amplification process. For analysis of the PCR results, 7 µl of each sample was subjected to electrophoresis on a 1.5% agarose gel and then visualized on a UV transilluminator.

DNA Sequencing and Analysis

Two groups of randomly selected PCR amplicons (5 from cows and 5 from aborted foeti) of *N. caninum* were purified according to the manufacturer's instructions for the Gene JET Gel Extraction Kit (Thermo Scientific K0691, Germany). Sequencing (forward and reverse) was performed in an automated DNA sequencer (ABI 3730XL, Applied Biosystems, USA). The results were analyzed, and new sequences were submitted to GenBank. ClustalW was used to align sequences via BioEdit software version 7.2.5 (**Kumar *et al.*, 2016**), and the sequences were compared with available DNA sequences of *N. caninum* in the GenBank database via the NCBI Basic Local Alignment Search Tool (BLAST). Phylogenetic analysis was carried out via the maximum likelihood method on the basis of the Tamura–Nei model (**Tamura and Nei, 1993**) with 1000 bootstrap replicates (**Felsenstein, 1985)**.

Histopathological examination

Brain and heart formalin-fixed tissues were grouped according to fetal age at abortion into 5- and 7-month age groups. Tissues that were positive by *N. caninum* PCR were examined histopathologically for alterations caused by the presence of the parasite in fetus tissues. Thin sections of 4 µm thickness were prepared and stained with hematoxylin and eosin (H&E) to visualize the pathological findings via light microscopy **(Bancroft and Gamble, 2008)**.

RESULTS

PCR results of cow blood and aborted foeti tested for the presence of *N. caninum* DNA

Neospora caninum DNA was successfully detected in the blood of cows subjected to recent abortion **(Fig.1)** on three of the five farms examined **(Table 1).** A high prevalence of *N. caninum* infection was detected in the infected farms (45.7%, 87.0% and 90.9%), with a 47.5% total prevalence in all the examined animals. In the *N. caninum*-infected farms, 50.0%, 75.0% and 100.0% prevalence rates were recorded in aborted foeti, with an overall prevalence of 64.0%.

Table 1: Blood and aborted foeti examination results for detection of *N. caninum* DNA by Nc5- PCR.

Farm	Blood samples (cows)	PCR results		Prevalence (%)	Aborted foeti	PCR results		Prevalence (%)
		+	-			+	-	
1	35	16	19	45.7	16	8	8	50.0
2	46	40	6	87.0	5	5	-	100.0
3	11	10	1	90.9	4	3	1	75.0
4	26	-	26	-	-	-	-	-
5	21	-	21	-	-	-	-	-
Total	139	66	73	47.5	25	16	9	64.0

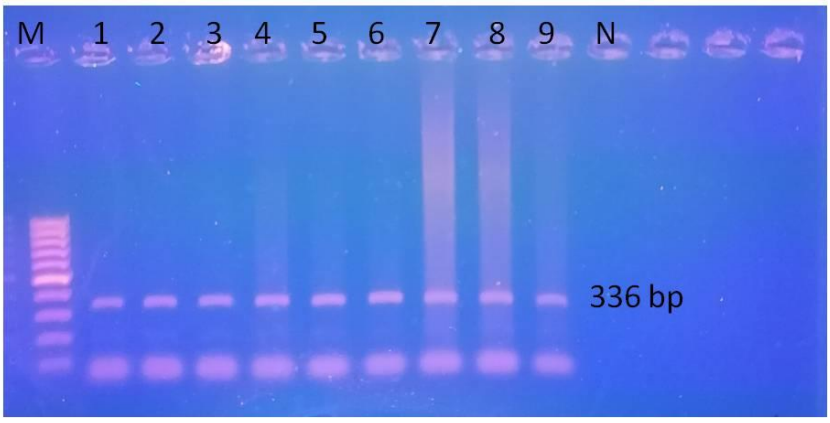

Fig. 1: Agarose gel electrophoresis of *N. caninum* PCR product of aborted cows' blood samples. M: 100 bp ladder; 1: positive control (336 bp); 2-9: *N. caninum* positive blood samples and N: negative control.

PCR examination of fetal tissues **(Fig.2)** revealed that *Neospora caninum* DNA was mostly detected in the brain, followed by the heart, liver, stomach contents and, finally, lung tissues **(Table 2).** Notably, all PCR-positive foeti were brain positive for the presence of *N. caninum* DNA. Moreover, 5-month-old foeti was the predominant age of abortion compared with other ages.

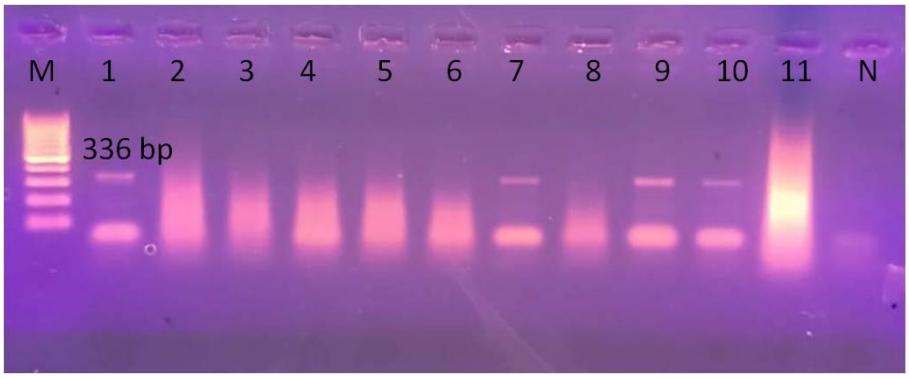

Fig. 2: Agarose gel electrophoresis of *N. caninum* PCR product of different fetal tissues. M: 100 bp ladder; 1: positive control (336 bp); 2-11: different tissue samples; 7, 8,9,10: *N. caninum* positive brain, liver, stomach contents and heart tissues respectively and N: negative control.

Table 2: Tissue samples examination results for presence of *N. caninum* DNA by Nc5- PCR in aborted foeti along with their age

Age of aborted foeti	Total number	Number of positive	Examined tissues				
			Brain	Heart	Liver	Lung	Stomach contents
5 M	20	13	+(13/13)	+(11/13)	+(10/13)	+(6/13)	+(7/13)
6 M	2	-	-	-	-	-	-
7 M	2	2	+ (2/2)	+ (2/2)	NE	NE	+(1/2)
8 M	1	1	+ (1/1)	NE	+ (1/1)	+ (1/1)	-
Total	25	16	16	13	11	7	8

NE: not examined

Sequencing results and phylogenetic analysis of detected *N. caninum* isolates

Alignments of the sequenced amplicons from aborted cows and foeti showed 100% identity with each other. One sequence from each group was submitted to GenBank and is available under the accession numbers OR939832.1 (*Neospora caninum* isolate ARRI1 NC5 marker genomic sequence, fetal brain isolates) and PP708713.1 (*Neospora caninum* isolate ARRI2 NC5 marker genomic sequence, cattle blood isolate). By the nucleotide basic local alignment search tool (nBLAST), we found that the two *N. caninum* nucleotide sequences presented 94.68% to 99.32% identity with previously published *N. caninum* sequences in GenBank. The highest similarity (99.32%) is shared with *N. caninum* isolates from pigs in China (accession no: MT340533.1) and the Liverpool isolate in the UK (accession no: LN714476.1). The sequences shared 98.98% homology with isolates from deer in Italy (accession no: KP715560.1), wolves in the USA (accession no: KF649848.1), and cattle in South Korea (accession no: FJ464412.1). Low similarities were shared with other isolates from dogs in Australia (accession no: KU253799.1, 98.64%), cattle in Italy (accession no: KP715561.1, 98.30%), sheep in Iraq (accession no: OP574221.1, 97.88%), cattle in Iran (accession no: MT955657.1, 97.95%), chickens in Brazil (accession no: EU073600.1, 96.67%), cattle in Italy (accession no: KP715563.1, 96.28%), pigs in China (accession no: MT340529.1, 95.33%), and Pol-B1 clones in Poland (accession no: EF463098.1, 95.02%). Notably, two isolates previously isolated from camels in Egypt (accession no: MF 980926.1, MF980927.1) presented 97.08% and 97.07% similarity with our isolates; however, their coverage percentages were 93% and 92%, respectively.

Multiple sequence alignment **(Fig.3)** revealed variation between our *N. caninum* sequences and the other sequences found in GenBank. A single nucleotide deletion in the Chinese pig *N. caninum* isolate was observed. This nucleotide is represented by a cytosine in our sequences, whereas it is guanine in the other aligned sequences. An additional single nucleotide polymorphism (SNP) is reported in our sequences (A>T). Other sequences show single or multiple SNPs and some exposed insertions of 6-nucleotide segments that do not exist in other sequences

(the Brazil chicken brain isolate, the China pig isolate, the Poland clone isolate, two repeats of the UK NcLIV strain and the Iran cattle isolate).

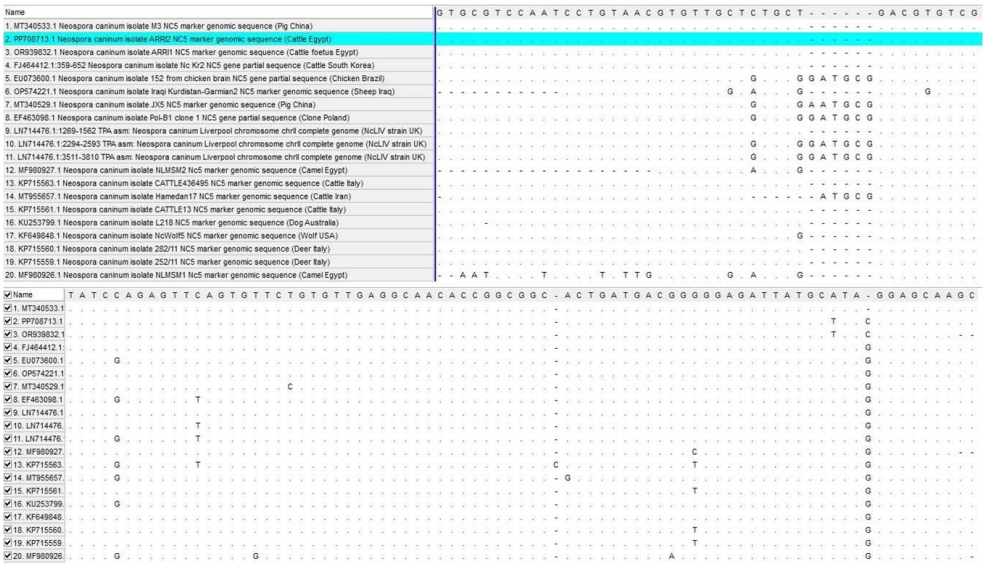

Fig. 3: Variations detected by multiple sequence alignments of obtained *N. caninum* sequences and some sequences retrieved from GenBank.

The Egyptian isolates from camels presented multiple SNP variations compared with our cattle isolates. A bootstrap phylogenetic tree was constructed using 15 partial sequences of the *N. caninum* Nc5 marker genomic sequence and one complete genomic sequence, as well as our two sequences **(Fig. 4).** Clustering of our sequences (indicated by red squares) with sequences of isolates from South Korea, China, the UK and Italy is shown. The sequences were distinct from those of isolates from Brazil, Iran, Iraq, and Poland in addition to the Egyptian camel isolates.

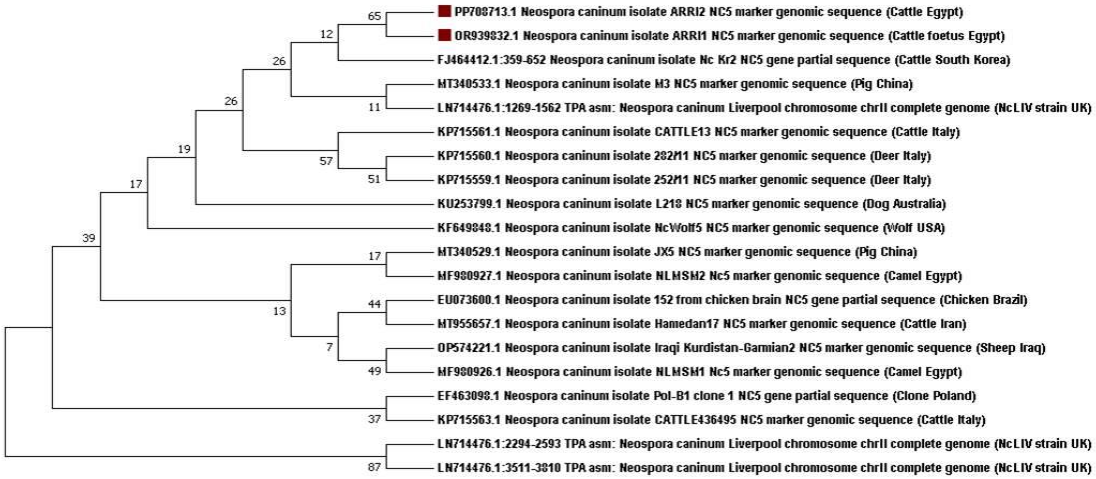

Fig. 4: The bootstrap phylogenetic tree of the Nc5 marker sequence. The tree was constructed from the two study *N. caninum* partial sequence isolates (indicated by the red square) with 15 partial sequences of *N. caninum* Nc5 marker genomic sequence and one complete genomic sequence.

Histopathological findings

In the 5-month-old group of aborted foeti, the lesion in the brain was characterized by microglia scattered throughout the brain, which condensed in the meninges and became submerged (**Fig.5 A)**. Lesions in heart tissues were myocarditis with mild lymphocytic infiltration between muscle fibers. Furthermore, *N. caninum*-like tissue cysts were identified **(Fig. 5B).** In the 7-month-old group of aborted foeti, the lesions in the brain tissue were microgliosis, pericellular edema and demyelination, and *N. caninum*-like tissue cysts were detected **(Fig. 6A).**

Examination revealed the presence of multifocal necrosis surrounded by mononuclear inflammatory cell infiltration **(Fig. 6B).** Congestion of the blood vessels, perivascular and pericellular edema together with perivascular mononuclear cell infiltration, and degeneration of most neurons were further observed **(Fig. 6C).** Heart tissue showed scattered RBCs between muscle fibers, and some muscle fibers showed necrotic changes **(Fig. 6D).**

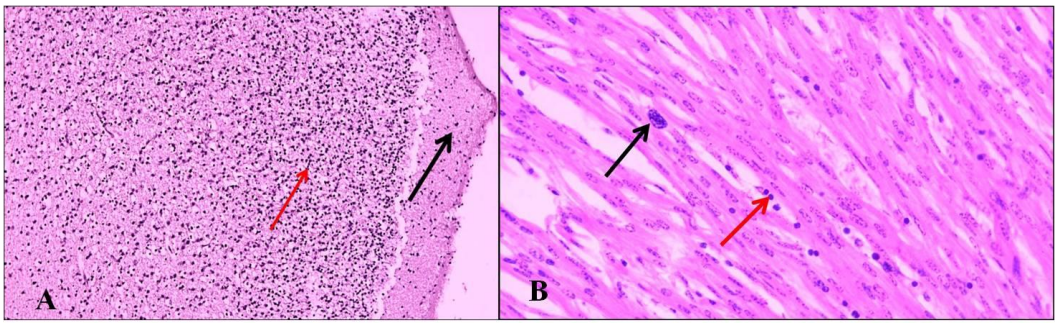

Fig. 5: lesions of 5 month-old *N. caninum* aborted bovine fetus tissues stained with H&E (x100). A: Brain tissue shows dense microgliosis in meninges (black arrow) and submeningeally (red arrow). B: Heart tissue with mild mononuclear cell infiltration between heart muscle fibers (red arrow) and *N. caninum*-like tissue cysts (black arrow)

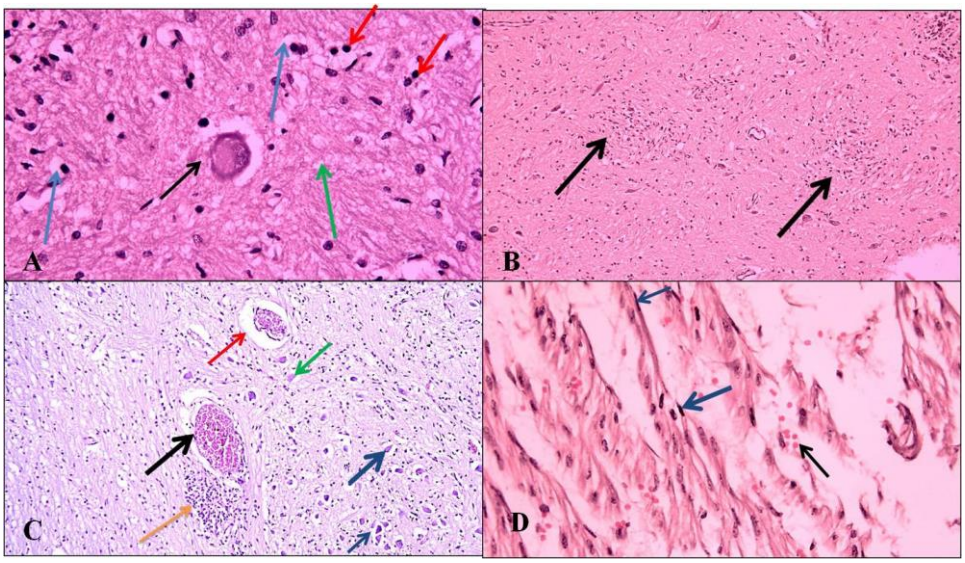

Fig. 6: Lesions of 7-month-old *N. caninum* aborted bovine fetus tissues stained with H&E (x100). A: Brain tissue shows *N. caninum*-like tissue cysts (black arrow), microgliosis (red arrow), pericellular edema (blue arrows), and demyelination (green arrow). B: Brain tissue with multifocal necrosis surrounded by mononuclear inflammatory cell infiltration (black arrows). C: Brain tissue with congested blood vessels (black arrow), perivascular and pericellular edema (red and blue arrows), perivascular mononuclear cell infiltration (orange arrow) and degeneration of most of neurons (green arrow). D: Heart tissue with RBCs scattered between muscle fibers (black arrow) and some of their nuclei show necrotic changes (blue arrow).

DISCUSSION

Neospora caninum is reported to be one of the most efficiently placenta-transmitted parasites among all known microbes in cattle, with up to 90% of cattle in some herds becoming infected (**Dubey *et al.*, 2006**). Most animals suffering from reproductive infections remain asymptomatic; therefore, these diseases are not managed (**Eaglesome and Garcia, 1997**). Moreover, such infected animals are reservoirs of infection to healthy animals, thus complicating the epidemiology and control of these infections. Therefore, it was necessary to use a sensitive method to detect the parasite in different clinical samples even if it was present in very small numbers.

Nucleic acid detection by PCR is a particularly effective approach for the detection of *N. caninum* in bovine blood and aborted foeti. It can amplify small quantities of parasite DNA to detectable levels even from low-quality tissue (autolyzed, mummified, or desiccated tissue). Approximately 3 genome copies of *N. caninum* can be detected via a well-founded conventional PCR test (**Baszler *et al.*, 1999**).

A good extraction technique should be quick, safe, and produce enough genomic DNA for further analysis in a sufficient quantity and of high quality (**Silva *et al.*, 2023**). Historically, techniques for

obtaining DNA from diverse biological sources have involved phases of separation and purification. A significant disadvantage of all of these techniques is the need for substantial amounts of biological samples to extract DNA (**Singh *et al.,* 2018**). In the present study, we employed blood samples from aborted cows for direct detection of *N. caninum* DNA, as the blood was confirmed to be a transport factor for *Neospora* tachyzoites between body tissues (**Okeoma *et al.,* 2004; Al-Kaabi *et al.,* 2023)**. It is likely that blood contains small amounts of parasitic DNA. Consequently, Chelex 100 (an ion-exchanging resin) was used to obtain sufficient amounts of genetic material for PCR, as recommended by **Singer-Sam *et al.,* (1989)**.

Chelex 100 serves as a model for the extraction procedure since it shields the DNA from the effects of boiling, while boiling is helpful for releasing DNA from small amounts of cells. Chelex-based methods are less likely to contain PCR inhibitors than extracts made by proteinase-aiding and phenol–chloroform separation. The possibility of operator-initiated DNA contamination or sample mixing will also be reduced by procedure simplicity, which is demonstrated by fewer steps in sample preparation and transfer (**Walsh *et al.,* 2013**). However, Chelex 100 resin is a quick and affordable method for extracting genomic DNA in addition to being a useful tool in studies with a large number of samples; debris in the DNA solution might cause DNA to degrade when stored for an extended period of time (**Singh *et al.,* 2018**).

The primer pair Np21/Np6 was selected for the current PCR study. It is an oligonucleotide primer that yields superior sensitivity in detecting *N. caninum* DNA without reducing specificity when tested against the DNA of closely related organisms, such as *Toxoplasma gondii*, *Sarcocystis* spp., and *Hammondia hammondi*. This pair has shown the highest practical sensitivity. It can identify a single parasite disseminated in two milligrams of brain tissue (**Yamage *et al.,* 1996**). The results of the PCR investigation of blood samples from aborted cows revealed a 47.5% total molecular prevalence of *N. caninum* infection. The results also revealed that two of the five farms suffering abortion were negative for *N. caninum* infection, which means that there may be other causes of abortion. A high prevalence was found on the other three farms, ranging from 45.7% to 90.9%, which indicates an outbreak of *N. caninum*-associated abortion on these farms.

Significant variations in *N. caninum* prevalence exist among and within countries, among regions, and even between dairy and beef cattle. However, variations in detection methods, study designs, and sample sizes should be taken into consideration (**Dubey *et al.,* 2007a**). The high prevalence rates of *N. caninum* infection in female cattle may indicate a significant risk of transplacental transmission. As *N. caninum* infections do not always result in abortion in pregnant animals, calves from PCR-positive cows may be susceptible to congenital infections and may serve as a possible source of parasite transmission and maintenance on these farms (**Japa *et al.,* 2019**). On the basis of fetal examination by PCR, *N. caninum*-associated abortion in dairy cattle was previously reported in different countries. In Iran, 13%, 11.9%, and 100% of examined foeti in different studies were PCR positive **(Razmi *et al.,* 2007; Salehi *et al.,* 2009; Razmi *et al.,* 2010)**. In Japan, 27% (**Ghanem *et al.,* 2009**), in China, 4% and 57.7% (**Zhang *et al.,* 2007; Yao *et al.,* 2009**), in Romania, 33% **(Suteu *et al.,* 2010)** and in Switzerland, 20.9% (**Reitt *et al.,* 2007**).

In the present study, 64.0% of the aborted foeti were positive for *N. caninum* via PCR, and all of them were aborted in the second term of gestation (5–8 months). A similar recent study in Brazil (**Da Costa *et al.,* 2022**) reported that the majority of abortion cases occurred in the sixth month of pregnancy; however, those with positive PCR results were mostly found in the fifth month, as we found in our study. The transient immune suppression of T lymphocytes recorded in *N. caninum* experimentally infected cattle could be responsible for the increased susceptibility of these animals to parasitaemia during that period (**Innes *et al.,* 2001**). Unfortunately, both naive and chronically *N. caninum*-infected cows suffer abortions. Although the pathogenesis is incompletely understood, placental destruction or liberation of maternal prostaglandins may result in luteolysis and subsequently abortion (**Dubey *et al.,* 2006**).

In this study, the brain was the most frequently PCR-positive tissue in the aborted foeti. This could be due to higher parasite loads corresponding to more parasite multiplication observed in the central nervous system tissue than in other tested organs in the fetus (**Almeria *et al.,* 2016**). This likely improves the capacity to detect parasitic DNA and establishes the brain as the preferred tissue for molecular detection. The study results were consistent with those of previous studies (**Almeria *et al.,* 2010; Pereira *et al.,* 2014; Almeria *et al.,* 2016**), which reported that the CNS of *N. caninum*-infected foeti aborted after 150 days of pregnancy. In addition, the heart was the second most common tissue for PCR detection, followed by the liver, stomach contents and then the lung. Some studies have suggested that the lung is a favorite tissue for *N. caninum* (**Pescador *et al.,* 2007; Rojo-Montejo *et al.,* 2011)**, whereas others have failed to detect parasite DNA in the liver of infected foeti after the fifth month of gestation (**Almeria *et al.,* 2010; Almeria *et al.,* 2016)**. On the other hand, **Regidor-Cerrillo *et al.,* (2014)** reported that all infected foeti had parasites in their liver tissues and that the liver and heart had greater

parasite loads than did the central nervous system after experimental infection in early gestation. In another study in Argentina, 54.4% of PCR-positive tissues were liver, 24.2% were CNS, 33.3% were placenta, and 10.5% were heart **(Moore *et al.*, 2008)**. All of these findings collectively demonstrate that the parasite can infect several organs via a dynamic system **(Almeria *et al.*, 2016)**. Consequently, it is critical to choose the appropriate fetal tissue for parasite detection on the basis of the age of abortion.

The histopathological alterations of the aborted foeti found in this study agreed with the *N. caninum* lesions that other investigations have documented **(Nematollahi *et al.*, 2013; Almeria *et al.*, 2016; Melendez *et al.*, 2021; Da Costa *et al.*, 2022**). Pathologists know from experience that various tissues in aborted foeti infected with *N. caninum* show significant cellular infiltration and necrosis. However, it is incorrect to assume that *N. caninum* is the source of an abortion outbreak if you base your diagnosis only on the findings of fetal histopathology or immunohistochemistry (IHC) (**Wouda *et al.*, 1997**). Our histopathological findings validated the PCR results and provided stronger evidence that *N. caninum* is the cause of abortion. During the investigation, tissue cysts, such as *N. caninum* cysts, were found in H&E-stained sections of brain and heart tissues; however, these cysts had to be confirmed with other tests, such as immunohistochemistry (IHC) or electron microscopy. The morphology of the detected cysts matches that of *Neospora* cysts described in cattle (**Morals *et al.*, 2001; Salehi *et al.*, 2009**).

To the best of our knowledge, this study is the first to perform sequencing of *N. caninum* isolates from cattle hosts. Multiple sequence alignments and phylogenetic analyses revealed variation between the study isolates and other published isolates from different regions and hosts. The isolate sequences were distinct from sequences previously isolated from camels in Egypt (**Ahmed *et al.*, 2017**). Isolates of *N. caninum* vary significantly because the genotypic and phenotypic traits of the species are not strictly conserved **(Al-Qassab *et al.*, 2010)**. The broad distribution, ability to reproduce sexually, and wide intermediate host range all contribute to this expected variety **(Calarco and Ellis, 2020)**.

CONCLUSIONS

In conclusion, this study emphasizes how urgently control strategies must be developed to reduce abortion and economic losses in the area. These findings further support the use of the PCR technique as a sensitive and accurate way to identify *N. caninum* infections in different samples. It is essential to assess the genomic sequence of isolates from different hosts and various geographic locations to create a sequence map and identify the diversity, pathogenesis, virulence, and source of *Neospora* infection.

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Author contribution

The study design and conception: WMAM, SZA, TSB, RMH. Sample collection: WMAM, RMH. Molecular experiments: RMH, TSB. Histopathology: HAA, RMH. Data analysis: WMAM, SZA, TSB, RMH. The original draft of the manuscript: RMH, TSB. Revision and editing: WMAM, SZA, HAA, TSB, RMH. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

REFERENCES

AHMED, N. E., AL–AKABWAY, L. M., RAMADAN, M. Y., EL-GAWAD, A., MOHAMED, S., and MOUSTAFA, M., 2017. Serological and PCR-sequencing assays for diagnosis of *Toxoplasma gondii* and *Neospora caninum* infecting camels in Egypt. Benha Veterinary Medical Journal, 33 (2), pp.200-210. https://doi.org/10.21608/bvmj.2017.30466

AL-QASSAB, S. E., REICHEL, M. P., and ELLIS, J., 2010. On the biological and genetic diversity in *Neospora caninum*. Diversity 2, 27. https://doi.org/10.3390/d2030411

ALMERÍA, S., ARAUJO, R., TUO, W., LOPEZ-GATIUS, F., DUBEY, J., P., and GASBARRE, L., C., 2010. Fetal death in cows experimentally infected with *Neospora caninum* at 110 days of gestation. Veterinary Parasitology, 169 (3-4), pp.304-311. https://doi.org/10.1016/j.vetpar.2009.12.044

ALMERÍA, S., 2013. *Neospora caninum* and wildlife. International Scholarly Research Notices. https://doi.org/10.5402/2013/947347

ALMERÍA, S., SERRANO-PEREZ, B., DARWICH, L., DOMINGO, M., MUR-NOVALES, R., REGIDOR-CERRILLO, J., CABEZÓN, O., PÉREZ-MAILLO, M., LOPEZ-HELGUERA, I., FERNÁNDEZ-AGUILAR, X., and PUIG-RIBAS, M., 2016. Foetal death in naive heifers inoculated with *Neospora caninum* isolate Nc-Spain7 at 110 days of pregnancy. Experimental Parasitology, 168, pp.62-69. https://doi.org/10.1016/j.exppara.2016.06.009

AL-KAABI, N. A. B. M., AL-RIKABY, R. S. A., and ALKAABAWI, N. A. M., 2023. Molecular detection and prevalence study of *Neospora caninum* isolated from blood of aborted cows in babylon province of Iraq. Online Journal of Animal and Feed Research, 13(5), pp. 410-415. https://doi.org/10.51227/ojafr.2023.57

BJERKÅS, I., MOHN, S. F., and PRESTHUS, J., 1984. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Z. Parasitenkd. 70, 271–274. https://doi.org/10.1007/BF00942230

BASZLER, T. V., GAY, L. J., LONG, M. T., and MATHISON, B. A., 1999. Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. Journal of Clinical Microbiology, 37 (12), pp.4059-4064. https://doi.org/10.1128/jcm.37.12.4059-4064.1999

BUXTON. D., MCALLISTER, M. M., and DUBEY, J. P., 2002. The comparative pathogenesis of neosporosis. Trends in Parasitology, 18 (12), pp.546-552. https://doi.org/10.1016/s1471-4922(02)02414-5

BERECZKY, S., MARTENSSON, A., GIL, J. P., and FARNERT, A., 2005. Rapid DNA extraction from archive blood spots on filter paper for genotyping of *Plasmodium falciparum*. American Journal of Tropical Medicine and Hygiene, 72(3), pp.249-251. https://doi.org/10.4269/ajtmh.2005.72.249

BANCROFT, J. D., and GAMBLE, M., 2008. Theory and practice of histological techniques (sixth edition). Elsevier Health Sciences. Pages xiii. https://doi.org/10.1016/b978-0-443-10279-0.50006-3

BAHRAMI, S., HAMIDINEJAT, H., FATEMI-TABATABAEI, S. R., and SARDARIFAR, S., 2018. Effect of natural neosporosis on bull sperm quality. Tropical Animal Health and Production, 50, pp.85-89. https://doi.org/10.1007/s11250-017-1403-8

COLLANTES-FERNÁNDEZ, E., RODRÍGUEZ-BERTOS, A., ARNÁIZ-SECO, I., MORENO, B., ADURIZ, G., and ORTEGA-MORA, L. M., 2006. Influence of the stage of pregnancy on *Neospora caninum* distribution, parasite loads and lesions in aborted bovine foetuses. Theriogenology, 65 (3), pp. 629-641. https://doi.org/10.1016/j.theriogenology.2005.06.003

CALARCO L., and ELLIS, J., 2020. Species diversity and genome evolution of the pathogenic protozoan parasite, *Neospora caninum*. Infection, Genetics and Evolution 84. 104444. https://doi.org/10.1016/j.meegid.2020.104444

DUBEY, J. P. 2003. Review of *Neospora caninum* and neosporosis in animals. The Korean Journal of Parasitology, 41(1), p.1. https://doi.org/10.3347/kjp.2003.41.1.1

DUBEY, J. P., KNICKMAN, E., and GREENE, C. E., 2005. Neonatal *Neospora caninum* infections in dogs. Acta Parasitologica 50(2), pp.176-179.

DUBEY, J. P. and SCHARES, G., 2006. Diagnosis of bovine neosporosis. Veterinary Parasitology, 140(1-2), pp. 1-34. https://doi.org/10.1016/j.vetpar.2006.03.035

DUBEY, J. P., BUXTON, D., and WOUDA, W., 2006. Pathogenesis of bovine neosporosis. Journal of Comparative Pathology, 134 (4), pp. 267-289. https://doi.org/10.1016/j.jcpa.2005.11.004

DUBEY, J. P., SCHARES, G., and ORTEGA-MORA, L., 2007a. Epidemiology and control of neosporosis and *Neospora caninum*. Clinical Microbiology Reviews, 20(2), pp.323-367. https://doi.org/10.1128/cmr.00031-06

DUBEY, J. P., and SCHARES, G., 2011. Neosporosis in animals—the last five years. Veterinary Parasitology, 180 (1-2), pp. 90-108. https://doi.org/10.1016/j.vetpar.2011.05.031

DA COSTA, L. S., WITHOEFT, J. A., BILICKI, J. V., MELO, I. C., SNAK, A., DAS NEVES, G. B., MILETTI, L. C., DE MOURA, A. B., and CASAGRANDE, R. A., 2022. *Neospora caninum*-associated abortions in cattle from Southern Brazil: anatomopathological and molecular characterization. Veterinary Parasitology: Regional Studies and Reports, 36, p.100802. https://doi.org/10.1016/j.vprsr.2022.100802

EAGLESOME, M. D., and GARCIA, M. M., 1997. Disease risks to animal health from artificial insemination with bovine semen. Revue Scientifique et Technique (International Office of Epizootics), 16 (1), pp. 215-225. https://doi.org/10.20506/rst.16.1.1017

FELSENSTEIN, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x

GHANEM, M. E., SUZUKI, T., AKITA, M., and NISHIBORI, M., 2009. *Neospora caninum* and complex vertebral malformation as possible causes of bovine fetal mummification. The Canadian Veterinary Journal, 50(4), p.389. https://doi.org/10.1016/j.anireprosci.2007.05.006

GABER, A., HEGAZY, Y. M., OREIBY, A. F., and AL-GAABARY, M. H., 2021. Neosporosis: a neglected abortifacient disease in Egypt, seroprevalence and farmers' knowledge, attitudes and practices. Journal of the Hellenic Veterinary Medical Society, 72 (3), pp. 3109-3116. https://doi.org/10.12681/jhvms.28500

INNES, E. A., WRIGHT, S. E., MALEY, S., RAE, A., SCHOCK, A., KIRVAR, E., BARTLEY, P., HAMILTON, C., CAREY, I. M., BUXTON D., 2001. Protection against vertical transmission in bovine neosporosis. International Journal for Parasitology, 31 (13), pp.1523-1534. https://doi.org/10.1016/s0020-7519(01)00284-3

JAPA, O., NUANGMEK, A., PRAKHAMMIN, K., and FLYNN, R. J., 2019. Prevalence of vertically transmitted *Neospora caninum* amongst beef cattle in Phayao, Thailand. Parasitology International, 70, pp. 98-101. https://doi.org/10.1016/j.parint.2019.02.008

KUMAR, S., STECHER, G., and TAMURA, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular Biology and Evolution, 33(7), pp.1870-1874. https://doi.org/10.1093/molbev/msw054

MORALES, E., TRIGO, F. J., IBARRA, F., PUENTE, E., and SANTACRUZ, M., 2001. Neosporosis in Mexican dairy herds: lesions and immunohistochemical detection of *Neospora caninum* in foeti. Journal of Comparative Pathology, 125 (1), pp.58-6 https://doi.org/10.1053/jcpa.2001.0477

MOORE, D. P., REGIDOR-CERRILLO, J., MORRELL, E., POSO, M. A., CANO, D. B., LEUNDA, M. R, LINSCHINKY, L., ODEON, A. C., ODRIOZOLA, E., ORTEGA-MORA, L. M., and CAMPERO, C. M., 2008. The role of *Neospora caninum* and *Toxoplasma gondii* in spontaneous bovine abortion in

Argentina. Veterinary Parasitology, 156(3-4), pp.163-167. https://doi.org/10.1016/j.vetpar.2008.06.020

MELENDEZ, P., ILHA, M., WOLDEMESKEL, M., GRAHAM, J., COARSEY, M., BAUGHMAN, D., WHITTINGTON, L., and NAIKARE, H., 2021. An outbreak of *Neospora caninum* abortion in a dairy herd from the State of Georgia, United States. Veterinary Medicine and Science, 7 (1), pp. 141-147. https://doi.org/10.1002/vms3.346

METWALLY, S., HAMADA, R., SOBHY, K., FREY, C. F., and FEREIG, R. M., 2023. Seroprevalence and risk factors analysis of *Neospora caninum* and *Toxoplasma gondii* in cattle of Beheira, Egypt. Frontiers in Veterinary Science, 10, p.1122092. https://doi.org/10.3389/fvets.2023.1122092

NEMATOLLAHI, A., MOGHADDAM, G. H., JAAFARI, R., HELAN, J. A., and NOROUZI, M., 2013. Study on outbreak of *Neospora caninum*-associated abortion in dairy cows in Tabriz (Northwest Iran) by serological, molecular and histopathologic methods. Asian Pacific Journal of Tropical Medicine, 6(12), pp.942-946. https://doi.org/10.1016/s1995-7645(13)60168-6

OKEOMA, C. M., WILLIAMSON, N. B., POMROY, W. E., STOWELL, K. M., and GILLESPIE, L., 2004. The use of PCR to detect *Neospora caninum* DNA in the blood of naturally infected cows. Veterinary Parasitology, 122 (4), pp. 307-315. https://doi.org/10.1016/j.vetpar.2004.06.001

PESCADOR, C. A., CORBELLINI, L. G., OLIVEIRA, E. C., RAYMUNDO, D. L., and DRIEMEIER, D., 2007. Histopathological and immunohistochemical aspects of *Neospora caninum* diagnosis in bovine aborted foeti. Veterinary Parasitology, 150(1-2), pp.159-163.. https://doi.org/10.1016/j.vetpar.2007.08.028

PEREIRA, G. R., VOGEL, F. S. F., BOHRER, R. C., DA NÓBREGA, J. E., ILHA, G. F., DA ROSA, P. R A., GLANZNER, W. G., CAMILLO, G., BRAUNIG, P., DE OLIVEIRA, J. F. C., and GONÇALVES, P. B. D., 2014. *Neospora caninum* DNA detection by TaqMan real-time PCR assay in experimentally infected pregnant heifers. Veterinary Parasitology, 199 (3-4), pp. 129-135. https://doi.org/10.1016/j.vetpar.2013.10.018

REITT, K., HILBE, M., VOEGTLIN, A., CORBOZ, L., HAESSIG, M., and POSPISCHIL, A., 2007. Aetiology of bovine abortion in Switzerland from 1986 to 1995–a retrospective study with emphasis on detection of *Neospora caninum* and *Toxoplasma gondii* by PCR. Journal of Veterinary Medicine Series A, 54(1), pp.15-22. https://doi.org/10.1111/j.1439-0442.2007.00913.x

RAZMI, G, R., MALEKI, M., FARZANEH, N., TALEBKHAN, G. M., and FALLAH, A. H., 2007. First report of *Neospora caninum*-associated bovine abortion in Mashhad area, Iran. Parasitology Research, 100, pp.755-757. https://doi.org/10.1007/s00436-006-0325-6

RAZMI, G. R., ZAREA, H., NASERI., Z., 2010. A survey of *Neospora caninum*-associated bovine abortion in large dairy farms of Mashhad, Iran. Parasitology Research, 106, pp.1419-1423. https://doi.org/10.1007/s00436-010-1820-3

ROJO-MONTEJO, S., COLLANTES-FERNÁNDEZ, E., REGIDOR-CERRILLO, J., RODRÍGUEZ-BERTOS, A., PRENAFETA, A., GOMEZ-BAUTISTA, M., and ORTEGA-MORA, L. M., 2011. Influence of adjuvant and antigen dose on protection induced by an inactivated whole vaccine against *Neospora caninum* infection in mice. Veterinary Parasitology, 175 (3-4), pp. 220-229. https://doi.org/10.1016/j.vetpar.2010.10.028

REGIDOR-CERRILLO, J., ARRANZ-SOLÍS, D., BENAVIDES, J., GÓMEZ-BAUTISTA, M., CASTRO-HERMIDA, J. A., MEZO M., PÉREZ, V., ORTEGA-MORA, L. M., GONZÁLEZ-and WARLETA, M., 2014. *Neospora caninum* infection during early pregnancy in cattle: how the isolate influences infection dynamics, clinical outcome and peripheral and local immune responses. Veterinary Research, 45,pp.1-15. https://doi.org/10.1186/1297-9716-45-10

SINGER-SAM J. 1989. Use of Chelex to improve the PCR signal from a small number of cells. In Forum for PCR Users (Vol. 3).

SALEHI, N., HADADZADEH, H., ASHRAFI, H. J., SHAYAN, P., and SADREBAZZAZ, A., 2009. Molecular and pathological study of bovine aborted foeti and placenta from *Neospora caninum* infected dairy cattle. Iranian Journal for Parasitology: 4 (3), pp.40-51.

ŞUTEU, O., TITILINCU, A., MODRÝ, D., MIHALCA, A., MIRCEAN, V., and COZMA, V., 2010. First identification of *Neospora caninum* by PCR in aborted bovine foetuses in Romania. Parasitology Research, 106, pp.719-722. https://doi.org/10.1007/s00436-009-1684-6

SINNOTT, F. A., MONTE, L. G., COLLARES, T. F., SILVEIRA, R. M., and BORSUK, S., 2017. Review on the immunological and molecular diagnosis of neosporosis (years 2011–2016). Veterinary Parasitology, 239, pp. 19-25. https://doi.org/10.1016/j.vetpar.2017.04.008

SINGH, U. A., KUMARI, M., and IYENGAR, S., 2018. Method for improving the quality of genomic DNA obtained from minute quantities of tissue and blood samples using Chelex 100 resin. Biological Procedures Online, 20, pp. 1-8 https://doi.org/10.1186/s12575-018-0077-6.

SELIM, A., ALSHAMMARI, A., GATTAN, H. S., MARZOK, M, SALEM, M., and AL-JABR, O. A., 2023. *Neospora caninum* infection in dairy cattle in Egypt: a serosurvey and associated risk factors. Scientific Reports, 13(1), p.15489. https://doi.org/10.1038/s41598-023-42538-8

SILVA, R. C. D., DE LIMA, S. C., DOS SANTOS REIS, W. P. M., DE MAGALHÃES, J. J. F., MAGALHÃES, R. N. D. O., RATHI, B., KOHL, A., BEZERRA, M. A. C., and PENA, L., 2023. Comparison of DNA extraction methods for COVID-19 host genetics studies. Plos One, 18 (10), p.e 0287551. https://doi.org/10.1371/journal.pone.0287551

TAMURA, K., and NEI, M., 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees.

Molecular Biology and Evolution, 10 (3), pp. 512-526. https://doi.org/10.1093/oxfordjournals.molbev.a040023

VAN MAANEN, C., WOUDA, W., SCHARES, G., VON BLUMRÖDER, D., CONRATHS, F. J., NORTON, R, WILLIAMS, D. J., ESTEBAN-REDONDO, I., INNES, E. A., MATTSSON, J. G., and BJÖRKMAN, C., 2004. An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. Veterinary Parasitology, 126 (4), pp.351-364. https://doi.org/10.1016/j.vetpar.2004.08.016

WOUDA, W., MOEN, A. R., VISSER, I. J. R., and VAN KNAPEN, F., 1997. Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. Journal of Veterinary Diagnostic Investigation, 9 (2), pp.180-185.https://doi.org/10.1177/104063879700900212

WALSH, P. S., METZGER, D. A., and HIGUCHI, R., 2013. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques, 54 (3), pp.134-139. https://doi.org/10.2144/000114018

YAMAGE, M., FLECHTNER, O., and GOTTSTEIN, B., 1996. *Neospora caninum*: specific oligonucleotide primers for the detection of brain" cyst" DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). The Journal of Parasitology, pp. 272-279. https://doi.org/10.2307/3284160

YAO, L., YANG, N., LIU, Q., WANG, M., ZHANG, W., QIAN, W. F., HU, Y. F., and DING, J., 2009. Detection of *Neospora caninum* in aborted bovine foeti and dam blood samples by nested PCR and ELISA and seroprevalence in Beijing and Tianjin, China. Parasitology, 136 (11), pp. 1251-1256. https://doi.org/10.1017/s0031182009990813

ZHANG, W., DENG, C., LIU, Q., LIU, J., WANG, M., TIAN, K. G., YU, X. L., and HU, D. M., 2007. First identification of *Neospora caninum* infection in aborted bovine foetuses in China. Veterinary Parasitology, 149 (1-2), pp. 72-76. https://doi.org/10.1016/j.vetpar.2007.07.013

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