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MOLECULAR DETECTION AND PREVALENCE STUDY OF *Neospora Caninum* ISOLATED FROM BLOOD OF ABORTED COWS IN BABYLON PROVINCE OF IRAQ

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ABSTRACT: Neosporosis is internationally documented as one of the most popular diseases in cattle that cause economic losses due to high levels of abortion cases. Although *Neospora caninum* has been recently classified as a new species, it is still sharing many features with *Toxoplasma gondii*. This study aimed to detect and Imaging *N. caninum* in the blood of aborted cows, and prevalence study of *N. caninum* infection based on age, region and month. Blood samples from 106 aborted cows were collected using the appropriate method. First, these samples were examined microscopically via blood smears using Giemsa dye to diagnose the *N. caninum* within RBC. A qPCR technique was carried out to detect accurately *18S rRNA* gene accurately. The results revealed that 65% of total aborted cases were positive for *18S rRNA* detection of *N. caninum*, although this parasite was found microscopically in 15% of blood smear samples. According to PCR results, the prevalence study showed that the highest rate of infection was signed in the Al-Qassim district (75%) followed by the Al-Mahaweel district (74%) and decreased in Western Hamza district (48%). According to study months, November recorded the peak of infection (88%), then August (71%), whereas July recorded the lowest percentage (50%). The statistical analysis revealed there was no significant difference between the subjected regions and study months based on (P<0.05). On the other hand, it was found that cows less than 3 years old were more susceptible to infection than those over 3 years old. the results revealed that 71% of infected cows were less than 3 years old, while 29% were at age over 3 years old with a significant difference (P<0.005). In conclusion, *N. caninum* can be detected through blood within RBC. Age and regional factors in cows play an important role in resisting infection with this pathogen.

Keywords: Cow, Neosporosis, N. caninum, Prevalence, 18S rRNA

INTRODUCTION

Neosporosis is a parasitic disease caused by *N. caninum*. It is recognised as an intracellular protozoan parasite of livestock distributed worldwide. In cattle, it is considered one of the main causes of abortion (Wei et al., 2022). Neosporosis has been related to epizootic and sporadic abortion in dairy herds worldwide. Since the discovery of Neosporosis, some studies have been conducted to assess the prevalence and to identify factors related to the disease, Prevalence's has been estimated in ranges between 16.8% and 70% (Waldner et al., 2004). In intermediate hosts, tachyzoites and tissue cysts are the infective stages (Mahajan et al., 2019). It has been reported in many countries with different prevalence rates since the disease was recognized in 1988 (Noori et al., 2019).

Morphology for parasite tachyzoites are ovoid, crescent or spherical in shape according to the phase of division and measures 3-7 × 1-5 µm in size (Speer et al., 1999), bradyzoites are slightly longer, slenderer than the tachyzoites, measuring 8.1 × 2 µm. parasite, capable of forming tissue cysts containing up to 100 parasites and Oocysts almost rounded measuring about 11.7 × 11.3 µm in diameter, surrounded by a smooth, colorless, 0.6-0.8 µm thick wall (Lindsay et al., 1999). Definitive hosts (dog, coyote, dingo) shed unsporulated oocysts in their feces, which sporulate within several days to become infectious to the intermediate host such as cow, sheep, goats or deer, water buffaloes, horse, etc. when they consume foods or water contaminated by them (Lindsay and Dubey, 2020; Venturoso et al., 2021). García et al. (2015) found that many cases of abortion in both dairy and beef cattle were caused by *N. caninum*. Cattle get infected due to feeding mostly on mixed, drinking from rivers, and existing in contact with other animals at the same farm. As observed in many reports, the second way to spread the infection is by consuming parasite oocysts or eggs contaminated food or water or grazing on contaminated pastures (Claude et al., 2017). In addition, the transmission of infection could be transplanted during pregnancy to the fetus from the infected mother (Jiménez-Pelayo, et al., 2019). Previous specific

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studies showed that the higher anti-*N. caninum* antibodies were found in cattle living on farms with dogs in comparison with those without dogs, suggesting the active involvement of dogs in the transmission of parasites to cattle (Gharekhani et al., 2020). The release of oocysts of *N. caninum* through dog faces could contaminate the food and water which become the source of infection (Ahmed et al., 2021). Billions of dollars were lost annually as reported in New Zealand and the US by the infection of Neosporosis, due to loss of fetus, milk yield, gaining weight, and time for rebreeding (Reichel et al., 2013).

The aim of our study is to detect *N. caninum* in the blood of aborted cows using the PCR technique and to study the prevalence of this infection in the Babylon province. Furthermore, the presence of *N. caninum* in red blood cells was also observed.

MATERIALS AND METHODS

Collection and examination processes for 106 blood samples of aborted cattle were carried out from different ages and regions of the Babylon province: (Al. Qassime, Al-Hilla, and Mahaweel Districts) as well as different periods from July 2022 to January 2023. The blood samples were collected especially from aborted cows, and 3 ml of venous blood (jugular vein) was taken in a 5 ml EDTA tube (AMEER et al., 2019).

Microscopic examination

Direct microscopic examination was performed to diagnose this parasite and to study the morphology of it. A small drop of blood was applied to the slide approximately 20 mm from one end. A spreader of another slide was placed on the blood drop at an angle of 20-30° and drawn back to another end to blood smear. After air drying, the sample was fixed with methyl alcohol for a minute and then stained with Giemsa stain for a minute again, wishing and drying were carried on after that, the samples were ready for microscopic examination (Fraser et al., 2010)

Molecular detection

Genomic DNA extraction from 400 µl blood was done using manufacturer instructions (QIA GENE KIT). The concentration of DNA was measured using a Nanodrop device at an absorbance of (260 /280) nm. PCR technique was performed on the collected samples, all PCR samples were prepared following the manufacturer protocol (1µl of 10 pmol forward and reverse primers, 8 µl genomic DNA, 12.5 Mastermix, and complete volume to 25 µl of nuclease-free water. Primers for 18S rRNA were self-designed with a predicted size (236 bp) to be used in this stage based on the NCBI database of Genbank: MT860359. The foreword primer sequence (NeoF) was (gtgtacggcgaagggactc), while the reverse primer sequence (NeoR) was (gccaagacatccattgctga). PCR programs were set according to the annealing temperature of each primer (94 ^{o}C for 5 minutes, for 40 cycles of 94 ^{o}C for 30 seconds, 50 ^{o}C for 25 seconds, 72 ^{o}C for 45 seconds, and a final extension at 72 ^{o}C for 8 minutes, with a final hold at 4 ^{o}C (THERMO-CYCLER; FISHER, GERMANY). A 1.5% agarose gel electrophoresis was prepared for checking PCR products using 1X Tris-Borate- EDTA (TBE buffer), and then the components were dissolved into 1 L of distilled H2O. They were run for 60 min/ 80 volts (Hube et al., 2005). DNA bands were compared with a 1500 bp DNA ladder to confirm the specific size of the 18S rRNA gene for the subjected parasite. Imaging was carried out using a UV Trans-illuminator for DNA detection.

Ethical regulation

It was obtained from the Faculty Scientific Committee (College of Veterinary Medicine, Al-Muthanna University, Iraq) numbered 202205 – Naer Abdulbari Madlool Alkaabawi.

RESULTS

Direct microscopic examination

All the collected blood samples of aborted cows were examined by direct smear method; it appeared that 15% of blood samples were suspected Neospora at the tachyzoite stage. These tachyzoites were ovoid and crescent in shape according to the phase of division and measurements of size (3-7 × 1-5 µm) using an ocular micrometer lance as shown in (Figure 1).

Detection of 18S rRNA of *N. caninum*

According to the results of the microscopic examination, all samples were run on PCR for 18S rRNA detection of *N. caninum* with PCR product size (236 bp). The results show that 65% of the total samples are infected with *N. caninum* (Figure 2).

Prevalence of *N. caninum* infection in aborted cows based geographical areas using PCR is at the highest rate in Al-Qassim and Al-Mahaweel districts which are 75% and 74%, respectively. Al-Hilla District records 63%. The lowest rate is in Western Hamza district which archives 48%. The statistical analysis (P>0.05) reveals that there is no significant difference (P=0.154) in the prevalence of parasites among geographical areas. These statistics are similar with no significant difference (P=0.239) to the prevalence of *N. caninum* infection according to the time of infection, although the

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infection rate was at the highest level during November (88%) (Figure 3). On the other hand, it is found that the *N. caninum* parasite is detected in (71%) of aborted cows aged 1-3 years group. Meanwhile, a ratio of 29% is identified in the aborted cows at age over 3 years group. This refers to a significant difference between these two groups based on statistical analysis at P<0.05 that recorded P<0.005.

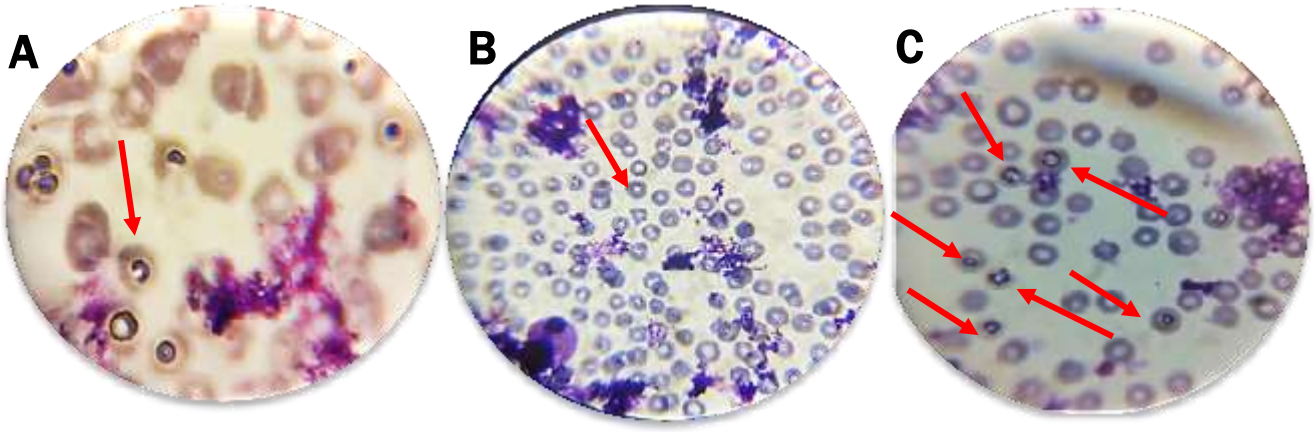

Figure 1 - Shows different stages of infection with *Neospora* caninum parasite inside the red blood cells at (40x) after dying by Giemsa stain. (A and B) pictures refer to tachyzoite phase, while (C) refers to heavy infection with *N. caninum* in red blood cells (40x)

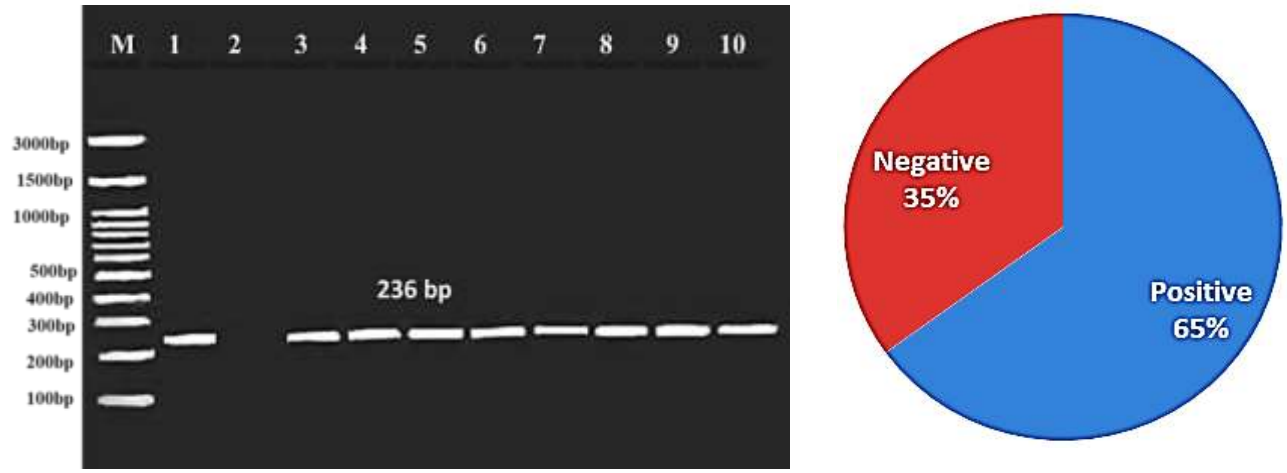

Figure 2 - A) It shows the PCR amplification results of the 18s rRNA gene of *N. caninum* isolated from aborted cow. The agarose gel picture shows the PCR product bands with a molecular weight of 236 bp. (M) refers to (3 Kbp) DNA ladder; 1) positive control; 2) Negative control. (1-9) PCR results of blood samples. B) shows the infection ratio with *N. caninum* over 106 samples.

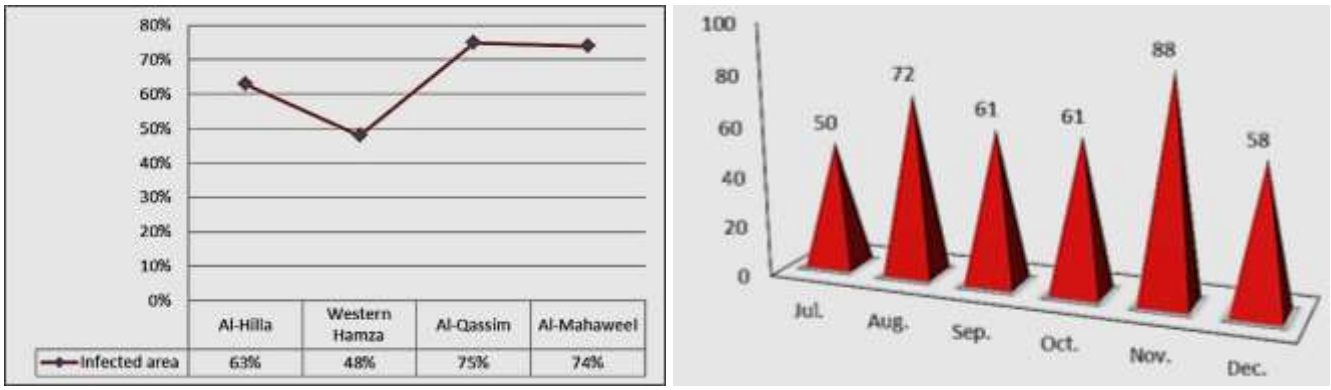

Figure 3 - Refers to the prevalence of *N. caninum* infection according to the region and the time of infection. It is clear to see that the highest ratio of infection is in Al-Qassim district, which records (75%) while November is the critical time of infection (88%).

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DISCUSSION

Neospora caninum is the main cause of neosporosis as a polysystemic disease that is an obligated intracellular parasite that belongs to the phylum Apicomplexa (Dubey and Schares, 2011). Neosporosis was misdiagnosed as toxoplasmosis due to sharing several morphological and biological features that are closely related to *Toxoplasma gondii* (Dubey et al., 2017). In 1984 was the first recognition of *N. caninum* in Norwegian dogs (Bjerkas et al., 1984), then a significant classification was done to consider it as a separate species from *Toxoplasma gondii* since 1988 (Dubey et al., 1988; Seltmann et al., 2020).

Among diagnostic techniques, the PCR is more sensitive and specific than other tests and less likely to be affected by autolysis or postmortem changes. In addition, it can be applied for the identification of *N. caninum* DNA in blood, semen, brain, spinal cord, different fetal fluids, embryonic tissues, and even oocysts in the faeces of the final host (Kamali et al., 2014).

The prevalence of *N. caninum* infections was the highest in the Al-Qassim and Al-Mahaweel districts which were 75% and 74.07% respectively, while in the Al-Hilla District was 63.33% and the lowest rate was in Western Hamza district which was 48%. The statistical analysis reveals that there was no significant difference in the prevalence of parasites among geographical areas (P>0.05). Globally, several various studies were carried out to detect the prevalence of bovine *N. caninum*, using different diagnostic tests that revealed significant variation in their prevalence between countries, regions as well as between herds as Semango et al. (2019) discuss in their paper. The results of this study were higher than those reported by Al-Gharban et al. (2017) that were (12.36%). However, it was nearly close to the findings of Japa et al. (2019), they found the diversity of *N. caninum* infection rates (16-50 %) in different districts of Phayao province in Thailand using PCR for placental samples of beef cattle. This success in the detection of DNA was expected because the blood was confirmed to be a transport factor for Neospora tachyzoites between body tissues (Okeoma et al., 2004). Neosporosis has a wide range of infection rates with the presence of contrast between the study's districts. This finding might be attributed to the inequality of applied techniques and/or their cut-off origin of evaluated herds and the probability of frequent exposure to sources of infection (Moura et al., 2011). As well as, the increase in *N. caninum* prevalence could occur because discrepancy of animal housing or poor hygienic management, herds that are involved in a study, increasing exposure to definitive host or intermediate hosts, and the contact, directly or indirectly, to adjacent endemic areas (Celik et al., 2013; Llano et al., 2018).

The prevalence of *N. caninum* infections was at a high rate within 1-3 year's old group which was 71%, while the lower rate of infection was at the age over 3 years old group, which was 29%. The statistical analysis using the T-test parameter showed that there was a significant difference between age groups (P<0.05). Furthermore, the results of the present study showed that the lowest per cent was in the age group years old which may be due to the fact that cows in this age (>3 years) have good resistance and immunity against *N.caninum*. These results agreed with Noori et al., (2019), who discussed that the vertical transmission of *N. caninum* might be the reason for increasing the infection in cows under 3 years old more than those increasing over 3 years based on his seroprevalence study. However, the results of Metwally et al. (2023) revealed that those aged (3-5) years old were more exposed to the infection with seropositive version, due to horizontal transmission. Moreover, the results of Razmi et al. (2006) and Mallah et al. (2012) also disagreed with the present data, when they announced that there is no significant difference between age groups (P>0.05). The age effect might be influenced by management practices such as replacement rate, and the cattle may be exposed to horizontal transmission, or by selective culling of seropositive animals (Bartels et al., 2006).

On the other hand, the results of this study revealed that the prevalence of *N. caninum* infection based on the time recorded that the highest rate of infection was in November (88 %) followed by 72% in August, while the lower prevalence was in July which was 50% (figure 3). The statistical analysis reveals no significant difference at (P>0.05) in aborted cows among studied months (figure 3). Distribution of *N. caninum* infection takes place all year round, the peak of infection was observed in Nov. with a percentage of 88%, this result was in agreement with (Ibrahim et al. 2012) who found the peak of Neospora infection in autumn and winter California. However, this result was in disagreement with Pitel et al. (2001) in France, who found the peak of Neospora infection in March-June. These differences in infection may be due to seasonal differences in parasite exposure and/or oocyst survival by providing suitable environmental conditions (temperature and humidity) Because of its close relationship with *T. gondii* it is assumed that the environmental resistance of *N. caninum* oocysts is similar to *T. gondii* oocysts that the degree of suitable temperatures for sporulation of oocysts was ranged between (22-30) C˚ (Dubey, 2007).

CONCLUSION

Overall, despite, that there are many sources to isolate *N. caninum* from the infected body, blood is the main linker to spread the pathogens into other body organs, and apparently, it is the best source for early diagnosis of infection. We can also conclude that the age factor plays a crucial role in resisting the infection, especially over 3 years old cows. However, the region and time factors do not make any difference in spreading the infection of *N. caninum* in the Babylon province of Iraq.

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DECLARATIONS

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Authors' contribution

Nawras Abdul bari Madlol Alkabi did the study conception, design, and monitored the experiments. Ra'afat Sabbar Abbas Al-Rikaby did all the preparations for sample collection, and the laboratory works involving the molecular part. Naer Abdulbari Alkaabawi prepared and read the molecular part of the laboratory work, interpreted the data and revised the final version of this paper.

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Conflict of interest

The authors have not declared any conflict of interest.

Consent to publish

We, give our consent for the publication of identifiable details, which can include photograph(s) and details within the text to be published in the OJAFR.

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