

Research Article

MOLECULAR DETECTION OF *Neospora caninum* FOR ABORTED WOMEN IN BABYLON PROVINCE, IRAQ

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Abstract

Blood samples of 112 aborted women collected to diagnosis *Neospora caninum* in Babylon province through Six month from August - 2022 to January - 2023. Two tests were used (Microscopic examination by Giesma staining and PCR assay). The aborted women were classified into sub-groups according to age, locality and months in order to study association of these risk factors with rate of infection. A total of 112 blood samples of aborted women were examined by direct method out of which 1 blood samples had suspected *Neospora* tachyzoites ($1 \times 5 \mu\text{m}$) with percent 0.98 %. The result of the study revealed that infection percent in aborted women by PCR was 71.42% in according to the geographical areas were in Al-Hilla (70 %), Western Hamza (70.37 %), Al-Qassim (85.71 %) and Al-Mahaweel (59.25 %) districts, the statistical analysis reveals that there was no significant ($P>0.05$), and depending on the home address the rate of infection in rural area was 80.3 % and in urban area was 58.69 % with Significant difference at $P<0.05$. The rate of infection in age group by PCR was highest in $>20 - 25$ (92.69 %), while the lower prevalence of infection was for the rate of infection in age group ($>35- 40$) (36 %) also with Significant difference at $P<0.05$. in case months. The prevalence of infection by PCR was Aug. (73.68 %), Sep. (52.63 %), Oct. (83.33 %), Nov. (88.88 %), Dec (68.42 %) and Jan (71.42 %), Notes the higher prevalence rate was in December month. No significant at $P<0.05$. The rate of *Neospora caninum* infection in Babylon is extremely high in aborted women. Also, positive case in aborted women with frequent abortion.

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1. Introduction

Neospora caninum is parasite that considered, the etiologic agent of the polysystemic disease neosporosis, is an obligate intracellular tissue cyst forming coccidian parasite of the phylum *Apicomplexa* (Dubey *et al.*, 2017).

Neospora caninum shares many morphologic and biologic features with its close relative *Toxoplasma gondii* Prior to its initial recognition in Norwegian dogs in 1984 and consequential classification as a distinct species in 1988 (Dubey *et al.*, 1988), many *N. caninum* infections were misdiagnosed as toxoplasmosis (Dubey and Schares *et al.*, 2011). The structures that have been identified for *N. caninum*:

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tachyzoites, bradyzoites (tissue cyst) and oocysts are similar to those of *Toxoplasma gondii* and other related apicomplexan parasites (Speer *et al.*, 1999). Tachyzoites are ovoid, crescent or spherical in shape according to the phase of division and measures $3 - 7 \times 1 - 5 \mu\text{m}$ in size (Speer *et al.*, 1999). Definitive hosts (dog, coyote, dingo) shed unpopulated 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 and horse. When they consume foods or water contaminated with them (McAllister *et al.*, 1998). Armengol *et al.* (2006) found that *N. caninum* causes abortion in both dairy and beef cattle, comparatively little is known about the disease in beef cattle. It causes neuromuscular signs in infected dogs and they may show encephalomyelitis and myositis which results in paralysis and also early death in puppies (Al-Qassab *et al.*, 2010). In aborted fetuses of cattle, multifocal non suppurative encephalitis, myositis, myocarditis, periportal hepatitis with or without focal hepatocellular necrosis were repeatedly observed microscopically (Jardine and Wells, 1995; Wouda *et al.*, 1997). Cattle can become infected either horizontally, through the ingestion of infectious oocysts that have been shed by the definitive hosts or vertically, through transplacental or congenital transmission from an infected pregnant mother to her fetus (Dubey *et al.*, 2007). Neosporosis is a serious disease in animals. The significant role of the disease in abortion and other reproductive failures in cattle is clear (Dubey *et al.*, 2005; Dubey *et al.*, 2007). The annual economic losses related to the Neospora infection have been estimated at more than US\$1.3 billion on a global scale (Santos *et al.*, 2016). The detection of this infection is possible by different laboratory methods such as serology and molecular biology in animals and humans (Dubey *et al.*, 2003; Dubey *et al.*, 2007). Human could become exposed to *N. caninum* by accidental ingestion of oocyst shed in the feces of canids definitive host or by consumption of undercooked meat that contains tissue cysts and concern is that *N. caninum* might be zoonotic disease because its close contact biological relationship to common zoonotic parasite *T.*

gondii and also Rhesus monkeys have been experimentally infected (Barr *et al.*, 1994; McCann *et al.*, 2008). A different level of the *Neospora* infection have been detected in humans' sera (Dubey *et al.*, 2007; Dubey *et al.*, 2011). *N. caninum* is an important cause of fatal infections through experimentally transferring to pregnant cases with the lesions closely resembling those caused by congenital toxoplasmosis (Lobato *et al.*, 2006).

2. Materials and Method

The study included collecting blood samples from 112 aborted women during August - 2022 to January - 2023 in Babylon province. These Blood samples were subjected to direct examination and molecular detection by PCR assay.

Collection of blood samples

Five ml of blood samples were taken from aborted women, each sample was divided into two part, 4 ml were put in tube in the EDTA tube was used for the purpose of extracting DNA and PCR assay and 1ml were put in EDTA tube for using to Microscopic examination by Geimsa stain.

Primers That Used in the Present Study

Neospora caninum isolate ITS fetuses small sub-unit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, complete sequence; and 5.8S ribosomal RNA gene, partial sequence. The PCR primers pairs of NP2 and NP6 for woman was designed in this study by using NCBI dbSNP database GenBank: MT860359.1 (Table - 1).

Statistical Analysis

Data were summarized, presented and analyzed using statistical package for social science (SPSS version 16) and Microsoft Office Excel 2007. For the determination of the significant difference among one way analysis ANOVA was used. A p-value <0.05 was considered statistically significant the difference were considered statistically.

Table - 1: Primers 18S rRNA used for the detection of *Neospora caninum* Integrated DNA Technologies

Primer	Sequence 5'-3'		Amplicon size	References
pairs of NP2 and NP6	F	GTGTACGGCGAAGGGACTC	236 bp	Gene bank code MT860359.1
	R	GCCAAGACATCCATTGCTGA		

3. Results and Discussion

Microscopic examination

The diagnosis of *Neospora caninum* in aborted women was determined by method Microscopic examination. Microscopic examination showed that examined by direct method out of which 1 blood samples had suspected *Neospora* tachyzoites with percent 0.98 % (Figure - 1). A rapid diagnosis may be made by microscopic examination of smears (Hill, 2002; Silveira, 2011). The use of a stained smear microscopy by Geimsa stain (thin smear) still remains the most available, easy to perform, and inexpensive, this technique can be done routinely in a variety of clinical settings and the Direct microscopy provides judging specimen quality, detecting a variety of organisms in clinical specimens (Misawa., 1999). The result of the present study is similar to the finding of

(Naot and Remington, 1980; Deslyper *et al.*, 2019). The presence of the tachyzoite in blood during the acute phase of the disease and leaving to the tissues during the chronic phase, Probably the reason for the fact that a positive result in the chronic cases that these case are with subacute phase and a few number of tachyzoite still exists in the blood. The women, a blood test was delayed immediately after the abortion, the test is done after a period of abortion has passed, or after it was decided to get pregnant again. Therefore, it is difficult to find this phase during the microscopic examination for women due to the delay in conducting laboratory tests, during this time, the parasite moved to the tissues and the proportion of its presence in the blood decreases.

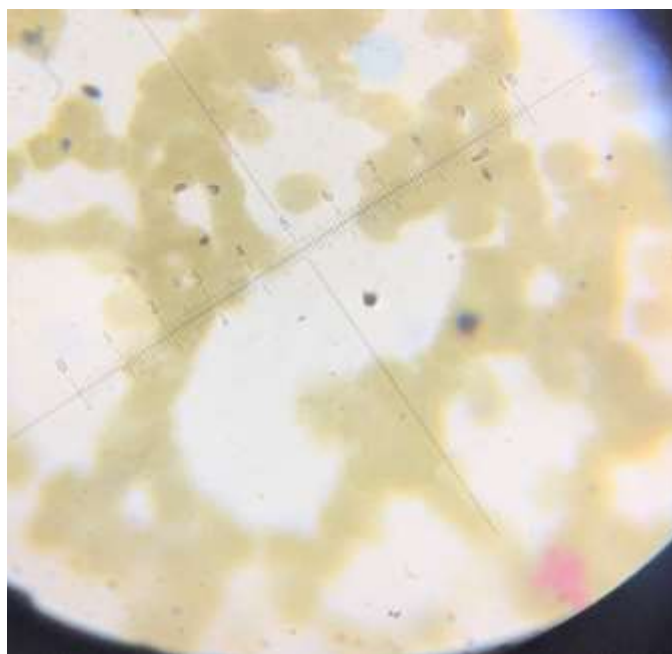


Figure - 1: The pictures show infection with *Neospora caninum* parasite in the blood smear of aborted women pigmented with Giemsa stain (40 x) and using Ocular micrometer lance

Rate of *Neospora caninum* infection in Aborted woman determined by PCR assay

PCR analysis is a fast, specific and sensitive method that obviates the time-consumption, possible loss of samples or strain selection problems associated (Fuentes *et al.*,

2001; Remington *et al.*, 2004; Khan *et al.*, 2006). The presence of positive cases in maternal blood probably indicates a recent infection or apparent parasitemia (Slawska *et al.*, 2005), as shown in Table - 2.

Table - 2: Rate of *Neospora caninum* in Aborted woman determined by PCR assay

Total No. samples	No. of positive samples	No. of negative samples	Percentage (%)
112	80	32	71.42

Association between infection rate of *Neospora caninum* and the geographical area of aborted woman determined by PCR assay

The rate of positive cases, infection was the highest in Al Qassim district and it was 24 out of 28 (85.71 %), followed Al Western Hamza district was 19 out of 27 (70.37%), and the rate in Al Hilla District was 21 out 30 (70 %), whereas in Al Mahaweel district it was 16 out of 27 (59.25 %). The lack of significant difference in rate, as shown in Table - 3.

The present study showed no significant impact of geographical variation. Agreed with another study performed by (Al-Ka'bi, 2016). In Iraq found no significant difference in rate of *Toxoplasma* in women in relation to variation in areas of residence may be explained by the fact that the study included areas that have nearly similar environmental and cultural outlines. On the other hand, the source of infection in different areas might be the major player that caused the absence of such significant association. In other words, the common source of infection is the encysted tissue parasite which is present in substantial proportion of imported consumed meat sources which is agreed with the finding of several authors with regard to the lack of significant association between locality or residence of women and the rate of infection with *Neospora*. Hajssoleimani *et al.* (2012)

concluded no significant effect for residence on prevalence of *T. gondii* in women in Iran. In Denmark, Lebech *et al.* (1993) stated that there was no significant difference in the prevalence of *Toxoplasma gondii* in women from different parts of Denmark. Another study performed by Zapata *et al.* (2005) in Costa Rica also found no significant association between locality and positivity in women. Similarly, Ramsewakl *et al.* (2008) found no significant impact of geographic variation on rate of *T. gondii* in women in Trinidad and Tobago. A literature review summarized reports of presence of *N. caninum* in patients with neurologic disorders pregnant women and healthy people, including blood donors in Spain (Dubey *et al.*, 2017; Calero-Bernal *et al.*, 2019). In the readings Neosporosis according to a residence area may be because lack of health education in areas, insufficient appropriate of treatment and difficulty of early diagnosis of symptoms and the lack of obvious symptoms. Regarding the high frequency of the *Neospora* infection of cows (up to 90%) should be regarded as a potential source of infection for humans, and the close similarity with the *T. gondii* infection, the possibility of *Neospora* posing a risk for pregnant women should receive special attention (Gharekhani *et al.*, 2020).

Table - 3: Rate of *N. caninum* infection and the geographical area in aborted woman determined by PCR assay

Geographical area	Total number	Positive cases	Percentage (%)
Al-Hilla	30	21	70
Western Hamza	27	19	70.37
Al-Qassim	28	24	85.71
Al-Mahaweel	27	16	59.25
Total	112	80	*71.42

*No significant difference at $P < 0.05$

Association between rate of *Neospora caninum* infection and distribution area depending on the home address of aborted woman determined by PCR assay

This study included the geographical distribution area of 112 aborted women illustrated in Table - 4, the aborted women were classified depending on the home address into rural which infection rate was 53 out of 66 (80.3 %) while the others habitat in urban rate 27 out 46 (58.69 %) with Significant difference at $P < 0.05$. For aborted women, showed that rural-

urban ration was higher in rural than in urban. The result of the present study is similar to the finding of Mohammed *et al.* (2019); Al-Jbouri *et al.* (2020); Romero *et al.* (2021). The results were compatible with study of Al-Jbouri *et al.* (2020) in Iraq (Babylon City) this finding might be regard to the fact that they were likely to be of low education about the routes of transmission and reflects houses contamination with feces (oocyst) and similar to the finding of the females are more interaction to oocyst *via* meat, vegetable in Kirkuk City, Iraq (Salman, 2014).

Table - 4: Rate of *N. caninum* infection in aborted woman according to residence area by PCR assay

Residence area	Total number	Positive cases	Percentage (%)
Urban	46	27	58.69
Rural	66	53	80.3
Total	112	80	71.42*

*Significant difference at $P < 0.05$

Association between the infection rate of *Neospora caninum* and age of aborted woman by PCR assay

The infection rate of *N. caninum* for aborted women in age group ($>20 - 25$) was 38 out 41 (92.69 %), while in age group ($>25 - 35$) was 29 out 46 (63.04 %) and in age group ($>35 - 40$) 9 out 25 (36 %), significantly different found at ($P < 0.05$), as shown in Table - 5. The results showed that the age distribution among *Neospora* infected women was between 20 and 25 years, which represents higher percentage of 92.69 %, while the lowest percentage of infection (36 %) in the aborted women was between 35 and 40 years (Table - 5). This finding was compatible to the results of a study in Al-Qadisiyah province, which found that 45.5

% of aborted women were at 25 - 34 years, while other study found the highest rate of infection in the age group (16 - 19 years) these results may be due to the cumulative effect of exposure to the infective stages of the parasite (Hadi *et al.*, 2016). This rate is approximately similar to the finding of the present study, registered the significant association between decrease age of the women and the prevalence of *Neospora* ≤ 30 years (55.1 %), 30 - 40 years (40.8 %) and in >40 years (4.1 %) (Duarte *et al.*, 2020b).

In Brazil (Duarte *et al.*, 2020a), the first data in the literature relating positivity to age in humans for *Neospora caninum* infected according to age group, considering the number of pregnant women positive for infected ≤ 20

years (33.3 %), 21 - 30 years (22.5 %) and in ≥ 31 (41.17 %). Changes in the maternal immune status occur during pregnancy to maintain fetal survival and this immunosuppression may leave pregnant woman more prone to infections (Weetman *et al.*, 1999; Yip *et al.*, 2006). Under healthy conditions, these infections are typically kept under control during pregnancy. However, the immature immune system of the fetus leaves it vulnerable to parasites that are able to cross the uteroplacental barrier (Barratt *et al.*, 2010). The transplacental hematogenic route is the most common route of maternal-fetal parasite transmission (Carrier *et al.*, 2012). This result was very nearly the same level as reported in Brazil ≤ 30 (55.1 %) years, 31 - 40 years (40.8 %) and >40 years (4.1 %) (Duarte *et al.*, 2020b), the positivity rate was in the present study the rate of 5 % found in cord blood samples, 7.92 % pregnant women infected for *N. caninum*, 6.7

% and in blood bank samples found 26.1 % positivity in HIV patients (Tranas *et al.*, 1999; Lobato *et al.*, 2006; Ibrahim *et al.*, 2009; Oshiro *et al.*, 2015). Several authors, registered the significant association between increasing age of the women and the prevalence of *T. gondii*. Of those authors are Sakikawa *et al.* (2012) in Japan, Kolbekova *et al.* (2007) in Czech Republic, Nowakowska *et al.* (2006) in Poland and Studenicova *et al.* (2006) in Slovakia. These rates are also less than that of the current study. If there is a possibility of vertical transmission in humans, we believe that the evolution and severity of the infection is dependent on the mother's gestational age and the virulence of the strain causing the infection, as occurs in other animal species (Buxton *et al.*, 2002; Al-Qassab *et al.*, 2010). Other previous studies have documented that PCR can detect the parasite DNA in blood samples of women before or during pregnancy (Chabber *et al.*, 2004).

Table - 5: Prevalence of *N. caninum* infections in aborted women by (PCR assay) according to the age group

Age	Total number	Positive	Percentage (%)
20 – 25 years	41	38	92.68
>25-35 years	46	29	63.04
>35-45 years	25	9	36
Total	112	80	71.42*

*Significant difference at $P < 0.05$

The infection rate of *N. caninum* of aborted woman according to month by PCR assay

According to months, the infection rate of *N. caninum* was higher in Dec. which was (88.88 %) (16 out of 18), followed by Oct. (83.33) (15 out of 18), Aug. (73.68 %) (14 out of 19), Jan. (68.42 %) (13 out of 19), Nov. (63.15 %) (12 out of 19) and Sep. (52.63 %) (10 out of 19). No significant difference at ($P < 0.05$) (Table - 6). This result closely agrees with (Al-Ka'bi, 2016), there is little if any researches that mention a relation between the seroprevalence of *T. gondii* in women and season of the years, the present study found no significant relation between the rate of infection in women and the

month of the year. The explanation for that can be viewed from the fact that the major source of infection in women is the tissue encysted form of the parasite in meat products, whereas environmental contamination with Oocysts plays a relatively minor role in women infection (Tenter *et al.*, 2000). The variations in the seropositivity rates found in several studies may be attributed to the study populations and the climatic and environmental factors of each region, as some authors have reported an association between climatic factors and risk factors for *N. caninum* infection in cattle (Dobrowolski *et al.*, 1996; Rinaldi *et al.*, 2005; Dubey *et al.*, 2007). The sporulation and survival of coccidial oocysts in the environment may be favored by temperature and humidity

(Dubey *et al.*, 2007). According to the months, there is no effect on the rate of the given infections relatively close, and ranged between 15 – 23 %, this may be because of the sources of infection are different in human populations and they depended on the differences in culture and eating habits (Gilot-Fromont *et al.*, 2009), oocyst can survive for 18 months in soil, in the ethanol 95 %, methanol 100 %, and formalin 10 % with no effectiveness its infectivity due to

environmentally resistant Oocysts (Miller *et al.*, 1972; Arranz-Solis *et al.*, 2023). Environmental conditions (humidity and temperatures), which are considered an important factor for increase resistant of oocysts in the soil or may be due to the increase the spread of insects, that play an important role as mechanical transmitter vectors for oocysts. Also, it was found that the infection influence by the differences between day and night temperature (Lan-Bi *et al.*, 2018).

Table - 6: The rate of infection *N. caninum* and in aborted woman According to month by PCR assay

Months	Total number	Infected cases	Percentage (%)
Aug.	19	14	73.68
Sep.	19	10	52.63
Oct.	18	15	83.33
Nov.	19	12	88.88
Dec.	18	16	68.42
Jan.	19	13	71.42
Total	112	80	

*No significant difference at $P < 0.05$

Distribution of aborted women according to frequency of previous abortion by PCR assay

The frequency of previous abortion was highest (47.5 %) in patients having two previous abortions, one previous abortions (27.5 %) and ≥ 3 previous abortions (25 %), respectively as shown in Table - 7. The current study did not found a significant relationship between the percentage of infection and the number of

abortions (Table - 7), although it reported high rates of infection among women who experienced double abortion. The current study was compatible with the study by Nariman *et al.* (2018); Mohammed *et al.* (2019) who found that the number of abortions to two times higher than the number of abortions for once, when toxoplasmosis infection in aborted women especially with recurrent spontaneous abortion in Babylon Province, Iraq.

Table - 7: Distribution of aborted women according to frequency of previous abortion by PCR assay

Frequency of previous abortion	Number of infected women (%)
1	22 (27.5)
2	38 (47.5)
≥ 3	20 (25)
Total	80

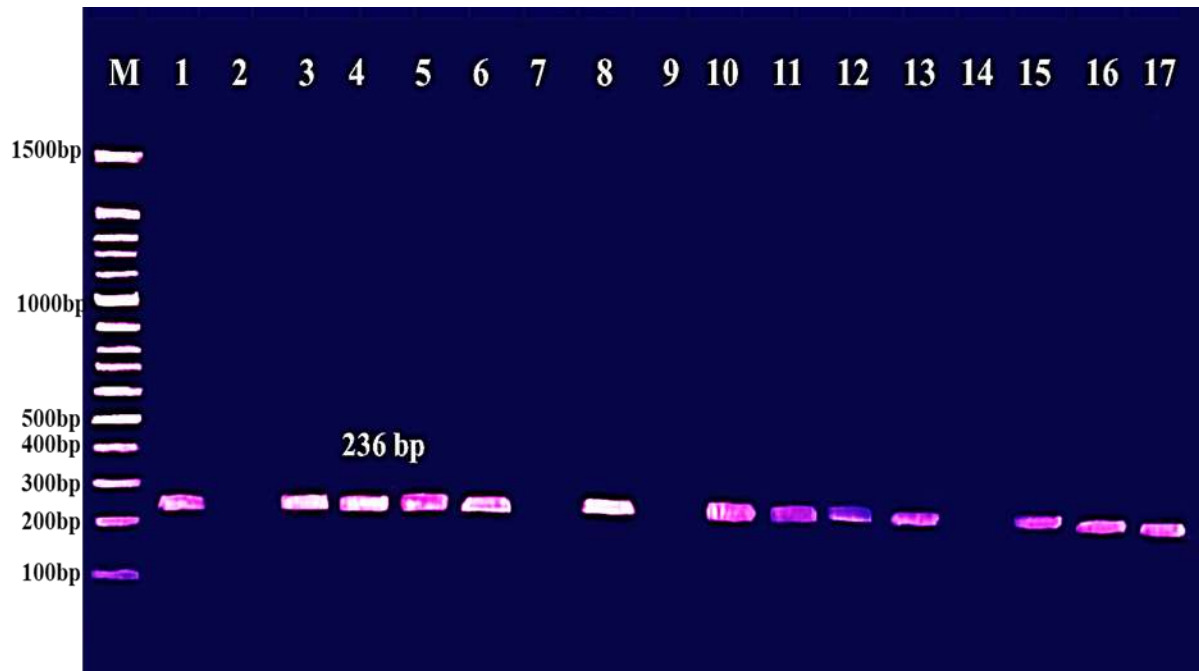


Figure - 3: It shows the PCR amplification results of 18s rRNA gene of *Neospora caninum* isolated from aborted women. Agarose gel picture appears the PCR product bands with molecular weight of 236bp.(M) refers to (1500bp) DNA ladder, (1) positive control, (2) Negative control. (4-17) PCR results of blood samples.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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