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Neospora caninum detection by nested PCR in domestic and stray dogs in Baghdad City

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Abstract---Neospora caninum a protozoan parasite causes abortions in cattle, as well as neurological disorders and reproductive problems in dogs. This study was conducted to detect Neospora caninum in fecal samples of stray and domestic dogs and determine the effects of sex, age, months and areas in the infection rate in Baghdad city by using molecular technique (Nested PCR– n PCR) during the period of the beginning of January to October (2021). The total infection rate was 10% (10/100). Higher infection rate 15.30% (8/52) was recorded in stray dogs than domestic dogs 4.16 % (2/48), and males than females (12.72% and 6.66%) respectively. The higher infection rate (7.70%) was recorded in the age group more than 5 years, while no infection rate (0.00%) was found in the age group less than 2 years. A higher infection rate (20.00%) was recorded in May while no infection rate (0.00%) was found in January and February. A significant ($P \leq 0.01$) difference was found in the infection rate among areas and the higher infection rate was recorded in Al-Shalaa (20.00%), while no infection rate (0.00%) was found in Al-Salam and Al-Gzalia areas. The phylogenetic analysis of 10 local isolates of N. caninum of both stray dogs (8 isolates) and domestic dogs (2 isolates) were recorded in the National Center for Biotechnology Information (NCBI) with accession numbers MN115378 , MN115379 , MN115380 , MN115381 , MN115382 , MN115383 , MN115384 , MN115385 , MN115386 and MN115387 and their compatibility with other global isolates were 99-100%. The present study was considered as the first molecular investigation of N.caninum among both stray and domestic dogs in Baghdad city, Iraq.

Keywords---neosporea, dogs, nested PCR, stray dogs, detection, Baghdad city.

Introduction

Neospora caninum is an obligate intracellular protozoan parasite belonging to phylum Apicomplexa (Dubey *et al.*, 2017). It is a heteroxenous parasite requiring more than one host to complete its life cycle (Dubey *et al.*, 2006). The parasite can cause severe disease in all ages of dogs but it is mostly found in puppies (Nishimura *et al.*, 2015). It causes neuromuscular, may show encephalomyelitis and myositis which results in paralysis and early death in puppies (Al-Qassab *et al.*, 2010). Neosporosis in dogs had been reported in Brazil (Langoni *et al.*, 2012), Germany (Basso *et al.*, 2009), Turkey (Yilmaz *et al.*, 2020), Iran (Motamedi *et al.*, 2020) and Iraq (Mallah *et al.*, 2012). Diagnostic molecular techniques are used for the detection of *N. caninum*, and they have been high levels of sensitivity and specific alternative to morphological methods (Orlandi and Lampel, 2000) and most PCR protocols are used to detect the *N. caninum* DNA from oocysts of dog or coyote feces (Gondim *et al.*, 2004). Due to lack of information about the molecular detection of *N. caninum* in dog's fecal samples and the effects of some risk factors in the infection rate in Baghdad city, this study was designed.

Materials and Methods

Collection of samples: One hundred fecal samples were collected from the stray dogs (52 samples) and domestic dogs (48 samples) into sterile containers and transported by a cooled box to Baghdad Veterinary Hospital, and stored at -20°C during the period from 1/ January / 2021 until 31/ October / 2021.

Stool DNA extraction: The DNA of fecal samples was extracted by using Presto™ Stool DNA extraction Kit (Geneaid Master Mix- Taiwan).

Nested Polymerase Chain Reaction: The first round of n-PCR was performed with both *N. caninum*-specific JB1 and JB2 primers and the second round n-PCR was conducted with SF1 and SF2 primers (Barratt *et al.*, 2008). The first reaction was carried out in 25 µl reaction mixtures having 5 µl of template DNA, 10 pmol of every respective primer, 12.5µL masters mixes (Geneaid Master Mix- Taiwan), and 3.5 µl distilled water. The first round of reaction was performed with primary denaturation at 95°C for 5 min followed by 40 times at 95 °C for 30 sec (denaturation), annealing at 60 °C for 30 sec, extension at 72 °C for 1 min, and last extension at 72 °C for 5 min. The second round of reaction was conducted with the same circumstances as the first round with exceptions, extension at 72 °C for 30 sec and 10 pmol of primers (SF1 and SF2). Products of PCR were electrophoresed by TAE buffer using 1.5% (w/v) agarose gel with ethidium bromide stain, and they were visualized by ultraviolet trans-illumination (Abdoli *et al.*, 2015).

Statistical Analysis: The data were analyzed by using Chi-square test for compare between the risk factors that effects in the infection rate and values of $P \leq 0.05$ and $P \leq 0.01$ were considered as statistically importance. (Al-Mohammed *et al.*, 1986).

Results

Infection rate

Nested PCR products of 100 samples were exhibited in distinct bands of 250 bp on 1.5% agarose gel and the total infection rate of *N. caninum* in dogs of the fecal samples examined was 10% (10/100). (Fig.1). Table (1) shows a higher infection rate of *N. caninum* in stray dogs 15.30% (8/52), than domestic dogs 4.16 % (2/48) with significant ($P \leq 0.01$) difference.



Figure (1): Agarose gel (1.5%) electrophoresis image that shows the Nested PCR products analysis of *ITS1* ribosomal gene of *Neospora caninum* from fecal samples of dogs. Where M: marker (100-1500bp). The lanes (1-10) show positive results at 250bp.

Table (1): Total infection rate of *N. caninum* in dogs

Dogs	No. of examined samples	Positive	Percentage (%)
Stray	52	8	15.30
Domestic	48	2	4.16
Chi-Square (χ^2)	32.62*		

* $P \leq 0.01$

Infection rate according to sex

Results of the present study showed a significant ($P \leq 0.01$) difference between male and female dogs. A high infection rate was recorded in males (12.72%), than in females (6.66%). (Table, 2)

Table (2): Infection rate of *Neospora caninum* dogs according to sex

Sex	No. of examined samples	Positive	Percentage (%)
Males	55	7	12.72
Females	45	3	6.66
Chi-Square (χ^2)	9.76*		

* $P \leq 0.01$ **Infection rate in domestic dogs according to age**

The result shows a significant ($P \leq 0.01$) difference in the infection rates of *N. caninum* among different age groups. The highest infection rate was recorded in the age group more than 5 years (7.70%) followed in the age group between 2-5 years (7.14%), while no infection rate (0.00%) was found in the age groups 6 months - 2 year and less than 6 months. (Table, 3)

Table (3): Infection rate of *Neospora caninum* in domestic dogs according to age

Age	No. of examined samples	Positive	Percentage (%)
≤ 6 months	10	0	0.00
≥ 6 months - 2years	11	0	0.00
> 2-5 years	14	1	7.14
> 5 years	13	1	7.70
Total	48	2	4.16
Chi-Square (χ^2)	19.30*		

* $P \leq 0.01$ **Infection rate according to months**

The infection rate of *N. caninum* was higher in May (20.00%), followed by April (18.18%), and there was no infection rate (0.00%) recorded in January and February with significant ($P \leq 0.01$) difference. (Table, 4)

Table (4): Infection rate of *Neospora caninum* in dogs according to months

Months	No. of examined samples	Positive	Percentage (%)
January	10	0	00.00
February	10	0	00.00
March	11	1	9.09
April	11	2	18.18
May	10	2	20.00
June	9	1	11.11
July	10	1	10.00
August	9	1	11.11
September	10	1	10.00
October	10	1	10.00
Chi-Square (χ^2)	30.9*		

* $P \leq 0.01$

Infection rate of *Neospora caninum* in dogs according to area

A significant ($P \leq 0.01$) difference was found in the infection rate of *N. caninum* between areas of study. The higher infection rate was recorded in Al-Shalaa 20.00% (4/20), while no infection rate (0.00%) was found in Al-Salam and Al-Gzalia. (Table, 5).

Table (5): Infection rate of *Neospora caninum* in dogs according to areas

Areas	No. of examined samples	Positive	Percentage (%)
Al-Shalaa	20	4	20.00
Abu Ghraib	21	4	19.04
Al-Salam	18	0	0.00
AlJihad	13	1	7.69
Palestine Street	14	1	7.14
AlGzalia	14	0	0.00
Chi-Square (χ^2)	66.09*		

* $P \leq 0.01$

DNA sequence and phylogenetic tree

Ten isolates of *N. caninum* from both stray dogs (8 isolates) and domestic dogs (2 isolates) were sequenced and analyzed by BLAST-NCBI program. The sequences were submitted to the NCBI Gen bank database under accession numbers: No. 1 (MN115378), No. 2 (MN115379), No. 3 (MN115380), No. 4 (MN115381), No. 5 (MN115382), No. 6 (MN115383), No. 7 (MN115384), No. 8 (MN115385), No. 9 (MN115386) and No. 10 (MN115387). These local isolates were compared for identities with the global NCBI- Gen Bank *N. caninum* isolates (AY582109.1) Spain, (AY4632123.1) New Zealand, (EF219139.1) USA, (HQ542299.1) Brazil, (JX857865.1) Scotland, (KC710321.1) Iran, (KC461932.1) Ethiopia, (KF536899.1) Chile, (MF802343.1) China, (MH356490.1) Tunisia and (MK 203863.1) Australia, which they were closely related with an identity 100% with No. 1, 2, 4, 6, 8 and 9, and 99% with No. 3, 5, 7, 10 local isolates. (Figure, 2)

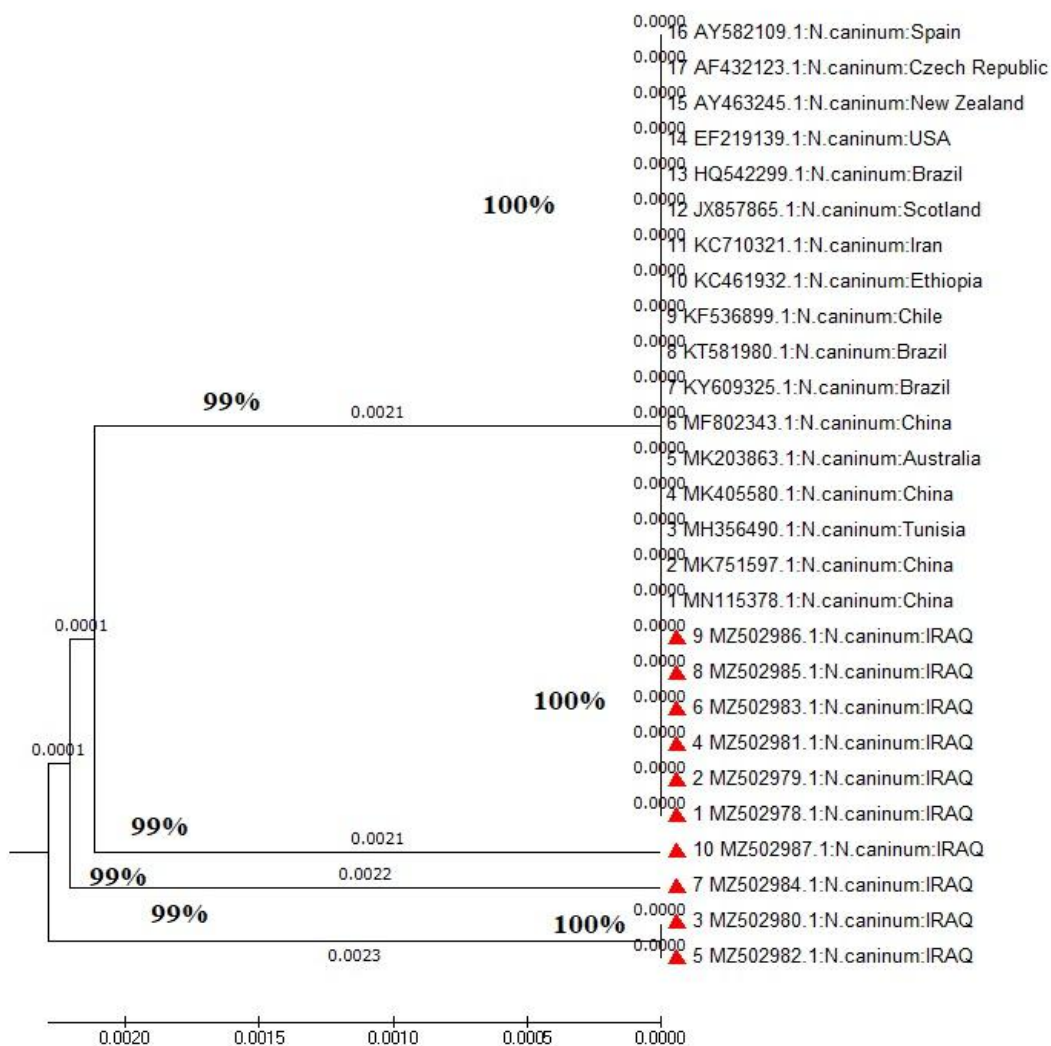


Figure (2): Phylogenetic analysis and identified *N. caninum* of the local isolates with the global NCBI isolates

Discussion

Diagnostic molecular techniques, such as PCR, offer a more sensitive and specific alternative to morphological methods (Adam *et al.*, 2000; Orlandi and Lampel, 2000). PCR-based genotyping systems are among the most useful tools applied to date, because they can be used very small quantities of DNA and allow the identification and tracking of individual strains and the determination of allele and genotype frequencies; For these reasons DNA markers have become the method of choice for defining differences in many eukaryotic genomes (MacLeod, 2004). One of the first repetitive sequences was reported in the *N. caninum* genome by Regidor-Cerrillo *et al.* (2006) and in an article authored by Calarco *et al.* (2018) reported that there are over 100 different identified strains of *N. caninum* isolates; the use of ribosomal DNA, ITS-1, and microsatellites have been

recommended for discriminating between *N. caninum* isolates (Al-Qassab *et al.* 2010). The total infection rate of *N. caninum* in dog fecal samples was 10.00% (10/100), which it was higher than that observed in Turkey 2.00% (Yilmaz *et al.*, 2020), and 3.80% (Düzlü *et al.*, 2014), while lower than that in Isfahan 24.40% (Motamedi *et al.*, 2020), in Tehran, Iran 35.00% (Pouramini *et al.*, 2016), and Brazil 42.90% (Langoni *et al.*, 2012). Previous studies conducted in Iraq done by Mallah *et al.* (2012) in AL-Muthana Province, recorded the infection rate was 1.60%. The variation in the infection rates between the results of the present study and the previous studies may be due to many risk factors associated with infection, including age, sex, particular breed, presence of an intermediate hosts, type of feeding, coinfections and climate that could be affects in the transmission, (sporulation and survival of oocysts) (Reichel *et al.*, 2007; Collantes-Fernández *et al.*, 2008; Anvari *et al.*, 2020). The results of the positive samples of *N. caninum* infection in stray dogs 15.30% (8/52), which is higher than of the domestic dogs 4.16 % (2/48) with significant ($P \leq 0.0$) difference, this results are close agreement with Wang *et al.* (2016), who recorded a significantly higher infection rate of *N. caninum* in rural dogs 18.17%, compared of dogs raised in urban areas (11.33%). This interesting finding may be attributed to the feeding habits like eating raw meat containing parasite cysts, differences in welfare, and living environments (Wang *et al.*, 2016). Infection of dogs in both urban and rural environments is likely to occur through feeding on raw or poorly cooked beef, and vertical transmission from bitches to the successive litters had also been described (Dubey *et al.*, 2007). However, dogs in rural / nature conservation areas are more probable to predate potentially infected small mammals and birds and may be have access to aborted fetal tissues from cattle, wild ruminants and the carcasses of the wild animals (Waap *et al.*, 2017). The present results shows a significant ($P \leq 0.01$) difference between male and female dogs in the infection of *N. caninum* a high infection rate was recorded in males than females with, that results are agreement with the study in China by Wang *et al.* (2016) and Adhami *et al.* (2020), while in Iraq, the higher infection rate was showed in females than males but without significant difference in water buffaloes (Al-Amery *et al.*, 2016); Also, disagree with Dwinata *et al.* (2018) in Indonesia, and in Poland (Gozdzik *et al.*, 2011). The difference in the infection rates between males and females may be related to the levels of sex hormones between both sexes (Gharekhani and Heidari, 2014). A significant ($P \leq 0.01$) difference in the prevalence of *N. caninum* infection among different age groups. The highest infection rate was recorded in the age group more than 5 years, followed by the age group between 2-5 years, while no infection rate was found in at the age groups less than 2 years (6 months - 2 years and < 6 months). The current serological data showed that there is a relationship between serological status and age groups in dogs. The positive seroprevalence of increasing in age above (5) years and this is agreement with results of Wang *et al.* (2016) who recorded the prevalence of infection in dogs increased significantly ($P \leq 0.05$) with increasing the age of animal; the highest infection was detected in the age group six-year-old or older dogs, followed in the age group 3-6 year, while the lower infection rate found in dogs less than 3 years. Also, in the other study that conducted in dogs in central Poland by Gozdzik *et al.* (2011), who found the prevalence of infection increase with age, less than 1 year old, 1- 4 years, 5-10 years, and the highest infection rate in the age group more than 10 years. Al-Majali *et al.* (2008) determined that the seroprevalence of parasite was significantly higher in sheep and goats older than 4 years of age

than in younger animals. Mallah, (2012) suggested that may be due to more ingestion of oocysts (horizontal transmission) that shedding by the final host. According to months, the prevalence of *N. caninum* was higher in May followed by April. No infection rate was recorded in January and February with significant ($P \leq 0.01$) difference. This result close agree with Al-Jumaily and Al-Rubaie (2019) who recorded in the local bread chickens different infection rates in different months which recorded an infection rates in May (25%) followed by April (20%), March (11.1%) and during the December, July and August a same infection rate (5%), in June the infection rate was 9.2%, but there was no infection rate was recorded in January and February. The difference between the present study and the previously reported may be related to the difference in the temperature and humidity. Jung *et al.* (2014) suggested that an increase in the infection rates in some months or seasons than others due to the horizontal transmission for both intermediate and final hosts. Our study was manifested a significant ($P \leq 0.01$) variation between areas of study. The higher infection rate of parasite is registered in Al-Shalaa area while no infection rate was recorded in Al-Salam and AlGzalia. This difference in the prevalence of *N. caninum* among areas in this study may be related to that the dogs living near or with the domestic animals such as cattle and sheep. In addition, some areas are near to the animal slaughterhouses that agree with Ghattof and Faraj (2015) who mention that an increase in the infection rate in these areas. The diversity may be due to the difference in the environmental conditions in the areas of the studies, or quality of the tests (sensitivity and specificity) that used for detection of the parasite (Moore *et al.*, 2002). Sequence analysis of the ITS1 is an important tool for species characterization and differentiation between *Neospora* and closely related parasites, especially between *Hammondia heydorni* and *N. caninum*. However, minor variations in ITS1 sequences are not a strong tool to infer differences in the genus of *Neospora* (Cabrera *et al.*, 2019). The level of genetic diversity detected in *N. caninum* is a little surprising, since sexual reproduction is considered uncommon in this species (Pérez-Zaballos *et al.*, 2005). Intrastrain variation of the ITS1 region appears to be common among isolates of *N. caninum*, which can cause sequencing problems and create inaccurate sequences (Gondim, *et al.*, 2004). It has been suggested that the high homology between strains of *N. caninum* from different geographical areas would be influenced by the movement of animals between different regions, resulting in the distribution of a relatively homogeneous and dominant strain (dos Santos, *et al.*, 2011). Little nucleotide variation in *N. caninum* isolates, and minor intra-strains differences seem to be a common finding, unlike the case of *T. gondii* (Gondim, *et al.*, 2004; Al-Qassab, *et al.*, 2010). The results of the present study was agree with Gondim, *et al.*(2004)who reported that ITS-1 region is not sufficiently variable in sequence for studying diversity within the genus *Neospora* and small differences had been reported in the ITS1 region among strains of North American and European compared among 6 strains, including 2 strains (NC-Illinois and NC-Bahia); Also no major differences were identified in sequence among 11 other *N. caninum* isolates (Barber *et al.*, 1995; Miller *et al.*, 2002; Slapeta *et al.*, 2002), but Cabrera *et al.* (2019) who was isolated four distinct strains and determined by microsatellite typing, these represent three unique genetic lineages, which distinct from those reported previously in the region or elsewhere; an unbiased analysis of the current worldwide genetic diversity of the parasite strains known, whereby six typing clusters can be resolved, revealed that three of the four

Uruguayan strains group closely with regional strains from Argentina and Brazil , the remaining strain groups in an unrelated genetic cluster, suggesting multiple origins of the local strains. In conclusion, *N. caninum* is considered spread in Baghdad city in high manner in stray dogs, females, some months, areas, synchronizing with increasing the age of dogs and identity of local isolates between 99%-100% with the clobber NCBI isolates.

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