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Serological and Molecular Detection of *Neospora Caninum* in Goats in Baghdad City

Haider Mosaed Mohammed [1]*, Haider Mohammed Ali Al-Rubaie [2]

[1, 2] Department of Parasitology, College of Veterinary Medicine, University of Baghdad, Baghdad, IraqEmail: haidar.mosaed1205a@covm.uobaghdad.edu.iq [1], header.m@covm.uobaghdad.edu.iq [2]

* *Corresponding author*

Abstract

Neospora caninum is a protozoan parasite and it has a wide range of hosts, domestic and wild ruminant species. This study was conducted to estimate the prevalence of *Neospora caninum* in local goats using Indirect ELISA and Nested PCR during the period from December / 2020 until April /2021 at Baghdad city using one hundred and nineteen blood samples and one hundred tissue samples (Brain, esophagus, diaphragm and heart) twenty five for each one. The total infection rate by ELISA was 5.04% (6/119). Higher infection rate was recorded in females [7.69% (3/39)], while lower infection rate was found in males [3.75% (3/80)] with significant difference (P≤0.01). Also, higher infection rate was recorded at an age group more than [12 20.00% (2/10)], while no infection rate (0.00%) was recorded in the age group less than 6 months with significant (P≤0.01) difference. Different infection rates were recorded in different Baghdad areas with significant (P≤0.01) difference; while the total infection rate by Nested PCR was 8% (8/100) and brain had a high infection rate (20%) than other organs (heart, esophagus and diaphragm) which showed the same infection rate (4%) with significant (P≤0.01) difference. The sequence of ITS1 gene of *N. caninum* local isolates was submitted in Genbank under the accession numbers MZ725531, MZ725532, MZ725533, MZ725534, and MZ725535 and their compatibility with other global isolates between 99-100%.

Keywords: Neosporosis, ELISA, PCR, Caprine, Iraq

Introduction

Neospora caninum is an obligate intracellular parasite that can infect and cause neosporosis in a wide variety of mammals (Jung *et al*., 2014). It is an Apicomplexan protozoan parasite which identified as a causative agent of reproductive problems in cattle worldwide (Dubey, 2003), goats, sheep and horses (Dubey, 1992; Daft *et al.*, 1997). The definitive hosts are dogs (Mcallister *et al*., 1998), coyotes (Gondim *et al*., 2004), and grey wolves (Dubey *et al*., 2011). Many mammals identifying as an intermediate hosts such as cows (Cabral *et al*., 2009; Serrano-Martínez*etal*., 2019), sheep (Panadero *et al*., 2010; Salaberry *et al.*, 2010), roe deer (Panadero *et al*., 2010), goats (Varaschin *et al*., 2012), alpacas (*Vicugnapacos*) and lamas (*Lama glama*), (Chávez-Velásquez *et al*., 2004). The principal mode of transmission in the intermediate host is believed to be vertical transmission (Dubey and Lindsay, 1996; Dubey, 2003a; Gondim *et al.* 2004a; Dubey and Schares, 2011) or ingestion of contaminated

colostrums (Davison *et al*., 2001) or through contaminated food and water (Thompson *et al*., 2001; Dubey *et al*., 2007) and horizontal transmission in the definitive host (Thompson *et al*., 2001; Dubey and Lindsay, 1996; Dubey *et al*., 2007).

The principle method of diagnosis *N. caninum* infection (aborted fetus) is by histopathology (O'Handley *et al*., 2002), isolation of viable parasite in variety of cell cultures and by bioassays (Dubey and Schares, 2006; Dubey *et al*., 2007), indirect fluorescence antibody test -IFAT (Chávez-Velásquez *et al*., 2004; Varaschin *et al*., 2012), indirect ELISA (Serrano-Martínez *et al*., 2019) and PCR (Varaschin *et al*., 2012; Serrano-Martínez *et al*., 2019). The economic, clinical, and epidemiological significance of neosporosis in goats is not yet clear (Jung *et al*., 2014). Hence, this study was performed for estimation the prevalence and molecular detection of *N. caninum* in Baghdad city.

Materials And Methods

Samples collection

One hundred and nineteen blood samples and one hundred tissue samples (Brain, esophagus, diaphragm and heart) twenty-five for each one from local goats during the period from December/2020 untill April /2021 at Baghdad city. The blood samples were collected from jugular vein using sterile syringes, transmitted to sterile tubes without anticoagulants, which kept and transferred in a cold box to the Laboratory of Parasitology / College of Veterinary Medicine / University of Baghdad. The blood samples were left for clotting and then centrifuged at 3000/ min. for 10 min. to adequate the sera and kept at -20ºC (**Coles, 1986**), which later used for Indirect Enzyme Linked Immunosorbent Assay analysis (ID. Vet 310 Innovative Diagnostics, NCS ver 0818 EN, France). The tissues sampled were kept under -20 ºC until used for DNA extraction and for Nested Polymerase Chain Reaction (Nested PCR).

Serology

Indirect enzyme-linked immunosorbent assay was applied for serological detection of *N. caninum* following the manufacture procedure of the company.

Molecular assay

The Nested PCR primers that used for detection *N. caninum* based on ITS1 region of ribosomal DNA gene that designed by Cabral *et al*. (2009) and provided by the Scientific Researcher Co. Ltd / Iraq (Table 1).

Table (1): Sequence of primers used to detect *N. caninum* based on ITS1 region of ribosomal DNA gene

PCR	Sequence 5'---3'		Product size (bp)	Reference
Conventional PCR rRNA gene	F	AGGAAGGAGAAGTCGTAACAAGG	500	**Cabral *et al*. (2009)**
	R	GAGCCAAGACATCCATTGC		
Nested PCR rRNA gene	F	CCTGTGAGTTGTATCGCCTTC	250	
	R	TCTCTTCCCTCAAACGCTATCC		

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The nested PCR technique was achieved for direct detection of *N. caninum* DNA, which based on ITS1 region of ribosomal DNA gene from goat tissue samples as the genomic DNA were extracted from tissue samples using the gSYAN DNA mini Extraction Kit (Geneaid. USA) and performed according to company instructions. The extracted genomic DNA was checked using the Nanodrop spectrophotometer (THERMO. USA).

The PCR master mixwas prepared by using (Go Taq ^{TM} Green PCR Master Mix) and this master mix done according to company instruction. After that, these PCR master mix tubes that mentioned in table above transferred into Exispin vortex centrifuge at 3000/ rpm for 3 min., and then placed in PCR Thermocycler following these conditions: 1 cycle for initial denaturation (95ºC / 5 min); 35 cycles for denaturation (95ºC / 30 sec), annealing (60ºC / 30 sec) and extension (72ºC / 2 min); and 1 cycle for final extension (72ºC / 5 min). After that, these Nested PCR master mix tubes transferred into Exispin vortex centrifuge and centrifuged at 3000 rpm for 3 min. Then placed in PCR Thermocycler. The PCR Thermocycler conditions were done by using conventional PCR Thermocycler system. The Nested PCR product of rRNA genes were sent to Macrogen Company in Korea in ice bag by DHL for performed the DNA sequencing by AB DNA sequencing system. The DNA sequencing analysis (Phylogenetic tree analysis) was conducted by using Molecular Evolutionary Genetics Analysis version 6. 0. (Mega 6. 0), and multiple sequence alignment analysis based ClustalW alignment analysis and the evolutionary distances were computed using the maximum composite likelihood method by phylogenetic tree UPGMA method. The phylogenetic tree were done between local isolates and *N. caninum* isolates NCBI-Blast related countries andThey were submitted into of NCBI-Genbank to get Genbank accession numbers.

Results

The total infection rate of *Neospora caninum* in goats by indirect ELISA in the present study was 5.04% (6/ 119). According to sex, infection rate of *N. caninum* by indirect ELISA reported that the higher infection rate of *N. caninum* was recorded in females [7.69% (3⁄39)], while a lower infection rate was found in males [3.75% (3/ 80)] with significant difference (P≤0. 01), (Table 2).

Table (2): Infection rate of *N. caninum* by indirect ELISA according to sex

Sex	No. of Examined Animals	Positive	Percentage (%)
Males	80	3	3.75
Females	39	3	7.69
Total	119	6	5.04
χ^2	**75. 67***		

***P≤0.01**

According to age, infection rate of *N. caninum* by indirect ELISA revealed no infection was recorded at an age group less than 6 months [rate 0.00% (0/ 14)], while in the age group between 6 to 12 months the infection rate 4.21% (4/ 95) was found, and finally the higher infection rate was recorded at an age group more than 12 months [20.00% (2/ 10)] with significant difference (P≤0. 01), (Table 3).

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Table (3): Infection rate of *N. caninum* according to age by indirect ELISA

Age /Months	No. of Examined Animals	Positive	Percentage (%)
≤ 6	14	0	0.00
6-12	95	4	4.21
≥ 12	10	2	20.00
Total	119	6	5.04
χ^2	**70. 79***		

***P≤0.01**

According to areas, infection rate of *N. caninum* by indirect ELISA revealed high infection rate of *N. caninum* was recorded in Assadar city [10% (1/10)] according to areas using indirect ELISA, while no infection rate was found in Sabaa Al-Borh, Al-Ameriya, Al-Ghazaliya and New Baghdad with significant difference (P≤0.01), (Table 4).

Table (4): Infection rate of *N. caninum* according to areas by indirect ELISA

Areas	No. of Examined Animals	Positive	Percentage (%)
Abu-Ghraib	30	2	6.66
Al-Taji	15	1	6.66
Sabaa Al-Borh	10	0.00	0.00
Al-Ameriya	5	0.00	0.00
Al-Shuela	15	1	6.66
AL-Ghazaliya	14	0.00	0.00
Al-Fadiliyah	15	1	6.66
New Baghdad	5	0.00	0.00
Assadar City	10	1	10.00
Total	119	6	5.04
χ^2	***48.84**		

*(**P≤0.01**)

The total infection rate of *N. caninum* by Nested PCR was 8% (8/100). The total infection rates of different organs showed that the brain had a high infection rate (20%) than other organs (Heart, Esophagus and diaphragm) which showed the same infection rate (4%) with significant (P≤0.01) difference (Table 5, Figure 1).

Table (5): Total infection rate of *N. caninum* in different organs by Nested PCR

Organs	No. of Examined Samples	Positive	Percentage (%)
Heart	25	1	4
Brain	25	5	20
Esophagus	25	1	4
Diaphragm	25	1	4
χ^2	40. 08*		

***P≤0. 01**

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Figure (1): Agarose gel electrophoresis image (1. 5%) that showed the Nested PCR product analysis of 18S ribosomal RNA (ITS1) gene in *Neospora caninum*from brain goats samples. Where The lane (1-24) shows the positive results (Lanes2, 17 and 21) at 250bp. M: marker (100-1500bp)

The DNA sequencing of the ITS1 gene of *N. caninum* by used the DNA extraction from the tissue samples of goats and performed by using phylogenetic tree analysis and compatible with the NCBI isolates. The local *N. caninum* isolates were submitted under the accession numbers MZ725531, MZ725532, MZ725533, MZ725534, MZ725535 in the GenBank. The compatibility of the local isolates between 99-100% with other global isolates (Table 6, Figure 2).

Table (6): NCBI-BLAST homology sequence compatibility between local *N. caninum* isolates and NCBI-BLAST related country isolates

No.	Accession	Country	Host	Compatibility (%)
1	ID: MK203863. 1	Australia	Dog	100
2	ID: KT581980. 1	Brazil	*Bos Taurus*	100
3	ID: KU253801. 1	Australia	*Canis familiaris*	100
4	ID: KF536906. 1	Chile	Bovine	100
5	ID: JN634857. 1	China	Cow	100
6	ID: MW022528. 1	Brazil	*Larus dominicanus*	100
7	ID: JF810965. 1	Brazil	Chicken	100
8	ID: HQ542299. 1	Brazil	Dog	100
9	ID: HQ323749. 1	Brazil	Goat	100
10	ID: EU564167. 1	Spain	Bovine	100
11	ID: EF219139. 1	USA	Dog	100
12	ID: AY463245. 1	New Zealand	Cow	100
13	ID: AY877364. 1	USA	Deer	100
14	ID: AF432123. 1	Czech Republic	Dog	100
15	ID: MW044667. 1	Brazil	*Sula leucogaster*	99
16	ID: U16160. 1	Sweden	*Canis familiaris*	99
17	ID: MG871214. 1	Poland	---------	100
18	ID: KY562727. 1	Tunisia	Sheep	100

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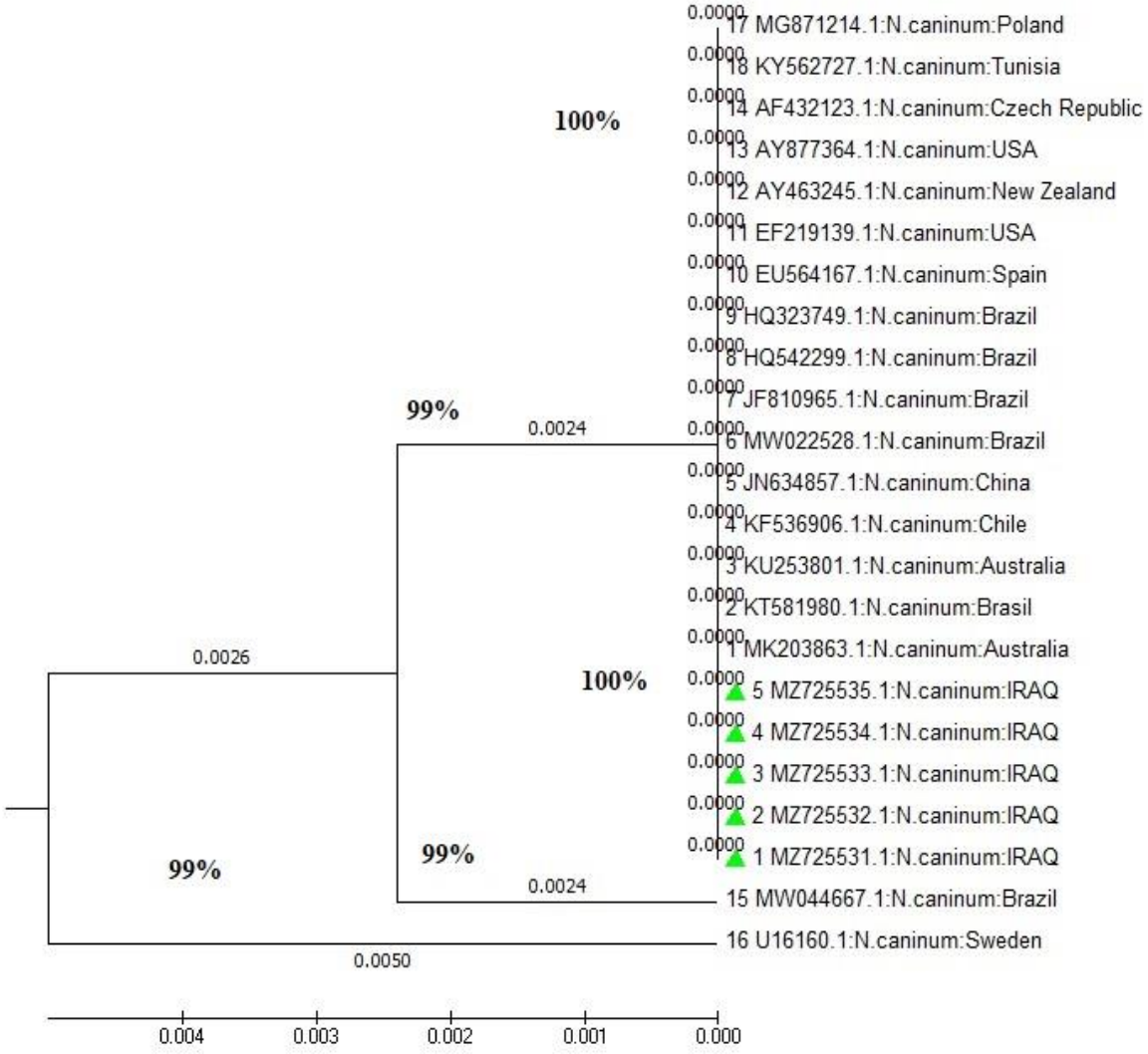

Figure (2): Phylogenetic tree analysis based 18S ribosomal RNA gene partial sequence in local *Neospora caninum* brain goat isolates that used for genetic relationship analysis. It was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in MEGA 6. 0 version. They are showed closed related to NCBI-BLAST *N. caninum* isolates at total identity between 99-100%.

Discussion

*Neospora caninum*is an obligate intracellular protozoan parasite of the phylum Apicomplexan, which preferably infects cattle as intermediate hosts, causing neosporosis, one of the main causes of cattle abortion and reproductive failure worldwide (Dubey and Schares, 2011). Although *Neospora* infection has also been described in other domestic and wild ruminant species (Chávez-Velásquez *et al*., 2004). Seroprevalence of parasite had been reported in various countries (Dubey and Schares, 2011), and had been detected in various animal hosts (Jung *et al*., 2014). The seroprevalence in goats reported in some countries, 5.6% in Iraq (Ghattof and Faraj, 2015); 17 out of 300 (5.7%) (Al-Majali *et al*., 2008), 2.0% (Abo-Shehada and Abu-Halaweh, 2010) andDiakoua *et al*. (2013) recorded 6.9% in Jordan; 18 out of 116 (15.5%) in Slovakia (Cobadiova *et al*., 2013); 26 out of 375 (6.9%) in Greece (Diakou *et al*., 2013; 6% in the Czech Republic (Bartova and Sedlak, 2012); 9% in Poland

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(Czopowicz *et al*., 2011); 3. 3% Faria *et al*. (2007), 15% (Uzeda *et al*., 2007) and 72 out of 667 (10.7%) (Andrade *et al*., 2013) in Brazil; 6.6% in Argentina (Moore *et al*., 2007). Jung *et al. (*2014) indicated a nationwide distribution of *N. caninum* among goats in native Korean goats (*Capra hircuscoreanae*) with a relatively low prevalence, by a commercial ELISA kit used to analyze 464 sera; four samples (0.9%) were found to be positive for *N. caninum* antibodies. The prevalence of positive antibodies 6.1% to *N. caninum* in goat (*Capra hircus*) by IFAT in Costa Rica from 81 examined animals (Dubey *et al*., 1996); in Sa˜o Paulo, Brazil were examined 394 animals by IFAT (6.4%) (Figliuolo*et al*., 2004) and in Taiwan no infection rate (0.00%) was recorded when examined 24 animals by IFAT (Ooi *et al*., 2000). Sharma *et al*. (2015) found the seroprevalence of parasite in sheep and goats in Grenada, West Indies by using an indirect ELISA multispecies kit in 18 of 138 sheep (13%) and 8 of 138 goats (5.8%). In addition, (Nie *et al*., 2018) who recorded in Tibetan sheep of 2187 sera 184 (8.4%) were seropositive by competitive – inhibition ELISA. The phylogenic studies showed that it was very closely related to *T. gondii* and it is placed as the sister group of this parasite (Hemphill *et al*., 1996). The infection of *Toxoplasma gondii* percentages of females were more than males in both horses and camels 21.1% and 20.2%, respectively (Asal and Al Zubaidy, 2016). The infection in young cats was higher than in adults (Kallo, 2002). Seropositive ELISA-IgG was demonstrated that 66 % of stray cats, female expressed 75% of seropositive by ELISA-IgG were higher than male (30%) and animals with average age 2 months showed high percentage of seropositive ELISA-IgG (100%) compared adult cat that expressed 63.82 % of seropositive (Jalil and Alwan, 2014).

The differences between the results of the present study and the previous studies may in agreement with the explanation before that the infection rate in the intermediate hosts affecting by the oocyst shedding which are largely unknown and difficult to investigate because of the costs involved in housing dogs in a secure facility and the low numbers of oocysts shed or oocyst shedding is erratic. Although, these results are not similar because of different serologic methods and different cutoff values used, none of the serologic tests used to detect antibodies have been confirmed based on recovery of the viable parasite in any host; therefore, the cutoff values used for serologic diagnosis are presumptive (Dubey *et al*., 2007). On same hand, that immunosuppressed dogs may shed more oocysts than immunocompetent dogs (Lindsay *et al*., 1999; 2001). Sharma *et al*. (2015) reported dogs might be considered as a source of infection, and it was found a positive correlation between the seropositive of farm dogs and increased seroprevalence in cattle, indicating a relationship between infections in dogs and in cattle (Wouda *et al*., 1999). Few studies were suggested an indication that there was a positive relationship between the presence of dogs on the farm and the seroprevalence (antibodies) of *N. caninum* in small ruminants (Soares*et al*., 2009; Abo-Shehada and Abu-Halaweh, 2010; Machado *et al*., 2011; Sharma *et al*., 2015). Although, the virulence strains of parasite may be effects in the infection rate because some strains are more pathogenic than others (Lindsay and Dubey, 1990; Lindsay *et al*., 1995; Atkinson *et al*., 1999; McInnes *et al*., 2006). However, Dubey *et al*. (2007) referred that caution should be used to evaluate the results because of differences in serologic techniques, study design, and sample size that used; so presumably, cows remain infected for life and transmitted the infection to their offspring for several consecutive pregnancies (Fioretti *et al*., 2003), or that may be intermittently (Boulton *et al*., 1995; Wouda *et al*., 1998; Guy*et al*., 2001). On the other hand,

the endogenous transplacental infection may decrease in the subsequent pregnancies due to an indication of immunity (Anderson *et al*., 1995; Dijkstra *et al*., 2003; Romero and Frankena, 2003). Although, exposure to *N. caninum* had been demonstrated in a large variety of wild and domestic animal species, viable parasites have been isolated from a limited number species, including cattle, bison, water buffaloes, white-tailed deer, sheep, and horses (Dubey and Schares, 2011). On the same way, Jung *et al*. (2014) reported that the lower infection rate of infection in their study might be attributed to differences in the following factors: the number of definitive hosts (dogs and/or other canids in the study area), climate, age, frequency of dogs defecating in the study area, farm management systems, and/or regional ecology hosts; high temperature and humidity favor faster sporulation and enhanced survival of oocysts in the environment; an accurate assessment of seasonal prevalence was difficult because antibodies against *N. caninum* can persist for several months. Also, minimal information was available on the presence of *N. caninum* hosts, grazing conditions, and farm management systems, all of which may have affected the seroprevalence and a larger sample size and more detailed information on goat breeding conditions would be needed to obtain more reliable data on seasonal variability and seroprevalence, and some epidemiological factors such as a favor temperature and humidity faster sporulation and enhanced survival of *N. caninum* oocysts in the environment and increases the risk of postnatal infection (Thurmond *et al*., 1995; Dubey *et al*., 2007). Although, the interspecies transmission has not been reported, risk of horizontal transmission from cattle to goats through a definitive host and vice versa should not be excluded (Jung *et al*., 2014) and the transmission can be occur through experimentally infected milk replacer or colostrum (Davison *et al*., 2001). These results were disagree with some previous reports that were mentioned no significant differences observed between female and male goats (Faria*et al*., 2007; Garcia-Bocanegra *et al*., 2012; Sharma *et al*., 2015; Topazio*et al*., 2014 ; Gharekhani *et al*., 2016). The difference between males and females in the infection rate may be related to the differences in hormone levels between them (Gharekhani and Heidari, 2014). It is also the difference between both sexes may be due to the horizontal transmission and exposure of older animals for a long period (Nie *et al*., 2018), while these results not reliable with some previous studies that mentioned before, there was no significant difference observed between males and females goats (Garcia-Bocanegra *et al*., 2012; Topazio*et al*., 2014; Gharekhani *et al*., 2016). These results were consistent with some previous study when the older animals were at high risk of being exposed to *N. caninum* (Cerqueira-Cezar *et al*., 2016), but disagree with Jung *et al.* (2014) who found no statistical significant difference in seroprevalence according to age that was analyzed (less than to one year, young ; more than or equal one year, adult ; and unknown), and Nie *et al*. (2018) who recorded in Tibetan sheep there was no statistical significance among age groups; but Al-Gharban *et al*. (2017) identified that cows with ≥3 years were being more infection with *N. caninum*. Chávez-Velásquez *et al*. (2004) found infection in the adult alpacas and llamas in Peru; for that seropositive in herds might be increase with age or gestation number and the horizontal transmission can be influence event (Jensen *et al*., 1999; Rinaldi *et al*., 2005). Al-Shaeli *et al*. (2020) mention the reasons for that may be due to the different investigation sites, different detection methods, breeds, various sample capacities, and referred that it is difficult to compare *N. caninum* prevalence between studies mention before, but these studies all confirmed that caprines are truly easy to be

infected. therefore, attention should be used when risk factor analysis obtained in a particular region or management system to another (Al-Shaeli *et al.,* 2020). Jung *et al*. (2014) were reported no statistically significant differences in seroprevalence according areas. The infection ratein dogs of different areas may be affects in the infection rate in the goats in the present study that was recorded before; the seroprevalence in dogs in Korea had been reported at 10.3% (35/340) and specifically in rural dogs at 21.6% (11/51) (Kim *et al*., 2003). The results of the present study are showed the compatibility with the world isolates previously recorded between 99-100%, that disagree with Cabrera *et al*. (2019) who isolated four distinct strains which 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.

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