Original Article

Molecular and Serological Study of *Neospora caninum* Infection among Dogs and Foxes in Sanandaj, Kurdistan Province, Iran

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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 aimed to investigate the serological and the molecular prevalence of *N.caninum* among foxes and dogs using indirect fluorescent antibody test (IFAT) and nested-polymerase chain reaction (PCR). For this purpose, 288 and 95 both fecal and serum samples of dogs and foxes were collected, respectively, from around industrial and traditional dairy flocks in different parts of Sanandaj, Kurdistan Province, Iran, from 2013 to 2015. The sera were examined using IFAT, and fecal samples were microscopically assessed for detecting *Neospora* oocyst and by nested-PCR. The findings revealed that *N.caninum* seroprevalence were 4.86% and 4.21% in dogs and foxes, respectively. In addition, no *Neospora* oocysts were found microscopically and by PCR. Since this study is the first serological and molecular investigation of *N.caninum* among both dogs and foxes in Sanandaj, the findings of indicated that stray dogs is a main source of *N.caninum* infection in dairy farms in Sanandaj, Iran.

Keywords: Dog, Fox, Indirect Fluorescent Antibody Test (IFAT), *Neospora Caninum*, Nested- polymerase chain reaction (PCR), Sanandaj, Iran

Étude Moléculaire et Sérologique de *Neospora caninum* chez des Chiens et des Renards dans le District de Sanandaj, Province du Kurdistan (Iran)

Résumé: *Neospora caninum*, un parasite protozoaire, provoque à la fois des avortements chez les bovins ainsi que des troubles neurologiques et des problèmes de reproduction chez les chiens. Dans cette étude, la prévalence sérologique et moléculaire de *Neospora caninum* a été étudiée chez les renards et les chiens par le biais de test d'immunofluorescence indirecte (IFA) et une nested-PCR. Pour cela, 288 et 95 échantillons fécaux et sériques de chiens et de renards ont été respectivement collectés entre 2013 et 2015 dans des troupeaux laitiers industriels et traditionnels dans différentes parties du district de Sanandaj, dans la province du Kurdistan. Les sérums ont été examinés avec le test IFA et par nested-PCR, alors que des échantillons de matières fécales ont été examinés au microscope pour détecter la présence d'oocyste de *Neospora* et. Les résultats ont révélé que la séroprévalence de *N. caninum* était respectivement de 4,86% et 4,21% chez les chiens et les renards. De plus, aucun oocyste de *Neospora* n'a été détecté au microscope et par PCR. Comme cette étude est la première enquête sérologique et moléculaire de *N. caninum* chez les chiens et les renards de Sanandaj, les résultats de l'étude ont indiqué que les chiens errants sont une source majeure d'infection à *N. caninum* dans les fermes laitières de Sanandaj.

INTRODUCTION

Neospora caninum is the main cause of both neuromuscular disorders in dogs and abortion in cattle, which leads to irreparable economic losses in the dairy industry (Wang et al., 2016). Moreover, the protozoan infections, such as *N. caninum* can cause an important disease in dogs (Dubey et al., 2005) since the parasite was at first emerged in the brain of a dog in 1988 (Yildiz et al., 2009). Naturally, contaminated dogs have been identified across the world (Vlaardingerbroek, 2013). Bovine neosporosis is regarded as one of the vital causes of abortion in cattle worldwide. Due to the high prevalence and rapid spread of this disease factor in cattle, *N. caninum* is considered a vital factor for bovine abortion (Bahrami et al., 2016). The worldwide economic influence of *N. caninum* infections/abortions has been estimated at 1.3 billion dollars annually (Okumu et al., 2016). Studies on the incidence of anti-*N. caninum* antibodies have shown that neosporosis has a much greater geographic distribution (Oliveira et al., 2004). Although dogs are important in the epidemiology of *N. caninum* reflecting environmental contamination (Sedlak and Bartova, 2006), there is a dearth of research reporting this fact that they are capable of shedding significant numbers of oocysts in their feces (King et al., 2012). However, the predominance of experimentally contaminated canids has led only to a mean number of oocysts (King et al., 2012). *N. caninum* causes mortality in dogs (Acosta et al., 2016), and they have been the first founded definitive hosts over *N. caninum*. Recently, coyotes (*Canis latrans*) have been additionally established according to definitive hosts on the parasite (Gondim et al., 2004). The dog has been recognized as an intermediate and definitive host for the parasite since it sheds oocysts among the faeces following the ingestion of *N. caninum*-contaminated tissues. Although dogs can be contaminated through the ingestion of infected tissues, the ascending pass of transmission can also be noticed across the placenta (i.e., transplacental transmission). Herbivores are intermediate hosts; however, they may be infected by the ingestion of circulating oocysts as definitive hosts with subclinical congenital infection (Vlaardingerbroek, 2013). Environmentally, the parasite resistant stage (i.e., oocyst) is excreted in the coyotes and dog feces through an unsporulated phase. Oocysts need at least 24 h to sporulate in the host (Dubey et al., 2007). Clinical and subclinical infections, including *N. caninum* among dogs are epidemiologically obvious since domestic dogs (*Canis familiaris*) are capable of bearing oocysts in the environment. As a result, they constitute a risk for the incidence of abortion-related to *N. caninum* in bovines (Oliveira et al., 2004). The most extreme symptoms of neosporosis among dogs taking place in congenitally contaminated puppies include predominant paralysis of the limbs and solid hyperextension, which leads to innovative paralysis and death. Moreover, difficult swallowing, jaw paralysis, muscular stiffness, dermatitis, peritonitis, atrophy, and cardiac failure are observed in dogs. In addition, the disease can progress to a generalized form, and adult and/or old puppies can also present with most extreme neosporosis, which result in deaths. Furthermore, reproductive disorders, such as abortion and stillborn, take place in female dogs due to neosporosis (Moura et al., 2012). Dogs of any age can develop clinical

neosporosis (Oliveira et al., 2004), and the neurological signs depend on the site parasitized (Oliveira et al., 2004). Determination of neosporosis may be reached by applying histology, immunohistochemical staining, polymerase chain reaction (PCR), and serology (Ghalmi et al., 2014). Moreover, serological tests have this benefit to be used ante mortem (Ghalmi et al., 2009). The serological screening of *N. caninum* in puppies is an indirect tool to assess environmental infections; therefore, all seropositive animals probably bear oocysts (Waap et al., 2017). Serologic tests are regarded as the vital techniques for *N. caninum* determination and consist of Indirect Fluorescent Antibody Test (IFAT), Enzyme-Linked Immunosorbent Assay, Direct Agglutination Test, or *Neospora* Agglutination Test. However, serological testing as an indirect diagnosis test has been subjected to significant changes; moreover, in the diagnosis of early or chronic contamination induced by cysts formation, the serology may result in negative parasite which is observed in the dog (Pouramini et al., 2017).

The confirmed reference technique for serological prognosis is IFAT. The IFAT is applied to many species and regarded the "gold standard", compared to other new techniques (Ghalmi et al., 2014). The amount of cutoff for IFAT varies over laboratories; however, the most frequently applied ones are 1:50 among puppies (Oliveira et al., 2004). Therefore, direct prognosis and rather sensitive methods, namely immunohistochemistry or PCR may be applied (Pouramini et al., 2017). There are no reports that *N.caninum* contaminates human beings; therefore, the incidence of antibodies over this factor has been pointed out in humans. Experimental results in primates showed congenital transmission of the protozoa and indicated that clinical neosporosis in primates was once comparable in conformity with congenital toxoplasmosis in human beings (Moura et al., 2012). Although many seroepidemiological and molecular investigations have been conducted on *N. caninum* among dogs in different parts of Iran, no study has

reported the prevalence of *N. caninum* among puppies in Kurdistan Province, Iran. Therefore, this study aimed to investigate the molecular, microscopic, and serological features of *Neospora* among dogs and foxes in Sanandaj, Iran.

MATERIAL AND METHODS

Sanandaj is the center of Kurdistan Province and is located in western Iran with latitude and longitude of 35 ° 18′ 53N and 46° 59′ 55E, respectively. The temperature in Sanandaj is between -8 to 35.1 in a variable scale, and the annual precipitation rate is between 6 and 27 mm monthly (Figure 1). In total, 288 blood and stool samples from the flock and stray dogs and 95 samples from foxes were collected from July 2013 to February 2015 in Sanandaj, Iran. It should be noted that the samples were taken from the dogs living in the industrial and traditional dairy flocks as well as the stray dogs or foxes roaming around the farme in the area. Subsequently, the sera were isolated and kept at -20 °C until examination.

Serological Tests. The antibody of *N.caninum* was identified in animal sera by IFAT. In performing the serological test, *N.caninum* tachyzoites (NC-1 strain) were applied as antigens (Langoni et al., 2012). Briefly, $1x10^4$ tachyzoites were used for 10 slides. The parasites were fixed on the with aceton and 20 μl of the diluted sera (1:50-1:800) was poured on the slides for 25 min at 37 °C. After washing, a FITC-conjugated anti-dog immunoglobulin G (IgG) (Sigma-Aldrich, St Louis, USA) was poured and incubated as mentioned above. In the next stage, the slides were mounted, and positive tachyzoites were observed under an epifluorescence microscope (400x) (Ghalmi et al., 2008). The selected serum cutoff was 1:50.

Microscopic Test of Stool Samples. A strainer (60 meshes) was employed to sieve fecal material using tap water for separation. The samples were mixed with 2.5% potassium dichromate (K2Cr2O7) for 10-14 days at room temperature and kept at 4 °C for more usage. To remove the K2Cr2O7, the oocysts were rinsed 4-6

times using tap water by centrifugation (1100×g) for 5 min. The standard technique on sucrose flotation was applied to separate the oocyst. One gram sample of feces was transmitted into 15 ml tubes combined with 14 ml concentrated sucrose, and centrifugation was implemented (1600×g) for 10 min. Totally, three drops were collected from the supernatant surface applying a loop and transferred to a slide for microscopic evaluation. The oocysts content of the concentrated sucrose solution was assessed using light microscopy at magnification 400 (Dalimi et al., 2014).

DNA Extraction. Stool sample homogenization was performed by 20 ml of phosphate-buffered saline. The phenol-chloroform extraction instructions were used to extract DNA from stool samples. The extracted DNA was precipitated by the addition of 0.1 volume of sodium acetate solution (3M, pH 5.2) and both volumes of 100% cold ethanol; moreover, it was stored at -70 °C for 60 min and centrifuged for 5 min at 13,000 g. The pellet was rinsed two times using 70% ethanol and re-suspended in distilled water (100 µl). The DNA concentration was assessed via a spectrophotometric test at 280/260 nm. Furthermore, the DNA was kept at -20°C until use (Abdoli et al., 2015).

Nested Polymerase Chain Reaction. The PCR was performed with both *N. caninum*-specific primers Np21plus and Np6plus (Müller et al. 1996). Additionally, the second turn nested-PCR was conducted with Np6 and Np7 primers. The first reaction was carried out in 20 µl reaction mixtures having 1 µl of template DNA, 10 pmol of every respective primer, 10 µl 2 × masters mixes (DFS Master Mix- BIORON GmbH), and 7 µl distilled water. Moreover, the first round of amplification reaction was performed with primary denaturation at 94 °C for 5 min followed by 40 times at 94 °C for 40 sec (denaturation), annealing at 62 °C for 40 sec, extension at 72 °C for 40 sec, and last extension at 72 °C for 10 min. The second stage of nested-PCR was conducted with the same reaction circumstances as the first, except for the utilization of an annealing temperature at 56 °C and 25 pmol of primers (Np7 and Np6). One microlitre from

the first round of PCR product was applied as the sample for nested-PCR. A negative control (double distilled water) and positive control (DNA from the NC5 strain of *N. caninum*) were involved for every reaction. In the next stage, five microliters of every product of PCR was electrophoresed (by TAE buffer) using 1.5% (w/v) agarose gel bear with safe stain, and it was visualized by ultraviolet trans-illumination (Abdoli et al., 2015).

Statistical Analysis. Statistical analysis was performed in SPSS software (version 19) through the Chi-square test (X^2) to compare the seroprevalence rates. A p-value of ≤0.05 was considered statistically significant. The 95% confidence intervals (95% CI) of seroprevalence rates were also calculated in this study.

RESULTS

The *N.caninum* IgG antibodies were detected in 4.86% (n=14/288) and 4.21% (n=4/95) of the dog and fox sera, respectively. Totally, 4.72% (n=7/148) and 5% (n=7/140) female and male dogs were serologically positive, respectively. However, serum positive levels were higher among male dogs, compared to that in female dogs with no significant differences. Moreover, two male (n=2/44) and female (n=2/51) foxes were found serologically positive with no significant differences. In the microscopic test of stool samples, no *Neospora* oocyst was observed, and no specific band was found in the molecular study of the stool specimens.

DISCUSSION

During three past decades, *N. caninum* has been widely studied as a veterinary pathogen due to its importance (Donahoe et al., 2015); however, there is little information regarding the parasite outbreak in foxes and dogs as the parasite final hosts. In the current study, the contamination rates of *N. caninum* were 4.86% and 4.21% in dogs and foxes, respectively. This study is considered as the first serological and molecular investigation on the infection among dogs in Sanandaj, Iran, and the first study on foxes in this

country. Regarding the prevalence of *N. caninum* among dogs in different regions of Iran, Gharakhani et al. (2014) reported a 27% infection rate among dogs in Hamadan province, Iran (Gharekhani and Heidari, 2014). Moreover, Gharedaghi (2012) reported 31% of seropositivity among stray dogs in Tabriz, Iran (Garedaghi, 2012). According to a study conducted by Hossein Nejad and Hosseini (2011), an infection rate was determined at 29% among dogs in the central and western parts of Iran (Hosseininejad and Hosseini, 2011). In the same line, the results of a study carried out by Khanmohammadi and Fallah (2011) estimated the prevalence rate at 10.6% among herding dogs in

Sarab, Iran (Khanmohammadi and Fallah, 2011). Furthermore, a high infection rate of 27% was reported by Yakhchali et al. (2010) among dogs in Urmia, Iran (Yakhchali et al., 2010), and Malmasi et al. (2007) revealed 33% incidence rate among dogs in Tehran, Iran. It can be seen that the incidence of *N. caninum* seropositivity (4.86%) among puppies in Sanandaj, Iran, is lower than that in other areas in this country (Table 1). Nevertheless, the predominant reasons concerning various results can be attributed to different serological tests and cutoff amounts, climatic variations, study design, and the incidence of canid frequency over the farms or around animals
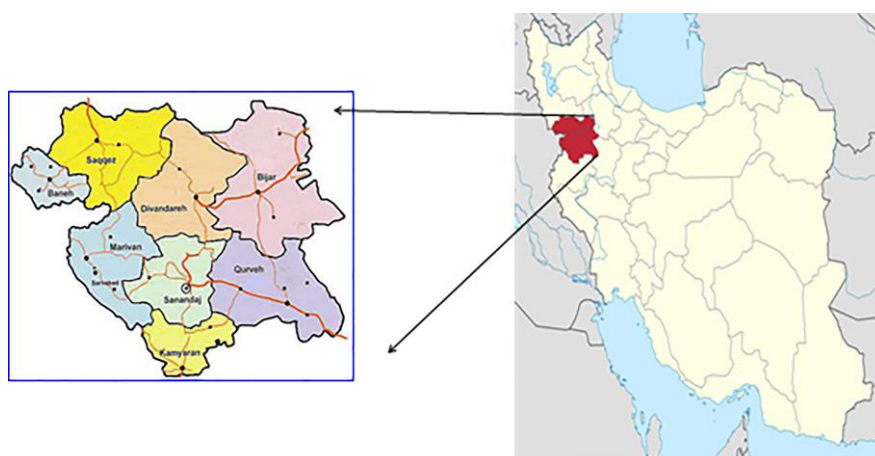

Figure 1. The geographic location of the study area (Sanandaj district, Kurdistan Province, Iran)

Table 1. A summary on the prevalence of Neospora caninum among dogs in different cities in Iran

Location and Year	Number of studied animals (N)	Serology (%)	Polymerase chain reaction (%)	Reference
Sanandaj, Kurdistan Province, western Iran	288	IFAT, 4/86	0	Current study
Hamadan Province, Northwestern Iran, 2009-2012	270	IFAT, 27	_	(Gharekhani and Heidari, 2014)
West and central parts of Iran, 2007-2009	548	Elisa, 29	_	(Hosseininejad and Hosseini, 2011)
Sarab District, East Azerbaijan Northwestern Iran	384	IFAT, 10/6		(Khanmohammadi and Fallah, 2011)
Mashhad, Khorasan Razavi Province, Eastern Iran, 2006-2008	174	_	1/14	(Razmi, 2009)
Tabriz, East Azerbaijan, Northwestern Iran, 2012	100	IFAT, 31	_	(Garedaghi, 2012)
Lorestan Province, Western Iran	428	-	2/1	(Dalimi et al., 2014)
Tehran, Tehran Province, Center of Iran, 2007	100	IFAT, 33	_	(Malmasi et al., 2007)
Tehran, Tehran Province, Center of Iran, 2007	103	IFAT, 19/4	_	(Haddadzadeh et al., 2007)
Urmia, West Azerbaijan Province Northwestern Iran	135	IFAT, 27	_	(Yakhchali et al., 2010)

(Gharekhani and Heidari, 2014). Furthermore, the findings revealed that the contamination rate was higher in males (5%) rather than females (4.2%). Additionally, the amount of contamination in male foxes (4.54%) was higher than that in female ones (3.92%); however, this difference was not significant (P>0.05). There were no significant differences among the results of the studies in terms of gender, which was consistent with the findings of other studies (Hosseininejad and Hosseini, 2011; Sharifdini et al., 2011; Gharekhani and Heidari, 2014). The findings of a study conducted by Moura revealed no relationship between gender and the incidence of antibodies over *N.caninum*. Similarly, no relationship was observed between gender and the incidence of anti-*N. caninum* antibodies in the studies conducted by Coskun et al. (2000) in Turkey and Oliveira et al. (2004) in Campo Grande, Brazil. In a similar vein, Jesus et al. (2006) in Bahia State, Brazil, found no relationship between gender and the frequency of antibodies anti-*N. caninum* (Moura et al., 2012). The findings of several studies revealed that neosporosis was observed among male dogs. Moreover, other studies showed that this finding was true for female dogs. The findings of other studies indicated no significant difference between males and females in terms of seropositivity. Obviously, male dogs are more frequently preferred in animal farms; therefore, they might have been contaminated more, compared to female dogs (Yildiz et al., 2009). Furthermore, in Iran, farmers mostly rear male dogs in their farms; accordingly, male dogs may be contaminated with *Neospora* more than the females (Hosseininejad et al., 2011; Khanmohammadi and Fallah, 2011). The findings of this study were also in line with 4.8% of the results obtained from a study conducted on foxes in Britain by Bartely et al. (Bartley et al., 2013). The incidence rates in previously performed studies were between 10.6% and 33%, and the findings showed that despite 4.86% seropositivity, there were no *N. caninum* oocysts in the stool samples. The diagnosis of neosporosis during life is difficult (Dubey et al., 2005). Specific antibody identification in

dog sera shows the contact ability of these animals with the pathogen; however, there is neither significant relationship with a bear of oocysts nor horizontal transmission danger or environmental infections. As a result, oocysts are rarely detected in the feces of dogs (Goździk et al., 2011). Another justification for the presence of antibodies in the sera of dogs may be attributed to habits, including the ingestion of raw meat consisting of parasite cysts; however, dogs ingest raw or poorly cooked meat by their properties (Goździk et al., 2011). Moreover, the consumption of raw beef may be considered as a risk factor for *N.caninum* contamination. The primer pairs Np21/Np6 and Np7/Np6 were used for PCR as the certain factors for *N. caninum* prognosis; however, *Neospora* oocyst was not found in fecal samples of dogs and *Neospora* DNA was not detected in the samples. In Iran, only two studies reported isolated *N. caninum* oocyst from fecal specimens among dogs in Mashhad and Lorestan Province, Iran. In a study conducted by Razmi et al. in 2009, out of 174 dogs, fecal specimens of two of them were infectious, and this rate was obtained at 1.2% in a study performed by Dalimi et al. (2014). In a study, *N. caninum* oocyst shedding was observed among dogs that were infected naturally (Razmi, 2009), and the oocysts were rare in the feces of dogs according to a study conducted by Goździk et al., 2011. Furthermore, in a study performed by Galmi et al., 8 dogs were found positive through IFAT and negative through PCR indicating that PCR was less sensitive than IFAT. It is worth mentioning that since a change occurs at the initial and final stages of infection, fewer oocysts are excreted in the feces (Stoker, 2013). However, *N. caninum* oocyst has to be isolated from related coccidian oocysts, including *Hamondia hydroni* and *Toxoplasma gondii* which are rarely found in feces (Stoker et al., 2013). Proper identification of the *N. caninum* oocysts in fecal samples is crucial (Mallah et al., 2012); therefore, it is suggested to utilize molecular methods to better recognize parasite infection among the dogs (Dalimi et al., 2014). Moreover, contaminated dogs over a specific area must be assumed as a risk

factor for *Neospora* infection in cattle (Goździk et al., 2011). *N. caninum* seropositivity in dogs is recognized for a recent or past touch over the parasite; however, it may not be related to the oocysts' shedding by the infected dogs. Consequently, the molecular methods are recommended to be used to improve the diagnosis among dogs. Truly, molecular techniques can detect coccidian oocyst species using PCR and sequencing (Dalimi et al., 2014). In Mashhad, Iran, 174 specimens were collected from 85 household dogs and 89 farm dogs during 2006 and 2008. Subsequently, the fecal samples were tested microscopically to detect *Hammondia Neospora*-like oocysts (HNLO). The HNLOs fecal samples were assessed using *N. caninum*-certain PCR, and two samples were found positive for *N.caninum* (Razmi 2009). In Costa Rica, Palavicini et al. (2007) gathered 263 fecal samples at intervals from 34 farm dogs. The fecal samples were tested microscopically for *N. caninum*-like oocysts through DNA detection applying PCR and bioassay. Additionally, the DNA *of N. caninum* was indicated using PCR in four samples of feces two times from one dog; however, the oocysts were not microscopically observed in this study (Dalimi et al., 2014).

In conclusion, the seropositivity rates of *N. caninum* were determined at 4.86% and 5% among dogs and foxes in Sanandaj, Kurdistan Province, Iran, respectively. In this regard, further investigations on dog populations are needed in Kurdistan to indicate the possible infection path in dogs and assess neosporosis seroprevalence in certain hosts in other areas of the country. The findings of this study revealed that farm dogs may be a risk factor for the contamination of *N. caninum* in dairy farms in Sanandaj, Iran. However, to confirm this hypothesis, further studies are recommended to be conducted on stillborn calves or aborted cattle.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

Authors' Contribution

Study concept and design: Adhami, Gh.; Dalimi, A.; Hoghooghi-Rad, N.

Acquisition of data: Adhami, Gh.; Fakour, Sh.

Analysis and interpretation of data: Adhami, Gh.; Dalimi, A.

Drafting of the manuscript: Adhami, Gh.; Dalimi, A.

Critical revision of the manuscript for important intellectual content: Dalimi, A.

Statistical analysis: Dalimi, A.

Administrative, technical, and material support: Dalimi, A.; Hoghooghi-Rad, N.

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