

Original Article

Molecular Detection of *Neospora caninum* in Infected Dogs of Isfahan, Iran

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Summary

The aim of the present study was to evaluate the presence of *N. caninum* in stool samples of one hundred sheltered and Indoor dogs in Isfahan province, Iran, by using the PCR method. It is believed that cattle infected by *N. caninum*, with acute or chronic forms, represent a higher risk of abortion than non-infected cows. The zoonosis aspects of Neosporosis is under investigation and infection with *N. caninum* in human is not reported yet. We collected Ninety stool samples from mixed shelter dogs of an animal shelter and ten more stool samples from Indoor dogs with the average age of 1-3 years old in districts of Isfahan province, Iran. The samples were selected randomly and examined by the PCR technique to analyze the extracted DNA from stool samples, and statistical analysis was performed by using SPSS software. Out of 90 mixed breeds sheltered dogs whose stools were examined by using PCR, 22 samples were positive (16 female dogs and six male dogs). The results of the PCR test were negative in Indoor dogs. The highest presence of *N. caninum* in stray/sheltered female dogs compared to male and Indoor dogs was found. In this research by the first time, we determined that the prevalence of *Neospora caninum* is comparatively high in dogs from Isfahan and the middle parts of Iran.

Keywords: Isfahan, Infected Dogs, *Neospora caninum*, Nested-PCR.

Introduction

Neospora caninum is well known as a coccidian protozoon that identified as one of the most important causes of abortion in cattle and neurological diseases in horses and dogs (Dubey and Lindsay, 1993; Dubey et al., 2006; Dubey and Schares, 2006). This parasite can

cause considerable economic losses in the livestock industry. Dog is known as a definitive host of *N. caninum* infestation (Basso et al., 2009). Growing evidence reported the clinical and subclinical infections of neosporosis in dog worldwide (Dubey and Lindsay, 1996; Lindsay and Dubey, 2000). Dogs can shed oocysts after the infestation in

their feces (Basso et al., 2001; Cavalcante et al., 2011). Subsequently, ingestion of oocysts can lead to the horizontal transmission of infection to cattle (Dubey et al., 2007; McAllister et al., 1998; Dubey, 2003; Dubey and Lindsay, 1996).

Vertical transmission is proposed as the major route of transmission, whereas the epidemiological aspect of the horizontal transmission seems to differ regionally. To detect *N. caninum* oocyst in the dog feces, various methods like microscopic and molecular approaches have been applied.

Microscopically, oocysts of *N. caninum* in the feces are incomprehensible from those of other coccidian like *Hammondia Hammondii*, *Hammondia heydorni*, and *Toxoplasma gondii* (Hill et al., 2001). In the contrary the molecular assays are determined as sensitive methods that specifically identify the *N. caninum* oocysts in the feces. In this connection, several special studies have been performed by some investigators (Slapeta et al., 2002; Dubey et al., 2004; Reichel et al., 2007; Palavicini et al., 2007; Razmi, 2009; Langoni et al., 2012; King et al., 2012). In the current study, a polymerase chain reaction (PCR) assay has been used to detect the Nc5 gene of *N. caninum* oocysts that isolated from feces samples.

Materials and methods

Sample collection

From January to March of 2018, ninety stool samples of mixed-breeds dogs from an animal shelter and ten more stool samples from indoor dogs in districts of the city of Isfahan, Iran, with the average age of 3 years were selected randomly. The stool samples were examined in the biologic research center of Islamic Azad University of Shahrekord, Iran.

Fecal material was sifted using a strainer (60 meshes) by tap water for separation. The samples were mixed with 2.5% Potassium dichromate (K₂Cr₂O₇) for 10-14 days at room temperature and stored at 4°C for subsequent use. After sporulation, the oocysts were washed 4-6 times with tap water by centrifugation (1100 ×g) for 5 min to eliminate the K₂Cr₂O₇. The standard sucrose floatation method was used for oocyst separation. Briefly, one gram feces transferred into 15 ml tubes mixed with 14 ml concentrated sucrose, and centrifugation was done (1600 ×g) for 10 min. Three drops were collected from the surface of the supernatant by a loop and transfer to a slide for microscopic examination. Oocysts in concentrated sucrose solution were evaluated by light microscopy at a magnification of at least 200 (Sabevarinejad et al., 2014).

DNA extraction

The oocysts were ruptured by 3-4 times freeze-thaw cycles. DNA was subsequently extracted from purified oocysts using CTAB protocol.

Cetyl trimethylammonium bromide (CTAB) is a cationic detergent that is used for extraction and purifying DNA from tissues. After purifying the DNA, the samples were resuspended in DNA extraction buffer (150 µl TE 10mM, pH: 8, 60 µl SDS10%) plus proteinase k, 20 mg/ml, and vortex the suspension then incubated for five h at 60-degree centigrade Cover night. The DNA was extracted with phenol-chloroform/isoamyl alcohol. The DNA was precipitated with absolute ice-cold ethanol. The genomic DNA concentration was demonstrated by spectrophotometric analysis at 280/260 nm. The DNA was stored at -20 °C until used for PCR assay (Sabevarinejad et al., 2014)

DNA amplification by PCR

In this method for increasing the sensitivity of the PCR exam, two pairs of primers were used. First, with one pair of primers using 15-30 cycles, certain sequences of the DNA were duplicated. Then the PCR product was moved to another tube and used as a sample, and by using the second primer pair, the second step of the PCR exam was performed. The primer sequences for detecting *N.caninum* in samples, were as followed: Np4 forward (5'-CCTCCCAATGCGAACGAAA-3') and Np7 reverse (5'-GGGTGAACCGAGGGAGTTG-3') targeting the Nc5 genomic of *N. caninum* (Thimothy et al., 1999).

Genomic DNA from *N. caninum* oocysts was added to tubes of a PCR master mix contains 2 mM MgCl₂, x10 PCR buffer (10 mM Tris-HCl pH=8.3, 50 mM KCl.) 200 µM dNTPs, 10 pm each primer and 2 U Taq DNA polymerase in a thermocycler Master Cycler gradient (Eppendorf Germany CO®). PCR was performed using the following cycling protocol (Thimothy et al., 1999): 95 °C for 5 min as initial denaturation, followed by 35cycles of 95°C for 30 sec (denaturation), 62 °C for 30 sec (annealing), 57 C for 30 sec (extension) and final extension of 72 °C for 1 min. Five microliters of PCR products were electrophoreses on a 1% agarose gel at 90 V for 1 hour. The gel was observed and photographed using a transilluminator (UVITEC). To estimate the size of the amplicons, a 100bp DNA ladder (Fermentas) was used (Thimothy et al., 1999).

Results

DNA amplification of the Nc5 gene produced a 340bp fragment for the *N. caninum* samples; however, after sequencing the Nc5 fragment of *N. caninum*, a total of 339 nucleotides were found.

Totally, 100 stool samples were examined with PCR technique for the presence of *Neospora caninum*, and statistical analyses were performed using the chi-squared test with SPSS software (version 25). Out of 90 mixed breeds sheltered dogs whose stools were

examined by using Nested PCR, 22 samples were positive (24.4%). They were 16 three years old female dogs and six male dogs. The results of the PCR test were negative in Indoor dogs. The highest presence of *N. caninum* in stray/sheltered female dogs compared to male and Indoor dogs was found. The present findings present that the prevalence of *N. caninum* is relatively high in dogs from the Isfahan province, Iran.

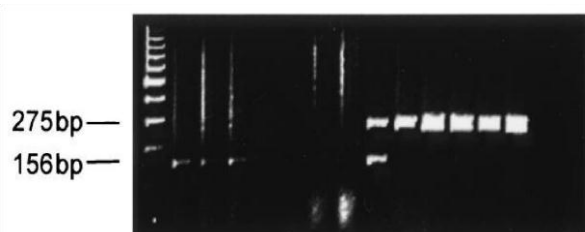


Fig. 1. Agarose gel of PCR products from positive cases for *Neospora* spp.

Discussion

The large number of studies on *N. caninum* seroprevalence have been conducted in various parts of the world. Of note, the *N. caninum* seropositivity in the dog is only indicated for past or recent contact with the parasite, but it cannot be related to the shedding of oocysts by infected dogs. Accordingly, in recent years for better detection of the infection in dogs, the use of molecular methods has been suggested. Molecular assays, based on PCR and sequencing, can assist to detect the species of coccidian oocysts (Reichel et al., 2007). The technique is considered as a sensitive and

specific method, which has been used for the detection of *Neospora* infection in dogs and other intermediate hosts. In a previous study, the isolation, biologic, and molecular attributes of *N. caninum* from three littermate dogs were discussed, which identified by PCR and sequence analyses (Dubey et al., 2004).

In a previous study in Australia, the extent of *N. caninum* infection was demonstrated in a total of 374 dogs (75 wild dogs and 299 Aboriginal community dogs) using a combination of microscopic, serological, and molecular techniques (King et al., 2012). Oocysts of *N. caninum* were found in the feces of two juvenile Aboriginal community dogs (2/132; 1.5%). Of the 263 dog sera tested, the true prevalence of *N. caninum* antibodies was 27.0%. In Iran, a total of 174 fecal samples was obtained from 89 farm dogs and 85 household dogs in Mashhad, the samples were evaluated for identification of *Neospora Hammondia* like oocysts, the samples were examined for *N. caninum* by specific PCR test, and two samples were reported positive (Razmi, 2009). According to Malmasi et al. (2007) study, *N. caninum* antibodies were observed in 10 (20%) of 50 household dogs and 23 (46%) of 50 farm dogs in Tehran, Iran. In another study conducted by Yakhchali et al. (2012) in Iran, 135 blood samples were collected from strayed dogs in Urmia, and the blood serums were tested for the presence of IgG antibodies using

IFAT test, from 135 total samples, 27% of them were positive for having anti-*Neospora* antibodies (Yakhchali et al., 2012). In another study in Iran, the prevalence of anti-*Neospora caninum* and *Toxoplasma gondii* antibodies were measured using the ELISA test by Hosseini et al. (2009). In this study, 548 blood samples were collected from strayed dogs in Charmahal and Bakhtiari, Isfahan, and Khuzestan provinces for 18 months. From 548 total blood serum samples, 29% were positive for having anti *N.caninum* antibodies and 26.8% for having anti *T.gondii* antibodies and 8.94% of samples for having antibodies against both parasites. In another study conducted by Hadadzade et al. (2007) in Tehran, Iran, the presence of the IgG antibody was examined by using the IFAT test. For this study, 103 blood samples were collected from 53 indoor dogs and 50 sheepdogs. The presence of IgG antibody was 11.3% in indoor dogs and 28% in sheepdogs.

Stray dogs usually comprise the most considerable population; meanwhile, working sheepdogs are used by farms frequently for guarding the properties, and to protect against wild animals. As in many countries, the owned dogs are not limited to the home area and are provided to roam freely with stray dogs. Most of these animals are infamous for their parasite harboring nature and, therefore could be a

notable source of infection for parasite transmission. In fact, the role of stray dogs, as well as wild canids in the spread of infection is not well understood.

The infection with *N.caninum* in humans is not reported yet, but the presence of the infection in other primates is proven. Serologic evidence show that human is underexposure of the microorganism. In research, in blood serums of 76 women with a history of abortion, the antibodies against *N.caninum* were not detected. In blood samples of farmers and blood donors from Ireland, there was no any antibody activity against *N.caninum* (Greene 2012).

In many countries, especially in developing countries where housed animals living close to wild carnivores, the relevant condition for *Neospora* transmission may dispense. In fact, studies on *Neospora* infection in stray dogs and wild canids are infrequent (McGarry et al., 2003, Gondim et al., 2004, King et al., 2010, 2012). Thus, conducting the epidemiological studies on *Neospora* infection regarding the role of stray dogs and wild canids could be an important subject for future investigations.

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