Short Communication

Molecular detection of Neospora caninum from naturally infected dogs in Lorestan province, West of Iran

Dalimi*, A., Sabavarinejad¹, Gh., Ghafarifar¹, F., Forouzandeh-Moghadam², M

1. Department of Medical Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

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ABSTRACT

Neospora caninum is a coccidian protozoan that causes abortion in dairy and beef cattle and neurological disorders in dogs and horses. To identify N. caninum oocysts in the dog feces the molecular approaches are known as sensitive methods that specifically detect the oocysts. In present study, a polymerase chain reaction (PCR) targeting N. caninum specific Nc5 genomic fragment was performed to identify the N. caninum DNA in the feces of naturally infected dogs of Lorestan province, West Iran. Fecal samples of dogs living in small dairy farms were collected. The samples were homogenized in 2.5% Potassium dichromate (K2Cr2O7) and stored at 4 °C. Genomic DNA was extracted from the feces using CTAB protocol. PCR assay and DNA sequencing were performed with specific primers. DNA amplification of the Nc5 formed a 340bp fragment for the N. caninum specimens; however, the fragment was 99% identical to the homologous sequences from Neospora caninum isolates. Totally, 9 positive samples of N. caninum were detected by PCR from 428 fecal specimens.

Keywords: Neospora caninum, Dog, Nc5, PCR, Lorestan, Iran

INTRODUCTION

Neospora caninum is a coccidian protozoan that recognized as one of the most important causes of abortion in dairy and beef cattle and neurological disorders in dogs and horses (Dubey & Lindsay 1993, Dubey et al 2006, Dubey & Schares 2006). This parasite causes significant economic losses in the livestock industry. Dogs are definitive host of N.caninum both experimentally and naturally (Basso et al 2009). Neosporosis was first detected in dogs in Norway by Bjerkas et al. (1984). The worldwide reports of clinical and subclinical infections of neosporosis in dogs were summarized by Dubey and Lindsay (1996) and Lindsay and Dubey (2000). Dogs shed oocysts after the infection (Basso et al 2001, Cavalcante et al 2011). Ingestion of oocysts from the feces of dog (horizontal transmission) or from the dam to the fetus (vertical transmission) are modes of transmission of infection to cattle (Dubey et al 2007, McAllister et al 1998, Dubey 2003, Schares et al 2005, Dubey & Lindsay 1996). Vertical transmission is considered as the major route of transmission, whereas the epidemiological importance of the horizontal transmission seems to vary regionally. To identify N. caninum oocyst in the dog feces different methods such as microscopic and molecular approaches have been applied. In the microscopic fecal examination, oocysts

*Author for correspondence.Email: dalimi_a@modares.ac.ir
of *N. caninum* are indistinguishable from those of other coccidian such as, *Hammondia heydorni*, *Hammondia hammondii* and *Toxoplasma gondii* (Hill et al 2001). In contrary the molecular techniques are known as sensitive methods that specifically detect the *N. caninum* oocysts in feces. In this regard, particular studies have been conducted 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 present study a polymerase chain reaction (PCR) technique for amplification of the Nc5 gene has been used to specifically detect *N.caninum* oocysts isolated from dogs' feces.

**MATERIALS AND METHODS**

**Sample collection.** A total of 428 fecal samples was collected from dogs that living in the small dairy farms in Lorestan province, West Iran. Fecal material was sieved through a strainer (60 meshes) using 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 further use. After sporulation, the oocysts were washed 4-6 times with tap water by centrifugation (1100 ×g) for 5 min to remove the K2Cr2O7. The standard sucrose floatation technique 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 taken from the surface of the supernatant using a loop and transfer to a slide for microscopic examination. Oocysts in concentrated sucrose solution were examined by light microscopy at a magnification of at least 200.

**DNA extraction.** The oocysts were ruptured by 3-4 times freeze – thaw cycles. DNA was subsequently isolated from purified oocysts with CTAB protocol and 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 5-15 h at 60 °C over night. The DNA was extracted with phenol chloroform/ isoamylalcohol. The DNA was precipitated with absolute ice cold ethanol. The genomic DNA concentration was determined by spectrophotometric analysis at 280/260 nm. The DNA was stored at -20 °C until used in PCR assay.

**DNA amplification by PCR.** PCR assay was performed with species specific primer pairs Np6+ ('5'-CTGCCCAGTCCAACCTACGTTCTTCTTCTCTCTC' and Np21+ ('5'-CCCAGTGCGTCCAATCCTGTAAC-3') (King et al 2010) targeting the Nc5 genomic of *N. caninum*. Genomic DNA form *N. caninum* oocysts was added to tubes of a PCR master mix contains 1.5 mM MgCl2, 1×PCR buffer (10 mM Tris-Hcl pH=9.0, 30 mM KCl,) 250 µM dNTPs, 10 pg each primer and 1 U Taq DNA polymerase in a thermo cycler (Bio-Rad®). (Gen Amp kit); PCR was validated and performed using the following cycling protocol: 95 °C for 5 min as initial denaturation, followed by 35cycles of 95°C for 30 sec (denaturation), 62 °C for 30 sec (annealing), 72 C for 30 sec (extension) and final extension of 72 °C for 10 min(13). Five microliters of PCR products were electrophoreses on a 1.5% agarose gel at 80 V for 1 h. The gel was visualized and photographed using a transilluminator (UVITEC). To estimate the size of the amplicons, a 100bp DNA ladder (Fermentas) was used.

**Nucleotide sequence analysis.** For each marker analyzed, the 25 amplicons of *N. caninum* was purified using Bioneer AccuPrep® Gel Purification Kit as specified by the manufacturer. Sequencing reactions were performed with both primers used for PCR and the results were analyzed on a 310 Automa DNA sequencer. The obtained Ne5 sequences were compared with those of *N. caninum*, already registrated in the GenBank™ database. All the comparison and alignments were conducted using the BLAST system (basic local alignment tool).

**RESULTS AND DISCUSSION**

DNA amplification of the Ne5 gene produced a 340bp fragment for the *N. caninum* specimens; however, after sequencing the Ne5 fragment of *N. caninum*, a total of 339 nucleotides were obtained.
Totally, nine positive samples of *N. caninum* were detected by PCR from 428 fecal specimens collected from dogs living in dairy farms. No variations of intrapopulation were observed for the Nc5 sequences of *N. caninum* samples. The sequences obtained in this study were compared with the existing homologues in the GenBank (Figure 2). The fragment of the Nc5 gene sequenced here is 99% identical to the homologous sequences from *Neospora caninum* isolate 152 from chicken brain NC5 gene, partial sequence (Sequence ID: gb EU073600.1) and *Neospora caninum* isolate PolBb1 clone 1 NC5 gene, complete sequence (Sequence ID: gb HM031965.1).

For *N. caninum* detection, the serological techniques have been used widely. In this regard, numerous studies on *N. caninum* seroprevalence have been conducted in several parts of the world which summarized by Lindsay and Dubey 2000 and Dubey 2003. On the other hand, the *N. caninum* seropositivity in the dog is only indicated for a past or recent contact with the parasite, but cannot be correlated to the shedding of oocysts by infected dogs. Consequently, in the recent years for better detection of the parasite infection in dogs, the use of molecular methods has been recommended. Molecular techniques, based on polymerase chain reaction (PCR) and sequencing, can help to identify the species of coccidian oocysts (Reichel et al. 2007). The technique is known as a sensitive and specific method and has been used for detection of *Neospora* infection not only in dogs but also in intermediate hosts. In a study conducted by Dubey et al. (2004) the isolation and biologic and molecular attributes of *Neospora caninum* from three littermate dogs are described. The isolates were identified as *N. caninum* by PCR and sequence analysis (Dubey et al., 2004). In the study of Langoni et al. (2012) which aimed to determine the occurrence of *N. caninum* infections in 50 dogs with neurological signs in Brazil. Tissues of positive animals were bioassayed in gerbils (*N. caninum*), and DNA was analyzed using the polymerase chain reaction (PCR). Specific antibodies were detected in 7 out of 50 samples (14%) for *N. caninum*. In the bioassay and PCR, 3 out of 7 (42.9%) samples were found positive for *N. caninum*. In the study conducted by King et al. (2012) in Australia, the extent of *N. caninum* infection was determined in a total of 374 dogs (75 wild dogs and 299 Aboriginal community dogs) using a combination of microscopic, molecular and serological techniques. Oocysts of *N. caninum* were observed in the faeces 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 collected from 89 farm dogs and 85 household dogs during 2006 and 2008 in Mashhad. Fecal samples of dogs were microscopically examined for detecting *Hammondia Neospora*-like oocysts (HNLO). The fecal samples with HNLO were examined by *N. caninum*-specific PCR. Two of the samples were reported positive for *N. caninum* (Razmi 2009). Palavicini et al. (2007) were collected at intervals a total 263 fecal samples from 34 farm dogs in Costa Rica. Fecal samples were examined for *N. caninum*-like oocysts microscopically, by DNA detection using the polymerase chain reaction (PCR), and by bioassay. *N. caninum* DNA was detected by PCR in four fecal samples, twice from one dog, but oocysts were not detected microscopically in these dogs (20). In intermediate hosts, the surveys of *N. caninum* and *T. gondii* infection in *Rattus rattus*, *Rattus norvegicus*, and *Mus musculus* captured in urban areas of São Paulo reveal a striking low frequency of occurrence of these infections (Muradian et al. 2012). In the study of Santos et al. (2010) in Brazil, the infection rates caused by *N. caninum*, *Hammondia sp.*, and *T. gondii* was investigated in beef cattle using a nested PCR for Toxoplasmatinae RDNA, followed by sequencing of the PCR products. After sequencing of the PCR products from all positive tissues, five sequences matched with *N. caninum* and two matched with *T. gondii*. Antibodies to *N. caninum* and *T. gondii* were found in 20% and 26% of the animals, respectively. According to the study conducted by Silva et al. (2009) on tissues from slaughtered goats in
Figure 1. PCR results on DNA from dog feces containing oocysts of Neospora caninum using primers targeting the Nc5 region. Ladder (L). Negative control (1), positive control (2) and Lanes 3-10: positive isolates (approximately 340 bp).

Figure 2. The partial sequences of NC5 gene of three isolates (1, 2, 3) of Neospora caninum isolated from dogs in Iran

Bahia, Brazil, after sequencing the PCR products from all positive tissues, a frequency of 3.92% (4/102), 1.96% (2/102) and 7.84% (8/102) were obtained for H. heydorni, N. caninum and T. gondii, respectively. In Iran, according to Malmasi et al. (2007) study, N. caninum antibodies were seen in 10 (20%) of 50 household dogs and in 23 (46%) of 50 farm dogs in Tehran (Malmasi et al. 2007). Moreover, a few serological surveys that carried out for detection of N. caninum infection indicated frequencies from 12.6 % to 32 % in cattle (Nourolahi Fard et al 2008, Youssefi et al 2009), 43.36% in buffaloes (Hamidi Nejat et al 2007) and 3.22% in camel (Hosseninezhad et al 2009). In the study area, stray dogs usually comprise
the largest population; meanwhile working sheepdogs are used by farms mostly for guarding the properties, and to protect against wild animals. As in many countries, the owned dogs are not restricted to the home area and are allowed to roam freely with the stray dogs. Most of these animals are notorious for their parasite harboring nature and thus, could be an important source of infection for parasite transmission. In fact, the role of stray dogs as well as wild canids in the spread of Neospora caninum is not well defined. In many countries particularly in developing countries where domesticated animals living close to wild carnivores, the appropriate situation for Neospora transmission may provide. Indeed, studies on Neospora infection in stray dogs and wild canids are scarce (McGarry et al 2003, Gondim et al 2004, King et al 2010, 2012) and lack of comprehensive information on epidemiology of infecting intermediate hosts. So, conducting the studies on the epidemiological aspects of Neospora infection considering the role of stray dogs and wild canids could be a good topic for future investigations.

Ethics

Hereby, I declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors have no conflict of interest.

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