Archives of Razi Institute, Vol. 69, No. 2, December (2014) 185-190

DOI: 10.7508/ari.2014.02.010

Short Communication

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

Dalimi*[1], A., Sabevarinejad[1], Gh., Ghafarifar[1], F., Forouzandeh-Moghadam[2], 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

Received 31 Jul 2013; accepted 10 Sep 2013

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 hammondi* 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 $K_2Cr_2O_7$. 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 sepectrophotometric 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'-CTCGCCAGTCCAACCTACGTCTTCT-3') 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 $MgCl_2$, 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 Nc5 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 Nc5 gene produced a 340bp fragment for the *N. caninum* specimens; however, after sequencing the Nc5 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 7out 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

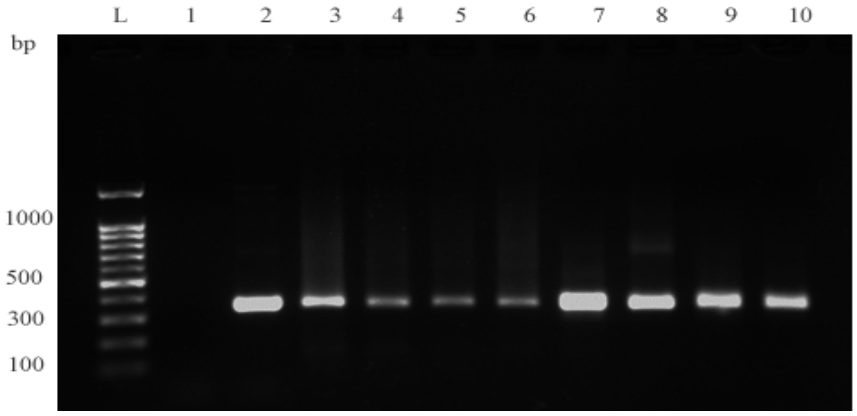

Figure 1. PCR results on DNA from dog feces containing oocysts of *Neospora caninum* using primers targeting the Nc5 region. Ladder (L). Negative control (1), positive control(2) and Lanes 3-10: positive isolates (approximately 340 bp).

```
1   CCCAGTGCGTCCAATCCTGTAACGTGTTGCTGTGCGTATGCGGACGTGTCGTTGTTGGG- 59
2   CCCA.....T...T------.....G..T- 53
3   CCCA.....T...T------.....- 53

1   CGCAGCCTGCGGCAGCAAGGCTCCTTTTTTGTTTGTGGCTATAGTGTGTGAACGGGTGAA 119
2   G.....T..... 113
3   ..... 113

1   CCGAGGGAGTTGGTAGCGGTGAGAGGTGGGATACGTGGTTTGTGGTTAGTCA--TTCGTC 177
2   .....--..... 171
3   .....--..... 171

1   ACGTTGAA--ATCAGCC-TGCGTCAGGGTGTGGACAGTGTGTCAATGATACTTATCGAGA 234
2   .....--.....-.....C... 228
3   .....--.....-..... 228

1   GTTCAGTGTTCTGTGTTGAGGCAACACCGGCGGCACTGATGACGGGGGAGATTA--TGC- 291
2   .....--...- 285
3   .....--...- 285

1   ATAGGGAGCAA-GCGGACGAGGGAAGGG-GC 320
2   .....A...-.....-.. 314
3   .....A.....-.. 315
```

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.

Acknowledgment

This study was supported financially by Tarbiat Modares University. The authors wish to thank Dr. Sadraei and Dr. Pirestani for their kind assistance.

References

Barber, J. S., van Ham, L., Polis, I. and Trees, A. J. (1997) Seroprevalence of antibodies to *Neospora caninum* in Belgian dogs. *The Journal of Small Animal Practice* 38, 15-16.

Basso, W., Venturini, L., Venturini, M.C., Hill, D.E., Kwok, O.C. and Shen, S.K. (2001). First isolation of *Neospora caninum* from the feces of a naturally infected dog. *Journal of Parasitology* 87(3):612-8.

Basso, W., Herrmann, D.C., Conraths, F.J., Pantchev, N., Vrhovec, M.G. and Schares G. (2009). First isolation of *Neospora caninum* from the faeces of a dog from Portugal. *Veterinary Parasitology* 159(2):162-6.

Bjerkås, I., Mohn, S.F. and Presthus, J. (1984). Unidentified cystforming sporozoon causing encephalomyelitis and myositis in dogs. *Zeitschrift für Parasitenkunde* 70: 271-274.

Cavalcante, G.T., Monteiro, R.M., Soares, R.M., Nishi, S.M., Alves Neto, A.F. and Esmerini P.d.O. (2011). Shedding of *Neospora caninum* oocysts by dogs fed different tissues from naturally infected cattle. *Veterinary Parasitology* 179(1–3):220-3.

Dubey, J.P. (2003). Review of *Neospora caninum* and neosporosis in animals. *Korean Journal of Parasitology* 41(1):1-16.

Dubey, J.P., Buxton, D. and Wouda, W. (2006). Pathogenesis of bovine neosporosis. *Journal of Comparative Pathology* 134(4):267-89.

Dubey, J.P. and Lindsay, D.S. (1993). Neosporosis. *Parasitology today* 9(12):452-8.

Dubey, J.P. and Lindsay, D.S. (1996). A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology* 67(1–2):1-59.

Dubey, J.P. and Schares, G. (2006). Diagnosis of bovine neosporosis. *Veterinary Parasitology* 140(1–2):1-34.

Dubey JP, Schares G, Ortega-Mora LM. (2007). Epidemiology and control of neosporosis and *Neospora caninum* . *Clinical Microbiology Reviews* 20 (2):323-67.

Dubey, J.P., Sreekumar, C., Knickman, E., Miska, K.B., Vianna, M.C., Kwok, O.C., Hill, D.E., Jenkins, M.C., Lindsay, D.S. and Greene, C.E. (2004). Biologic, morphologic, and molecular characterisation of *Neospora caninum* isolates from littermate dogs. *International Journal for Parasitology* 34(10):1157-67.

Gondim, L.F., McAllister, M.M., Pitt, W.C. and Zemlicka, D.E. (2004). Coyotes (Canis latrans) are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* 34(2):159-61.

Hamidi Nejat, H. and Gorani, S. (2007). Occurrence of *Neospora caninum* antibodies in water buffaloes (Bubalus bubalis) from the southwestern region of Iran. *Bulletin of the Veterinary Institute in Puławy* 51(2):233-235.

Hill, D.E., Liddell, S., Jenkins, M.C. and Dubey, J.P. (2001). Specific detection of *Neospora caninum* oocysts in fecal samples from experimentally-infected dogs using the

polymerase chain reaction. *Journal of Parasitology* 87(2):395-8.

Hosseininezhad, M., Pirali Kheyrabadi, K.h. and Hosseini, F. (2009). Seroprevalence of *Neospora caninum* infection in camels (*Camelus dromedarius*) in Isfahan province, center of Iran. *Iranian Journal of Parasitology* 4(4):61-64.

King, J.S., Brown, G.K., Jenkins, D.J., Ellis, J.T., Fleming, P.J., Windsor, P.A. and Slapeta, J. (2012). Oocysts and high seroprevalence of *Neospora caninum* in dogs living in remote Aboriginal communities and wild dogs in Australia. *Veterinary Parasitology* 8;187(1-2):85-92.

King, J.S., Šlapeta, J., Jenkins, D.J., Al-Qassab, S.E., Ellis, J.T. and Windsor, P.A. (2010). Australian dingoes are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* 40(8):945-50.

Langoni, H., Matteucci, G., Medici, B., Camossi, L.G., Richini-Pereira, V.B. and Silva, R.C. (2012). Detection and molecular analysis of *Toxoplasma gondii* and *Neospora caninum* from dogs with neurological disorders. Revista da Sociedade Brasileira de Medicina Tropical 45(3):365-8.

Lindsay, D.S. and Dubey, J.P. (2000) Canine neosporosis. *Journal of Veterinary Parasitology* 14: 1-11.

Malmasi, A., Hosseininejad, M., Haddadzadeh, H. and Badii, A. (2007). Bahonar A. Serologic study of anti-*Neospora caninum* antibodies in household dogs and dogs living in dairy and beef cattle farms in Tehran, Iran. *Parasitology Research* 100(5):1143-5.

McAllister, M.M., Dubey, J.P., Lindsay, D.S., Jolley, W.R., Wills, R.A. and McGuire, A.M. (1993). Dogs are definitive hosts of *Neospora caninum* . *International Journal for Parasitology* 28(9):1473-8.

McGarry, J.W., Stockton, C.M., Williams, D.J. and Trees, A.J. (2003). Protracted shedding of oocysts of *Neospora caninum* by a naturally infected foxhound. *Journal of Parasitology* 89(3):628-30.

Muradian, V., Ferreira, L.R., Lopes, E.G., Esmerini Pde, O., Pena, H.F., Soares, R.M. and Gennari, S.M. (2012). A survey of *Neospora caninum* and *Toxoplasma gondii* infection in urban rodents from Brazil. *Journal of Parasitology* 98(1):128-34.

Nourolahi Fard, S.R. Khalili, M and Aminzadeh, M. (2008).Prevalence of antibodies to *Neospora caninum* in cattle in Kerman province south east, Iran. *Veterinarski arhive* 78(3):253-259.

Palavicini, P., Romero, J.J., Dolz, G., Jimenez, A.E., Hill, D.E. and Dubey, J.P. (2007). Fecal and serological survey of *Neospora caninum* in farm dogs in Costa Rica. *Veterinary Parasitology* 149(3-4):265-70.

Razmi, G. (2009). Fecal and molecular survey of *Neospora caninum* in farm and household dogs in Mashhad area, Khorasan province, Iran. *Korean Journal of Parasitology* 47(4):417-20.

Reichel, M.P., Ellis, J.T. and Dubey, J.P. (2007). Neosporosis and hammondiosis in dogs. *The Journal of Small Animal Practice* 48, 308–312.

Santos, S.L., de Souza Costa, K., Gondim, L.Q., da Silva, M.S., Uzêda, R.S., Abe-Sandes, K. and Gondim, L.F. (2010). Investigation of *Neospora caninum* , *Hammondia sp*., and *Toxoplasma gondii* in tissues from slaughtered beef cattle in Bahia, Brazil. *Parasitology Research* 106(2):457-61.

Schares, G., Pantchev, N., Barutzki, D., Heydorn, A.O., Bauer, C. and Conraths, F.J. (2005). Oocysts of *Neospora caninum* , Hammondia heydorni, Toxoplasma gondii and Hammondia hammondi in faeces collected from dogs in Germany. *International Journal for Parasitology* 35(14):1525-37.

Silva, M.S., Uzêda, R.S., Costa, K.S., Santos, S.L., Macedo, A.C., Abe-Sandes, K., Gondim, L.F. (2009). Detection of Hammondia heydorni and related coccidia (*Neospora caninum* and Toxoplasma gondii) in goats slaughtered in Bahia, Brazil. *Veterinary Parasitology* 26;162(1-2):156-9.

Slapeta, J.R., Modry, D., Kyselova, I., Horejs, R., Lukes, J. and Koudela B. (2002). Dog shedding oocysts of *Neospora caninum* : PCR diagnosis and molecular phylogenetic approach. *Veterinary Parasitology* 109(3-4):157-67.

Youssefi, M.R., Arabkhazaeli, F. and Tabar Molla Hassan, A. (2009). Seroprevalence of *Neospora caninum* infection in rural and industrial cattle in Northern Iran. *Iranian Journal of Parasitol* 4(1):15-18