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▣ BRIEF COMMUNICATION

Fecal and Molecular Survey of *Neospora caninum* in Farm and Household Dogs in Mashhad Area, Khorasan Province, Iran

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Abstract: *Neospora caninum* is an important cause of abortion in dairy cattle worldwide. Dog is the definitive host for *N. caninum* and can infect dairy cattle. The aim of this study is to determine the prevalence of *Neospora* oocysts in feces of dogs from dairy farms. A total of 174 fecal samples was collected from 89 farm dogs and 85 household dogs during 2006 and 2008. Fecal samples of dogs were microscopically examined for detecting *Hammondia Neospora*-like oocysts (HNLO) by Mini Parasep®SF fecal parasite concentrator. HNLO were microscopically detected in 4 fecal samples (2.2%). The fecal samples with HNLO were examined by *N. caninum*-specific PCR. Two of the samples were positive for *N. caninum*. The 2 positive fecal samples were selected for inoculation to calves. Two inoculated calves were seronegative by ELISA for 4 months post-infection. This is the first report of finding *N. caninum* DNA in feces of farm dogs in Mashhad area, Iran.

Key words: *Neospora caninum*, oocyst, dog, Iran

Neosporosis is a major cause of abortion in cattle [1-3]. There are 2 transmission routes for *Neospora caninum* in cattle. The first route is vertical or transplacental transmission. In cattle, transplacental transmission from infected dams to offspring appears to be the major route of infection [4]. Prenatally infected but health calves remain persistently infected and can pass infection to their own offspring. This leads to endogenous transplacental transmission of the infection through successive pregnancies and cattle generation [3]. The second route is referred to as horizontal or postnatal transmission that occurs in cattle after ingesting sporulated *N. caninum* oocysts [4]. Dogs and coyotes are definitive hosts for *N. caninum* [5,6]. Dogs can transiently shed oocysts upon ingestion of *N. caninum* infected tissues of intermediated hosts [3]. In some epidemiological studies of dairy herds, the presence of farm dogs either was a risk factor for seropositivity in cattle [2]. In Iran, there are a few reports on the seroprevalence of *N. caninum* infection in cattle and dogs [7-10]. Also, *N. caninum* has been recognized as an important agent of abortion in dairy cattle in Iran [11]. In these studies, correlation of seropositivity of dairy cattle to abortion was shown; however, the role of farm dogs in spreading *Neospora* infection in dairy farms of Iran has not been investigated. It seems that the presence of farm dogs could be associated with abortion due to *N. caninum* infection in dairy cattle. The aim of this study is to investigate the potential role of dogs as a source of *Neospora* oocysts shedding in infection of dairy cattle in this area and to demonstrate cyclical oral transmission of *N. caninum* between dogs and cattle.

The study was done in Mashhad area, capital city of the Razvai Khorasan province, situated in the northeast of Iran. The Razavi Khorasan province is located in northern temperature zone. The climate is semi-arid with cold winters and moderate summer. This area has an estimated 25,000 cattle on 110 dairy farms.

A total of 174 fecal samples were collected from 89 farm dogs and 85 household dogs during 2006 and 2008. Samples were kept at cold condition until laboratory examinations took place. Samples were examined by Mini Parasep®SF faecal parasite concentrator (Diasys Europe Ltd.). Briefly, the lid was unscrewed and 3.3 ml of 10% buffered formalin was added to the mixing tube, and a pea-sized (0.4 g) fecal sample was introduced by using the spoon on the end of the Parasep. The sample was mixed thoroughly with the Parasep spoon. The Parasep was immediately sealed by screwing the filter thrimble and conical tube. The mixture was vortexed and the Parasep was then inverted to allow the mixture tube filtered through the filter thimble. The Parasep was then centrifuged at 600 g for 1 min. The mixing chamber and filter thimble were unscrewed and discarded.

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All the liquid above the sediment was poured off and 1 ml water was added to the sediment. The sediment was re suspended with water by shaking. The sediment then was pipetted to a slide for microscopic examination. If the sample had oocysts, the oocysts of feces were measured with a calibrated ocular micrometer using bright-field microscopy. The oocysts with a diameter of 11.5 ± 1.5 μm and exhibiting morphology similar to non-sporulated *T. gondii*-oocysts were considered to be positive for *Hammondia Neospora*-like oocysts (HNLO) [12-14]. For sporulation of HNLO, the positive sample was reexamined from feces by a combined sedimentation and flotation procedure as described by Schares et al. [15]. Then, the number of isolated oocysts was estimated by microscopic examination of 10 μl samples using a Neubaur hemocytometer. The fecal samples containing oocysts were mixed with 2% potassium dichromate in a Petri dish and incubated at room temperature for 3-5 days.

Oocysts of *N. caninum* are morphologically indistinguishable from those of *Hammondia heydorni* and *Toxoplasma gondii* [12,15]. Thus, it was necessary to do molecular methods, such as PCR for differentiating oocysts of *N. caninum* from those of *H. heydorni* and *T. gondii*. Therefore, NC5-PCR was used since a high diagnostic value of this method has been demonstrated in European interlaboratory evaluation [16]. First, suspensions from sporulated oocysts were washed by repeated centrifugation in distilled water to remove potassium dichromate. Then, the oocysts were ruptured by 2-3 freeze-thaw cycles. DNA was subsequently isolated from purified oocysts with the DNeasy-kit according to the manufacturer's instructions (Cinagen, Tehran, Iran). After that, DNA amplification was performed as described by Müller et al. [17] using the primer pair $Np6^+$/$Np21^+$. Briefly, 2 oligonucleotide PCR primers were used to detect *N. caninum* for the PCR ($Np6^+$): 5′-CTCGCCAGTCAACCTACGTCTTCT-3′, and the reverse ($Np21^+$): 5′-CCCAGTGCGTCCAATCCTGTAAC -3′ were used for amplification reaction. The 50 μl reaction mixture contained; 2 μl of template DNA, 5 μl of 10 × PCR buffer (CinnaGene Inc., Tehran, Iran), 1 μl $MgCl_2$, 0.2 mM each of dATP, dGTP, and dCTP, 0.4 mM dUTP (CinnaGene), 1.25 units of Tag DNA polymerase (CinnaGene) and 20 *p*mol of each P1 and P2 primers (CinnaGene) for PCR and double distilled H_2O were added up to 50 μl. The amplification conditions for *N. caninum* included an initial enzyme activation of denaturation at 95℃ for 5 min, 40 cycles with denaturing at 94℃ for 60 sec, primer annealing at 63℃ for 60 sec and extension at 74℃ for 3.5 min, followed by final extension at 74℃ for 10 min. PCR Products were then chilled at 4℃. The final PCR products were subjected to electrophoresis in a 1.5% agarose gel with TBE buffer. Samples positive for *N. canium* produced visible bands at 337 bp in the PCR product.

Two dairy calves (male, 5-7 months) were purchased from the farm of Faculty of Veterinary Medicine, Ferdowsi University of Mashhad. Each calf was confirmed to be seronegative for *N. caninum* using a commercial ELISA kit (Bio-x Diagnostics, Belgium). Then, 2 fecal samples positive in PCR were selected for inoculation to calves. Prior to inoculation, potassium dichromate was washed out with tap water after 3 times centrifugation (1,100 g, 10 min) and the pellet was resuspended in 10 ml tap water. Finally, 2 calves were orally inoculated with approximately 50-100 HNLO oocysts. Blood was collected from the

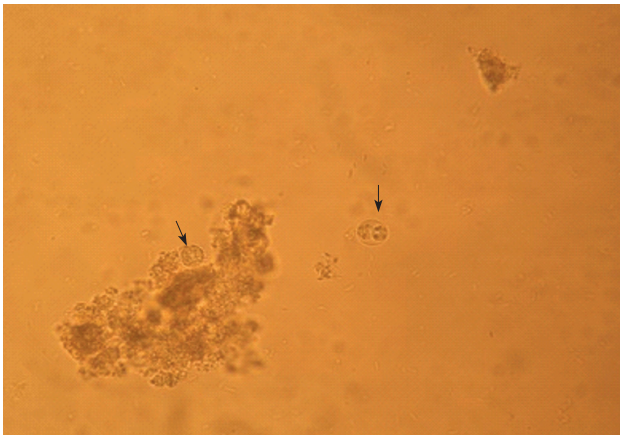

Fig. 1. Unsporulated (left arrow) and sporulated (right arrow) oocysts of *Hammondia Neospora*-like oocysts (HNLO). ×400.

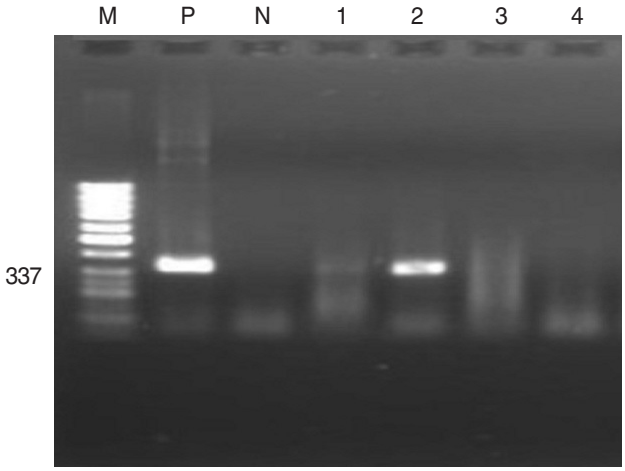

Fig. 2. Detection of *Neospora caninum* by PCR with positive bands at 337 bp. M, marker of 50 bp ladder; P, positive control; N, negative control; lanes 1-4, samples.

Table 1. Diagnostic techniques to detect *Neospora caninum* oocysts in fecal samples of dogs

	Fecal examination		*N. caninum*-specific PCR		Bioassay in calves	
	No. examined	No. HNLO* positive	No. examined	No. positive	No. examined	Results
Farm dogs	89	4	4	2	2	Negative
Household dogs	85	0	0	0	0	0
Total	174	4	4	2	2	Negative

**Hammondia Neospora*-like oocysts.

jugular vein every 2 weeks for 4 months. For preparing the serum, blood was centrifuged at 1,000 g for 10 min, and the serum was collected and stored at -20℃ until use. The collected sera were tested by a commercial ELISA kit (Bio-x Diagnostics).

From 174 dogs examined, HNLO was microscopically detected in 4 fecal samples (3.48%) (Fig. 1). The number of HNLO in examined fecal samples was low (5-10 oocysts per gram). Four samples with HNLO were tested by *N. caninum*-specific PCR. Two samples were positive for *N. caninum* (Fig. 2). The positive dogs were males and 1 of them was 2 years old and the other 4 months old. The calves fed sporulated HNLO remained healthy and *N. caninum*-specific serological analysis of serum samples collected 3 to 16 weeks after infection was negative (Table 1).

Many seroepidemiological studies were done for detecting *Neospora caninum* infection in dogs worldwide [2]. Seropositivity in dogs is a primarily indicator for a past or recent contact with the parasite, but cannot be correlated to shedding of oocysts. The majority of dogs shedding *N. caninum* oocysts after experimental infection do not seroconvert in serologic examination [12,13,18-20]. Therefore, it is important to properly identify the *N. caninum* oocysts in fecal samples. So far, there are only a few reports of *N. caninum* oocyst shedding by naturally infected dogs [21-24]. In this study, HNLO were found only in 4 fecal samples of farm dogs, and also, DNA of *Neospora* was detected in 2 of them by *N. caninum*-specific PCR. The DNA of *Neospora* or *N. caninum* oocysts in the fecal samples may be due to feeding of fresh and uncooked infected meat. For confirmation, it was need to isolate parasites in bioassay examination or cell culture. Gerbils are highly susceptible to infection with oocysts of various *N. canium* isolates [14]. Trees et al. [25] observed that the inoculation of approximately 1 *N. caninum* oocyst was sufficient to induce a *N. caninum*-specific antibody response in gerbils. However, because of unavailability of gerbils during the study, we used calves in bioassay examinations. Some studies were shown that challenge with 300-600 oocysts of *N. caninum* could induce *N. caninum*-specific antibodies in calves [20, 25-27]. In the present study, all calves were remained seronegative to *N. caninum* before and after oral inoculation of oocysts from positive fecal samples. It seems that the dose of *Neospora* oocysts may have been too low to induce immune responses or there may have been no *Neospora* oocysts in the given fecal samples.

In conclusion, HNLO were found only in farm dogs and 2 of fecal samples revealed positive reactions to *Neospora*-PCR. This result shows that the presence of farm dogs may be a risk factor for *N. caninum* infection in dairy farms in Mashhad area, Iran. However, to confirm this hypothesis, a further molecular characterization of extracted DNA and isolation of parasites in gerbil bioassay or in cell culture is needed.

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