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A molecular and serological study of *Neospora caninum* infection in pigeons from southwest Iran

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ABSTRACT

Neospora caninum is a protozoan parasite with worldwide distribution, mainly implicated as responsible for bovine abortion. There are indications that the presence of birds on cattle farms could be associated with the increase in seroprevalence and abortions related to *N. caninum*. The present study reports the serological (*Neospora* agglutination test) and molecular (PCR) presence of *N. caninum* in pigeons. From the 102 samples analyzed, 31 samples (30.39%) were seropositive for *N. caninum* and the overall molecular prevalence of *N. caninum* in the brains of the same pigeons was 9.8% (10/102). This is the first report of detection of *N. caninum* in Iranian pigeons. The results indicate soil contamination due to *N. caninum* oocysts, because pigeons feed from the ground, and suggest that the meat from the pigeons may be an important source for infection of dogs.

Key word: *Neospora caninum*, Neospora agglutination test (NAT), PCR, pigeons, Iran

Introduction

Neospora caninum is a heteroxenous cyst-forming coccidian, closely related to *Toxoplasma gondii* (DUBEY et al., 2002). In cattle, neosporosis is manifested by reproductive failure, which includes abortion and neural signs in neonatal calves (DUBEY and LINDSAY, 1996; DUBEY, 1999). Due to its high prevalence in cattle, *N. caninum* has emerged as an important cause of bovine abortion (ANDERSON et al.,

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2000), and neosporosis has been recognized as an economically important disease with a considerable impact on the livestock industry (TREES et al., 1999). Advances concerning the *Neospora* life cycle have proved dogs to be both intermediate and definitive hosts (McALLISTER et al., 1998), and cattle and other animals to be natural intermediate hosts (DUBEY, 1999). Although *N. caninum* has been widely described in mammals, the role of birds in the parasite's life-cycle is still obscure. It has been shown that chickens may be a permissive intermediate host for *N. caninum* since parasite DNA has been detected in tissues from birds kept outdoors (COSTA et al., 2008). Recently, *N. caninum* has been demonstrated in a few species of naturally infected birds, in particular in domestic chickens (*Gallus domesticus*), sparrows (GONDIM et al., 2010; BAHRAMI et al., 2015), in the common raven (*Corvus corax*) (COSTA et al., 2008; MOLINA LOPEZ et al., 2012) and in some wild birds (DARWICH et al., 2012). It is known that birds are preyed upon by canids, and could be a strong source of infection to the parasite's definitive hosts (McGUIRE et al. 1999; GONDIM, 2006). Furthermore, investigation of infection in birds is a useful way to access environmental contamination with oocysts, since different avian populations feed directly on the ground and are continuously exposed to oocyst ingestion (DUBEY et al., 2002; WAAP et al., 2008). Experimental infections have established that pigeons may be susceptible to experimental infection by *Neospora,* and were shown to be potential intermediate hosts of the parasite (McGUIRE et al. 1999). There is currently no information available about the natural neosporosis in pigeons. To our knowledge only one previous study in China showed the prevalence of *N. caninum* in pigeons (DU et al., 2015). In that sense, this work aimed to evaluate the presence of *N. caninum* in pigeons, using serological and molecular tools for the first time in Iran.

Materials and methods

Animals. The study included 102 pigeons that were referred to the veterinary hospital of faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran, by practitioners between December 2013 and August 2014, for further diagnosis and therapy. All the pigeons included in the present study exhibited nervous signs such as circling, incoordination, ataxia, opisthotonus and leg paralysis. Samples of blood were collected by jugular vein puncture, using a 1- mL syringe with a 26- G needle. The pigeons were scarified ethically, and brain tissue was collected from each bird. Sera and brains were kept at -20 °C.

Serological examination. The *Neospora caninum* agglutination test (NAT) was performed in 96 round-bottom-well microplates, according to the method previously described for toxoplasmosis (DESMONTS and REMINGTON, 1980). In brief, 50 μL of 0.2 M 2-mercaptoethanol in PBS was distributed into each well, and sera were diluted two-fold up to 128, starting at 1:2. In the present study, the tachyzoites of *N. caninum*

NC-1 isolate were used. Parasites were resuspended in alkaline buffer (7.02 g NaCl, 3.09 g H3BO3, 24 mL of 1 N NaOH, 4 g bovine plasma albumin (fraction V), with enough distilled water to bring the volume to 1 l; pH 8.7) and their concentration was adjusted at $2 \times 10^4/\mu L$. After the sera had been diluted, 50 µL *N. caninum* antigen suspensions were distributed into each well. Plates were gently agitated to allow for complete mixing and were then incubated overnight at 30 °C. A clear-cut button-shaped deposit of parasite suspension at the bottom of the well was interpreted as a negative reaction, and a complete carpet of agglutinated organisms was considered positive. Each assay included two negative controls and one positive control. A serum sample obtained from a rabbit with an experimental *N. caninum* infection was selected as the positive control. Those samples with doubtful results were re-tested.

DNA extraction and PCR. DNA was extracted using a genomic DNA purification kit (SinaClon Bioscience, Iran) from 40mg homogenized brain tissue. For detection of *N. caninum*, primers targeting Nc5 gene were selected from the literature (KANG et al., 2009). The primers (Bioneer, South Korea) used in the reaction were the forward primer Np 21 with the sequence 5'- CCCAGTGCGTCCAATCCTGTAAC-3' and the reverse primer Np 6 with the sequence 5'- CTCGCCAGTCAACCTACGTCTTCT-3', yielding a 338 bp product. All PCR were performed in a 25 µL reaction containing 12.5 µL Taq DNA polymerase master mix Red (Amplicon, Denmark), 1 µM primers and 50 ng DNA templates. PCR cycling included an initial denaturation at 94 ºC for 4 min, followed by 30 cycles of denaturation at 94 ºC for 50s, annealing at 56 ºC for 50s, extension at 72ºC for 60s. This was followed by a final extension at 72 ºC for 5 min. PCR reactions included a negative control, consisting of the reaction mix and 2 µL of DNase/RNase-free water instead of DNA, and a positive control consisting of a DNA sample from the *N. caninum* tachyzoites (NC- 1 isolate). To determine the possibility of cross reactions with the related protozoan, *Toxoplasma gondii*, the whole tachyzoites of these parasites were also analyzed by the same primers in all PCR analyses. PCR products were electrophoresed in 1.5% agarose (SinaClon Bioscience, Iran) in Tris-acetate-EDTA (TAE) buffer, stained with Green Safe stain (SinaClon Bioscience, Iran) and visualized under ultraviolet light. Positive samples showed a band of approximately 338 bp. Amplified fragments corresponding to the size predicted for *N. caninum* were purified using a PCR purification kit (Fermentase, Lithuania) and were sent for sequencing (Sequence Laboratories, Goettingen, Germany). The obtained sequences were compared with those of *N. caninum*, already registered in the GenBank™ database. All the comparison and alignments were conducted using the nBLAST system (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Antibodies to *N. caninum* were found in 30.39% of pigeons. The NAT titer that should be considered specific for diagnosis of neosporosis in birds was not estimated. Although for most serological surveys a NAT titer of 1:25 is used as the cut-off, occasionally *N. caninum* has been isolated from birds with a lower titer. Therefore, we have stated all titers in Table 1.

Table1. Seroprevalence of *N. caninum* in pigeons from southwest of Iran and their molecular results

Test	No. tested	No. of sera with NAT titers						
		1:2	1:4	1:8	1:16	1:32	1:64	1:128
NAT	102	-	3	6	5	11	4	2
PCR	102	-	1	2	2	3	-	-

In the present study, samples were considered positive if they produced a band approximately 338 bp, similar to that of the positive control (Fig. 1). The overall molecular prevalence of *N. caninum* in pigeons was 9.8% (10/102). Positive samples were sequenced in order to verify the positive result. All sequenced samples were found by BLAST analysis to be closest to the *N. caninum* Nc 5 gene in GenBank, with a similarity of ≥98% (GenBank™ accession nos. EF 202080.1, EF 202082.1, EU 073599.1 and KF 649846.1).

Based on the results, 10, 20, 20 and 40 percent of molecular positive samples were from seropositive pigeons, with titers 1:4, 1:8, 1:16 and 1:32, respectively. Results of PCR in seropositive pigeons with different titers are presented in Table 1. Out of 10 positive samples from PCR, one specimen was from a seronegative pigeon.

Discussion

Age-related prevalence data indicate that most dogs become infected with *N. caninum* after birth; a higher prevalence has been documented in older versus younger dogs (DUBEY et al., 2007).The ingestion of infected tissues is the most likely source of infection for carnivores. Theoretically, tissues of any animal containing tissue cysts may be a source of infection for dogs. Tissues of infected prey of dogs may represent a logical source of infection, but no viable parasite has been isolated from potential dog prey, such as birds and rodents (DUBEY and SCHARES, 2011). Recently, infected birds were suggested as a possible source of infection for *N. caninum* infection in dogs. Antibodies to *N. caninum* were found in 23.5% of 200 free-range and 1.5% of 200 indoor chickens from Brazil, and *N. caninum* DNA was detected in 6 out of 10 seropositive chickens

(COSTA et al., 2008). Experimentally, chickens older than one week inoculated with *N. caninum* tachyzoites intraperitoneally developed a transient infection. The susceptibility of chicken eggs for *N. caninum* infection was recently confirmed by MANSOURIAN et al. (2009) using embryonated broiler chicken eggs. GONDIM et al. (2010) found *N. caninum* DNA in 3 of 40 sparrows (*Passer domesticus*) from Brazil.

In the present study, antibodies to *N. caninum* were found in 30.39% of pigeons by the NAT test. 2-mercaptoethanol destroys both specific and non-specific IgM antibodies and NAT detects only IgG antibodies (DESMONTS and REMINGTON, 1980). However, IgG antibodies appear during the first 2 weeks after infection with *N. caninum*, so the efficiency of the NAT in the serological diagnosis of neosporosis is not likely to be compromised (DUBEY et al., 1996; ROMAND et al., 1998). It has been proved that NAT may be applied directly as described to all host species, whose serum samples may be analyzed within the same test run, with comparable patterns of agglutination (ROMAND et al., 1998). In contrast, ELISA and IFAT require the use of anti IgG conjugates that must be prepared from various animal species necessarily different from that of the infected host. Moreover, these latter assays also require time consuming standardization of conjugates, depending on the species of the infected host. Other drawbacks associated with IFAT may be the rather subjective interpretation of some patterns of the fluorescence of tachyzoites, resulting in poor reproducibility between laboratories (DUBEY et al., 1997). Furthermore, the wide spectrum of *N. caninum* antigens used in the different ELISAs currently available accounts for important variations between the results obtained, thus resulting in discrepant results for low antibody titers (DUBEY et al., 1997). It has been proven that NAT appears to be reliable for the detection and quantitation of IgG antibodies to *N. caninum* in various host species. In contrast to other serological tests currently available, NAT is less expensive, easy to read and to perform, and requires a minimum of laboratory equipment and materials. Therefore, this test could be suitable for epidemiological animal screening in field studies, as well as for evaluation of humoral responses to *N. caninum*.

In the present study, the primer pair Np21/Np6, as the sensitive primers for *N. caninum* detection, was used for PCR study. Five primer pairs (Np21/Np6, Np21/Np4, Np7/Np4, Np7/Np6, and Np5/Np6) were previously tested (YAMAGE et al., 1996) for their specificity using genomic DNA from closely related organisms as a template. The Np21/Np6, Np21/Np4 and Np7/Np4 pairs were found to be specific to *N. caninum* when tested against *T. gondii*, *Sarcocystis* spp. and *Hammondia hammondi*. Acceptable results were only shown for the primer pair Np21/Np6. This primer pair demonstrated the highest methodical sensitivity, whereas a pair of Np5/Np6 had a cross-reaction with *Sarcocystic cruzi*. Therefore, the primer pair Np21/Np6 was subsequently used. The overall molecular prevalence of *N. caninum* in pigeons was 9.8%, and 10, 20, 20 and 40 percent of molecular positive samples were from seropositive pigeons, with 1:4, 1:8,

1:16 and 1:32 titers, respectively. Out of 10 positive samples from PCR, one specimen was from a seronegative pigeon. NAT examination of pigeons detected a much higher prevalence of *N. caninum* compared with PCR. There are two reasons to justify the higher prevalence of *N. caninum* by NAT compared with PCR. One reason is the presence of shared antigens in *Toxoplasma gondii and N. caninum,* which was demonstrated by the cross-reactivity in the NAT. Therefore, it is not possible to say whether the antibodies in 30.39% of pigeons were due to 1 or both parasites (NISHIKAWA et al., 2002). Also, *Neospora* may be detected by PCR in the tissues of naturally infected chickens in the chronic phase onwards, but we expect serological procedures to detect antibodies during the acute phase of infection before division or localization in tissues (McGUIRE et al., 1997). A third reason is that *N. caninum* is focally located in the brain tissue, and PCR detection might produce a false negative if the brain tissue is not homogenously sampled.

Overall, experimental infections have established that pigeons may be susceptible to infection, produce specific IgG antibodies, and are potential intermediate hosts of the parasite (McGUIRE et al., 1999; MINEO et al., 2009). In a recent study, a high prevalence of *N. caninum* DNA (30% of 210 samples) was observed in brain tissue from pigeons in Changchun, Jilin (China) (DU et al., 2015). The high prevalence of *N. caninum* infection in pigeons could be related to the pigeon's diet. Pigeons commonly feed directly from the ground and are probably exposed to *N. caninum* after ingestion of the parasite oocysts from the soil. The results we obtained indicate soil contamination due to *N. caninum* oocysts, because pigeons feed from the ground, and suggest that the meat from the birds might be an important source for dog infection by *N. caninum*.

Natural infection of pigeons with *N. caninum* seems to have great economic importance, because of their increasing population in urban and rural areas, and cosmopolitan distribution. Pigeons may serve as a source of infection for other animals, including canids (WOODS et al., 2003), so their efficiency in transmitting *N. caninum* infection needs to be investigated.

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SAŽETAK

Neospora caninum praživotinja je proširena diljem svijeta koja pretežito uzrokuje pobačaj u goveda. Upozorava se da prisutnost ptica na goveđim farmama može biti povezana s povećanjem serološke prevalencije i broja pobačaja uzrokovanih tom vrstom. U ovom je istraživanju *N. caninum* dokazana u golubova serološki testom aglutinacije i molekularno lančanom reakcijom polimerazom. Od 102 pretražena uzorka 31 (30,39%) je bio serološki pozitivan na prisutnost *N. caninum*, a molekularna prevalencija u mozgu istih golubova iznosila je 9,8% (10/102). To je prvo izvješće o dokazu *N. caninum* u iranskih golubova. Rezultati pokazuju da je onečišćenje tla oocistama *N. caninum* izvor uzročnika za golubove jer se oni hrane na tlu. Također se upozorava na činjenicu da meso golubova može biti važan izvor zaraze za pse.

Ključne riječi: *Neospora caninum*, aglutinacijski test, lančana reakcija polimerazom, golubovi, Iran