Molecular and serological detection of *Neospora caninum* in multiple tissues and CSF in asymptomatic infected stray dogs in Tehran, Iran

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Key words:
dog, indirect ELISA, *Neospora caninum*, neosporosis, nested-PCR

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Received: 5 April 2016
Accepted: 21 June 2016

Abstract:

BACKGROUND: *Neospora caninum* is an Apicomplexan parasite. It causes paralysis and neuromuscular disorders in dogs and abortion in cattle. Although contamination with *N. caninum* is common in stray dogs, most of the dogs are infected with subclinical neosporosis. **OBJECTIVES:** The objective of this study was to evaluate the presence of *N. caninum* in multiple tissues and cerebrospinal fluid (CSF) using Nested-PCR technique. Furthermore, the *N. caninum* specific antibody was detected in serum of examined dogs by indirect enzyme-linked immunosorbent assay (ELISA). **METHODS:** Forty-two stray dogs of mixed breeds captured from districts of Tehran, Iran, were examined physically and euthanized. A commercially indirect ELISA kit was used to detect the anti-*N. caninum* antibodies in sera. Nested PCR was applied to analyze the extracted DNA from brain, skeletal muscle, CSF, liver, spleen and mandibular lymph nodes for Nc5 gene. **RESULTS:** Indirect ELISA assay for *N. caninum* antibody was positive in a seven year-old male dog (2.22%). Out of 42 stray dogs whose multiple organs were examined using Nested PCR, 15 samples (35%) were positive. The highest presence of *N. caninum* was found in skeletal muscle with 30% (13/42) frequency, followed by CSF (26.2%) (11/42), brain (19%) (8/42), liver (7.14%) (3/42), lymph node (4.62%) (2/42) and spleen samples (0/42). **CONCLUSIONS:** These results suggest that the highest presence of *N. caninum* is seen in skeletal muscle, CSF and brain in asymptomatic infected dogs respectively. Nested-PCR could be considered a sensitive method to detect *N. caninum* in subclinical infected dogs.

Introduction

Neospora caninum is a pathogen protozoan from Apicomplexan phylum. *N. caninum* is morphologically very similar to *Toxoplasma gondii*. Domestic dogs and some wild

canids are definitive hosts and the cattle are the intermediate host. The Oocysts are shed by infected dogs after ingestion of infected tissues with *N. caninum* cysts (1-5).

Serologic assays in serum or in CSF are the main methods of *N. caninum* diagnosis and include indirect fluorescent antibody test (IFAT) and ELISA (6), Direct Agglutination Test (DAT) or Neospora agglutination test (NAT) (7). Nevertheless, serology is an indirect diagnosis test, has considerable variations and also in case of early or chronic infection with cysts formation, the serology could be negative even if the parasite is present in the dog. Furthermore, some weak serologic cross-reactivity may occur between *T. gondii* and *N. caninum* antigens. Therefore, direct diagnosis and highly sensitive methods such as immunohistochemistry or PCR can be used (6). In comparison, PCR has been reported to be more sensitive to immunohistochemistry as some cross-reactivity could occur with immunocytochemistry depending on the antibodies used (6). Besides Classical PCR, Semi-nested or nested PCR was also developed to increase the sensitivity. (8-10).

N. caninum infection causes paralysis and neuromuscular disorders in dogs. Although *N. caninum* contamination is common in stray dogs, clinical manifestation is uncommon and most of the dogs are infected with subclinical and asymptomatic neosporosis (11, 12). Chemotherapy or immunosuppressive drugs can reactivate subclinical form of the disease to clinical neosporosis (13-15). Subclinically infected dogs can transmit the parasite to their pups (16-19). Detection of the infection in asymptomatic dogs, especially in females is crucial for prevention of vertical transmission to newborns; therefore, diagnosis of the asymptomatic dogs remains essential and the results of this study can be used for proper diagnosis of this parasite.

The aim of this study was to determine the levels of *N. caninum* infection in some organs/tissues of asymptomatic stray dogs using Nested-PCR and ELISA, in order to compare the positive results in different infected organs.

Materials and Methods

Sample collection: This study was performed under dog population control program and under permission and guidelines of the ethic committee of the University of Tehran. Forty-two stray dogs (22 male /20 female) of mixed breeds, ranging from one to seven years old, were captured from districts of Tehran, Iran, from November 2014 to September 2014. The captured dogs were first physically examined and their details were recorded. None of the dogs had any clinical symptoms associated with neosprosis. Blood samples (10ml) were then drawn from jugular vein. The samples were centrifuged at 3000×g for 10 min. Separated sera were stored at -20 oC until testing. The stray dogs were then euthanized and necropsies were performed. Samples were collected from individual organs using sterile scalpels, to minimize potential contamination of the tissues. Collected samples consisted of brain (from left hemisphere, 5-10 g), skeletal muscle (Biceps femoris), CSF (foramen magnum space), liver, spleen and mandibular lymph nodes were removed and the organs were stored at -20 °C until DNA extraction. Samples were maintained in PBS buffer containing 2% antibiotic (Penicillin and Streptomycin)-antimycotic (Amphotericin B) solution (Gibco BRL, Paisley, UK) and stored at −20 °C until being used for

DNA extraction and PCR amplification. The DNA obtained from cultured tachyzoites (Nc1) was used as positive control.

ELISA assay: For serological analysis, a commercially indirect multi-species ELISA kit (IDvet Co., France) for the detection of anti-*N. caninum* antibodies in serum was used according to the manufacturer's instructions. S/P of 0.5 was considered as cut-off based on the instructions of the manufacturer.

DNA Extraction: Each of the brain, skeletal muscle, liver, spleen and mandibular lymph nodes samples was first finely chopped using a sterile blade, and then powdered by being crushed in liquid nitrogen in a mortar. Furthermore, the powdered tissues were diluted in distilled water and homogenized.

DNA was extracted from the homogenized tissues and CSF samples using DNA extraction kit (MBST, Iran). The extraction procedure was performed according to the manufacturer's protocol.

DNA amplification by Nested-PCR: The sequence of the primer pair used in this study to amplify Nc5 gene of *N.caninum* was adapted from that used in a research carried out by Müller and colleagues (1996). Np6+ (5' gggtgtgcgtccaatcctgtaac3') and Np21+ (5' ctcgccagtcaacctacgtcttct3') primers were used to amplify a 328bp fragment of Nc5 gene (20). The product of the fist PCR reaction using Np6+ and Np21+ primers was then used as a template to run the second PCR reaction using Np7 (5'gggtgaaccgagggagttg3') and Np10(5'tcgtccgcttgctccctatgaat3'). The PCR product the size of the second reaction was 198bp.

The PCR reaction mixture consisted of 1µg template DNA, 2mM MgCl2, 5µl 10×reaction buffer, 10pmol of each reverse and forward primer, 200µM dNTP and 1U of Taq DNA polymerase. The thermal steps of the PCR reaction was as follows: 5 min incubation at 95 °C to denature double strand DNA, 35 cycles of 45s at 95 °C (denaturing step), 45s at 64 °C (annealing step) and 45s at 72 °C (final extension step). The PCR product of the first reaction was diluted 1:10 in distilled water prior to being used as the template in the second reaction (Nested-PCR). The thermal steps of the second PCR reaction using Np7/Np10 primers were similar to the first reaction.

PCR products were electrophoresed on 2% agarose gel followed by ethidium bromide staining. PCR products were visualized using UV illuminator.

Sequencing and Nucleotide sequence analysis: To confirm the specificity of the primers used in this study, Nc5 Nested-PCR specificity, amplicons of the second PCR reaction were sent to be sequence by Takapouzist Co, Iran. The obtained sequences were analyzed and edited using Finch TV software (GeospizaInc, Seattle, USA) and Bioedit software version 7.7.9 (mBio, Inc., North Carolina, USA), respectively.

The obtained nucleotide sequences (Nc5 gene) were blasted and compared with those already registered in the GenBank™ database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

ELISA assay: From 42 serum samples of stray dogs that were tested by indirect ELISA kit, 1 (2.22%) of them (a seven year old male) was positive for antibodies (IgGs) against *N. caninum*.

Gene Expression of *N. caninum* Nc5 in stray dogs: The data obtained from PCR

Table 1. Summary of PCR and ELISA test carried out on various samples obtained from stray dogs.

Sample number	Age (year)	Sex	ELISA assay(S/P)	Brain	Muscle	CSF	Liver	Spleen	Mandibular lymph nodes
1	2	Female	-(0.24)	+	+	+	-	-	-
2	7	Male	+(1.01)	+	+	-	-	-	-
3	3	Female	-(0.16)	-	+	-	-	-	-
4	3	Male	-(0.18)	-	+	-	-	-	-
5	3	Female	-(0.16)	+	+	+	-	-	-
6	3	Female	-(0.16)	-	+	+	-	-	-
7	4	Female	-(0.18)	+	+	+	+	-	+
8	5	Male	-(0.17)	-	+	+	-	-	-
9	3	Male	-(0.18)	+	-	+	+	-	-
10	3	Male	-(0.20)	-	-	+	-	-	-
11	2	Male	-(0.17)	-	+	+	-	-	-
12	4	Male	-(0.19)	-	+	+	-	-	-
13	2	Female	-(0.22)	+	+	-	-	-	-
14	6	Male	-(0.20)	+	+	+	+	-	+
15	1	Male	-(0.17)	+	+	+	-	-	-

analysis are illustrated in Fig. 1. *N. caninum* Nc5 gene was expressed in brain, skeletal muscle, CSF, liver, spleen and mandibular lymph nodes samples obtained from 15 out of 42 stray dogs (35%).

The presence of *N. caninum* was highest in skeletal muscle with 30% (13/42) positive PCR test. In the other organs, 19% of brain (8/42) and 26.2% of CSF (11/42) samples tested positive. The numbers of liver and mandibular lymph nodes samples were relatively lower, 7.14% (3/42) and 4.76% (2/42), respectively. None of the spleen samples (0/42) tested positive for Nc5 gene expression. The data obtained from Nested-PCR are presented in Table 1.

Sequencing and nucleotide sequence BLAST (BLASTn): Sequencing the amplified fragment confirmed the specificity of the used primers. Quality of the sequenced Nc5 gene fragment was confirmed by Finch TV software (Geospiza Inc., Seattle, USA). No variations of intra population were observed for the Nc5 sequences of *N. caninum* samples. Nucleotide sequences of partial Nc5 gene of *N. caninum* were used as the entry data to NCBI BLAST search to compare and verify with the existing paralogues in the GenBank. The fragment of the Nc5 gene sequenced in this study was 96% identical to the homologous sequences from *N. caninum* (Sequence ID: GQ899206, AJ271354, U17345, U16159, L24380 and U03069).

Discussions

This study aimed to estimate the serological prevalence of *N. caninum* infection in serum and the molecular prevalence in some organs/tissues of asymptomatic stray dogs in Tehran province, Iran.

Serological assays show widespread exposure to the *N. caninum* in Asia, Europe, Africa and America (11). Several studies have reported the prevalence of *N. caninum* by serological methods in Iran. In Meshkin-Shahr District (Northwestern Iran) from 171 domestic dogs, 52 cases (30.4%) had antibodies against *N. caninum* in their sera

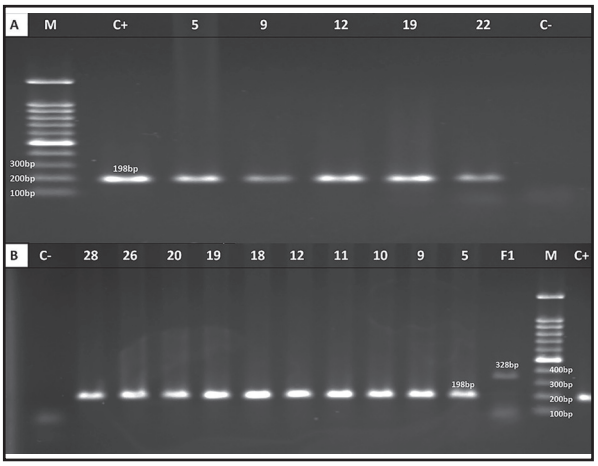

Figure 1. Detection of *Neospora caninum* using Nested-PCR. The applied primers targeted the Nc5 gene (A: Brains, B:Muscle) at -198bp. M, marker of 100bp ladder; C+, positive control; C-, negative control, Numbers (lanes 3-8 (A)& 4-14(B)); samples; F1, a sample of first round Nested-PCR.

which was detected by indirect ELISA (21). Furthermore, antibodies against *N. caninum* were detected in 36 (27%) of 135 dogs in Urmia, Iran using IFAT (22). Haddadzadeh et al., found that 20 of 103 (19.4%) cases they studied in Tehran were seropositive using IFAT. They have also reported that the infection rate in farm dogs was higher (28%) than in urban dogs (11.3%) (23). In household dogs and dogs living in dairy and beef cattle farms of Tehran, antibodies against *N. caninum* were detected in 10 (20%) of 50 household dogs and in 23 (46%) of 50 farm dogs using IFAT (24). The present study is the first study to investigate the presence of *N. caninum* in some organs of asymptomatic stray dogs in Iran using molecular technique (Nested-PCR) alongside serological detection method (ELISA). In the present study, only 1 of 42 stray dogs was seropositive using indirect ELISA. This dog was one of the 15 dogs (6.67%) that was positively assessed by Nested-PCR. Furthermore, the other 27 dogs that tested negative for Nested-PCR were also seronegative. Therefore, Nested-PCR might indicate a better sensitivity compared to ELISA for *N. caninum* detection in asymptomatic stray dogs. Serology is an indirect diagnosis test that has several variations. Moreover, in case of early or chronic infection with cysts formation, serology test could be negative even if the parasite is present in the dog (25). Furthermore, some weak serologic cross-reactivity may occur between *T. gondii* and *N. caninum* antigens. Therefore, direct diagnosis and highly sensitive methods such as immunohistochemistry or PCR based detection methods can be more suitable (6). In comparison, PCR has been reported to be more sensitive to immunohistochemistry as some cross-reactivity could occur in immunocytochemistry depending on the antibodies used (6).

In our study, as previously mentioned, 15 dogs from 42 dogs that were examined with Nested-PCR method had positive results at least in one tissue sample for each dog. Skeletal muscle (13/15), CSF (11/15) and brain (8/15) had significant higher positive results than liver (3/15) and mandibular lymph nodes (2/15). As confirmed by the results obtained using Nested-PCR in this study, skeletal muscle and brain are the major site of *N. caninum* infection and are suitable organs to detect the parasite (26-28).

Ghalmi et al. detected *N. caninum* in liver and spleen of 28 (32.2%) of 87 asymptomatic pound dogs in Algeria using PCR technique. From 28 dogs tested for Nc5 gene expression, 14 (50%) expressed Nc5 in both organs, 11 (39.28%) in spleen and 3 (10.72%) in liver. 19 of these dogs were seronegative using IFAT and only 8 seropositive dogs were seen in PCR positive dogs (25). Although Ghalmi et al. only tested liver and spleen using PCR which were shown to have the lowest infection in present study.

Therefore, it is possible that the other organs such as brain and muscle were infected and underestimated in the study reported by Ghalmi et al. Therefore, the organ/tissue chosen to be tested using PCR might be critical in making the correct diagnosis. Similarly, all the positive samples in PCR test did not test positive using ELISA technique in present study. Also, the study of Castaneda-Hernandez shows that the results of Nested-PCR were significantly greater than serology results in asymptomatic sheep. The seroprevalence was 5.5% and the prevalence of the *N. caninum*'s DNA in sheep's blood was 25% using Nested-PCR (29).

Ferroglio et al. showed that in samples obtained from 233 wild rodents 19 muscle, 6 kidney and 4 brain samples tested positive for Nc5 expression using PCR. Their study demonstrated that results obtained only based on the brain samples may potentially lead to an underestimation of the levels of infection as the majority of positive PCR samples were skeletal muscle, similar to the finding of present study (30).

Moreover, with respect to the presence of *N. caninum* in brain, CSF and muscle, this parasite should be considered in the differential diagnosis of neuromuscular disorders of dogs. The present study demonstrated that based on the Nested-PCR results and the data obtained using ELISA, the stray dogs in Tehran province are infected with *N. caninum* without any symptoms.

Serology can underestimate the real carrier of *N. caninum* in asymptomatic dogs. The main benefit of PCR technique to detect *N. caninum* in comparison with serology tests is to directly detect the presence of the DNA. However, incapacity to perform the test on living dogs is the main disadvantage of this technique (31).

In conclusion, Serology tests can underestimate the carrier of *N. caninum* in asymptomatic dogs compared to PCR. However, cross-reactivity between Leishmania species or *T. gondii* and *N. caninum* antigens may occur. Skeletal muscle, CSF and brain are the most infected tissues in dogs with *N. caninum* respectively. Nested-PCR is a potential alternative for the serology methods to detect *N. caninum* in asymptomatic infected dog.

Acknowledgements

The study was supported financially by the Faculty of Veterinary Medicine, University of Tehran.

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مجله طب دامی ایران، ۱۳۹۶، دوره ۱۱، شماره ۲، ۱۱۲-۱۰۵

بررسی مولکولی و سرولوژیک تک‌یاخته نئوسپورا کنینوم در بافت‌های مختلف و مایع مغزی-نخاعی در سگ‌های ولگرد آلوده به شکل تحت بالینی نئوسپروزیس

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(دریافت مقاله: ۱۷ فروردین ماه ۱۳۹۵، پذیرش نهایی: ۱ تیر ماه ۱۳۹۵)

چکیده

زمینه مطالعه: نئوسپورا کنینوم یک تک‌یاخته کوکسیدیایی از خانواده سارکوسیستیده است. این تک‌یاخته باعث ایجاد مشکلات عصبی_عضلانی در سگ‌ها و سقط در گاو می‌شود. اکثر موارد آلودگی به این تک‌یاخته در سگ‌ها، باعث ایجاد عفونت‌های تحت‌بالینی می‌شود. **هدف:** در این مطالعه نتایج سرولوژی و مولکولی در بافت های مغز، عضلات اسکلتی، کبد، طحال، عقده‌های لنفاوی تحت فکی و مایع مغزی_نخاعی در مبتلایان به شکل تحت‌بالینی نئوسپورا کنینوم در سگ‌های ولگرد مورد بررسی و مقایسه قرار گرفته است. **روش کار:** معاینه بالینی بر روی ۴۲ قلاده سگ ولگرد از نژاد مخلوط در استان تهران پس از ثبت مشخصات انجام شد. در ابتدا نمونه برداری از خون و مایع مغزی_نخاعی به عمل آمد و پس از معدوم سازی به روش بدون درد نمونه‌های مغز، عضلات اسکلتی، کبد، طحال و عقده‌های لنفاوی جمع آوری و تا زمان انجام آزمایشات در دمای $-20\ ^{\circ}C$ نگهداری شد. از کیت تجاری به روش الایزای غیر مستقیم جهت انجام آزمایش سرولوژی و از تکنیک Nested-PCR بر روی ژن Nc۵ جهت بررسی مولکولی استفاده شد. **نتایج:** از ۴۲ نمونه سرم مورد بررسی با کیت الایزای غیر مستقیم، در یک قلاده سگ نر هفت ساله (۲/۲۲٪) عیار آنتی بادی وجود داشت. همچنین با استفاده از روش Nested-PCR، در ۱۵ قلاده (۳۵٪) در حداقل یک نمونه، آلودگی به نئوسپورا کنینوم مشاهده شد. از میان نمونه‌های بررسی شده، بیشترین میزان آلودگی به ترتیب در عضلات اسکلتی (۳۰٪)، مایع مغزی-نخاعی (۲۶/۲٪)، مغز (۱۹٪)، کبد (۷/۱۴٪) و عقده های لنفاوی تحت فکی (۴/۷۶٪) مشاهده گردید. در هیچ یک از نمونه‌های بدست آمده از طحال آلودگی مشاهده نشد. **نتیجه‌گیری‌نهایی:** در عضلات اسکلتی بیشترین میزان آلودگی با استفاده از روش مولکولی مورد تشخیص قرار گرفت. در عین حال بر اساس نتایج حاصل روش مولکولی از حساسیت به مراتب بیشتری نسبت به روش سرولوژی برخوردار است.

واژه های کلیدی: سگ، الایزا، نئوسپورا کنینوم، نئوسپوروز، Nested-PCR

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