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Detection of Non-spermatozoal Cells of *Neospora caninum* in Fresh Semen of Naturally Infected Bulls

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ABSTRACT

***Background*:** *Neospora caninum* (*N. caninum*) is a cyst-forming coccidian parasite closely related to *Toxoplasma gondii* which has emerged as an important cause of reproductive failure in cattle worldwide. Routes of *Neospora* transmission include transplacental infection through tachyzoites, ingestion of tissues harbouring cysts and oral uptake of sporozoite-containing oocysts. Transplacental transmission seems to be very efficient for *N. caninum* in naturally infected cattle and plays a major role in the maintenance and spread of the disease. Other sources of vertical transmission, such as cow to calf transmission via pooled colostrum or milk could also be possible but until now this has not been proven in naturally infected cattle. The possibility of *N. caninum* transmission via semen requires profound repercussions on the trade of cattle's semen. In the present study, fresh non-extended semen of bulls was evaluated with naturally-acquired neosporosis for the presence of *N. caninum* by means of PCR.

***Materials, Methods & Results*:** Serum samples were analyzed for antibody activity to *N. caninum* by using the commercially available ELISA kit. Sera samples were obtained before the first semen sampling and after the last one (days 0 and 60). Thirteen seropositive and five seronegative bulls were selected for the next steps of the study. Genomic DNA was extracted from non-spermatozoal cells, purified tachyzoites (6 x 10^8 cells) of *N. caninum* (NC-1 strain), *T. gondii* (RH strain) (6 108 cells) and fish (rainbow trout) semen (2 x 10^6 cells) based on Chomezynski extraction method. PCR was performed using DNA with varying concentrations obtained from purified tachyzoites of *N. caninum* or *T. gondii* and from the 54 samples collected at the breeding centre. Genomic DNA of the *N. caninum* tachyzoites was digested with HindIII and 1 µg of digested DNA was labeled using a biotin labeling kit. Using primers Rep-F and Rep-R, a product with expected molecular weight of 213 bp was recorded. No cross-reaction was observed with other DNA tested from fish semen and/or a closely related species, *T. gondeii*. The result of Southern blot analysis showed no hybridization of *N. caninum* oligoneucleotide probes to PCR product of prolactin gene. Based on the results of PCR assay developed in this study, the parasite repetitive sequences were detected in the collected semen samples (a total of 39 samples) of all seropositive bulls (13 animals).

***Discussion*:** *N. caninum* is mainly spread by transplacental transmission from cow to calf. Recently, the seroprevalence of *N. caninum* infection in breeding bulls has been shown to be moderate, and the possibility of bulls playing a role in the horizontal transmission of *N. caninum* has been raised. In this regard, semen will require extensive removal of such materials from genomic DNA prior to amplification and it might be beyond the potential of the current kits to isolate DNA. In the present work, non-spermatozoal cells were isolated from the fresh semen via a density gradient centrifugation and subsequently washed and then subjected to DNA extraction by a silica based method. In this approach, it was first attempted to isolate the suspected cells carrying the parasite genome, and then minimize the inhibitory effects of the fresh semen and finally increase the concentration of the extracted DNA. Based on the results of this approach, observing the parasite signal in all semen samples from the seropositive bulls became feasible by the aforementioned PCR assay or what was developed in this study. In the present study, the detection of *N. caninum* DNA in non-spermatozoal cells semen of naturally infected bulls is reported for the first time which can probably be used as a diagnostic tool. Whether venereal transmission plays a role in the spread of bovine neosporosis needs to be determined, since *N. caninum* is one of the major causes of abortion in cattle.

Keywords: bull, Neospora caninum, non-spermatozoal cells, semen.

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INTRODUCTION

Neospora caninum is a cyst-forming coccidian parasite closely related to *Toxoplasma gondii* which has emerged as an important cause of reproductive failure in cattle worldwide [5,6]. Neosporosis in bovines is a common cause of abortion and congenitally infected calves may show neural signs after birth [1,6]. *N. caninum* is mainly spread by transplacental transmission from cow to calf [4]. Recently, the seroprevalence of *N. caninum* infection in breeder bulls was shown to be moderate [3], and the possibility of bulls to play a role in the horizontal transmission of *N. caninum* has been suggested [11] The possibility of *N. caninum* transmission via semen could imply profound repercussions on cattle semen trade. Several studies have been carried out to investigate the possibility of venereal transmission in bovine neosporosis. [8,13,15]. The occasional detection of the parasite genome in semen samples of infected cattle was already reported [13].

Many tests have been used to detect the infectious agents in bovine semen. Polymerase chain reaction (PCR) has been shown to be a useful tool to identify some pathogens, like BHV-1, present in semen [11]. Recent research about neosporosis has focused on the development of fast and sensitive PCR systems to detect *N. caninum* in cattle tissues [8], but not in semen.

In the present study, non-spermatozoal cells of fresh semen of bulls were evaluated with naturally-acquired neosporosis for the presence of *Neospora caninum* genome by means of a newly developed PCR assay.

MATERIALS AND METHODS

ELISA

Blood samples were obtained from 18 bulls which were 1-5 years old: 13 seropositive and 5 seronegative bulls were selected for the next steps of the study. Sera samples were obtained before the first semen sampling and after the last one (days 0 and 60). Serum samples were analyzed for antibody using the commercially available ELISA kit[1] for *N. caninum*. Results were calculated as a corrected sample to positive (S/P) ratio ($OD_{Sample_}$ODNeg)/ ($OD_{Pos\_}OD_{Neg}$) _ 100) and expressed in percentage. Two repetitions from each sample were performed. Sera with positive reaction were conducted to further analysis.

Parasites

N. caninum (NC-1 strain) tachyzoites were grown *in vitro* using Vero cell monolayers in RPMI-1640 supplemented with 2% fetal bovine serum and a mixture of 50 U/mL penicillin and 50 mg of streptomycin. The cultures were incubated at 37ºC, 5% CO_2 [14]. Tachyzoites were frozen at -80 ºC until required for further processing.

Preparation of bovine semen and non-spermatozoal cells

Fifty-four samples were examined; the samples were collected by using artificial vagina from 13 seropositive and 5 seronegative bulls maintained at Sheikh Hassan breeding centre; Tabriz, Iran,. Three consecutive semen samples with 30-day intervals were obtained from each animal and sent to the laboratory in refrigerated boxes. For preparation of the non-spermatozoal cells, the semen samples were diluted with an equal volume of PBS. The diluted semen specimens' underlay was a 21% cushion of Nycodenz in PBS. After centrifugation at 850 g for 30 min, non-spermatozoal cells were harvested from the top of the cushion. Using a hemocytometer, cells were counted and approximately 10^7 cells were used for DNA extraction.

DNA extraction

Genomic DNA was extracted from non-spermatozoal cells, purified tachyzoites (6 x 10^8 cells) of *N. caninum* (NC-1 strain), *T. gondii* (RH strain) (6 x 10^8 cells) and fish (rainbow trout) semen (2 x 10^6 cells) based on Chomezynski extraction method. Briefly, in a 1.5 mL tube, a 25 µL volume of fresh semen, or purified tachyzoites were added to 200 µL of a solution containing 5% Chelex 100 (w/v) in ultrapure water, 2 µL of 10 mg ml^{-1} proteinase K[2] and 7 µL of DTT3. The mixture was incubated at 56ºC for 45 min, followed by centrifugation at 13000 *g* for 10 s, incubation in a boiling water bath for 8 min and subsequent centrifugation at 13000 *g* for 3 min. DNA concentration was measured in 260 and 280 nm[4]. Electrophoresis of each DNA sample on 0.5% agarose gel in 1X TBE buffer was undertaken to check the integrity of the DNA. An aliquot of total DNA was produced from each sample and stored at -20ºC until required for analysis.

PCR assay

Primers and thermal cycling conditions

The primers were designed based on the available sequence on gene bank (accession number X84238.1) and by using Primer[3] software. The primers Rep-F (5 GGG TGA GGA CAG TGT GTC AA -3) and Rep-R (5- TCC CAT ACC TGG ATG CTT

TC-3) were predicted to produce a 213 bp region of *N. caninum* genome. A reported primer pair (NP6 & NP21) was also included in the study [11]. Furthermore, two primers HL033 (5- CGA GTC CTT ATG AGC TTG ATT CTT -3) and HL035 (5- GCC TTC CAG AAG TCG TTT GTT TTC -3) that target part of the bovine prolactin gene were considered as an internal control [2].

Amplification

PCR was performed using DNA with varying concentrations obtained from purified tachyzoites of *N. caninum* or *T. gondii* and from the 54 samples collected at the AI centre. The gradient PCR at different annealing temperatures (55 to 65ºC) and varying concentration of $MgCL_2$ (1 to 3 mM) were conducted in a Mastercycler gradient4. The PCR consisted of 35 cycles, with each cycle consisting of 60 s at 95ºC, 60 s at 56ºC and 60 s at 72ºC. A final extension time of 6 min at 72ºC was included at the end of the last cycle.

Limit of detection

Serial 10-fold dilutions of purified tachyzoites of *N. caninum* or *T. gondii* containing 100 to 0.1 ng/µL DNA were tested by PCR to determine the sensitivity of the test. Specificity of the PCR test was determined by testing extracted DNA from purified tachyzoites of *T. gondii* containing 100 to 0.1 ng/µL DNA (Table 1). In order to better assess the specificity of the PCR test, extracted DNA from fish semen was used in test in combination with different dilutions of extracted DNA from tachyzoites of *N. caninum* (Table 1).

Analysis of PCR products by Dot-Southern blotting

The PCR product from the *N. caninum* PCR was excised from a 1% agarose gel and purified using PCR DNA Extraction Kit from agarose gels[5] according to the manufacturer's instructions. The PCR product was denatured (1.5 M NaCl, 0.5 M NaOH) and the denatured PCR products in different concentrations (1ng, 10ng 100pg, 10pg, 1pg and 100pg) were immobilised on nylon membrane[6]. Genomic DNA of the *N. caninum* tachyzoites was digested with HindIII and 1 µg of digested DNA was labeled using a biotin labeling kit[7] according to the manufacturer's instructions. Empirically, random decamers are annealed to a denatured template DNA molecule and new strands are synthesized by the klenow fragment, in the presence of Biotin-dUTP. During this reaction, the biotinylated nucleotides are incorporated into a newly synthesized complementary DNA strand. As a result, DNA fragments of any length can be uniformly

Table1. Different concentration of *Neospora caninum* DNA used for PCR assay development.

No.	*N. caninum* DNA (ng/PCR tube)	*T. gondii* DNA (ng/PCR tube)	Fish semen DNA (ng/PCR tube)	Total DNA (ng/PCR tube)
S1	10	0	0	10
S2	1	0	0	1
S3	0.1	0	0	0.1
S4	0.01	0	0	0.01
S5	0	100	0	100
S6	0	10	0	10
S7	0	1	0	1
S8	0.1	10	0	10.1
S9	0.1	100	0	100.1
S10	0.1	0	10	10.1
S11	0.1	0	100	100.1
S12	0.1	100	100	200.1

Serum samples were analyzed for antibody using the commercially available ELISA kit1 for *N. caninum.*

labeled. Biotin-labeled DNA is detected with Biotin-Strepavidin detection system[8].

RESULTS

ELISA and PCR assay status to *N. caninum* of the animals are shown in Table 2.

Optimization of PCR assay

Using primers Rep-F and Rep-R, a product with expected molecular weight of 213 bp was recorded. The PCR assay was optimized for the detection of *N. caninum* employing various annealing temperatures and $MgCl_2$ concentrations. The results of PCR optimization experiments indicated that the PCR at an annealing temperature of 61°C was optimal for the primer pair Rep-F and Rep-R and in a Taq polymerase buffer containing 2mM $MgCl_2$ (data not shown). The optimized conditions of PCR consisted of an initial denaturation of 5 min at 94ºC followed by 35 cycles of 94 for 1 min, 60ºC for 1 min, 72ºC for 1 min and a final extension at 72ºC for 6 min.

Detection limit of PCR assay

The detection limit of the assay was determined to be to 0.1 ng/pcr tube of DNA using 10-fold serially diluted genomic DNA of *N. caninum* (Figure 1). No cross-reaction was observed with other DNA tested from fish semen and/or a closely related species, *T. gondii* (Figure 1).

Table 2. ELISA and PCR results for *Neospora caninum* from eighteen bulls.

No.	Age	ELISA	PCR						ELISA
			First assay		Second assay		Third assay		
			Np21 & Np6	Rep-F & Rep-R	Np21 & Np6	Rep-F & Rep-R	Np21 & Np6	Rep-F & Rep-R	
1	2.5	+	+	+	+	+	+	+	+
2	2	+	+	+	+	+	+	+	+
3	3.5	+	+	+	+	+	+	+	+
4	2	+	+	+	+	+	+	+	+
5	4.5	+	+	+	+	+	+	+	+
6	2	+	+	+	+	+	+	+	+
7	1.5	+	+	+	+	+	+	+	+
8	3	+	+	+	+	+	+	+	+
9	3	+	+	+	+	+	+	+	+
10	4.5	+	+	+	+	+	+	+	+
11	4	+	+	+	+	+	+	+	+
12	2.5	+	+	+	+	+	+	+	+
13	5	+	+	+	+	+	+	+	+
14	2	-	-	-	-	-	-	-	-
15	3.5	-	-	-	-	-	-	-	-
16	1.5	-	-	-	-	-	-	-	-
17	4	-	-	-	-	-	-	-	-
18	4	-	-	-	-	-	-	-	-

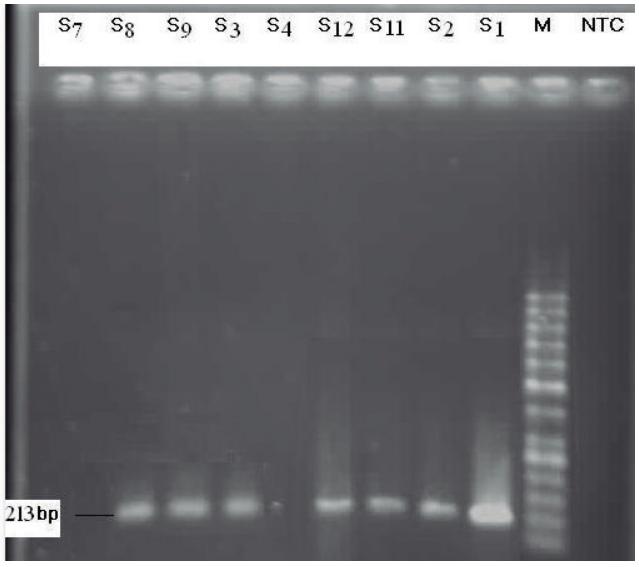

Figure 1. PCR amplification products (PAPs) with Neospora caninum specific primer pair. M: DNA marker; NTC: non template control; S1, S2, S3 & S4: PAPs with 10 ng, 1 ng, 0.1 ng and 0.01 ng DNA from *Neospora caninum* respectively; S7: PAPs with 0 ng DNA from N. caninum and 10 ng DNA from *Toxoplasma gondii*; S8 and S9: PAPs with 0.1 ng DNA from *N. caninum* in the presence of 10 and 100 ng DNA from *T. gondii*, respectively; S11: PAPs with 0.1 ng DNA from *N. caninum* and 100 ng DNA from fish semen; S12:PAPs with 0.1 ng DNA from *N. caninum*, 100 ng DNA from *T. gondii* and 100 ng DNA from fish semen.

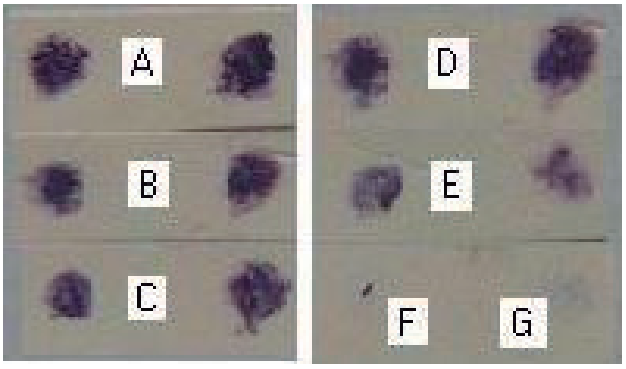

Figure 2. Southern blotting, A: With 10 ng of PCR amplification products (PAPs) with *Neospora caninum* specific primer pair; B: with 1 ng of the PAPs; C: with 100 pg of the PAPs; D: with 10 pg of the PAPs; E: with 1p of the PAPs; G: with 100 pg of the PAPs and F: with 100 pg of PCR product of prolactin gene.

Dot-Southern blotting

The Biotin labeled probes were able to identify the different concentrations of PCR product of *N. caninum* down to 1 pg (Figure 2). As a negative control, we used PCR product of prolactin gene. The result of Southern blot analysis showed no hybridization of *N. caninum* oligonucleotide probes to PCR product of prolactin gene (Figure 2)

DISCUSSION

As a new try we developed and standardized PCR assay to investigate the presence of the parasite in fresh semen of the bulls. The designed primers were found to be highly specific for *N. caninum*. Several PCR assays have now been adapted for the detection of *N. caninum* [2,7,9,10,12,16]. The parasite possesses repetitive sequences. Since these segments are present in multiple copies, the idea of targeting them appears straightforward. Indeed, repetitive sequences of DNA increase PCR amplification sensitivity because there are several copies of them in each cell [9]. Kaufmann *et al*. [9] and Yamage *et al*. [16] reported observation of the parasite signal in PCR with as little as 100 pg and 10 pg parasite template DNA, respectively. A highly repeated DNA sequence in *N. caninum* genome was the target of the PCR described by them. The detection limit of the PCR assay in this study was 100 pg which is in line with the report of Kaufmann *et al*. [9]. Even in the presence of excessive amount of genomic DNA from *T. gondii*, the assay was able to find the parasite signal in PCR. The results for templates amounting less than 100 pg were inconsistent. The sporadic detection of *N. caninum* DNA in semen straws of the serologically positive bulls, may be explained by the low parasite number of the parasite template in the semen of chronically affected bulls which are affected by the sensitivity of the PCR assay via the standard dilution used to prepare the extended semen straws [3]. Of course, this might provide an inadvertent protection of the venereal transmission of the disease. Based on the results of a SYBR Green I Real - Time PCR, maximum parasite numbers observed in positive semen samples from naturally infected bulls were 10 parasites/mL of semen, equivalent to an ejaculation containing approximately 100 parasites/mL [8,13].

In these three reports above mentioned, semen samples were conducted to spinning for harvesting cellular fraction of the samples (fresh or frozen) and DNA was extracted from this fraction. Substances such as semen, as a condense biological fluid, contain polysaccharides, proteases, cell lysis products, or other substances interfering with the amplification reaction. In this regard, semen will require extensive removal of such materials from genomic DNA prior to amplification and it might be beyond the potential of current kits to conduct DNA isolation. In the present work, via a density gradient centrifugation, non-spermatozoal cells were isolated from the fresh

semen and subsequently washed and then subjected to DNA extraction by a silica-based method. With this approach, it was attempted first to isolate the suspected cells that carry the parasite genome, then minimize the inhibitory effects of the fresh semen and finally to increase the concentration of the extracted DNA. Based on the results of this approach, the parasite signal in all semen samples from the seropositive bulls was observable by the aforementioned PCR assay [9], or what was developed in this study. Moreover, further studies are needed to determine the effect of using *N. caninum* infected semen to inseminate heifers or cows. However, these results should be considered in the broader perspective of safety in AI centers and for management of seropositive bulls. Since artificial insemination is being used to introduce new genetics in bovine herds and also for better control of the product quality with regard to transmission of diseases, it would be interesting to determine whether the semen of infected bulls represents a potential source of *N. caninum* introduction in the herd.

In the present study, the detection of *N. caninum* DNA in non-spermatozoa of the semen of naturally infected bulls was reported for the first time which can probably be used as a diagnostic tool for the infection. Whether venereal transmission plays a role in the spread of bovine neosporosis, needs to be determined since *N. caninum* is one of the major causes of abortion in cattle.

SOURCES AND MANUFACTURERS

[1]IDEXX Laboratories, Westerbrook, ME, USA.
[2]Merck, Darmstadt, Germany.
[3]Biorad, Hercules, CA, USA.
[4]Biophotometer plus, Eppendorf, Germany.
[5]Cinnaclone, CinnaGen Co., Karaj, Iran.
[6]Sensiblot Plus Nylon Membrane, Fermentase, Ukraine.
[7]Biotin Decalabel DNA labeling kit, Fermentase, Ukraine.
[8]Biotin Chromogenic Detection kit, Fermentase, Ukraine.

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