

Seminested PCR for Diagnosis of *Neospora caninum* Infection in Cattle

Short Communication

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Summary

Neospora caninum, an apicomplexan protozoan, is regarded as a major cause of abortion and stillbirth in cattle in countries world-wide. The ability to detect *N.caninum* in tissue samples can be a useful detection diagnostic tool for use in the study of the pathogenicity, immunoprophylaxis, and treatment of *Neospora* infection. However, molecular biology is one of the most sensitive tools for detecting protozoa in infected tissue samples. Specific semi-nested PCR was designed based on specific ITS1 and 5.8S rRNA genomic DNA for detection of parasite in infected tissues. The designed PCR detected four of six aborted fetal brain samples infected by *N.caninum*. Our results revealed that PCR with selected primers gave a 357bp product in examined samples and confirmed the presence of *N.caninum* DNA in infected fetal brains. This is the first report that demonstrated the reliability of PCR-based assay to identify *N.caninum* infection in Iran.

Key words: *Neospora caninum*, PCR, protozoa, cattle, abortion, diagnosis

Introduction

Neosporosis, caused by *Neospora caninum* (*N.caninum*), is an apicomplexan protozoan has a worldwide distribution, and a major cause of reproductive failure associated with abortion among cattle. Infection is common and may frequently be vertical transmission with no signs of disease (Kritzner *et al* 2002). Since its first

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recognition in dogs in Norway (Bjerkås *et al* 1984) and the description of a new genus and species *N.caninum* (Dubey *et al* 1988), neosporosis has emerged as a serious disease of cattle and dogs worldwide. The infectious organisms can cause significant losses in farm ruminant production as a result of abortion, embryonic damage or maternal infertility (Dubey & Lindsay 1996, Dubey 1999). Life cycle of the protozoan is typified by three infectious stages: tachyzoites, tissue cysts, and oocysts. Tachyzoites and tissue cysts are the stages found in the intermediate hosts and they occur intracellularly. Domestic dogs are the only known definitive host for *N.caninum* (Dubey *et al* 2002).

The diagnosis of neosporosis is difficult, due to non-specific clinical signs in cattle and there are no parasitological examinations which can help to recognize neosporosis (Atkinson *et al* 2000, Wouda *et al* 1997, Wouda 2000, Barr *et al* 1995, Bjorkman & Ugglå 1999). There is no effective vaccine against bovine neosporosis or therapeutic agents that can be used to control the proliferation/replication of *N.caninum* in cattle (Innes *et al* 2002, Tou *et al* 2004). Examination of the serum from an aborting cow is only indicative of exposure to *N.caninum* and histologic examination of the fetus is necessary for a definitive diagnosis of neosporosis (Dubey 2003, Sadrebazaz *et al* 2004). Although immunohistochemical demonstration of *N.caninum* in lesions is the best evidence for etiology of abortion at the present time but it is not much sensitive method (Dubey 2003). Therefore, highly sensitive diagnostic methods are important to control the infection. *N.caninum* DNA can be detected by PCR in formalin-fixed, paraffin-embedded bovine aborted brain tissue. However, the efficiency of the diagnosis by PCR is dependent on the laboratory, stage of the autolysis of the fetus, and sampling procedures (Dubey 2003).

In this study, we tried to optimize a sensitive semi-nested PCR test for detecting genomic DNA of *N.caninum*, and also to examine frozen aborted bovine fetal tissue samples for the presence of DNA of the parasite.

Materials and Methods

Sample. Six frozen bovine aborted fetuses were prepared from the Khorasan province (Razi Ins., Mashhad).

DNA extraction. DNA was extracted from 50mg of thawed brain tissues by using proteinase K digestion, phenol-chloroform purification followed by ethanol precipitation (Sambrook *et al* 1989). As positive and negative controls DNA of *Neospora*, *Toxoplasma*, *Babesia* and *Theileria* were also extracted.

Semi-nested PCR. Oligonucleotide primers for *N.caninum* ITS1 and 18S rRNA sequence (GenBank accession no. AY463245) were designed to amplify a 357bp DNA fragment. The *N.caninum* Nc1 forward primer spans nucleotides 111 to 129 (5'- AGC GTG ATA TAC TAC TCC C -3'), Nc2 reverse primer spans nucleotides 446 to 467 (5'- CGA GCC AAG ACA TCC ATT GCT G -3') and Nc3 semi-nested PCR primer spans nucleotides 209 to 227 (5' GTG TGT GCA TAT ATC CGG G 3') (Figure 1). The PCR mixture of 50µl contained 0.1-1.0µg of target DNA, 2mM MgCl₂, 10×reaction buffer (50mM KCl, 10mM Tris-HCl [pH8.3], 10pmol of each PCR primer, 200µM each dNTP, and 1U of *Taq* DNA polymerase (CinnaGen, Iran). PCRs were performed in a thermocycler (Techgene-Techne, Germany) for 35cycles of denaturation at 94°C for 30S, annealing at 55°C for 45S, and extension at 72°C for 60S. For semi-nested PCR, second-round primers Nc2 and Nc3 were used 2µl of amplicon solution from first-round Nc1-Nc2 PCR amplification as target DNA with the same PCR mixture subjected to 35cycles of denaturation at 94°C for 30S, annealing at 55°C for 45S, and extension at 72°C for 60S. Amplicons were resolved on a 2% agarose gel stained with ethidium bromide and photographed under UV light. Positive (*Neospora* DNA) and negative controls (no DNA) as well as irrelevant templates (*Toxoplasma*, *Babesia* and *Theileria* DNAs) were included in each PCR run. Positive samples were tested at least three times for showing reproducibility of the specific PCR. Amplification products were analyzed by electrophoresis through a 2% agarose gel for the specific *N.caninum* PCR.

Restriction digestion. The PCR products were digested with endonuclease *Hinf*I (Fermentas, Germany) according to the manufacturer's instruction.

Analysis. Sequences and primers analyzed using "GeneRunner" program and homology studies were performed by "Blast n" on line program.

Results and Discussion

DNA was successfully extracted from frozen aborted bovine fetuses using proteinase K digestion and further phenol-chloroform purification method.

Semi-nested PCR. The sequences of ITS1 and 5.8S rRNA genomic DNA have been selected for designing primers (Figure 1).

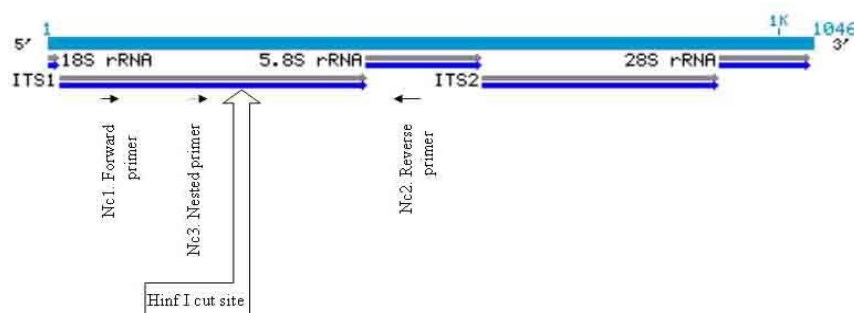


Figure 1. *N.caninum* 18S ribosomal RNA (18S rRNA), internal transcribed spacer 1 (ITS1), 5.8S ribosomal RNA (5.8 rRNA), internal transcribed 2 (ITS2), and 28S ribosomal RNA (28S rRNA) sequences shown in graph as well as used primers locations and directions (Nc1, Nc2 and Nc3,) and restriction site for *Hinf*I

Homology analysis of the selected primers using "Blast n" demonstrated specificity of the designed primers. The primers Nc1 and Nc2 used to amplify 357bp of the above sequences. Moreover, Nc2 and Nc3 amplified 259bp of the same regions (Figure 2). The semi-nested PCR demonstrated that four of six aborted fetal brain samples were infected by *N.caninum*. Figure 2 shows the specific PCR results visualized by gel electrophoresis analysis. PCR testing included the amplification of *N.caninum* DNA as well as of the phylogenetically closely related organism

protozoan (*Toxoplasma*, *Babesia* and *Theileria*) with potential diagnostic importance (Figure 2).

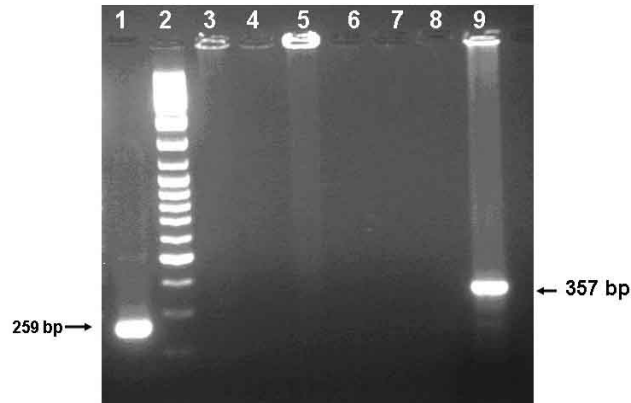


Figure 2. Specificity of the PCR for detecting *N.caninum* infection using various apicomplexan parasites DNAs as irrelevant templates (lanes 3-7). Lane 1, semi-nested PCR product by Nc2 and Nc3 primers; lane 2, 100bp DNA ladder; lane 3, *Theileria annulata* DNA from infected blood; lane 4, *T.annulata* DNA from vaccine cell line; lane 5, *T.lesoquardi* DNA; lane 6, *Babesia ovis* DNA; lane 7, *Toxoplasma gondii* DNA; lane 8, negative control (no DNA); and lane 9, specific PCR for *N.caninum* DNA (by primers Nc1 and Nc2). Left and right arrows show specific 259 and 357bp Nc2-Nc3 and Nc1-Nc2 PCR products respectively

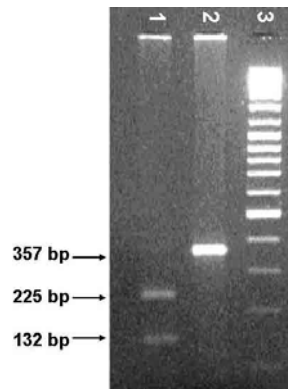


Figure 3. A 2% agarose gel electrophoresis of the *Hinf I*-digested PCR product from *N.caninum*. The bands of 132bp and 225bp on the gel (lane 1) indicate that specific PCR fragment was successfully amplified by Nc1 and Nc2 primer pair. Lane 1, the *HinfI* digested PCR product; lane 2 undigested PCR product; and lane 3, 100bp DNA ladder

Restriction enzyme analysis. ITS1 and 5.8S rRNA genomic sequence of *N.caninum* contains a unique restriction cut site for endonuclease *Hinf*I at nucleotide 243. Digestion of PCR products with this enzyme produced two fragments of 225 and 132bp in length (Figure 3), which confirms specificity of the PCR.

The present study demonstrates the utility of PCR-based assay to identify *N.caninum* infection in spontaneously aborted bovine fetuses. All tissue samples from aborted fetuses were tested by semi-nested PCR in order to determine whether nested PCR would increase the sensitivity of detecting *N.caninum* in an individual tissue over that of standard PCR. False-negative and inconsistent results due to very low parasite numbers in the sample could be detectable only by nested PCR. PCR has been reported as a procedure for highly sensitive detection and infectiveness of *N.caninum* through the amplification in bovine samples (Bretagne *et al* 1993, Burg *et al* 1989, Lally *et al* 1996, Muller *et al* 1996, Yamage *et al* 1996). The designed specific PCR analyses showed that a nested PCR procedure (to increase sensitivity and specificity) was necessary to detect *N.caninum*-infected fetuses. There are up to 1000 times increased efficiency at generating second-round amplicons (Jackson *et al* 1992). Regarding to the *N.caninum* specific PCR, demonstration by gel electrophoresis of amplification products from reactions on aborted fetal brain materials was often substantially hampered by the existence of a background smears at the predicted location of the diagnostic band (Muller *et al* 1996, and Ellis *et al* 1999). In these cases again, a semi-nested PCR between positive and negative results was much facilitated by applying a respective restriction digestion test (Figures 2 and 3)

The aborted fetal brain has been shown as a reliable tissue for PCR analysis and a nested-PCR procedure may be used to increase sensitivity and specificity to detect *N.caninum*-infected fetuses. Our results are consistent with the previous studies showing tissue parasites detected most frequently in brain by PCR (Yamage *et al* 1996, Dubey and Lindsay 1996, Wouda *et al* 1997, Dubey 2003). In another study,

Collantes-Fernandes used molecular detection of parasite DNA with PCR in different organs of the mice for assessing treatment efficiency (Collantes-Fernandes *et al* 2002). PCR-positive fetus detected in the frozen tissue samples exhibits the PCR being more sensitive than the gold standard tests; histopathology and immunohistochemistry (Baszler *et al* 1999). The present study, PCR detection of *N.caninum* DNA worked well on frozen tissue, which increased the practical application of a PCR-based assay. When interpreted in conjunction with significant histopathologic changes in aborted fetal tissues, PCR could provide a valuable confirmatory tool to diagnose *N.caninum* abortion in cattle. In conclusion, our study demonstrated that the reliability of PCR-based diagnosis of *N.caninum* was much improved when using semi-nested PCR as confirmation test for detection of respective DNA amplification products. It is the first report that demonstrated the capability of the PCR-based assay to detect *N.caninum* infection in aborted bovine fetuses in Iran.

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