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# Isolation of *Neospora caninum* from an aborted fetus of seropositive cattle in Iran

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**ABSTRACT**

*Neospora caninum* was isolated from the brain of aborted 4-month-old bovine fetuses in Tehran, Iran. From *Neospora* seropositive dairy cattle, cerebral tissues from aborted fetuses were inoculated interaperinoneally into immunosuppressed Balb/C mice. After 60 days, positive ELISA titer for *Neospora caninum* was determined in one group of mice, which had been inoculated with the brain of the 4-month-old aborted fetus and *Neospora* tachyzoite-like organisms were detected in the peritoneal exudates of the mice. *Neospora caninum* tachyzoites were isolated in Vero cell culture after inoculation with mice peritoneal exudates within 37 days. The identity of the parasite was confirmed using the nested-PCR method in the brain tissue of the aborted calf, peritoneal exudates and brains of the positive mice and cell culture. The nucleotide sequence from the brain and peritoneal exudate of the inoculated mice was designated as Nc-Iran and has been deposited in the GenBank database under the accession number FJ655914. This is the first report dealing with the isolation of *N. caninum* in Iran.

**Key words:** *Neospora caninum,* cattle, brain, mice, cell culture, Iran

## Introduction

*Neospora caninum* is an apicomplexan protozoan that was first described and isolated from puppies with congenital encephalomyelitis (BJERKAS et al., 1984; DUBEY et al., 1988). The parasite has since been recognized as a major cause of abortion in cattle worldwide as well as a causative agent of neuromuscular disease in dogs (BJERKAS et al., 1984). *N. caninum* was first isolated from a dog following *in vitro* cultivation of the

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brain homogenates using Vero cells (DUBEY et al., 1988). Since then, *in vitro* isolation of *N. caninum* from dogs or aborted bovine fetuses has been reported from several countries (BARBER et al., 1993; BARBER et al., 1995; CONRAD et al., 1993; YAMANE et al., 1997). Many attempts have been made to obtain viable *N. caninum* isolates by bioassays (*in vivo* inoculation) in mice or directly in cell culture; however, the isolation *in vitro* of this parasite is arduous and often unsuccessful. Successful isolation of *N. caninum* is however, difficult due to the low numbers and/or viability of parasites and observation of parasites in primary culture can often take more than one month (DAVISON et al., 1999; OTTER et al., 1995).

In Iran, there have been some studies on the seroprevalence of *N. caninum* infection in different hosts (SADREBAZZAZ et al., 2004; RAZMI et al., 2006 and 2007; MALMASI et al., 2007; HADDADZADEH et al., 2007; NOUROLLAHI FARD et al., 2008; SALEHI et al., 2010). These results could be an indication that bovine neosporosis is one of the important causes of bovine abortion in Iran, leading to great economic losses. In spite of its economic importance to the dairy industry, research on neosporosis has been impeded by the lack of parasite isolates in Iran. This paper describes the successful isolation of *N. caninum* from an aborted bovine fetus in Iran and characterization of the isolated parasite by ELISA, PCR and sequence analysis of the Nc-5 gene.

## Materials and methods

*Sampling*. From four Holstein dairy herds in the vicinity of Tehran, 12 brains of aborted fetuses from ELISA seropositive dams, which were checked by an IDEXX Herdcheck-ELISA commercial kit (SALEHI et al., 2010), were selected randomly and used for isolation of *Neospora caninum*. All samples were immediately transported to the laboratory on ice. The brains were removed aseptically and half of them (one hemisphere) were kept in cold PBS containing antibiotics (1000 IU/mL of penicillin and 100 μg/mL of streptomycin) for one day until isolation.

*Isolation of Neospora caninum.* A portion of the each brain was placed in PBS buffer containing 2% antibiotic-antimycotic solution (Gibco BRL, Paisley, UK) and maintained at 4 ºC until they were used for mice inoculation. Before inoculation, at least one sample of 20 mg from the removed brain portions was analyzed to confirm the presence of the parasite in the tissues by nested-PCR on the Nc-5 gene described below. Five grams of brain portions were homogenized, filtered through a sterile gauze (150 μm) and centrifuged at 1350g for 15 min. The supernatant was discarded and 0.6 mL of the sediment suspended in PBS with 2% antibiotic-antimycotic solution (Gibco BRL) was inoculated intraperitoneally into two female Balb/c mice (Pasture institute, Tehran, Iran) given 10 μg/mL dexamethasone (Farayand Danesh Company, Tehran, Iran) in drinking water ad libitum for 10 days after inoculation, as described by ROMAND et al.

(1998). The mice were maintained at the Animal House of the Veterinary Faculty of the University of Tehran with irradiated feed and water ad libitum, and examined daily for the development of neosporosis clinical signs. After 60 days, blood samples were taken from the tails of the mice for serological analysis. Seropositive mice were sacrificed, and their peritoneal cavity washes were used for *in vitro* cultivation. The 24 h Vero cell monolayer growing in DMEM, supplemented with 100 IU penicillin G, 100 μg streptomycin per mL and 10% fetal calf serum, was used for inoculation. The medium was removed 24 h after inoculation and replaced with fresh medium. The cell cultures were examined daily with an inverted microscope for the presence of parasites. DNA isolated from the brain tissue of aborted calves, peritoneal exudates, brains of inoculated mice and inoculated cell culture were analyzed by PCR using specific primers for *N. caninum*.

*Genetic characterization of N. caninum isolates.* Total DNA was extracted from 300 μL of homogenized suspension using a QIAGEN DNeasy Tissue kit (Qiagen, GmbH, Germany) according to the manufacturer's instructions. Nested-PCR on Nc-gene region of *N. caninum* was carried out with four oligonucleotides Np21+ and Np6+ primers and internal primers, Np7, as a forward primer (5′GGGTGAACCGAGGGAGTTG3′) and Np10 as a reverse primer (5′TCGTCCGCTTGCTCCCTATGAAT3′) were designed using available sequence data for the Nc-5 gene. To avoid carryover of contaminating nucleic acids, each step of the procedure was performed in separate rooms. PCR reactions were performed for 35 cycles, each cycle including denaturation at 94 °C for 45 Sec, annealing at 64 °C for 45 Sec, and extension at 72 °C for 45 Sec. For nested PCR, second-round primers Nc7 and Nc10, and 1 μL of the first-round PCR product (as a target DNA) were used with the same mixture and procedure. DNA from *N. caninum* (Razi Vaccine and Serum Research Institute, Mashhad, Iran) was used as PCR positive controls. To identify false-positive results, negative control reactions (sterile water) were added to each set of PCR procedures. The secondary amplification product was visualized as a 198 bp band by using 2% agarose gel electrophoresis in 0.5X TBE buffer and ethidium bromide staining.

*DNA sequencing.* Positive PCR products of the brain and peritoneal exudates of inoculated mice were purified using an MBST PCR Purification kit (MBST, Iran) according to the manufacturer's instructions and sequenced at the Kawsar Biotech Company, Iran, with the internal primers (Np7/Np10), using an ABI Big Dye Terminator Cycle Sequencing kit according to the manufacturer's instructions. Sequences were analyzed using SeqEd v1.0.3 (Applied Biosystems).

*Serological analysis.* Serologic analysis for the inoculated mice was done by the Pasteur Laboratory (Tehran, Iran) according to their procedure. Briefly, 96 wells plates (Maxisorp, Nunc, Wiesbaden, Germany) were coated overnight at 4 °C with 100 μL of Nc-extract (0.7 μg/mL), diluted in 0.1 M carbonate-bicarbonate buffer, PH = 9. To block non-specific binding of antibodies, the plates were incubated with 200 μL of PBS containing

3% BSA and 0.2% Tween20 at RT for 2 hours. Serum samples were diluted 1:50 in PBS containing 3% BSA and 0.2% Tween20 (BSAT) and incubated for 2 hours at room temperature. After washing 3 times with BSAT, anti-mouse IgG alkaline phosphatase-conjugated (Promega, Germany), and goat anti-mouse alkaline phosphatase- conjugated IgG1 and IgG2a, (Promega, Germany) respectively were added at a dilution of 1:1000 in BSAT and incubated for 2 hours at room temperature. Following three washes in BSAT, the wells were subsequently incubated with 100 μL of 1 mg/mL p-nitrophenil-phosphate-disodium in 10% diethanolamine containing 0.5 mM MgCl2, PH = 9.8. After developing the color (30 minutes at room temperature), the plates were read in an ELISA microplate reader at a wavelength of 405 nm. The cut off value was 0.185.

**Results**

DNA of *Neospora caninum* was detected in 12 brains of aborted fetuses (Fig. 1). After 60 days only one group of mice (two female BALB/c mice) inoculated with one of the fetal brains showed ELISA antibody titer against *Neospora caninum* (0.295). Also *N. caninum*-like organisms were seen in the peritoneal exudates of seropositive mice microscopically using a Giemsa stain. The specific *N. caninum* PCR products were obtained from the brain and peritoneal fluid of the seropositive mice (Fig. 1). The nucleotide sequence from these isolates was designated as Nc-Iran and has been deposited in the GenBank database under the accession number FJ655914. The *N. canium* speicies-specific DNA sequence was: GGTTTGTGGTTAGTCATTCGTCACGTTGAAATCAGCCTGCGTCAGGGTGT GGACAGTGTGTCAATGATACTTATCGAGAGTTCAGTGTTCTGTGTTG AGGCAACACCGGCGGCACTGATGACGGGGGAGATTATTCATAGGGAGCA AGCGG.

After 37 days of inoculation of Vero cell culture with the peritoneal fluid of the infected mice, *Neospora* tachyzoites were observed in the cell culture microscopically (Fig. 2). DNA fragments of the expected size (198 bp) were amplified from the harvested tachyzoite from the cell culture (Fig. 1).

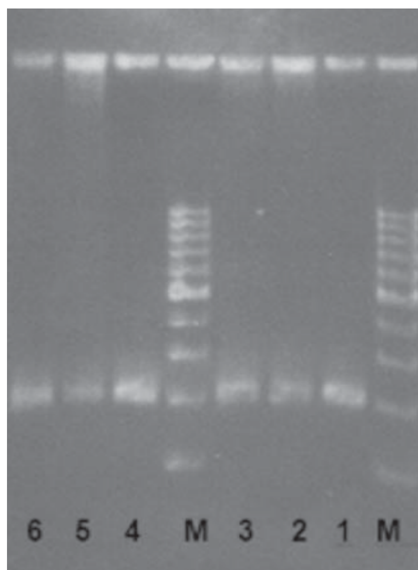


Fig. 1. Detection of *N. caninum* using nested-PCR. M: Marker, 1: positive control, 2: Fetus brain, 3: Fetus brain, 4: Mouse brain, 5: mouse peritoneal exudate, 6: Tachyzoite in cell culture

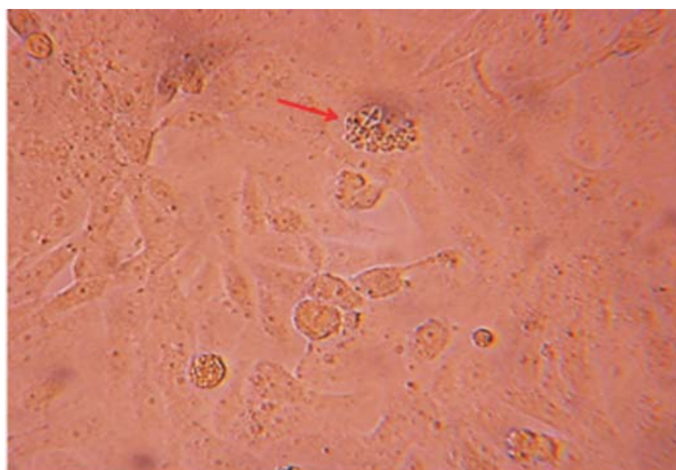

Fig. 2. Aggregation of *Neospora caninum* tachyzoites in cell culture; ×40

**Discussion**

This study describes the first isolation of *N. caninum* from aborted fetus of cattle in Iran. Although many attempts have been made, isolation of *N. caninum* from aborted bovine fetal tissues has been extremely difficult, depending upon the degree of postmortem autolysis of the submitted cases and limited numbers of tissue cysts or tachyzoites present in the tissue (CONRAD et al., 1993). To increase the success of isolation chemically immuno-compromised mice, athymic mice, or gamma interferon knocked-out mice have been used to isolate *N. caninum* from intermediate hosts (YAMANE et al., 1998; PIERGILI FIORETTI et al., 2000; KOYAMA et al., 2001; MILLER et al., 2002; CANADA et al., 2004; VIANNA et al., 2005). In this study immuno-compromised mice (by dexamethasone) were used to increase the success of isolation in cell culture, because the parasite load expected in brain tissues from aborted fetuses could be low. The inoculation of suspected tissue into immuno-compromised mice, as a previous step of inoculating cell culture, is more efficient than the direct inoculation of infected tissues, for isolation of *N. caninum* (REGIDOR-CERILLO et al., 2008). Moreover, serology analysis of pregnant cattle by ELISA confirms exposure to *N. caninum*. Also, prior to mice inoculation, PCR analysis of brain tissues from the aborted fetuses collected for isolation confirmed the presence of the parasite and ruled out isolation failure due to inoculation of uninfected brain samples.

Most isolation methods including the inoculation of trypsin- or non-trypsin-treated brain homogenates onto the cell culture can be toxic to the monolayer cell cultures and can reduce the chance of isolation *in vitro* (REGIDOR-CERILLO et al., 2008). In this study peritoneal fluids from treated mice were directly inoculated into the cell culture, which can be maintained on the monolayer for 24 h without toxic results. This method is similar to the study by REGIDOR-CERILLO et al. (2008). BASSO et al. (2009) reported isolation of *Neospora caninum* from brain, heart and peritoneal exudates of mice inoculated with oocysts, derived from feces of a naturally infected 8-year-old male stray boxer and observed tachyzoites in the cultures on 7, 8 and 3 d.p.i. with brain, heart homogenates and peritoneal washing, respectively. CANADA et al. (2002) inoculated the fetal brain homogenate interaperitoneally, first into outbred Swiss Webster mice given dexamethasone, and then co-inoculated the peritoneal exudates from these mice with mouse sarcoma cells in the peritoneal cavity of mice given dexamethasone. *Neospora caninum* tachyzoites were seen in the peritoneal exudate of the second passage.

The amount of time required for the parasite to be visualized in cell culture could be affected by the density of tachyzoites in the peritoneal cavity fluid and the proliferation and/or adaptation of parasites *in vitro* (REGIDOR-CERILLO et al., 2008). In Japan (YAMANE et al., 1997) tachyzoites of *N. caninum* were detected in cell cultures 49 days after inoculation with brain homogenate from a 2-week-old calf However, in Korea (JAE-HOON et al., 2000) the parasites were first observed on days 45 and 56 post-inoculation with brain homogenate originating from a newborn calf and an aborted fetus. In the present case,

tachyzoites were first seen in cell cultures 37 days after inoculation. This is in agreement with the findings of CONRAD et al. (1993), who reported the detection of parasites at 15 and 34 days after inoculation of cell cultures. In Sweden extracellular tachyzoites of *N. caninum* were first seen in Vero cell culture 56 days after the inoculation of brain homogenate from a stillborn calf (STENLUND et al., 1997), and in Brazil tachyzoites were detected 50 days after brain inoculation (LOCATELLI-DITTRICH et al., 2003). It has been reported that tachyzoite densities in the brains of infected mice are higher than those observed in aborted bovine fetuses or congenitally infected calves (YAMANE et al., 1998). This could explain the relatively early detection of tachyzoites in the cell cultures inoculated with the mouse peritoneal exudates in the present study.

The nucleotide sequence from this isolate had homology with other isolates from other countries and the minimal differences were related to different kinds of strain and geographic situation. This report describes a suitable isolation method for *N. caninum*. Further research into the genetic variation of the present isolates is recommended.

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**SAŽETAK**

Protozoon *Neospora caninum* bio je izdvojen iz mozga četveromjesečnog ploda podrijetlom od serološki pozitivne krave u Teheranu (Iran). Pripravak moždanog tkiva pobačenog ploda bio je inokuliran intraperitonejski u imunološki oslabljenih miševa Balb/C. Nakon 60 dana, miševi su bili pretraženi pri čemu su dokazana specifična protutijela imunoenzimnim testom kao i tahizoitima slični organizmi u peritonejskom eksudatu. Tahizoiti su bili izdvojeni na staničnoj kulturi Vero tijekom sljedećih 37 dana. Identifikacija je provedena metodom ugniježđene lančane reakcije polimerazom korištenjem DNA iz mozga pobačenog ploda i mozga naknadno inokuliranih miševa. Sekvencija materijala iz mozga i peritonejskog eksudata pohtanjena je u bazi GenBank (FJ655914). Ovo je prvi dokaz o izdvajanju protozoona *Neospora caninum* u Iranu.

**Ključne riječi:** *Neospora caninum,* govedo, mozak, miš, stanična kultura, Iran