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Survey for the presence of *Neospora caninum* in frozen bull's semen samples by PCR assay

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PEER REVIEW

Peer reviewer

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Comments

This is a well conducted study in which the authors evaluated the occurrence of *N. caninum* in frozen semen of Iranian bulls based on molecular tools.

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ABSTRACT

Objective: To evaluate the occurrence of *Neospora caninum* (*N. caninum*) DNA using polymerase chain reaction (PCR) technique in frozen semen samples of Iranian bulls that were used for artificial insemination.

Methods: In this study, 57 frozen bull's semen samples were collected randomly from artificial insemination centers and genomic DNA was extracted. For detection of *pNC-5* gene of *N. caninum* by oligonucleotide primers were amplified using PCR technique and 1% agarose gel electrophoresis used for visualization of amplified PCR products.

Results: The results of this study present 6 of 57 (10.53%) as positive samples. This show high presence of *N. caninum* infection and display importance of frozen semen samples of bulls, which were used for artificial insemination, in the spread of bovine neosporosis in Iran. These results present PCR as suitable technique for fast detection of this protozoan parasite in semen specimens.

Conclusions: According to these results control and eradication programs, for example prevent vaccinations, as reduction of economic losses caused by this protozoan infection in Iranian cattle seems to be necessary.

KEYWORDS

Artificial insemination, Bull, Iran, *Neospora caninum*, PCR, Semen

1. Introduction

Neospora caninum (*N. caninum*) is a global distributed protozoan obligate intracellular parasite classified in the kingdom Protista, phylum Apicomplexa, order Eucoccidiorida, and family Sarcocystidae. It is considered as the most important causative agent of repeated abortions in dairy cattle in numerous countries involving Iran and has got negative economic impact for their breeding, including reduced milk production, those attributed to a lengthened calving interval, falling stock value and an elevated culling rate among others[1-3]. *N. caninum* was the first time detected by Bjerkas *et al*. in a boxer dog litter in Norway[4].

N. caninum (neosporosis) life cycle includes canines as final hosts and ruminants, horses and several other species as intermediate hosts. Dogs (*Canis familiaris*), coyotes

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(*Canis latrans*) and grey wolves (*Canis lupus*) are usual final hosts of the parasite[1,2,5]. The coyotes shed in feces of the final host may perhaps infect the environment and serve as an origin of infection. Though, the most significant way is vertical transmission of the parasite from the mother to the fetus during mid gestation stage, resulting in obstinately infected offspring which seem clinically normal. Also, there is a possibility of postpartum infection by colostrum or milk[6,7]. Congenital transmission has been shown in other animals, for example sheep, goats, dogs, horses, rodents and rhinoceros, as well[7].

N. caninum has been shown to have two-host life cycles in which dogs and coyotes are the only recognized final hosts[1]. A wide range of intermediate hosts involving farm and free-living animals. Proof of usual infection have been found in cattle, sheep, deer, goats, horses, water buffalo and a rhinoceros[8,9]. Trial infections have been attained in cats, rats, dogs, mice, foxes, goats, sheep, cattle, coyotes, pigs, gerbils and rabbits[5]. One of the most important goals of cattle examination for *N. caninum* infection is to detect the potential cause of abortion. According to previous goal, it must be considered other infectious and noninfectious diseases as a cause of abortion. Abortions due to *N. caninum* can be manifest as endemic and/or epidemic pattern and have been described in herds of cattle[10].

In addition, *N. caninum* may cause disease in calves infected during pregnancy[11]. At birth such calves may have neurological signs, be underweight, lacking ability to rise, or have no clinical signs[5,12]. *N. caninum* infection may also reduced milk production and shortened production life due to early culling[10]. Afterwards, naturally occurring ovine and bovine neosporosis has been reported globally, including Japan, South America, Australia, Switzerland, Italy, Spain, New Zealand and Iran[3,13-18].

The probability of *N. caninum* transmission by semen can indicate great depth repercussions on cattle semen trade. Artificial insemination is a significant procedure for the development of cattle production and millions of doses of frozen bovine semen are traded once a year all over the world. This fact increase possibility of many bovine diseases spreading[19].

Rapid diagnosis of neosporosis is significant for control and treatment for the reason that *N. caninum* is a significant agent in bovine abortion. Numerous tests have been used to detect the infectious agents in bovine semen such as microscopic agglutination test, enzyme linked immuno sorbent assay, immuno fluorescence assay, polymerase chain reaction (PCR) and other methods[3,20,21]. PCR provides a specific and sensitive test for *N. caninum* DNA that possibly present in tissue samples and body fluids for example vaginal secretion, saliva, amniotic or seminal fluid, and cerebrospinal fluid of aborted fetus. The sensitivity of PCR means that very small amounts of preliminary material are necessary and there are fewer false negative results[13,22]. The occasional detection of the parasite genome in semen samples of infected cattle was previously reported[23]. The increases of infections of contaminated semen have made necessary the precise identification of all microorganisms[24].

Frozen semen samples of bulls and artificial insemination is a significant way for transmission of *N. caninum* infection in Iranian cattle. The goal of current study was to detect *N. caninum* DNA in frozen semen samples of bulls that were used in Iranian artificial insemination centers.

2. Materials and methods

2.1. Sample collection

In present study, 57 frozen semen samples of bulls were collected randomly from artificial insemination centers in various parts of Iran, between August 2013 and September 2013. Semen samples were diluted according to standard procedures and sent to the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in a cooler with ice packs. Each of these samples was stored at −20 °C for further use.

2.2. DNA purification

Genomic DNA was extracted from frozen semen samples using a DNA extraction kit (Cinagen, Tehran, Iran) according to manufacturer's protocol. The used concentrations were from DNA manufacturer's protocol, as well. The total DNA was measured at 260 nm optical density according to the technique described by Sambrook and Russell[25]. Only DNA by A260/280 ratios of 1.0 was kept for PCR analysis. The extracted DNA of each sample was kept frozen at −20 °C until used.

2.3. PCR procedures

The oligonucleotide primers (*pNC-5*-F: 5′-CCT CCC AAT GCG AAC GAA-3′ and *pNC-5*-R: 5′-GGG TGA ACC GAG GGA GTT G-3′) described by Baszler *et al*. were used in this research for firm decision of the presence of *pNC-5* gene

of *N. caninum* in bull's semen samples[26]. PCR was carried out in 25 μL total reaction volumes, each containing 100 ng of target DNA, 0.2 pmol/L of each primer, 2.5 μL of 10× PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 0.1% Triton X-100), 1.5 mmol/L MgCl , 200 mmol/L DNTPs and 1 unit of *Taq* DNA polymerase (CinnaGen, Iran). PCR was performed in a Gradient Palm Cycler (Corbett Research, Australia). In PCR reaction *Neospora* DNA was used as the positive control. A negative control was made by adding 1 μL of sterile ultrapure deionized water. The protocol of PCR amplification reaction was 5 min of pre-denaturing at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 57 °C and 1 min extension at 72 °C and then by a final extension at 72 °C in 5 min.

2.4. Analysis of PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mmol/L, Boric acid 5.5 g 2 mmol/L, ethylene diamine tetraacetic acid (pH 8.0) 4 mL of 0.5 mol/L ethylene diamine tetraacetic acid (pH 8.0), join all components in sufficient H_2O and stir to dissolve). Aliquots of 10 μL of PCR products were applied to the gel. Constant voltage of 80 V in 30 min was used for products separation. The DNA fragment size was compared by a standard molecular weight (100 bp DNA ladder of Fermentas, Germany). After electrophoresis, the amplicons were visualized by ultraviolet light after ethidium bromide (5 μg/mL) staining and photographed were obtained in UVIdoc gel documentation systems (UK).

2.5. Statistical analysis

Data analysis was performed by employing Statistical Package for Social Science (SPSS version 10.0).

3. Results

DNA was by successful manner extracted in high quality from frozen semen samples. Study of PCR products for presence of *pNC-5* gene of *N. caninum* on 1% agarose gel revealed a 275 bp (base pairs) fragment (Figure 1).

Samples which produced a band of the expected size (275 bp) were considered as positive and matched to the positive control but no product was observed for negative control. *N. caninum* DNA was detected in 6 (10.53%) of 57 frozen semen samples and 51 (89.47%) of 57 frozen semen samples was negative for *N. caninum*. The results of this study showed the high occurrence of *N. caninum* infection in frozen bull's semen samples that were used for artificial insemination in Iranian Insemination Centers.

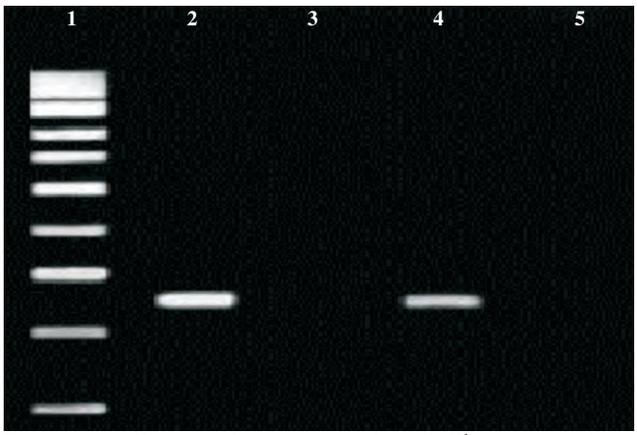

Figure 1. PCR detection of *N. caninum* in frozen bull's semen samples on agarose gel electrophoresis.
Line 1 is molecular mass ladder (100 bp from Fermentas, Germany); line 2 is positive control (*Neospora* DNA); line 3 is PCR negative control (no DNA); lines 4 are positive sample (275 bp) of frozen semen samples; and line 5 is a negative sample.

4. Discussion

N. caninum is a causal agent of abortion all over the world[27,28]. It is considered that *N. caninum* lives into central nervous system. Dogs (and possibly foxes) as final hosts shedding oocysts in feces. For the intermediate hosts like cattle, the most important is congenital infection after ingested oocysts whilst transmission between animals does not occur[29]. In California it is estimated that losses reach about US$35 million per year[30].

The purpose of current study was to detection of *N. caninum* infection in frozen semen samples of bulls used in artificial insemination centers in Iran. The results showed 6 (10.53%) frozen semen sample infected by *N. caninum*.

Many studies were performed about neosporosis infection in bull's semen and described its mutual relation by reproductive failure, reduced conception rates, abortion, milk yield, rebreeding and expenses associated by diagnosis of the diseases in cattle. In a serological study in Spain, beef farms had a lower prevalence of *N. caninum* infection than dairy farms being 55% and 83%, respectively[31]. Proof has been shown in New Zealand where beef cattle had a lower prevalence of *N. caninum* infection in comparison to dairy cattle[32]. The study of Baszler *et al*. in 1999 on detection of *N. caninum* in fetal tissues from spontaneous bovine abortions

by PCR presented higher sensitivity of PCR in comparison to immunohistochemistry[26].

In Europe, *N. caninum* was detected by PCR in the brains of 21% of 242 aborted fetuses in Switzerland[33]. Although in Spain, the prevalence of *N. caninum* DNA was 15.3%[34]. The highest prevalence of *N. caninum* in bovine fetuses, by PCR, was obtained in Mexico (80%; 35/44)[35]. A similar prevalence by our study was found in China, where Yang *et al*. found *N. caninum* DNA in 31.3% of the bovine fetuses[36].

The study of Habibi *et al*. in Iran for diagnosis of *N. caninum* infection in cattle semi-nested PCR was designed based on specific ITS1 and 5.8S rRNA genomic DNA for detection of parasite in infected tissues. Their study is the first report which demonstrates the reliability of PCR-based assay to identify *N. caninum* infection in Iran[37]. The study of Sharifzadeh *et al*. (PCR assay for detection of *N. caninum* in fresh and frozen semen samples of Iranian bulls) showed results as only 30 of 175 (17.14%) semen samples in Iranian bulls were infected with *N. caninum* and the protozoan DNA was detected in 17 (9.71%) and 13 (7.43%) of fresh and frozen semen samples, respectively[3]. The second article we used was by Jozani *et al*. detection of non-spermatozoal cells of *N. caninum* in fresh semen of naturally infected bulls[21]. Razmi *et al*. in 2007 showed first report of *N. caninum*-associated bovine abortion using PCR, complemented with histopathology and immunohistochemistry (IHC) technique in Mashhad area, Iran[38]. Their study indicated that neosporosis is an important cause of abortion in dairy cattle of Iran[38].

According to Cobádiová *et al*., first molecular detection of *N. caninum* in European brown bear (*Ursus arctos*) and presence of *N. caninum* DNA was established in 24.4% (11/45) of 45 muscle, liver, or spleen samples of brown bear tested in different locations of Central Slovakia[39]. In Romania, Şuteu *et al*. reports molecular detection of *N. caninum* abortion in dairy cattle from different historical regions[40].

In Costa Rica neosporosis diagnosed and associated by bovine abortion has been reported since 1996[41,42]. One of the most effective ideas of preventing and controlling neosporosis would be by vaccination; nonetheless developing an effective vaccine presents numerous challenges. An effective vaccine against *N. caninum* should protect in an opposite position fetal loss and prohibit vertical transmission. Serological discrimination intermediate to vaccinated and infected animals would need to be available for infection control[28].

There are numerous different types of vaccines to prevent infectious diseases. Several may be broadly categorized as: killed, modified live and chemically or physically altered vaccines by each having advantages and disadvantages. A killed vaccine has been developed for the prevention of *N. caninum*-induced abortion. It has the following advantages over all others. It cannot revert to the virulent form of the disease, has slight risk of inducing abortion and the vaccine organism does not spread to other animals[43]. The killed vaccine is stable in storage and is an excellent stimulant of passive antibodies in colostrums. Notwithstanding the listed advantages, the killed vaccine has its pitfalls. It may not provide a long-lasting immunity to the animal and can cause allergy and vaccination reactions. The killed vaccine may not be effective in the presence of passive colostral immunity[44].

In study of Ortega-Mora *et al*., *N. caninum* DNA was detected in non-extended fresh semen samples and frozen extended semen straws by nested-PCR[20]. The molecular method for detection of *N. caninum* DNA was used in their study similar to current research.

The research of Munhoz *et al*. in Brazil showed that the abortion was provoked by the protozoan *N. caninum*, though this is the first report concerning cattle in the northeast region of São Paulo state[45].

In conclusion, the results of our study and earlier researches present that frozen semen samples, which used for artificial insemination in Iranian Insemination Centers, plays an important role in the spread of bovine neosporosis. Preventing the entrance of *N. caninum* to cattle is a significant aim of control programs. Based on this data, analysis of bull's semen samples that used for artificial insemination to control and prevention of *N. caninum* infection is very important. The final examinations by high efficiency for diagnosis of *N. caninum* infection in bull's semen samples to reduce the economic losses of this protozoan infection such as abortion, lost milk and meat in dairy industries, it appear to be necessary.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

The protozoan parasite *N. caninum* is recognized as important infectious cause of abortion, neonatal mortality and reduced milk production in cattle, worldwide. Annually, the median global economic losses of the disease it causes, neosporosis, are estimated around 1 300 million US $ for the dairy industry. The continuous follow-up of the cattle herds with valuable diagnostic tools like molecular methods, with special emphasis on the quality of the bull semen which can harbor the parasite, can contribute greatly to the disease control.

Research frontiers

The research was achieved to provide data on the occurrence of *N. caninum* in frozen bull semen, used for artificial insemination in Iran, using molecular tools.

Related reports

Information's from the scientific literature regarding the presence of *N. caninum* in frozen bull semen used for artificial insemination are scanty. Therefore, additional studies using new generation, rapid, specific and sensitive diagnostic tools for *Neospora* infection are still required.

Innovations and breakthroughs

The authors demonstrated that the frozen bull semen can constitute an important way for the dissemination of neosporosis in the cattle herds. Moreover, the results highlight the importance of the analysis of bull's semen samples for *N. caninum* before artificial insemination, in order to avoid the spread of the bovine neosporosis.

Applications

Results of the current survey showed that the PCR technique can be considered a valuable diagnostic tool for the evidence of *N. caninum* in frozen bull's semen samples, highlighting, at the same time, the necessity of implementing of eradication and control programs in Iranian cattle herds. .

Peer review

This is a well conducted study in which the authors evaluate the occurrence of *N. caninum* in frozen semen of Iranian bulls based on molecular tools. The results are noteworthy as important contribution to the knowledge of molecular epidemiology of bovine neosporosis.

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