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Detection of *Neospora caninum* in ovine abortion in Iran

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Abstract The present study was designed to assess the importance of ovine neosporosis in abortion of Iraninan sheep. Seventy aborted foetuses and dams from ovine dairy farms in northwest of Iran were analyzed to investigate the role of *Neospora caninum* (*N. caninum*) in ovine abortion. Diagnosis of the infection was determined by serology and polymerase chain reaction (PCR). A total of 70 aborted dairy ovine were blood sampled and used to evaluate serological status for *N. caninum* infection by enzyme-linked immunosorbent assay (ELISA) and extracted DNA from the same aborted foetuses were subjected to PCR. Data were compared using Kruscal-Wallis test. From A total of the 70 sheeps, four (5.7 %) of the dams were seropositive. DNA from aborted foetuses was extracted primarily from placenta and CNS tissues. Extracted DNA from foetuses were analyzed using PCR with primers $Np21^{+}$ and $Np6^{+}$. Out of the 70 ovine fetuses 8.5 % were considered to be infected by PCR. This study confirms the importance of *N. caninum* as an important cause of ovine abortion in northwest of Iran.

Keywords *Neospora caninum* · Abortion · Ovine

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Introduction

Neosporosis, caused by the protozoan *N. caninum*, is an important cause of bovine abortion (Anderson et al. 1991), and neurological alterations in dogs (Barber and Trees 1998). It can also cause abortion or neonatal mortality in other animal species, including sheep, goats, horse, and deer (Dubey 2003). *N. caninum* was first described as a natural infection in sheep in a congenitally infected lamb in England (Dubey et al. 1990). Subsequently, naturally occurring ovine neosporosis has been reported in Japan, South America and Switzerland (Koyama et al. 2001; Hassig et al. 2003; Moore et al. 2005). Although *N. caninum* was shown to cause mortality in new born lambs and congenital infection in naturally exposed sheep, it is not regarded as a significant cause of abortion in sheep (Dubey et al. 1990; Dubey and Lindsay 1990; Buxton et al. 1998; Helmick et al. 2002).

Natural infection in sheep and goat is uncommon and only a few cases of abortion or congenital disease have been reported (Barr et al. 1992; Dubey et al. 1992; Dubey and Lindsay 1996; Lindsay et al. 1995; Corbellini et al. 2001; Dubey 2003). The role of *N. caninum* as a cause of natural abortion in small ruminants needs to be investigated, since their experimental inoculation with *N. caninum* during pregnancy causes a condition very similar to that observed in cattle (Buxton et al. 2002). In Iran, several serological surveys have allowed the detection of antibodies to *N. caninum* in cattle herds from different areas of the country (Sadrebazzaz et al. 2004), but no data are available about the infection in sheep.

Diagnosis of *N. caninum* is difficult, due to the vague nature of early clinical signs and low numbers of parasites in infected tissues (Ellis et al. 1999). Furthermore clinical signs and pathological lesions in sheep are similar to those induced in them by *Toxoplasma gondii*. Therefore, specific

serological tests such as the ELISA for the dam and specific direct tests such as PCR for the fetus are prerequisites to reliably confirm the diagnosis of the infectious agent causing abortion, which may have already been indicated by histopathology. Thus the objective of the present study were to determine the presence of antibodies to *N. caninum* in serum samples from dams by ELISA and presence of the parasite genome in different foetal tissues from the same sheep by PCR.

Materials and methods

Animals

During breeding season 2009–2010, 15 flocks, with reported abortions in late gestation period, were investigated. Tissue and blood samples were collected from 70 ewes with abortion. The animals belonged to two breeds, including Ghezel and Makuii.

Serum samples

70 Blood samples were collected from 70 aborting ewes when abortion was identified. Blood samples were taken using disposable needles (venoject). All samples were immediately transported to the diagnostic laboratory. Serum was removed after centrifugation at 1,000 × *g* for 10 min. All sera were divided equally into two micro tubes and stored at −20 °C until the test.

Serology

All sera were tested for antibody activity to *N. caninum* by using the commercially available ELISA kit (IDEXX Laboratories) coated with *N. caninum* antigen. Instead of the HRP conjucated anti-bovine IgG recommended in the commerical kit, HRP conjugated rabbit anti-bovine IgG was used to assay the sheep sera. A checker board titration was applied to obtain the optimal concentration of the HRP conjugated anti–ruminant IgG. Results are calculated as a corrected sample to positive (S/P) ratio ($OD_{Sample} - OD_{Neg}$)/($OD_{Pos} - OD_{Neg}$)−100) and expressed in percent. Positive control sera were perpared from three sheep infected experimentally by *N. caninum* infected sheeps. All serum samples were analyzed twice.

DNA extraction of tissues

Diagnostic specimens were also collected from brain, liver, gastric content and placenta of the aborted foetuses for genomic DNA isolation. Approximately, 5–10 g of each sample were taken selectively from different anatomic regions of the mentioned tissues were homogenized and powdered separately under liquid nitrogen, transferred to microtubes, and then stored at −20 °C until further use. Total DNA was extracted from approximately 200 mg powdered tissues using a *Aquaprep DNA Tissue kit* (Bioneer, S. Korea) according to manufacture instructions. DNA concentration was measured in 260 and 280 nm (Biophotometer plus, eppendorf, Germany). Electrophoresis of each DNA sample on 0.5 % agarose gel in 1X TBE buffer was undertaken to check the integrity of the DNA. A 60 µl aliquot of total DNA was produced from each sample and stored at −20 °C until required for PCR analysis.

PCR primers

Neospora caninum specific primer pair; $Np21^+$ Forward (3′ AAC ACT ACG ACT TGC AAT CC 5′) and $Np6^+$ Reverse (3′ GGT TCC TTA GGA CTC CGT CG 5′) that anneals to repetetive region of the parasite genome were used for molecular diagnosis of the parasite (Muller et al. 1996). Furthermore, two primers were included; BA1 Forward (5′ GAG AAG CTG TGC TAC GTC GC 3′) and BA2 Reverse (5′ CCA GAC AGC ACT GTG TTG GC 3′) that target a part of the Beta actin gene were considered as an internal control.

Polymerase chain reaction (PCR)

All PCR reactions were performed in a 20 µl volume with 1 µl of sample containing 100 ng DNA, 0.4 µl of 0.2 mM dNTPs mix, 1.4 µl of 3.5 Mm $MgSo_4$, 2 µl of PCR buffer, 0.12 µl of 0.6 U of platinm Taq polymerase, 0.4 µl of 0.2 Mm of each primer. PCR was performed in a thermocycler (primus 96, MWGA, GmBH, Germany) with the following conditions: Initial denaturation at 95 °C for 5 min, followed by 40 cycle at 94 °C for 1 min, 63 °C for 1 min and 72 °C for 2 min, with a final extension of 72 °C for 10 min. A non template control (water blank) and a positive control DNA from *N. caninum* (NC-5 strain) included in each PCR run. Amplification products were analyzed by electrophoresis through a 2 % agarose gel and stained with ethidium bromide.

Statistical analysis

Datas were compared using Kruscal-Wallis test with the SPSS version 19 program. The differences were not significant among the two groups at the confidence interval of 95 %.

Results

The average gestational time of all registered abortions varied considerably (range 1–5 months). *N. caninum* was

detected by means of PCR in six aborted ovine foetuses from different ewes. A placentitis was observed in the same cases (Data not shown). Of the 70 aborted foetuses and their dam sampled, four (5.7 %) of the dams were seropositive for antibodies to *N. caninum.*

DNA was successfully extracted from all samples and Beta actin gene was shown in all tissue samples (brain, liver, lung, gastric content and placenta). Aborted fetuses from the same seropositive and two seronegative ewes had *N. caninum* DNA at least in one type of their tissues (Table 1).The PCR demonstrated that four of six aborted foetal brain samples were infected by *N. caninum*. The primers $Np21^+$ and $Np6^+$ were used to amplify a 328 bp fragment of the repetitive region of the parasite genome (Fig 1).

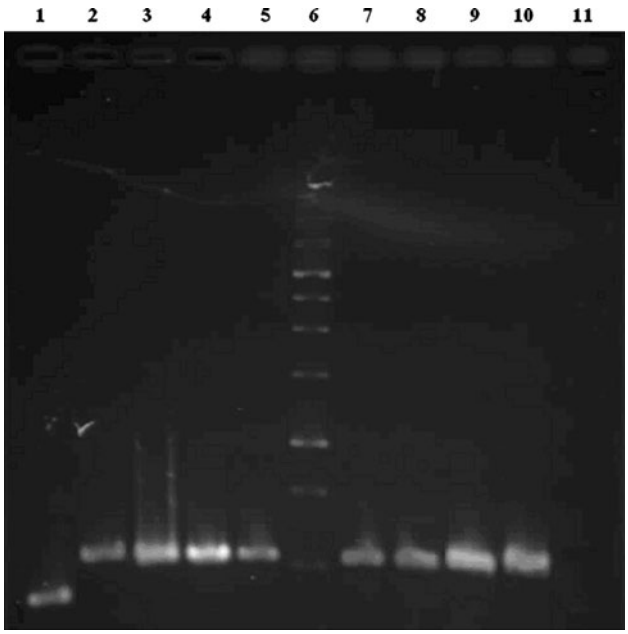

Fig. 1 Gel electrophoresis image showing PCR products (from *left to right*): *Lane 1* beta actin gene; *Lane 2–5* positive placenta samples; *Lane 6* molecular size marker; *Lane 7–9* positive brain samples; *Lane 10* positive control; *Lane 11* negative control

Discussion

Presence of *N. caninum* antibodies in aborted and healthy dairy cattle has been detected (Sadrebazzaz et al. 2004; Razmi et al. 2006), but there has been no information about ovine abortion associated with *N. caninum* by complex techniques in Iran. In our study, four of the 70 dams were diagnosed as seropositive by *N. caninum* using ELISA. Similar results were found in Brazil by Figliuolo et al. (2004) who, showed sero–prevalence to be less than 10 %. In contrast, in Brazil Soares et al. (2009) and Vogel et al. (2006) found a sero–prevalence of 1.81 % and 3.2 % *N. caninum,* respectively. In Italy, Gaffari et al. (2006) in a large study of 1,010 sheeps without abortion, reported a sero-prevalence of about 2 %. On the other hand, in Great Britain, in aborting sheep only sporadic 0.45 % sero–prevalence of *N. caninum* was found (Helmick et al. 2002). A relatively high *N. caninum* sero-prevalence (10.3 %) was observed in Switzerland (Hassig et al. 2003) and in the Czech Republic, (12 %) (Bártová et al. 2009). The variability of these results may be due to the common practice of running sheep with beef cattle which is common in Iran, the use of farm working dogs in these flocks, age, differences in management conditions, environment and the serological techniques employed. In our study, no significant correlation was demonstrated in infection rates in different ages in herds. These results are in agreement with Sadrebazzaz et al.(2004) who reported no significant difference in seropositivity for different age groups of cattle. It seems the relationship between age and sero-prevelance in ovine neosporosis is speculative. Some reports suggest that *N. caninum* sero-prevalence in pure breed can be higher than crossbreed, suggesting that imported animals have a greater neosporosis risk than local breeds (Guimarães et al. 2004; Akca et al. 2005). But in our study, we did not found any significant differences among sheep breeds included in the present study.

In this study, *N. caninum* DNA was detected in only 8.5 % of ovine foetuses. Our study is higher than that of the 2 % of the ovine from Italy (Masala et al. 2007). Based on the results of PCR using extracted DNA from placenta, it

Table 1 The results of PCR assay in different fetal tissues of dams with antibodies to *N. caninum*

No.	Breed	Ewe #	Ewe age	Elisa status	PCR ($Np21^+$ and $Np6^+$) result in: fetal				
					Brain	Liver	Lung	Gastric content	Placenta
1	Makui	190	2.5	+	+	–	–	–	+
2	Makui	4261	3	+	+	–	–	–	+
3	Makui	0052	2	+	–	–	+	–	+
4	Ghezel	27	2	+	–	–	–	–	+
5	Ghezel	1613	2	–	+	–	–	–	+
6	Ghezel	7830	2.5	–	+	–	+	–	+

might be concluded that PCR amplification of target gene to detect *N. caninum* DNA in placenta samples was considered a valuable tool for the diagnosis of abortion caused by *N. caninum*. In the present study,100 % of *N. caninum* of fetal placenta were diagnosed by PCR. This value was different as reported in other studies. Masala et al. (2007) reported that 9 ovine placentae (11.8 %) yielded positive PCR agents. In addition, there are two hypotheses to explain the complete absence or the presence of only small numbers of organisms in the placenta of *N. caninum* seropositive ovine: (1) The infection occurs in mid gestation and at the time of parturition *N. caninum* organisms are no longer present in the placentas, (2) *N. caninum* has a tropism for the nervous tissues and is not prevalent in placenta (Bergeron et al. 2001).

Concerning fetal diagnosis, detection of compatible lesions by histology and parasites by PCR in brain (as well as heart and liver) are the best choices for fetal diagnosis.

Histopathological studies have shown that tissue cyct occurs mostly in CNC. In this study, the second highest frequency was observed in PCR using extracted DNA from fetal brain. Our study is consistent with previous studies showing tissue parasites detected most frequently in brain by PCR (Dubey and Schares 2006).

The parasite was not shown in any of the DNA samples from gastric content. Since the parasite spread through trans–placental transmission to the foetus, and it needs to become enclosed in parasitophorous vacuoles, where they divide rapidly by endodyogeny, the entrance of the tachyzoites to gastric content might be uncommon. Thus, this result emphasizes the observation that many sections from a number of tissues may need to be tested in order to detect some parasitic infection (Jenkins et al. 1997). Any study of this type suffers from the possibility that parasites may happen to be absent in the portion of tissue tested and will not therefore be detected. Our results show that homogenization of the tissue before sampling may overcome this limitation when using a PCR as a tool for diagnosis of the parasite. These results emphasized the necessity of combining these complementary techniques to enhance detection of *N. caninum* infections in ewes and aborted foetuses.

Two of the PCR positive aborting fetuses were negative by ELISA. These negative conclusions, could be due to the limited immuno–competence in sheeps or the short interval between infection and foetal death, or can be a phenomenon of antibody falling titer just after abortion.

Since the titers of antibody may be affected by abortion, PCR had a crucial role in diagnosis of the parasite in foetal tissues and placenta of aborted fetus is the best site for detection of the parasite by PCR. In the present study, a good agreement between ELISA and PCR was observed, and in our study, PCR was used as the sensitive technique.

In conclusion, our findings confirmed the serology and molecular results of other studies about *N. caninum* infection and it seems to support the hypothesis that Neospora infection is associated with ovine abortion in Iran. Based on these results, it may be prudent to prevent dogs from ingesting placentas and aborted foetuses from seropositive ovine.

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References

Akca A, Gokce HI, Guy CS, Mcgarry JW, Wiliams DJL (2005) Prevalence of antibodies to *Neospora caninum* in local and imported cattle breeds in the Kars province of Turkey. Res Vet Sci 78:123–126

Anderson ML, Blanchard PC, Barr BC, Dubey JP, Hoffmann RL, Conrad PA (1991) *Neospora*–like protozoan infection as a major cause of abortion in California dairy cattle. J Am Vet Med Assoc 198:241–244

Bártová E, Sedlák K, Literá kI (2009) *Toxoplasma gondii* and *Neospora caninum* antibodies in sheep in the Czech Republic. Vet Parasitol 161:131–132

Barber JS, Trees AJ (1998) Naturally occurring vertical transmission of *Neospora caninum* in dogs. Int J Parasitol 28:57–64

Barr BC, Anderson ML, Woods LW, Dubey JP, Conrad PA (1992) *Neospora*–like protozoal infections associated with abortion in goats. J Vet Diagn Invest 4:365–367

Bergeron N, Girard C, Pare′ Julie, Fecteau Gilles, Robinson J, Baillargeon P (2001) Rare detection of *Neospora caninum* in placentas from seropositive dams giving birth to full-term calves. J Vet Diagn Invest 13:173–175

Buxton D, Maley SW, Wright S, Thomson KM, Rae AG, Innes EA (1998) The pathogenesis of experimental neosporosis in pregnant sheep. J Comp Pathol 118:267–279

Buxton D, McAllister M, Dubey JP (2002) The comparative pathogenesis of neosporosis. Trends Parasitol 18:546–552

Corbellini LG, Colodel EM, Driemeier D (2001) *Granulomatous encephalitis* in a neurologically impaired goat kid associated with degeneration of *Neospora caninum* tissue cysts. J Vet Diagn Invest 13:416–419

Dubey JP (2003) Neosporosis in cattle. J Parasitol 89: Supplement, (in press)

Dubey JP, Lindsay DS (1990) *Neospora caninum* induced abortion in sheep. J Vet Diagn Invest 2:230–233

Dubey JP, Lindsay DS (1996) A review of *Neospora caninum* and neosporosis. Vet Parasitol 67:1–59

Dubey JP, Schares G (2006) Diagnosis of bovine neosporesis. Vet Parasitol 140(1–2):1–34

Dubey JP, Hartley WJ, Lindsay DS, Topper MJ (1990) Fatal congenital *Neospora caninum* infection in a lamb. J Parasitol 76:127–130

Dubey JP, Acland HM, Hamir AN (1992) *Neospora caninum* (Apicomplexa) in a stillborn goat. J Parasitol 78:532–534

Ellis JT, McMillan D, Ryce C, Payne S, Atkinson R, Harper PAW (1999) Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA. Int J Parasitol 29:1589–1596

Figliuolo LPC, Kasai N, Ragozo AMA, de Paula VSO, Dias RA, Souza SLP, Gennari SM (2004) Prevalence of anti-*Toxoplasma*

gondii and anti-*Neospora caninum* antibodies in ovine from Sao Paulo State. Brazil Vet Parasitol 123(3–4):161–166

Gaffari A, Giacometti M, Tranquillo VM, Magnito S, Cordioli P, Lanfranchi P (2006) Serosurvey of roe deer, chamois and domestic sheep in the Central Italian Alps. J Wild Dis 42: 685–690

GuimarãesJS JR, Souza SL, Bergamaschi DP, Gennari SM (2004) Prevalence of *Neospora caninum* antibodies and factors associated with their presence in dairy cattle of the north of Paraná state. Brazil Vet Parasitol 124(1–2):1–8

Hassig M, Sager H, Reitt K, Ziegler D, Strabel D, Gottstein B (2003) *Neospora caninum* in sheep: a herd case report. Vet Parasitol 117(3):213–220

Helmick B, Otter A, McGarry J, Buxton D (2002) Serologicalinvestigation of aborted sheep and pigs for infection by *Neospora caninum*. Res Vet Sci 73(2):187–189

Jenkins MC, Wouda W, Dubey JP (1997) Serological response over time to recombinant *Neospora caninum* antigens in cattle after a neosporosis-induced abortion. Clin Diagn Lab Immunol 4: 270–274

Koyama T, Kobayashi Y, Omata Y, Yamada M, Furuoka H, Maeda R, Matsui T, Saito A, Mikami T (2001) Isolation of *Neospora caninum* from the brain of a pregnant sheep. J Parasitol 87:1486–1488

Lindsay DS, Rippey NS, Powe TA, Sartin EA, Dubey JP, Blagburn BL (1995) Abortions, fetal death, and stillbirths in pregnant pygmy goats inoculated with tachyzoites of *Neospora caninum*. Am J Vet Res 56:1176–1180

Masala G, Porcu R, Daga C, Denti S, Canu G, Patta C, Tola S (2007) Detection of pathogens in ovine and caprine abortion samples from Sardinia, Italy, by PCR. J Vet Diagn Invest 19:96–98

Moore DP, Leunda MR, Zamorano PI, Odeo′n AC, Romera SA, Cano A, de Yaniz G, Venturini MC, Campero CM (2005) Immune response to *Neospora caninum* in naturally infected heifers vaccinated with inactivated antigen during the second trimester of gestation. Vet Parasitol 130:29–39

Muller N, Zimmermann V, Hentrich B, Gottstein B (1996) Diagnosis of *Neospora caninum* and Toxoplasma gondii infection by PCR and DNA hybridization immunoassay. J Clin Microbiol 34:2850–2852

Razmi GR, Mohammadi GR, Garrosi T, Farzaneh N, Fallah AH, Maleki M (2006) Seroepidemiology of *Neospora caninum* infection in dairy cattle herds in Mashhad area. Iran Vet Parasitol 135:187–189

Sadrebazzaz A, Haddadzadeh H, Esmailnia K, Habibi G, Vojgani M, Hashemifesharaki R (2004) Serological prevalence of *Neospora caninum* in healthy and aborted dairy cattle in Mashhad. Iran Vet Parasitol 124:201–204

Soares HS, Ahid SMM, Bezerra ACDS, Pena HFJ, Dias RA, Gennari SM (2009) Prevalence of anti-*Toxoplasma gondii* and anti-*Neospora caninum* antibodies in sheep from Mossoró, Rio Grande do Norte. Brazil. Vet Parasitol 160(3–4):211–214

Vogel FSF, Arenhart S, Bauermann FU (2006) Anticorpos anti-*Neospora caninum* em bovinos. ovinos e ubalinos no Estado do Rio Grandedo Sul Cienc Rural 36:1948–1951