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ORIGINAL PAPER

Evaluation of bovine abortion associated with *Neospora caninum* by different diagnostic techniques in Mashhad, Iran

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Abstract Twelve aborted foetuses (gestational ranged from 4–9 months) and dams from dairies cattle farms in (Mashhad) Iran were analysed to investigate the participation of *Neospora caninum* in abortion. Diagnosis of the infection was determined by histopathology, serology (indirect fluorescent antibody test [IFAT]) and semi-nested polymerase chain reaction (PCR). A total 33% of bovine foetuses were considered to be infected by PCR technique. Microscopic lesions consistent with *N. caninum* infection in foetal brains were observed in 25% of the samples, whereas 33% were positive using IFAT (with a cut-off titre of 1:20). This study confirms the importance of *N. caninum* as an important cause of abortion in Iran.

Introduction

Neospora caninum is a protozoan parasite that is a primary abortifacient in cattle, and antibodies to *N. caninum* has been identified from many regions of the world (Dubey and Schares 2006) including Iran (Sadrebazzaz et al. 2004; Razmi et al. 2006). Cattle with antibodies to *N. caninum* are more to abort than seronegative cows. Foetus may die in utero, resorbed, mummified, autolyzed or stillborn or born alive with or without clinical signs (Dubey 2003).

Bovine neosporosis can be diagnosed in cattle using indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay and in foetuses using methods such as histopathology (HP), immunohistochemistry (IHC) and polymerase chain reaction (PCR; Dubey and Schares 2006). In Iran, results based on seroprevalence of *N. caninum* in aborted dairy cattle showed that neosporosis should be regarded as a cause of economic loss in dairy cattle (Sadrebazzaz et al. 2004; Razmi et al. 2006). The objective of the study was to analyse through detection *N. caninum* as a cause of bovine abortion in dairy cows by IFAT, semi-nested PCR and HP in Mashhad, Iran.

Materials and methods

Study site

The study was carried out in Mashhad, center of Khorasan-Razavi province, Iran, particularly in dairy cattle.

Sampling

The study was made during 2003–2005. Twelve aborted foetuses (with 4 to 9 months of age) were obtained. Foetuses and dams' fluids and blood were sampled and centrifuged at 1,000×*g* for 10 min to eliminate cellular debris and stored at −20°C until tested. Brain tissue samples from aborted foetuses were obtained. Tissue samples were sized from 0.5 to 2 cm^3 and fixed in 10% buffered formalin (pH 7.2).

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Serology

A total of 12 foetal sera and fluids and 12 dams' sera were tested in Razi Serum and Vaccine Research Institute (Mashhad branch) for antibodies to *N. caninum* using the IFAT slides, in which whole *N. caninum* tachyzoites (VMRD, Pullman, 99163 WA, USA) and *Toxoplasma gondii* tachyzoites (Biogen®, Iran) was used as antigens and a fluorescent-labelled antisera to IgG1 and IgG2 as conjugate. A cut off ratio of 1:200 for dams' sera and 1:20 for foetal fluids and sera were defined as positive.

Histopathology

Brain tissue samples from aborted foetuses fixed in buffer formalin (10%) were processed by histological preparation including final embedding in paraffin wax. Two sections in each samples (5 μm) were cut for a total of 12 foetuses' brains. All sections were stained with haematoxylin and eosin. Diagnosis of *N. caninum* was made on the observation of the non-suppurative encephalomyelitis characterized by micro-focal non-suppurative infiltration, perivascular cuffing and glial proliferation.

Semi-nested PCR

Brain samples, a total of six foetuses seropositive for *N. caninum*, were analysed using a semi-nested PCR. The dams of these foetuses had been also tested seropositive.

DNA was extracted from 50 mg 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 *N. caninum*, *T. gondii*, *Babesia* spp. and *Theileria* spp. were also extracted (50 μl contained 0.1–1.0 μg of target DNA). Amplification of the internal transcribed spacer 1 (ITS1) region and 18S rRNA sequence (GenBank accession no. AY463245) of *N. caninum* were designed to amplify a 357-bp DNA fragment with oligonucleotide primers. 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′). The PCR mixture of 50 μl contained 0.1–1.0 μg of target DNA, 2 mM $MgCl_2$, 10× reaction buffer (50 mM KCl, 10 mM Tris–HCl [pH 8.3]), 10 pmol of each PCR primer, 200 μM of each deoxyribonucleotide triphosphate and 1 U of *Taq*DNA polymerase (Cinagen®, Iran). PCRs were performed in a thermocycler (Techgene, Techne, Germany) for 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 60 s. For semi-nested PCR, second-round primers Nc2 and Nc3 used 2 μl of amplicon solution from first-round Nc1–Nc2 PCR amplification as target DNA with the same PCR mixture subjected to 35 cycle of denaturation at 94°C for 30 s, annealing at 55°C for 45 s and extension at 72°C for 60 s. Amplicons were resolved on a 2% agarose gel stained with ethidium bromide and photographed under UV light. Positive (*N. caninum* DNA) and negative controls (no DNA) as well as irrelevant templates (50 μl contained 0.1–1.0 μg of target DNA of *T. gondii*, *Babesia* spp. and *Theileria* spp.) were included in each PCR run. Positive samples were tested at least three times for showing reproducibility of the specific PCR. Amplification products were analysed by electrophoresis through a 2% agarose gel for the specific *N. caninum* PCR. The PCR products were digested with endonuclease *Hinf*I (Fermentas, Germany) according to the manufacturer's instruction.

Sequences and primers were analysed using the "GeneRunner" programme, and homology studies were performed by "blastn" (blastn, nucleotide–nucleotide basic local alignment search tool) online programme.

Results

Serology

Of the 12 aborted foetuses and their dam sampled, six (50%) of the dams were seropositive, and five (41%) of their aborted foetuses were seropositive for antibodies to *N. caninum*. There were five cases with a positive serology in both the dams and its foetuses. All of the 12 dams and foetuses were seronegative for antibodies to *T. gondii*.

Histopathology

In six foetuses aborted by seropositive dams, the lesions consistent with *N. caninum* were observed in three foetuses. Lesions included neural oedematous, congestion, perivascular oedematous, mild status spongiosis, focal gliosis, perivascular cuffing and granulomatose foci surrounded by mononuclear inflammatory cells (lymphocytes and hystocytes). In two foetuses' brains, *N. caninum*-like tachyzoites were observed.

Semi-nested PCR

DNA was successfully extracted from frozen brains of aborted foetuses using proteinase K digestion and further phenol–chloroform purification method. Homology analysis of the selected primers using "blastn" demonstrated specificity of the designed primers. The primers Nc1 and Nc2 were used to amplify 357 bp of the above sequences.

Moreover, Nc2 and Nc3 amplified 259 bp of the same regions. The semi-nested PCR demonstrated that four of six aborted foetal brain samples were infected by *N. caninum*. ITS1 and 5.8S rRNA genomic sequence of *N. caninum* contain 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 132 bp in length, which confirms specificity of the PCR.

Discussion

In the 12 aborted bovine foetuses analysed by *Neospora*, diagnostic procedures include serology, HP and PCR techniques. Presence of *N. caninum* antibodies in aborted and healthy dairy cattle was detected (Sadrebazzaz et al. 2004; Razmi et al. 2006), but there was no information about bovine abortion associated with *N. caninum* by complex techniques in Iran. In our study, 6 of the 12 dams were diagnosed as seropositive by *N. caninum* using IFAT, and four (33%) foetuses of six infected dams were considered as positive by IFAT. In this study, 33% of *N. caninum* foetal infection cases were diagnosed by semi-nested PCR (Habibi et al. 2005); this value was the same as reported in Mexico, 34% diagnosed by HP (Morales et al. 2001). Nested PCR were developed and used in studies on pathogenesis of bovine neosporosis. This PCR was used to examine aborted foetuses in other countries, and it was used for first time in Iran. The specifically designed PCR analyses showed that nested PCR procedure (to increase sensitivity and specificity) was necessary to detect *N. caninum* infected foetuses. There is an up to 1,000 times increased efficiency at generating second-round amplicons (Jackson et al. 1992).

Our study are consistent with previous studies showing tissue parasites detected most frequently in brain by PCR (reviewed in Dubey and Schares 2006). In our study, 25% of aborted foetuses showed suggestive lesions of neosporosis by HP, which was lower than our PCR results (33%), in Morales et al. (2001) and in Sager et al. (2001) by HP and PCR. In HP, neural and perivascular oedematous, perivascular cuffing, focal gliosis, granulomatose foci surrounded by mononuclear inflammatory cells and *N. caninum*-like tachyzoites were considered as indicative of infection by *N. caninum*. (Dubey et al. 2006; Dubey and Schares 2006). PCR methods generally had a higher sensitivity than IHC methods, and also a high specificity (Van Maanen et al. 2004).

In the present study, good agreement between HP, IFAT and PCR was observed, and in our study, PCR was used as the sensitive technique. Because of among the ITS1 regions of *T. gondii*, *N. caninum*, *Babesia* spp. and *Theileria* spp., there are number of sequence differences that allow the establishment of specific PCRs. In our study, the aborted foetal brains have 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 foetuses. In conclusion, the presence of *N. caninum* infection in aborted foetuses in dairies studied was demonstrated using IFAT, HP and semi-nested PCR in Iran, which was similar to those recorded in previous studies in Mexico (Morales et al. 2001) and the higher percentages of foetal infection in UK (10–13%; Otter et al. 1995; Schock et al. 2000).

However, it is necessary to carry out further studies especially in definitive hosts to determine vertical or horizontal transmission modes in the study area to develop adequate epidemiology and preventive management.

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References

Dubey JP (2003) Neosporosis in cattle. J Parasitol (89Suppl):S42–S46

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

Dubey JP, Buxton D, Wouda W (2006) Pathogenesis of bovine neosporosis. J Comp Pathol 134:267–289

Habibi GR, Hashemifesharaki R, Sadrebazzaz A, Bozorgi S, Bordbar N (2005) Seminested PCR for diagnosis of *Neospora caninum* infection in cattle. Arch Razi Ins 59:55–64

Jackson DP, Hayden JD, Quirk P (1992) Extraction of nucleic acid from fresh and archival material. In: McPherson JJ, Quirk P, Taylor GR (eds) PCR: a practical approach. Oxford University Press, New York, NY, pp 29–50

Morales E, Trigo FJ, Ibarra F, Puente E, Santacruz M (2001) Neosporosis in Mexican dairy herds: lesions and immunohistochemical detection of *Neospora caninum* in fetuses. J Comp Pathol 125:58–63

Otter A, Jeffrey M, Griffiths IB, Dubey JP (1995) A survey of the incidence of *Neospora caninum* infection in aborted and stillborn bovine fetuses in England and Wales. Vet Rec 136: 602–606

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(2):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(3–4):201–204

Sager H, Fischer I, Furrer K, Strasser M, Waldvogel A, Boerlin P, Audigé L, Gottstein B (2001) A Swiss case-control study to assess *Neospora caninum*-associated bovine abortions by PCR, histopathology and serology. Vet Parasitol 102:1–15

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 1659

Schock A, Buxton D, Spence JA, Baird A, Low JC (2000) Histopathological survey of aborted bovine fetuses in Scotland with special reference to *Neospora caninum*. Vet Rec 147:687–688

Van Maanen C, Wouda W, Schares G, von Blumröder D, Conraths FJ, Norton R, Williams DJL, Esteban-Redondo I, Innes EA, Mattsson JG, Björkman C, Fernández-García A, Ortega-Mora, LM, Müller N, Sager H, Hemphill A (2004) An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. Vet Parasitol 126:351–364