Acta Veterinaria (Beograd), Vol. 52. No. 5-6, 393-400, 2002.

UDK 619:616.993.19:636.2

THE FIRST DETECTION OF NEOSPORA CANINUM DNA IN BRAINS OF CALVES IN POLAND

WISNIEWSKI M**, CABAJ W*, MOSKWA B*, WEDRYCHOWICZ H */**

* *W. Stefanski Institute of Parasitology PAS, Warszawa, Poland* ** *Department of Parasitology, Warsaw Agricultural University, Warszawa, Poland*

(Received 17, August 2002)

The recognition of prevalence of Neospora caninum infections in Poland is very poor. Vertical transmission of N. caninum has been shown to be a major route of infection in cattle. In the present study we examined the utility of PCR methods in detecting Neospora caninum DNA in brains of calves born from mothers with a high titer of anti-Neospora antibodies. Conventional and nested PCR reactions were performed on DNA extracted from brain tissue of Neospora-suspected calves using primers complementary to sequences of ITS1 in Neospora caninum rRNA genes. Conventional PCR reactions employing a set of primers complementary to sequences flanking ITS1 using 10 ng of template DNA gave a single product of approximately 350 bp. Following single tube nested PCR a product of approximately 150 bp appeared.

Key words: Neospora caninum; PCR; vertical transmission

INTRODUCTION

In the past decade, the protozoan parasite *Neospora caninum* has become increasingly recognized as an important cause of reproductive failure in dairy and beef cattle (Anderson *et al.* 2000). *N. caninum* is a well host-adapted, apicomplexan protozoan parasite which principally infects dogs and cattle world-wide, causing neuromuscular disease and abortion, respectively (Dubey and Lindsay 1996, Hemphill 1999). Two methods for the transmission of the infection in cattle have been proposed: horizontal transmission which utilizes a two-host life cycle whereby the cow is infected from ingestion of coccidial oocyst stages shed by the definitive host or vertical transplacental transmission. Experimental infections have confirmed that the dog is the definitive host for the parasite. There is epidemiological evidence that the dog has a role in the prevalence of the infection but, as yet, there is no confirmation that the dog is the source for natural infections in cattle (Anderson *et al.* 2000). Neosporosis is now recognised as one of the most frequently diagnosed causes of bovine abortion world-wide, including Europe (Dubey and Lindsay 1996; Hemphil *et al.* 2000). *Neospora* abortion typically occurs in mid gestation with a mean age of 5.5 months (range 3.5-8 months). The most characteristic lesion in the foetus is focal encephalitis, characterized by necrosis and inflammation. Although most aborted foetuses are autolyzed, *Neospora* organisms and/or the characteristic lesions may be found in the brain

(Anderson *et. al.* 2000). Vertical transmission occurs because foetal infection frequently does not result in abortion but the foetus survives to be a persistently infected animal. Moreover, vertical transmission may occur over several generations and is thought to be a major factor contributing to the persistence of *Neospora* in the herd (Anderson et al. 1997, 2000, Hietala and Thurmond 1999, Reichel and Ellis 2002). At birth, congenitally infected calves may have neurologic signs, be underweight, unable to rise, or have no clinical signs. Congenitally infected calves have a higher rate of abortion, particularly during their first pregnancy, and a high rate of vertical transmission to their offspring (Anderson *et al.* 2001)

In Poland, the recognition of prevalence of *N. caninum* infections is still very poor. Cabaj *et al.* (2000, 2001) reported the occurrence of anti-*Neospora caninum* antibodies in sera of dairy cows from farms in central Poland . Out of the total 227 sera samples tested in these studies 47(20.7%) were found to have antibodies to *N. caninum.*

The diagnosis of neosporosis abortion or congenital neosporosis is difficult. Specific diagnosis can be accomplished by examination of foetal fluids for *N. caninum* antibodies, detection of the parasite in foetal tissues by immuno- histochemistry, and detection of *N. caninum* DNA in foetal brains. Histological and immunohistochemical methods allow direct assessment of pathomorphological changes caused by infection, while serological tests, such as ELISA, are useful in determining whether an animal has been infected with *N. caninum* (Jenkins *et al.* 2002). However, there are reports that confirmation of *N. caninum* infection by immunohistochemistry has relatively low sensitivity and the molecular detection assays such as PCR are superior because of higher sensitivity and specificity in identifying *N. caninum* in animal tissues (Jenkins *et al.* 2002). In the present study we examined the utility of PCR methods in detecting *N. caninum* DNA in the brains of calves born with neurological disorders from mothers reported to be *Neospora*-positive (Cabaj *et al.* 2000, 2001).

MATERIAL AND METHODS

Animals

Blood samples were collected from cows that had aborted in the period 1998-2001. and the anti-*N. caninum* antibodies were estimated using a commercial ELISA test.

Seven dams with high antibody titers were monitored during pregnancy. The offspring consisted of 7 calves which were born with neurological disorders and died within a few months after birth. Their brains were removed and preserved immediately after death for DNA extraction. In addition, one sample of brain tissue taken from a six-month old foetus several hours after abortion was tested by PCR.

Estimation of anti-Neospora antibody levels by ELISA

The tests were performed according to the manufacturer's instructions (IDEXX Laboratories Inc., Westbrook, Maine, USA). Briefly, bovine sera diluted 1:100 were tested in duplicate using plates coated with *Neospora* antigen. The plates were incubated for 30 min at room temperature, washed 5 times in phosphate/Tween 20 solution and incubated with anti-bovine IgG:HRPO conjugate for

30 min. After being washed again, the plates were incubated with a substrate solution (TMB) in the dark. The reaction was stopped with 0.125 hydrofluoric acid and the optical density was measured at 630 nm using an automated plate reader (EL*800, Bio-tek, Instruments Inc). The results were expressed as S/P ratios : (Sample – Negative Control OD values)/ (Positive Control - Negative control OD values). Serum samples with S/P ratios of less than 0.5 were classified as negative for *Neospora* antibodies

DNA preparation

DNA was prepared from the brain tissue of 7 calves using the QIAamp tissue kit (Qiagen) as detailed below. The brain tissue was homogenised and digested according to the manufacturer's instructions with 180 µl ATL buffer for every 25 mg brain tissue. The lysate was incubated at 55°C overnight, after which 400 µl of lysate solution was removed and further purified by column chromatography according to the manufacturer's instructions.

PCR and nested PCR

The PCR reaction was based on primers specific to ITS1 of *N. caninum* rRNA encoding genes (Ellis *et al.* 1999).

The reaction conditions for the PCR included 5 ng or 10 ng of brain DNA, 1xPCR buffer, 1.75 mM $MgCl_2$; 0.2mM each dNTP, 50 µM of NS2 (5'CAT-GTGGATATTTTGCA) and NR1 (5'AAACTCCTGGAAGTTAAAG); 0.1 mM NF1 (5'GCGTGATATACTACTCCCTGT) and SR1 (AAATAACGGTGTGGGAAAA) and 0.8 units of Taq DNA polymerase in a total reaction volume of 50 µl.

Classic PCR tests were performed using external (NF1 and SR1) or internal primers (NS2 and NR1). The amounts of DNA template used were 10 ng and 5 ng respectively.

PCR was performed using a MJ Research thermocycler.

PCR products were run on 2% agarose gels which included molecular weight markers (Gibco BRL, 100 bp DNA Ladder).

RESULTS

Table 1 shows anti-*Neospora* antibody levels in dams and their offspring. In sera of six out of seven cows *Neospora* specific antibody levels were 2.5-6.5 times higher than the minimum S/P ratio for positive samples (0.5). In sera of calves the antibody levels were similar or higher than in their mothers.

PCR tests revealed the presence of *Neospora* DNA in the brains of all seropositive calves and the amount of amplicons obtained were similar in all samples from the seropositive calves. The sample from the brain of a six-months old foetus did not give a positive reaction when 10 ng of DNA was used as the template but appeared positive when a higher amount of sample DNA was used. DNA isolated from brain of a healthy calf did not produce any amplicon (results not shown). Conventional PCR reactions were initially performed on DNA extracted from brain tissue of *Neospora*- suspected calves using a set of primers complementary to sequences flanking ITS1 in *N. caninum* rRNA genes. As a result of the reaction using 10 ng of template DNA a product of approximately 350 bp was obtained (Figure 1). PCR performed with a set of primers complementary

Table 1. The level of antibodies specific to *N. caninum* antigens in sera of cows and their offspring

No. of cow-calf	*Neospora* antibody level in serum of cow (ELISA S/P ratio)	*Neospora* antibody level in serum of calf (ELISA S/P ratio)
1	2.31	1.8
2	2.58	3.67
3	3.29	2.43
4	1.76	2.76
5	3.23	4.19
6	1.31	1.33
7	0.75	3.47

to the internal sequences of ITS1 gave a 150 bp product in all samples. However, additional products also appeared in all samples (Figure 2). When DNA from the same samples was amplified using the single tube nested PCR method, a single product of approximately 150 bp appeared (Figure 3).

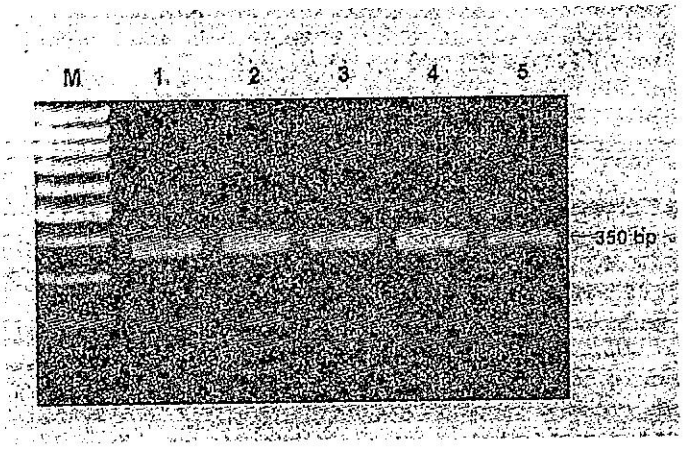

Figure 1. PCR performed using primers complementary to flanking sequences of N. caninum ITS1. M- Molecular-weight marker. Lanes 1& 5 -DNA from *N. caninum* infected Vero cells; Lane 2 - DNA from brain of aborted 6 month fetus; Lanes 3 & 4 –DNA from brains of calves born from dams seropositive to *Neospora*.

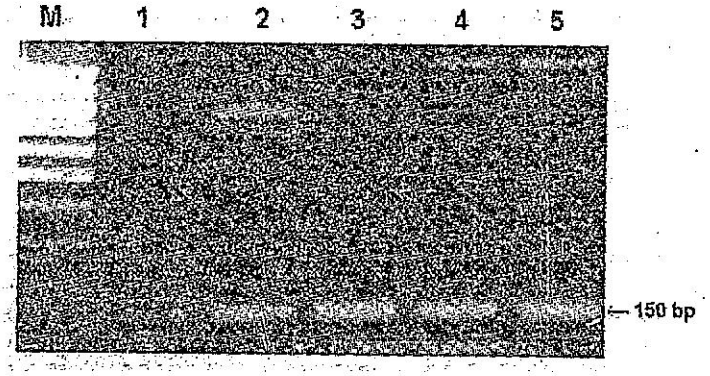

Figure 2. PCR performed with primers complementary to internal sequences of N. caninum ITS1 . M- Molecular-weight marker. Lanes 1& 5- DNA from *N. caninum* infected Vero cells; Lane 2 - DNA from brain of aborted 6 month fetus; Lanes 3 & 4 - DNA from brains of calves born from dams seropositive to *Neospora*.

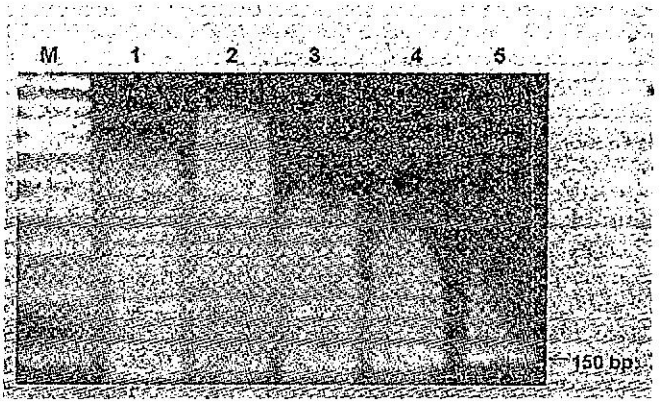

Figure 3. Single tube nested PCR. Further explanations as in Fig. 1.

DISCUSSION

The results of this study have confirmed the presence of *N. caninum* infection in Polish farms and strongly suggest the occurrence of vertical transmission of the parasite from seropositive cows to their offspring. Although the seropositive dams gave birth to full-term calves, the calves survived only a few months. Corbellini *et al.* (2002) reported a strong association between seropositivity to *N. caninum* and abortion, as seropositive cows were 3.3 times more likely to abort than seronegative cows .It is quite possible that other factors may be involved in the promotion of abortion by *N. caninum* infected dams. For instance, some recent epidemiological data suggest that concomitant infections with bovine pestivirus may increase the risk of abortion (Reichel and Ellis 2002).

The amplicons obtained using DNA templates from the brains of the seropositive calves were similar to those in previous studies. Ellis *et al.* (1999), using the same sets of primers and an identical reaction protocol, obtained a slightly smaller product of 146 bp. However, Schock et al. (2001) reported molecular polymorphism between different isolates of *N. caninum.* The spacer regions in the rDNA are good sources of DNA for diagnostics as they show high sequence heterogeneity even between closely related species like Toxoplasma gondii and *N. caninum* (Homan *et al.* 1997). For single tube nested PCR, in order to ensure amplification specificity, Ellis *et al.* (1999) selected primers from regions showing consistent sequence variation between *Neospora* and *Toxoplasma.* The selection process was performed using alignments of the ITS1 sequences from *N. caninum* and *T. gondii* (Ellis *et al.* 1999).

The results obtained in the present study demonstrate the usefulness of PCR, complemented by serology, for the specific diagnosis of bovine neosporosis. PCR tests may prove extremely suitable for epidemiological investigations. Interesting results were obtained by Bergeron *et al.* (2001) who investigated placentas of full-term calves born to seropositive cows, using immunohistochemistry and PCR assay for the presence of *N. caninum.* In sixteen placentas examined, *Neospora caninum* was not identified by immunohistochemistry, but two placentas from seropositive dams were positive for *N. caninum* by PCR. These results not only suggest that placentas of full-term calves from seropositive cows may be a potential source of *N. caninum* for farm dogs but also confirm that molecular detection assays such as PCR are superior because of their higher sensitivity and specificity in identifying *N. caninum* in animal tissues. The next step in development of molecular diagnosis tools for diagnosis and evaluation of pathogenicity of *N. caninum* infection may be a quantitative PCR. This kind of PCR allows estimation of the number of parasite cells per gram of host tissue. Such information is very important for evaluation of the severity and pathogenicity of the infection. A preliminary report has already appeared (Collantes-Fernandez et al. 2002). The authors developed a real-time PCR assay for the quantitative detection of *N. caninum* in infected host tissues. The assay uses the double-stranded DNA-binding dye SYBR Green I to continuously monitor product formation. Oligonucleotide primers were designed to amplify a 76-bp DNA fragment corresponding to the Nc5 sequence of *N. caninum.* A similar method was developed to quantify the 28S rRNA host gene in order to compare the parasite load of different samples and to correct for the presence of potential PCR-inhibiting compounds in the DNA samples. Assay specificity was confirmed by using DNA from the closely

related parasite *Toxoplasma gondii.* The applicability of the technique was successfully tested in aborted bovine fetuses classified into negative or positive *Neospora*-infected animals according to the observation of compatible lesions by histopathological study.

Since there are no proven control methods for the prevention or treatment of neosporosis, the control efforts must focus on reliable and sensitive diagnosis of congenital infections in order to reduce the number of infected animals in the herd and to minimize the opportunity for postnatal transmission from the environment. It should be stressed that for the sensitive and specific diagnosis of neosporosis, techniques based on amplification and detection of *N. caninum* DNA are most useful.

Address for correspondence:
Professor H. Wedrychowicz,
W. Stefanski Institute of Parasitology PAS,
Twarda 51/55, 00-818 Warszawa, Poland;
e-mail:halinwe@twarda.pan.pl

REFERENCES

1. *Anderson ML, Andrianarivo AG, Conrad PA,* 2000, Neosporosis in cattle *Anim Reproduct. Sci* 60, 417-31
2. *Anderson ML, Reynolds JP, Rowe JD, Sverlow KV, Packham AE, Barr BC, Conrad PA,* 1997, Evidence of vertical transmission of Neospora sp. infection in dairy cattle. *J. Am Vet Med Assoc,* 210, 1169-72
3. *Bergeron N, Girard C, Pare J, Fecteau G, Robinson J, Baillargeon P,* 2000, Rare detection of Neospora caninum in placentas from seropositive dams giving birth to full-term calves. *J Vet Diag Invest,* 13, 173-5
4. *Cabaj W, Choromanski L, Rodgers A, Moskwa B, Malczewski A,* 2000, Neospora caninum infections in aborting dairy cows in Poland. *Acta Parasitol,* 45, 113-4
5. *Cabaj W, Moskwa B, Choromanski L, Pastusiak K, Wojdan W,* 2001, Prevalence of Neospora caninum in dairy cattle in Poland .*Abstracts 2001WAAVP, Stresa, Italy,* 18.
6. *Collantes-Fernandez E, Zaballos A, Alvarez-Garcia G, Ortega-Mora LM.* 2002, Quantitative detection of Neospora caninum in bovine aborted fetuses and experimentally infected mice by real-time. *J Clin Microbiol,* 40, 1194-8
7. *Dubey JP, Lindsay DS,* 1996, A review of Neospora caninum and neosporosis. *Vet Parasitol,* 67, 1-59
8. *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 - 6
9. *Guy CS, Williams DJL, Kelly DF, McGarry JW, Guy F, Bjorkman C, Smith RF, Trees AJ,* 2001, Neospora caninum in persistently infected, pregnant cows: spontaneous transplacental infection is associated with an acute increase in maternal antibody. *Vet Rec,* 149, 443-53
10. *Hemphill, A,* 1999, The host-parasite relationship in neosporosis. *Adv Parasitol.,* 43; 47-104
11. *Hemphill A, Gottstein B, Conraths FJ,* 2000, A European perspective on *Neospora caninum. Int J Parasitol,* 30, 877-924
12. *Homan WL, Limper L, Verlaan M, Borst A, Vercamen M, Knapen FV,* 1997, Comparison of the internal transcribed spacers, ITS1, from *Toxoplasma gondii* isolates and *Neospora caninum. Parasit Res,* 83, 285-9
13. *Hietala SK, Thurmond MC,* 1999, Postnatal Neospora caninum transmission and transient serologic responses in two dairies. *Int J Parasit,* 29, 1669-76
14. *Jenkins M, Baszler T, Bjorkman C, Schares G, Williams D* 2002. Diagnosis and seroepidemiology of Neospora caninum-associated bovine abortion. *Int J Parasit,* 32 631-6

15. *Reichel MP, Ellis JT,* 2002, Control options for Neospora caninum infections in cattle - current state of knowledge. *NZ Vet J,* 50, 86-92
16. *Shock A, Innes EA, Yamane I, Latham SM, Wasling JM,* 2001, Genetic and biological diversity among isolates of *Neospora caninum. Parasitology* 123, 13-23

PRVI SLUČAJ DETEKCIJE DNK POREKLOM OD NEOSPORA CANINUM U MOZGU TELADI U POLJSKOJ

WISNIEWSKI M, CABAJ W, MOSKWA B i WEDRYCHOWICZ H

SADRŽAJ

U ovom radu su izneti rezultati ispitivanja prisustva DNK poreklom od *Neospora caninum* iz mozga teladi čije su majke imale visok titar anti - Neospora antitela. Primenjivane su konvencionalna i "nested" metoda PCR a kao prajmeri su korišćeni komplementarni sekvenci ITS1 u *Neospora canininum* rRNK genima. U konvencionalnoj PCR reakciji dobijen je sa 10 ng templata DNK jedan produkt od 350 bp dok je sa drugom metodom njegova veličina iznosila 150 bp.