# Detection of *Neospora caninum* in aborted bovine fetuses and dam blood samples by nested PCR and ELISA and seroprevalence in Beijing and Tianjin, China

L. YAO, N. YANG, Q. LIU*, M. WANG, W. ZHANG, W. F. QIAN, Y. F. HU *and* J. DING

*Key Laboratory of Zoonosis of Ministry of Agriculture and National animal Protozoa Laboratory, College of Veterinary Medicine, China Agricultural University, Beijing 100193, China*

(*Received 15 April 2009; revised 12 June 2009; accepted 15 June 2009; first published online 7 August 2009*)

SUMMARY

*Neospora caninum* infection is a significant cause of abortion in cattle. We investigated the tissue distribution of *N. caninum* in aborted bovine fetuses and dam blood samples by a nested PCR assay, and compared the nested PCR with ELISA in the diagnosis of *N. caninum* infection. In total, 26 aborted fetuses and 813 blood samples were collected from 8 dairy herds in Beijing ($n=212$) and Tianjin ($n=601$), China. Fifteen fetuses (57·7%) were tested *N. caninum*-positive by the nested PCR. *N. caninum* DNA was detected from the brain of 52%, kidneys of 22%, skeletal muscle of 18%, and heart of 4% of the aborted fetuses. The PCR-positive cases (55%, 11/20) were higher than seropositive cows (40%, 8/20) in a subset of 20 fetuses, but the PCR results of blood samples of the 20 cows were all negative. The seroprevalence of the 813 samples was 15·5% (43·4% of samples from Beijing, 5·7% of samples from Tianjin), compared to the PCR-positive blood samples of 0·9%. Our study showed that the nested PCR is a valuable diagnostic tool for the primary diagnosis of *N. caninum* in aborted fetuses, while ELISA is the preferred assay for testing blood samples collected from cows. The two assays are complementary in determining whether abortions are associated with *N. caninum* infection in cattle.

Key words: *Neospora caninum*, ELISA, nested PCR, abortion, bovine fetus.

## INTRODUCTION

*Neospora caninum* is an obligate intracellular apicomplexan protozoan parasite with a worldwide distribution. It was first reported in dogs (Dubey *et al*. 1988). The parasite can infect a broad range of warm-blooded animals (Anderson *et al*. 2000; Dubey, 2003; Dubey and Lindsay, 1996; Moore, 2005), but only dog (McAllister *et al*. 1998) and coyote (Gondim *et al*. 2004) are the definitive hosts. Cattle are the intermediate host, and *N. caninum* infection causes repeated abortions, stillbirths and vertical infection of calves (Anderson *et al*. 2000; Crawshaw and Brocklehurst, 2003; Huang *et al*. 2004).

Since the first isolation of *N. caninum*, a range of serological assays has been developed for detecting infections in dogs, cattle and a variety of other potential host species. The serological assays include the indirect fluorescent antibody test (IFAT), several enzyme-linked immunosorbent assays (ELISAs), *Neospora* agglutination test (NAT), and immunoblotting (IB). All the serological assays are based on tachyzoite antigens (Dubey and Schares, 2006) and measure antibody levels in serum samples collected from live animals, but none of the assays can detect *N. caninum* infection in aborted fetuses. In addition, the serological assays suffer the disadvantage of low specificity or sensitivity depending on the cut-off value for the assay or due to cross-reactivity with other parasites. PCR assays, which are highly specific and sensitive for the detection or identification of organisms, have been developed to detect *N. caninum* infection. Most published PCR methods are used to detect *N. caninum* DNA in the tissues of aborted fetuses or adult animals, although body fluids, such as amniotic fluid (Ho *et al*. 1997) and cerebrospinal fluid (Buxton *et al*. 2001; Peters *et al*. 2000; Schatzberg *et al*. 2003), blood (Okeoma *et al*. 2004; Ferre *et al*. 2005), milk (Moskwa, 2003) and semen (Caetano-da-Silva *et al*. 2004; Ferre *et al*. 2005; Ortega-Mora *et al*. 2003), can also be analysed for *N. caninum* by PCR.

Our laboratory has developed a recombinant tNcSRS2 protein-based ELISA, which was used in the investigation of the seroprevalence of *N. caninum* infection in cattle, water buffaloes, yaks and foxes in Beijing, Tianjin, Shanxi, Shandong, Heilongjiang, Xinjiang and Qinghai (Yu *et al*. 2007; Liu *et al*. 2007, 2008). *N. caninum* in aborted fetuses and dams were confirmed by serology, PCR, histology and immunohistochemistry (Zhang *et al*. 2007). In the present study, we used a nested PCR assay by amplifying the *N. caninum*-specific Nc5 gene to detect *N. caninum*

* Corresponding author: Tel: +86 10 62734496. Fax: +86 10 62732804. E-mail: qunliu@cau.edu.cn

*Parasitology* (2009), **136**, 1251–1256. 
doi:10.1017/S0031182009990813 Printed in the United Kingdom

infection in aborted fetuses of dairy cows in Beijing and Tianjin and compared the assay with ELISA.

## MATERIALS AND METHODS

### *Biological materials*

Twenty-six aborted fetuses of Holstein cows were collected from 8 dairy herds in Beijing and Tianjin, China between March and September 2008. Twenty-five of the fetuses were fresh, and 1 was mummified. Tissue samples of the brain, heart, lung, liver, spleen, kidney and skeletal muscle (gluteal muscle) of the fetuses, and placenta were stored frozen (−20 °C) before analysis. Blood samples were collected from the jugular vein of cows (including 20 dams at the time of abortion) from the same herds. A total of 813 blood samples was collected, and 601 samples were from Tianjin and 212 from Beijing. Aliquots of the clotted blood samples were used for DNA isolation and sera from the rest of the blood samples were stored frozen at −80 °C until analysis by ELISA as described by Liu *et al*. (2007) using the recombinant tNcSRS2 protein as the coating antigen.

### *DNA isolation*

After thawing, the fetal tissue (5 g) was homogenized, and incubated in a DNA extraction buffer (10 mM Tris–HCl, pH 7·4, 10 mM NaCl, 25 mM ethylenediaminetetraacetic acid, pH 8·0, 0·1% sodium dodecyl sulfate, pH 7·2 and 100 mg/ml proteinase K) overnight at 55 °C. DNA was extracted by the phenol and chloroform method, centrifuged and resuspended in distilled water at −20 °C. DNA of the blood samples was extracted by the same method.

### *PCR amplification*

The nested PCR primers were designed to amplify 2 fragments (about 328 bp and 224 bp) of the *N. caninum* Nc5 gene (GenBank Accession no. X84238). The external primer pairs were Np6+ (5′CTCGCAGTCAACCTACGTCTTCT3′) and Np21+ (5′CCCAGTGCGTCCAATCCTGTAAC 3′) (Muller *et al*. 1996), and the internal primers were Np9 (5′ GTTGCTCTGCTGACGTGTCGTTG 3′) and Np10 (5′ CTCAACACA GAACACTG-AACTCTCG 3′) (McInnes *et al*. 2006). A fragment of the bovine $\beta$-actin gene (Genbank AY141970) was amplified as the internal control using primers and amplification conditions described previously (Gilbert *et al*. 2007).

The primary PCR was carried out in a total volume of 25 $\mu$l consisting of 12·5 $\mu$l 2× *EasyTaq* PCR SuperMix (TransGene), 10 pM of each primer, 25 ng of DNA templates and double distilled water. An Nc-1 strain of *N. caninum* was used as the positive control, and distilled water as the negative control.

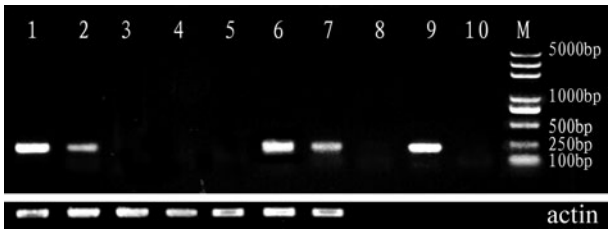


Fig. 1. Electrophoresis of the nested PCR products of the brain (1), heart (2), lung (3), liver (4), spleen (5), kidney (6) and gluteus (7) of an aborted fetus, *Toxoplasma gondii* (8), a Nc-1 strain of *N. caninum* (positive control) (9), and water (negative control) (10). M: DL2000 plus. Bovine $\beta$-actin was amplified as an internal control for the DNA extraction procedure.

*T. gondii* was used to test the specificity of the nested PCR. The PCR program started with an initial predenaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 20 s and extension at 72 °C for 30 s, and by a final extension of 72 °C for 10 min. The templates for the secondary PCR were 0·1 $\mu$l of the primary PCR products and cycling conditions were the same as for the primary PCR.

The PCR products were electrophoresed on a 1% agarose gel and the amplified DNA fragment was detected under UV light. Positive products were sequenced by Invitrogen (Beijing, China), and the sequences were analysed by BLAST against gene sequences deposited in the GenBank.

### *Data analysis*

The ELISA and nested PCR results were analysed by the *kappa* statistics using the SPSS software (Statistical Analysis System, Version 13.0). A $\kappa$-value between 0·4 and 0·6 indicates moderate agreement, a value between 0·6 and 0·8 indicates good agreement, and a value between 0·8 and 1·0 indicates almost perfect agreement. Chi-square analysis was used to compare *N. caninum* infection rates in the aborting and non-aborting cows.

## RESULTS

Nc5 fragments of the expected size of about 328 bp and 224 bp were amplified by the nested PCR by the external and internal primers, respectively, from the positive samples and positive control (Fig. 1). Sequence analysis of the PCR products showed that the amplified fragments were more than 99% homologous to the Nc5 gene of *N. caninum* (GenBank Accession no. X84238).

Fifteen (57·7%) out of 26 aborted fetuses were tested positive by the nested PCR. *N. caninum* specific DNA was detected from 13 brain samples (52%, 13/25), 1 heart sample (4%, 1/24), 5 kidney samples (21·7%, 5/23), 4 gluteus samples (18·1%, 4/22) and 1 placenta sample (33·3%, 1/3) (Table 1).

https://doi.org/10.1017/S0031182009990813 Published online by Cambridge University Press

Table 1. Nested PCR of aborted fetus and ELISA and PCR of corresponding dam blood samples

| Fetus no. | Age of fetus (day) | PCR positive tissue | ELISA/PCR of dam blood | Fetus no. | Age of fetus (day) | PCR positive tissue | ELISA/PCR of dam blood |
|---|---|---|---|---|---|---|---|
| 1 | 150 | B | +/− | 14 | 122 | — | −/− |
| 2 | 119 | B | +/− | 15 | 159 | B | −/− |
| 3 | 140 | — | −/− | 16 | 143 | — | −/− |
| 4 | 141 | — | −/− | 17 | 246 | B | NS |
| 5 | 133 | B | −/− | 18 | 151 | B,K,G | NS |
| 6 | 197 | B | −/− | 19 | 86 | B,P | NS |
| 7 | 115 | — | +/− | 20 | Unknown | — | NS |
| 8 | 153 | — | NS | 21 | 271 | — | −/− |
| 9 | 160 | B | +/− | 22 | 194 | K,G | −/− |
| 10 | 152 | B | +/− | 23 | 159 | — | −/− |
| 11 | 78 | B | +/− | 24 | 101 | — | −/− |
| 12 | 250 | K | NS | 25 | 187 | B,K,G | +/− |
| 13 | 191 | — | −/− | 26 | 156 | B, H, K | +/− |

NS, no sample; B, brain; K, kidney; G, gluteus; P, placenta; H, heart.

Among the 15 positive fetuses determined by the nested PCR, only 3 were tested positive by the primary amplification alone, suggesting that the secondary amplification increased the sensitivity of the PCR method.

No Nc5 fragment was amplified from blood samples of 20 aborting dams, although 8 samples tested positive by the ELISA for *N. caninum* antibodies and 11 of the 20 corresponding aborted fetuses were confirmed positive by the nested PCR.

The ELISA and nested PCR results were analysed by the *kappa* statistics (Table 2). The *kappa* statistical analysis of the ELISA of dam blood samples and nested PCR analysis of the corresponding fetal tissues showed moderate agreement between the two methods ($\kappa$ value 0·5098). The difference was not significant. One case was ELISA positive (dam blood) but PCR negative (fetal tissues). This abortion might be caused by an acute re-infection of *N. caninum* before the *N. caninum* tachyzoites were present in fetal tissues and detectable by the nested PCR. Unfortunately there was no information on the abortion history of the dam.

Analysis of 601 samples collected from Tianjin showed a seroprevalence of 5·7% (34/601) and PCR positive rate of 1·2% (7/601) (Table 3). In total, 39 cows were tested *N. caninum*-positive by PCR or ELISA. The abortion rate was 16·9% (95/562) for the *N. caninum*-negative cases, and 25·6% (10/39) for the positive cows. The abortion risk of *N. caninum* positive cows was 1·5 times than that of *N. caninum* negative animals. Two samples were both ELISA and PCR positive. One of the dams had been pregnant twice, but both fetuses were aborted at 138 and 135 days, respectively, while the other cow had given birth to 3 live calves with no abortion history.

The seroprevalence of *N. caninum* in cows from Beijing was 43·4% (92/212), and not one blood sample was PCR positive. Further analysis of samples from Beijing was not performed because of incomplete records for the animals.

Table 2. Nested PCR of aborted fetuses and ELISA of dam blood samples

| | ELISA of dam sera | | |
|---|---|---|---|
| Nested PCR of fetus | Positive* | Negative* | Total* |
| Positive | 7 (35) | 4 (20) | 11 (55) |
| Negative | 1 (5) | 8 (40) | 9 (45) |
| Total | 8 (40) | 12 (60) | 20 (100) |
| $\kappa$ value | 0·5098; 75% (15/20) | | |

* % in brackets.

The *kappa* value of the 813 blood samples was 0·014, and the nested PCR and ELISA results were significantly different in detecting *N. caninum* infection.

## DISCUSSION

*N. caninum* causes abortions in cattle and is an economically very significant parasite in the dairy industry (Liao *et al.* 2005). Definitive diagnosis of *N. caninum* infection in bovine abortion cases is made by histological examination of fetal tissues and immunochemical staining or detection of *N. caninum*-specific genes by PCR.

PCR has been used for the detection of *N. caninum* since 1996. Several genes including Nc5, 18S rDNA, 28S rDNA, ITS1 and 14-3-3 have been used as the target genes in detecting *N. caninum*. Of these genes, the Nc5 gene, first identified in 1996 (Kaufmann *et al.* 1996; Yamage *et al.* 1996) and not found in the genome of closely related parasites such as *T. gondii*, *Sarcocystis scruzi*, or *Hammondia hammondi*, is the most commonly used target gene for the detection of *N. caninum*. Therefore, the primer pairs

https://doi.org/10.1017/S0031182009990813 Published online by Cambridge University Press

Table 3. Nested PCR and ELISA results of blood samples from dairy herds in Beijing and Tianjin

| | | Tianjin | | | Beijing | | |
|---|---|---|---|---|---|---|---|
| | | ELISA | | | ELISA | | |
| | | Positive | Negative | Total | Positive | Negative | Total |
| Nested PCR | Positive | 2 | 5 | 7 | 0 | 0 | 0 |
| | Negative | 32 | 562 | 594 | 92 | 120 | 212 |
| | Total | 34 | 567 | 601 | 92 | 120 | 212 |
| $\kappa$ value | | 0·014; 84·13% (684/813) (all blood samples) | | | | | |

Np6+/Np21+ (Muller *et al*. 1996) and Np9/Np10 amplifying fragments of the Nc5 gene (McInnes *et al*. 2006) were chosen for the nested PCR in our study. PCR, especially nested PCR, is highly sensitive and specific for parasite identification if the primers are based on genes unique for the target species. The specificity of our nested PCR was demonstrated by the negative results tested with the closely related parasite, *T. gondii* and the negative controls, and the sensitivity was confirmed by the positive control samples. The DNA extraction procedure of *N. caninum* from fetal tissues was validated by the amplification of the bovine $\beta$-actin gene by PCR.

Of 26 aborted fetuses from dairy herds in Beijing, 15 fetuses were tested positive by the nested PCR, while only 3 were positive by PCR with the external primers only, suggesting that the nested PCR is more sensitive than the single-round PCR. In contrast to our findings, an interlaboratory comparison of *N. caninum* detection methods showed no difference in diagnostic sensitivity between single and nested PCR assays (van Maanen *et al*. 2004). In that study, the nested PCR was based on primers for the internal transcribed spacer-1 (ITS1) sequence, while the single-round PCR assays amplified the Nc5, SSU-rRNA or 5.8S-RNA gene. Since their study compared methods between laboratories, it was suggested that different DNA extraction methods used by different laboratories might have affected the diagnostic performance of the tests.

We detected a greater number of *N. caninum*-positive cases by the nested PCR analysis of fetal tissues than by ELISA of dam blood samples. This suggests that the nested PCR analysis of aborted fetuses is a more sensitive method for detecting *N. caninum* infection than analysis of antibodies in dam sera by ELISA. Since aborting cows with a *N. caninum*-infected fetus may not be seropositive, we recommend that seronegative abortion cases should be confirmed by analysis of fetal tissues by a more sensitive method such as the nested PCR. *N. caninum* DNA was not present in blood of the abortion cases, and only 7 out of 813 (0·86%) blood samples from aborting and non-aborting cases were found to contain *N. caninum* DNA by the nested PCR. However, negative PCR results of blood samples do not exclude *N. caninum* infection. The PCR analysis findings of bovine blood samples are consistent with the current understanding of the *N. caninum* life cycle that few *N. caninum* life stages are present in the blood of intermediate or definitive hosts.

Among all blood samples tested by both PCR and ELISA, 5 seronegative samples were found to contain *N. caninum* DNA determined by the nested PCR. There are several explanations for these findings. Firstly, the PCR-positive cases without specific antibodies to *N. caninum* may be in the early stage of infection, and blood samples were collected prior to the development of antibodies. Secondly, the sensitivity of ELISA depends on the cut-off value of the assay kit, and is compromised by the specificity required for the assay. Animals with low antibody levels may be tested negative by ELISA because of the low sensitivity of the assay. Thirdly, seronegative conversion may occur in *N. caninum*-infected cattle. It was also possible that some animals may be immunotolerant to *N. caninum* infection.

The distribution pattern of *N. caninum* in the host is not yet clear, but a number of studies showed that the central nervous system (CNS) is the most common tissue infected by *N. caninum*, followed by the heart, lung, and kidneys (Ho *et al*. 1997; Buxton *et al*. 1998; Gottstein *et al*. 1998; Baszler *et al*. 1999; Collantes-Fernandez *et al*. 2006). This distribution pattern was also observed in our study using the nested PCR. *N. caninum* DNA was detected in the brain of 52%, kidneys of 22%, skeletal muscle of 18%, and heart of 4% of 26 aborted fetuses. One out of 3 placentas collected was positive. None of the liver tissues were tested positive. Different distribution patterns of *N. caninum* in non-neural tissues have been reported. It is believed that the distribution of *N. caninum* varies between animals, and also depends on the time of infection during gestation and fetal age (Collantes-Fernandez *et al*. 2006). Understanding of the exact distribution pattern and the life cycle of *N. caninum* requires further research.

Our study showed that the nested PCR using primers for the Nc5 gene can be a valuable diagnostic

https://doi.org/10.1017/S0031182009990813 Published online by Cambridge University Press

tool for the primary diagnosis of *N. caninum* infection in aborted fetuses, and is more sensitive than the analysis of antibodies in the dam serum by ELISA in the determination of whether abortions are associated with *N. caninum* infection. PCR and ELISA play complementary roles in the detection of *N. caninum* infection in cattle.

ACKNOWLEDGEMENTS

We are grateful to Dr Jin Zhu (Therapeutic Goods Administration, Australia) for his assistance in the preparation of the manuscript.

FINANCIAL SUPPORT

This study was supported by the Natural Science Foundation of China (30871861 and 30571391), Natural Science Foundation of Beijing (6082014), the National Special Research Programs for Non-Profit Trades (Agriculture) (200803017) and the earmarked fund for Modern Agro-industry Technology Research System.

REFERENCES

**Anderson, M. L., Andrianarivo, A. G. and Conrad, P. A.** (2000). Neosporosis in cattle. *Animal Reproduction Science* **60–61**, 417–431.

**Baszler, T. V., Gay, L. J., Long, M. T. and Mathison, B. A.** (1999). Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. *Journal of Clinical Microbiology* **37**, 4059–4064.

**Buxton, D., Maley, S. W., Wright, S., Thomson, K. M., Rae, A. G. and Innes, E. A.** (1998). The pathogenesis of experimental neosporosis in pregnant sheep. *Journal of Comparative Pathology* **118**, 267–279.

**Buxton, D., Wright, S., Maley, S. W., Rae, A. G., Lunden, A. and Innes, E. A.** (2001). Immunity to experimental neosporosis in pregnant sheep. *Parasite Immunology* **23**, 85–91.

**Caetano-Da-Silva, A., Ferre, I., Aduriz, G., Alvarez-Garcia, G., Del-Pozo, I., Atxaerandio, R., Regidor-Cerrillo, J., Ugarte-Garagalza, C. and Ortega-Mora, L. M.** (2004). *Neospora caninum* infection in breeder bulls: seroprevalence and comparison of serological methods used for diagnosis. *Veterinary Parasitology* **124**, 19–24.

**Collantes-Fernandez, E., Rodriguez-Bertos, A., Arnaiz-Seco, I., Moreno, B., Aduriz, G. and Ortega-Mora, L. M.** (2006). Influence of the stage of pregnancy on *Neospora caninum* distribution, parasite loads and lesions in aborted bovine foetuses. *Theriogenology* **65**, 629–641.

**Crawshaw, W. M. and Brocklehurst, S.** (2003). Abortion epidemic in a dairy herd associated with horizontally transmitted *Neospora caninum* infection. *Veterinary Record* **152**, 201–206.

**Dubey, J. P.** (2003). Review of *Neospora caninum* and neosporosis in animals. *Korean Journal of Parasitology* **41**, 1–16.

**Dubey, J. P., Carpenter, J. L., Speer, C. A., Topper, M. J. and Uggla, A.** (1988). Newly recognized fatal protozoan disease of dogs. *Journal of the American Veterinary Medical Association* **192**, 1269–1285.

**Dubey, J. P. and Lindsay, D. S.** (1996). A review of *Neospora canimum* and neosporosis. *Veterinary Parasitology* **67**, 1–59.

**Dubey, J. P. and Schares, G.** (2006). Diagnosis of bovine neosporosis. *Veterinary Parasitology* **140**, 1–34.

**Ferre, I., Aduriz, G., Del-Pozo, I., Regidor-Cerrillo, J., Atxaerandio, R., Collantes-Fernandez, E., Hurtado, A., Ugarte-Garagalza, C. and Ortega-Mora, L. M.** (2005). Detection of *Neospora caninum* in the semen and blood of naturally infected bulls. *Theriogenology* **63**, 1504–1518.

**Gilbert, I., Bissonnette, N., Boissonneault, G., Vallee, M. and Robert, C.** (2007). A molecular analysis of the population of mRNA in bovine spermatozoa. *Reproduction* **133**, 1073–1086.

**Gondim, L. F., McAllister, M. M., Pitt, W. C. and Zemlicka, D. E.** (2004). Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **34**, 159–161.

**Gottstein, B., Hentrich, B., Wyss, R., Thur, B., Busato, A., Stark, K. D. and Muller, N.** (1998). Molecular and immunodiagnostic investigations on bovine neosporosis in Switzerland. *International Journal for Parasitology* **28**, 679–691.

**Ho, M. S., Barr, B. C., Rowe, J. D., Anderson, M. L., Sverlow, K. W., Packham, A., Marsh, A. E. and Conrad, P. A.** (1997). Detection of *Neospora* sp. from infected bovine tissues by PCR and probe hybridization. *Journal of Parasitology* **83**, 508–514.

**Huang, C. C., Ting, L. J., Shiau, J. R., Chen, M. C. and Ooi, H. K.** (2004). An abortion storm in cattle associated with neosporosis in Taiwan. *Journal of Veterinary Medical Science* **66**, 465–467.

**Kaufmann, H., Yamage, M., Roditi, I., Dobbelaere, D., Dubey, J. P., Holmdahl, O. J., Trees, A. and Gottstein, B.** (1996). Discrimination of *Neospora caninum* from *Toxoplasma gondii* and other apicomplexan parasites by hybridization and PCR. *Molecular and Cellular Probes* **10**, 289–297.

**Liao, M., Xuan, X., Huang, X., Shirafuji, H., Fukumoto, S., Hirata, H., Suzuki, H. and Fujisaki, K.** (2005). Identification and characterization of cross-reactive antigens from *Neospora caninum* and *Toxoplasma gondii*. *Parasitology* **130**, 481–488.

**Liu, J., Cai, J. Z., Zhang, W., Liu, Q., Chen, D., Han, J. P. and Liu, Q. R.** (2008). Seroepidemiology of *Neospora caninum* and *Toxoplasma gondii* infection in yaks (*Bos grunniens*) in Qinghai, China. *Veterinary Parasitology* **152**, 330–332.

**Liu, J., Yu, J., Wang, M., Liu, Q., Zhang, W., Deng, C. and Ding, J.** (2007). Serodiagnosis of *Neospora caninum* infection in cattle using a recombinant tNcSRS2 protein-based ELISA. *Veterinary Parasitology* **143**, 358–363.

**McAllister, M. M., Dubey, J. P., Lindsay, D. S., Jolley, W. R., Wills, R. A. and McGuire, A. M.** (1998). Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **28**, 1473–1478.

**McInnes, L. M., Ryan, U. M., O'handley, R., Sager, H., Forshaw, D. and Palmer, D. G.** (2006). Diagnostic significance of *Neospora caninum* DNA detected by PCR in cattle serum. *Veterinary Parasitology* **142**, 207–213.

**Moore, D. P.** (2005). Neosporosis in South America. *Veterinary Parasitology* **127**, 87–97.

**Moskwa, B., Cabaj, W., Pastusiak, K. and Bien, J.** (2003). The suitability of milk in detection of *Neospora caninum* infection in cows. *Acta Parasitologica* **48**, 138–141.

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

**Okeoma, C. M., Williamson, N. B., Pomroy, W. E., Stowell, K. M. and Gillespie, L.** (2004). The use of PCR to detect *Neospora caninum* DNA in the blood of naturally infected cows. *Veterinary Parasitology* **122**, 307–315.

**Ortega-Mora, L. M., Ferre, I., Del-Pozo, I., Caetano-Da-Silva, A., Collantes-Fernandez, E., Regidor-Cerrillo, J., Ugarte-Garagalza, C. and Aduriz, G.** (2003). Detection of *Neospora caninum* in semen of bulls. *Veterinary Parasitology* **117**, 301–308.

**Peters, M., Wagner, F. and Schares, G.** (2000). Canine neosporosis: clinical and pathological findings and first isolation of *Neospora caninum* in Germany. *Parasitology Research* **86**, 1–7.

**Schatzberg, S. J., Haley, N. J., Barr, S. C., Delahunta, A., Olby, N., Munana, K. and Sharp, N. J.** (2003). Use of a multiplex polymerase chain reaction assay in the antemortem diagnosis of toxoplasmosis and neosporosis in the central nervous system of cats and dogs. *American Journal of Veterinary Research* **64**, 1507–1513.

**Van Maanen, C., Wouda, W., Schares, G., Von Blumroder, D., Conraths, F. J., Norton, R., Williams, D. J., Esteban-Redondo, I., Innes, E. A., Mattsson, J. G., Bjorkman, C., Fernandez-Garcia, A., Ortega-Mora, L. M., Muller, N., Sager, H. and Hemphill, A.** (2004). An interlaboratory comparison of immunohistochemistry and PCR methods for detection of *Neospora caninum* in bovine foetal tissues. *Veterinary Parasitology* **126**, 351–364.

**Yamage, M., Flechtner, O. and Gottstein, B.** (1996). *Neospora caninum*: specific oligonucleotide primers for the detection of brain ''cyst'' DNA of experimentally infected nude mice by the polymerase chain reaction (PCR). *Journal of Parasitology* **82**, 272–279.

**Yu, J., Xia, Z., Liu, Q., Liu, J., Ding, J. and Zhang, W.** (2007). Seroepidemiology of *Neospora caninum* and *Toxoplasma gondii* in cattle and water buffaloes (*Bubalus bubalis*) in the People's Republic of China. *Veterinary Parasitology* **143**, 79–85.

**Zhang, W., Deng, C., Liu, Q., Liu, J., Wang, M., Tian, K. G., Yu, X. L. and Hu, D. M.** (2007). First identification of *Neospora caninum* infection in aborted bovine foetuses in China. *Veterinary Parasitology* **149**, 72–76.

https://doi.org/10.1017/S0031182009990813 Published online by Cambridge University Press