

NEOSPORA CANINUM AS CAUSATIVE-PATHOGEN OF ABORTION IN CATTLE

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ABSTRACT

Neosporosis, caused by infection with the intracellular protozoan *Neospora caninum*, is recognized as one of the major causes of infectious abortion in cattle. Since abortion is the only clinical sign associated with *N. caninum* infection, diagnosis is complicated, however it is crucial to understand the epidemiology of the disease, and to undertake measures to reduce losses to the cattle industry. In Israel the diagnosis of neosporosis is based on the detection of specific antibodies in aborted dams, or in the body fluids of aborted fetuses. The presence of antibodies in sera of aborted dams indicates exposure to *N. caninum*, but does not necessarily point to the causative agent of the abortion. In the present study sera from 7,951 aborted dams and body fluids from 446 fetuses were analyzed by the indirect fluorescent antibodies test (IFAT). *N. caninum* seropositivity was found in 51.4% of the dams, and in 18.2% of the fetuses. In addition, primers were designed for the detection of *N. caninum* DNA, by a specific nested PCR (nPCR) test, in brain tissues from aborted fetuses. The primers do not react with other closely related apicomplexa species. The performance of both molecular and serological assays was evaluated in 97 samples of fetuses. Comparable results were observed in 57 negative and 20 positive samples, while 20 remaining samples showed positivity in only one of the assays. In addition, 26 paired dam and aborted fetuses were tested. Of these, 15 samples obtained from dams or fetuses were found to be negative by both tests, 5 seropositive dams had positive fetuses in at least one of the assays, and 6 samples from seronegative dams were found to be positive in at least one of the fetus' assays. The results obtained show high prevalence of *N. caninum* in cattle in Israel and emphasize the necessity for the application of several methods for a reliable diagnosis of neosporosis as a cause of abortion.

INTRODUCTION

Neosporosis is caused by the intracellular protozoan parasite *Neospora caninum* and is known as one of the major causes of infectious abortion, stillbirth and reproductive failure in cattle worldwide, including Israel (1, 2, 3, 4, 5). Cattle infected with *N. caninum* exhibit no clinical signs other than abortion. In the absence of typical clinical signs of infection, diagnosis and control of neosporosis is complicated. The mechanisms that cause abortion have not yet been fully elucidated (6). In Israel diagnosis of neosporosis is based on the detection of specific *N. caninum* antibodies on the aborting cow or in the body fluid of the aborted fetus. Presence of serum antibodies in dams indicates exposure to *N. caninum*, (7) but is not necessarily associated with the reproductive failure; on other hand, seronegative results do not exclude the presence of infection. (8). Non-aborting seropositive dams have been reported worldwide, including in Israel (5, 9). It is documented that only some of infected dams abort, while most of their calves are born infected but healthy (10). It has been widely reported that the level of abortion rate in *N. caninum* sero-positive dams is 3.5 to 7 times higher than in their seronegative counterparts (11, 12).

Molecular methods allowing detection of specific *N. caninum*

DNA in tissues of aborted fetuses have been applied for complementary diagnosis of neosporosis. The detection of *N. caninum* DNA provided evidence of intra-uterine transmission of parasites from dams to offspring (13, 14, 15, 16, 17). In the present study, aborting dams and aborted fetuses were examined using either serology or molecular methods in order to evaluate whether the two techniques complement each other thus allowing for a more reliable diagnosis of neosporosis.

MATERIALS AND METHODS

Samples

Sera from 7,951 aborting cows and thoracic fluid from 446 aborted fetuses, from farms with history of abortion, were submitted for *Neospora caninum* diagnosis between 2006-2009. Brain tissues from 98 aborted fetuses were tested by nested PCR (nPCR), and 26 paired samples from dams and their respective fetuses were examined by both nPCR and indirect fluorescent antibody test (IFAT).

Indirect fluorescent antibody test (IFAT)

Serum from dams and thoracic liquids from aborted fetuses were examined for the presence of antibodies to *N. caninum*

by IFAT as previous described (18). The cut-off was 1:200 and 1:80 for dams and fetuses, respectively (19, 4).

DNA extraction and purification from brain tissues

Twenty milligrams of brain tissue collected from aborted fetuses were used for DNA purification. The ground tissues were digested with Proteinase K overnight at a temperature of 56° C, and then DNA purification was performed using Qiagen DNeasy Tissue Kit (QIAGEN, USA). Samples were frozen and stored at - 20°C until use. For positive control *N. caninum* from the Israeli isolate NcIs491 were grown in Vero cells, and parasites were obtained after destruction of monolayer cells and subsequent release of parasites from the cells (5). The media containing parasites and cell debris were collected, parasites were separated from cells by differential centrifugation at 70x g for 5 minutes, and the supernatant containing parasites was passed to a new tube, centrifuged again at 1000x g for 20 minutes. The pelleted parasites were washed in PBS at least 3 times by centrifuge at 1000g for 20 minutes. The specificity of the nPCR test to *N. caninum* was evaluated using DNA from *Toxoplasma gondii* (RH strain) parasites and *Besnoitia besnoiti*, taxonomically related tissue cyst-forming coccidian parasite.

Nested PCR (nPCR) amplification

Two sets of primers were designed for nPCR, based on DNA sequence of *N. caninum* (GenBank Accession number X84238). The nPCR reaction was performed with an external set of primers: forward primer 5': CTG CTG ACG TGT CGT TGT TG-3' represent nucleotide (nt) position 476 of referred sequence and reverse primer 5': CAT CTA CCA GGC CGC TCT TC-3' reverse sequence of nt position 1014, and an internal set of primers: forward primer 5': GCG TCA GGG TGA GGA CAG TG-3' nt position 631 of referred sequence and reverse primer 5': CTC TCC GTT CGC CAG CAG TG -3' reverse sequence of nt position 910.

The PCR reaction contained: 1µl of genomic DNA, each primer concentration 4µg and 2x Ready Mix (AB gene) containing: DNA Polymerase Thermoprime Plus (0.3125 units), 18.75 mM Tris-HCl (pH 8.8), 5mM (NH₄)₂SO₄ 0.375 mM MgCl₂, 0.0025% Tween 20 and 0.05mM dNTP mix in final volume of 50 µl. The cycling protocol, following preheating of 95°C for 3 minutes were: 30 cycles of denaturizing at 94° C for 30 seconds, annealing for 30 seconds at 56° C for the external primers and 57° C for the internal primers, extension at 72°C for 40 seconds and a final extension at 72°C for 5 minutes. The nPCR was performed using 1µl of the first reaction as a template. After amplification the reaction products were visualized on 1.5% agarose gel.

RESULTS

Out of the 7,951 sera from aborted cows tested for *N. caninum* antibodies, 4,104 (51.4%) were seropositive with titers ranging from 1:200 to >1:3200, and 3,847 samples (48.4%) were seronegative (Figure 1). Among seropositive cows the titers found were: up to 1:400 in 24.8% (1,018 samples), between 1:800

to 1:1600 in 22.7% (933 samples) and ≥1:3200 in 52% (2, 153 sample). The results obtained by the IFAT in 446 aborted fetuses were: 18.2% (81 samples) positive and 81.8% (365 samples) negative. As shown in Figure 1, the titer in seropositive fetuses ranged from 1:80 to 1:1280. Distribution of antibody titers in fetuses was 23.5% (19 samples) up to 1:160, 39.5 % (32 samples) from 1:320-1:640 and ≥1:1280 in 37% (30 samples).

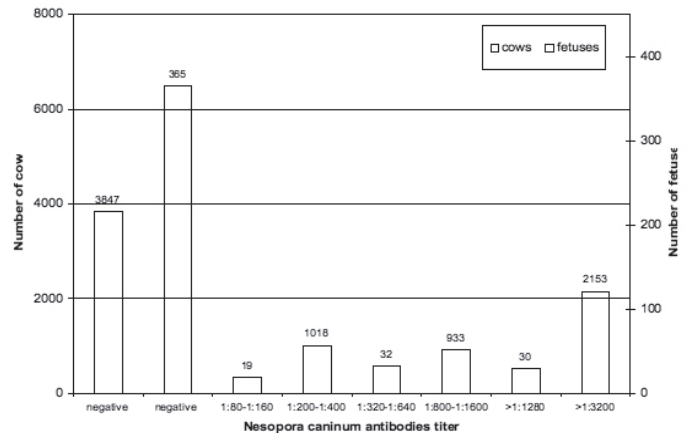


Figure 1. Distribution of *N. caninum* antibodies in 7951 dams and 446 aborted fetuses.

As shown in Figure 2, the primers designed for nPCR successfully detect *N. caninum* from aborted infected brain and no amplification was detected when DNA from *T. gondii* or *B. besnoiti* were applied. Ninety seven fetuses examined by IFAT and nPCR, showed comparable results. A complete agreement was detected in the assays in 20 positive and 57 negative samples. In 21 out of 98 samples tested, 11 samples were positive by the nPCR, but negative in IFAT, and 10 samples were found positive by IFAT, but negative by nPCR (Figure 3).

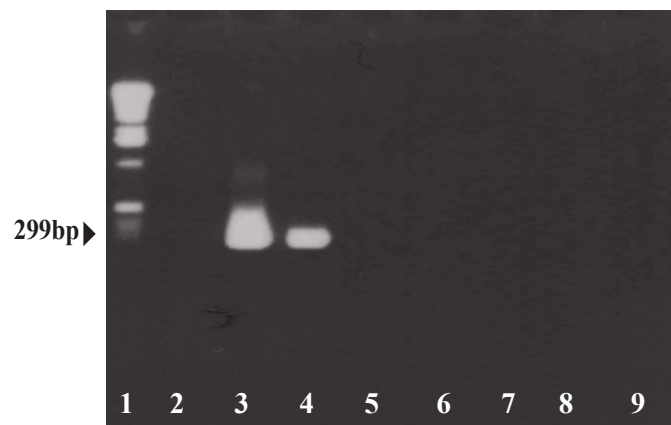


Figure 2. Specificity of the nPCR. Lanes: 1-Marker, 2 Negative control, 3- *N. caninum* – positive control, 4- *N. caninum* positive abortion brain, 5-7 Negative brains from aborted fetuses, 8- *Toxoplasma gondii* and 9- *Besnoitia besnoiti*.

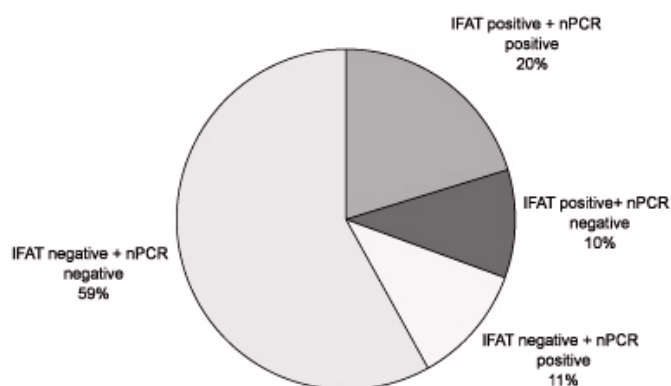


Figure 3. Results of nPCR from brain tissues and IFAT from thoracic fluids of 98 aborted fetuses.

Results of paired samples from 26 dams and their respective fetuses are summarized in Table 1. Fifteen fetuses (57.7%) from serological negatives dams were found negative in both nPCR and IFAT tests. All seropositives dams and 6 out of 21 seronegative dams produced fetuses positive in at least one of the fetal assays.

Table 1. Comparison between tests of the dams with respectively aborted fetuses

Aborted fetuses test IFAT / nPCR	IFAT of dams	
	Positive	Negative
positive / positive	1	3
positive / negative	3	0
negative / positive	1	3
negative / negative	0	15

DISCUSSION

In the present study high seroprevalence to *N. caninum* was observed in cattle from herds with history of abortions. From the dams tested 51.4% were seropositive to *N. caninum* indicating high exposure to the parasite. Similar seroprevalence levels has been reported in cattle worldwide (4). In Portugal, Korea and Argentina reports of seroprevalence of *N. caninum* antibodies among cattle suffering abortion were 46%, 48.7% and 64.5%, respectively (20, 21, 22). A highly efficient transmission rate (up to 95%) from dams to fetuses has been reported worldwide (8, 23). Although about 50% positivity was detected in cows, seroprevalence among fetuses was 18.2%. In Brazil infection in aborted fetuses reported was 39.1% (24), and in Switzerland about 21% (25). Among the seropositive cows examined, 52% exhibited *N. caninum* antibodies with titers $\geq 1:3200$, these results indicate that *N. caninum* might be the causative pathogen of abortion. According to Dubey *et al.* (26); McAllister *et al.* (27); Schares *et al.* (23,28) dams aborting due to

neosporosis have higher titers of those infected but non-aborting.

The primers designed for nPCR in this study specifically detected *N. caninum* 251 bp fragment from DNA of aborted fetuses, and no DNA from *T. gondii* and *B. besnoiti* was amplified. The detection of *N. caninum* DNA in aborted fetuses by PCR and nPCR was described previously (14, 25, 29, 30). Examination of the aborted fetuses resulted in 79% agreement between nPCR and IFAT. The discrepancy found in negative IFA results but positive by nPCR, or on the other hand, positively tested by IFA, but negative in nPCR, was described in other studies when using various methods (15, 31, 32). There were several explanations for the differences observed using various assays. According to Dubey *et al.* (31), negative serological results in infected fetuses were influenced by the age of fetus, level of exposure and time between exposure and abortion. It has also been found that lack of antibodies in the fetuses might give inconclusive results concerning absence of infection, since the fetus might be infected in the late gestation, leaving insufficient time for antibody production (4). Negative results in nPCR in seropositive fetuses might result as the distribution pattern of parasites in the host tissue which is as yet unclear. Furthermore the distribution of parasites might vary between animals, and the parasite load might be influenced by the stage of gestation and age at which the fetus was aborted (33). In addition, the brain tissues although considered to be the most suitable for the detection of *N. caninum* DNA by PCR (14, 32), it is possible that examination of additional organs would lead to increased reliability of the diagnosis. On the other hand, as shown by Yao *et al.*, (32), PCR applied to various tissues including brain, heart, lung, liver, spleen, kidney and skeletal muscle, resulted in a total of 15 positive samples tested from various tissues, while only 2 were negative from brain, but positive from kidney and gluteus.

Examinations of the paired samples showed that all seropositive dams produced positive fetuses by at least one of the assays, while among seronegative samples from dams, positive fetuses were detected in 23%. The results provided evidence of vertical transmission of *N. caninum* and emphasize the high rate of transmission of parasites from dams to their offspring. Studies with dams and asymptomatic progenies were summarized by Dubey *et al.*, (4), with seropositivity in progeny ranging from 30.8% to 100% among seropositive dams, and from 0% to 23.5% in progenies from seronegative dams. Similar finding on seropositive fetuses born from seronegative dams were reported (12, 25, 34). It was shown that most cows that abort have *N. caninum* antibodies at the time of abortion, therefore seronegative dams are unlikely to be involved in the *Neospora*-induced abortion (34, 8). On the other hand, this possibility can not be ruled out since during pregnancy, antibody levels fluctuate (35), and their levels may fall below the threshold sensitivity of the test (36). Therefore it has been suggested that application of more than one diagnostic test would be more reliable to associate abortion to the *N. caninum* infection. The results obtained here showed that in addition to the conventional IFAT, nPCR is a complementary

tool for an improved and confirmative diagnosis of *N. caninum* in fetuses.

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