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ORIGINAL PAPER

A survey of *Neospora caninum*-associated bovine abortion in large dairy farms of Mashhad, Iran

G R Razmi · H Zarea · Z Naseri

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Abstract *Neospora caninum* is an apicomplexan protozoon causing abortion in cattle worldwide. The present study was designed to assess the importance of this parasite for causing abortion in dairy farms in the Mashhad area of Iran. Of the aborted bovine fetuses, 151 were collected from dairy farms between 2006 and 2008. First, brain samples were examined by polymerase chain reaction (PCR) for the presence of *N. caninum* DNA, diagnosis was complemented with immunohistochemistry (IHC) and fetal serology (ELISA). Twenty-two (14.5%) of bovine fetuses were considered to be infected with *N. caninum* with at least one diagnostic technique being positive. PCR yielded 18 (11.9%) positive out of 151 brain samples. Only 52 brain samples were suitable for IHC examination, and *N. caninum* organism was detected in six (11.5%) of these 52 brain samples. Fetal fluids (n=151) were assessed with a *N. caninum*-ELISA, resulting in 15 (9.9%) seropositive fetal fluids samples. In the present study, a good agreement was observed between PCR and ELISA, and a fair agreement between PCR and IHC. The results indicated that abortion due to *N. caninum* infection is prevalent among large-size dairy farms in the Mashhad area of Iran, and that different complementary diagnostic techniques should be used to increase the chance to detect *N. caninum*.

G. R. Razmi · H. Zarea
Department of Pathobiology, School of Veterinary Medicine, Mashhad, Iran

Z. Naseri
Excellence Research Center for Ruminantal Abortion and Neonatal Mortality, Ferdowsi University of Mashhad, Mashhad, Iran

Present Address:
G. R. Razmi (✉)
Department of Pathobiology, School of Veterinary Medicine, Ferdowsi University of Mashhad,
Mashhad P.O. Box: 91775-1793, Iran
e-mail: razmi@ferdowsi.um.ac.ir

Introduction

Neosporosis has been described an important cause of abortion in cattle worldwide (Dubey et al. 2007; Conraths and Gottstein 2007). In cattle, trans-placental transmissions from infected dams to their offspring appear to be the major natural route of infection. Prenatally infected but healthy calves remain persistently infected and can pass the infection to their own offspring. (Conraths and Gottstein 2007). *Neospora caninum* can be transmitted postnatally (horizontally and laterally) by ingestion of tissues infected with tachyzoites or tissue cysts or by ingestion of food or drinking water contaminated by sporulated oocysts (Dubey et al. 2007). Recently, the terms "exogenous transplacental transmission" and "endogenous transplacental transmission" have been proposed to describe more precisely the origin of the transplacental infection of the fetus (Trees and Williams 2005). Endogenous transplacental transmission occurs in a persistently infected dam after recrudescence of the infection during pregnancy, while, exogenous transplacental transmission occurs after a primary, oocyst-based, infection of a pregnant dam (Trees and Williams 2005; Wouda 2007).

Most neosporosis-induced abortions occur at 5−6 months gestation. Fetuses may die in uterus, be resorbed, mummified, autolyzed, stillborn, born alive with clinical signs, or born clinically normal but chronically infected. Neosporosis-induced abortions occur year-round.

Dairy cattle with *N. caninum* antibodies (seropositive) are more likely to abort than seronegative cows and this applies to both dairy and beef cattle. However, up to 95% of calves born congenitally infected from seropositive dams remain clinically normal (Dubey 2005; Dubey et al. 2007; Wouda 2007). Since the discovery of *N. caninum,* many diagnostic tests have been developed to help in diagnosing this parasitic infection. They include serologic tests, immunohistochemistry (IHC) staining, and polymerase chain reaction (PCR; Haddad et al. 2005; Dubey and Schares 2006). To assess the situation in a herd with regard to abortions due to infection with *N. caninum*, there is a general agreement to apply a combination of diagnostic techniques (Conraths and Gottstein 2007) In Iran, seroepidemiological studies have shown that the prevalence of *Neospora* infection is relatively high in dairy cattle (Sadrebazzaz et al. 2004; Razmi et al. 2006; Nourollahi Fard et al. 2008) and dogs (Malmasi et al. 2006; Haddadzadeh et al. 2007). *N. caninum* infection was also detected in the brains of aborted fetuses by PCR and IHA methods (Razmi et al. 2007). The aim of present study was to determine the role of *N. canium* infection plays in abortion in large size dairy farms, by comparatively applying PCR, IHC, and fetal serology.

Materials and methods

The study was carried out in the Mashhad area of the Razvi Khorasan province, located in the northeast of Iran. The climate is semi-arid with cold winters and moderate summers. The area has an estimated 25,000 cattle on 110 dairy farms. The herd size varies from farm-to-farm with a range of 30−2000 cattle. The most common cattle breed is Holstein-Friesian. There are many dairy farms with large size that have over 500 dairy cattle and have an important role in the production of high volumes of milk in this region. This study was performed over a 2 year period in some Holstein-Friesian dairy herds with large size. The herds were selected on the basis of a known history of *N. caninum* infection and abortion as performed in a previous study (Razmi et al. 2006). In all dairy farms selected, all animals were bred by artificial insemination; all animals were free from tuberculosis and brucellosis, as shown by yearly tests. All farms had no dogs.

Sample collection

A total of 151 aborted bovine fetuses at different stages of gestation were collected from some large size dairy farms during 2006 and 2008. First, the age of the submitted fetuses was estimated by crown-rump length. Then, fetal fluids were also collected by using sterile syringes. After that, skull was opened under aseptic condition, and then one half of the brain was sampled for PCR and another half for immunhistopathological examination. Collected fetal samples were centrifuged at 2,000 rpm for 10 min and stored at −20°C until used.

DNA-isolation and PCR

One half of the brain was homogenized with a stirrer, and DNA was extracted from 1 g homogenate sample using the DNA™ Kit (Cinnagen Inc., Iran) according to the manufacturer's recommendations. PCR was done as described by Müller et al. 1996. The Oligonucleotide primers used were NP21plus (5′CCCAGTGCGTCCAATCCTGTAAC3′) and Np6plus (5′CTCGCCAGTCAACCTACGTCTTCT3′).

Immunohistochemistry

Brain tissue samples were fixed in 10% neutrally buffered formalin and embedded in paraffin. Sections (5 μm) were cut and were immunohistochemically examined. A diagnostic kit based on anti-*N. Caninum* monoclonal antibody labelled with fluorsecein isothiocyanate was applied for the detection of *N. canium* in tissue section (Bio-x Diagnostic kit). Briefly, before adding the monoclonal antibody, slides were deparaffinised via xylene, reverse graded ethanol and water, then incubated with with protinas (P8038 Sigma 50 mg/ml in TBS) and finally rinsed with PBS. In the FITC-labelled mAb was diluted 1:20 in PBS-Evans Blue solution. Incubation with the conjugate was for 1 h at room temperature. Subsequently, the labeled sections were rinsed with PBS. After air frying and subsequent application of the mounting medium, the slides were examined in a Ziess fluorescent microscope.

Serology

Fetal fluid samples were analyzed for antibody activity to *N. caninum* by using a commercially available ELISA kit (IDEXX Laboratories). Briefly, fetal fluid samples were diluted 1:100 in phosphate buffered saline solution, pH7.4, with 0.05% Tween-20, and analyzed for the presence of IgG antibodies specific for *N. caninum* by ELISA as recommended by the manufacturer's instructions. Sera with absorbance values above the cut-off level of 0.20 were considered positive according to manufacturer instructions. Each sample was tested in duplicate.

Statistical analyses

The agreement between the different tests was expressed as κ value. The agreement as: fair if κ values ranged between 0.2 and 0.4, moderate if κ values ranged between 0.4 and

Table 1 Results of PCR, IHC and fetal serology of 22 aborted bovine fetuses that were found infected with *N. caninum* (by at least one of the techniques used)

Fetus no	Age of fetus (month)	PCR result	IHC	Fetal serology
1	8	+	NS	+
2	9	−	NS	+
3	Calf (1 day)	+	NS	+
4	8	+	NS	−
5	6	+	NS	−
6	5	+	+	−
7	6	+	NS	−
8	9	+	+	+
9	5.5	+	−	−
10	Calf (3 days)	+	NS	+
11	9	+	NS	+
12	7	+	+	+
13	5	+	NS	+
14	8	−	+	−
15	8	+	+	+
16	7	+	−	+
17	8	−	−	+
18	9	+	−	+
19	9	+	NS	+
20	6	+	NS	+
21	7	−	+	+
22	9	+	NS	−

NS no sample available to be histologically sectioned NS

0.6, substantial if values ranged between 0.6 and 0.8, and good if the values exceeded 0.8 (Petrie and Watson 2006).

Results

A total of 151 aborted fetuses were collected from selected farms with large cattle populations, all geographically located in the Razavi Khorasm province. From the aborted fetuses, 98% were older than 5 months with a mean of 7.5 months. *Neospora* infection was detected in 22 (14.5%) of aborted fetuses when based on a positive finding by at least one of the diagnostic techniques used (Table 1), two samples were diagnosed as positive when combining all three methods together (Table 2). By using PCR, 18 (11.9%) out of 151 brains of aborted fetuses were positive. Among all cases investigated, only 52 brains were suitable for IHC examination, the other brains were morphologically not any more suitable to be processed for histological sectioning by IHC. *N. caninum* infection was detectable in seven (11.5%) of the 52 investigable brain samples. A poor agreement was observed between IHC with PCR (κ values, 0.184). Fetal fluids of 151 aborted fetuses were examined for antibodies specific to *N. caninum* by ELISA. *N. caninum* antibodies were found in 9.9% (15 out of 151) of fetal fluids samples. A good agreement was seen between ELISA and PCR (κ values 0.69). The agreement between ELISA and IHC was fair (κ values, 0.259).

Table 2 Results of *N. caninum* detection in aborted bovine fetuses by comparison of the combined different techniques

Total	Percentage	Positive No	Kind of technique
151	11.9	18	PCR
52	11.5	6	IHC
151	9.9	15	Fetal serology
52	7.6	4	PCR and IHC
151	7.9	12	PCR and fetal serology
52	7.6	4	IHC and Fetal serology
52	3.8	2	PCR and IHC and fetal serology

Discussion

The present study combined PCR, IHC and fetal serology to examine aborted bovine fetuses for the presence of *N. caninum*. *Neospora* infection was overall diagnosed in 14% of bovine fetuses, based on at least one positive results out of the three different diagnostic techniques used. This percentage was very similar to the rate previously reported in the same area (Razmi et al. 2007). Other studies carried out in the USA and European countries indicate that 12–42% of aborted fetuses from dairy cattle were infected with *N. caninum* (Dubey 2005). One of our objectives of this study was to compare the results obtained by different diagnostic tests. Among these methods, PCR plays an important role as it can detect *N. caninum* DNA in the body tissues of aborted fetuses or other intermediate hosts exhibiting morphological conditions not anymore suitable for other techniques such as IHC (Dubey and Schares 2006). So far, several PCR methods have been used to detect *N. caninum* infection in dairy cattle. Based on the report of European Cooperation in Science and Technology (Müller et al. 1996), the NC5-PCR demonstrated a high diagnostic value and has been generally accepted by many groups performing routine and epidemiological diagnosis of neosporosis (van Maanen et al. 2004; Mattsson and Müller 2007). In this study, 11.2% of brain samples were positive by NC5-PCR.

Although IHC could, in technical terms, not be performed on all brain samples, *N. caninum* was detected in six (11%) of the 52 IHC-investigated fetuses. A relatively poor agreement was found between IHC and PCR. Other studies had already documented low agreements between in IHC and PCR (Gottstein et al. 1998; Sager et al. 2001; van Maanen et al. 2004). In the present study, we detected specific antibodies against *N. caninum* in 9% of fetal fluids, and a good agreement was observed between fetal serology and PCR, and also a fair agreement with IHC. Several other studies had yielded low sensitivities by fetal serology using IFAT (Barr et al. 1995; Wouda et al. 1997; Gottstein et al. 1998). However, finding specific antibodies against *N. caninum* is dependent on the fetal age, and especially in older fetuses' fetal serology can be of significant help to diagnose an *N. caninum* infection in fetuses, more specifically in fetuses aged 5 months or older (Ortega-Mora et al. 2006). In this study, 98% of the collected of bovine fetus were older than 5 months, and this may be the reason why the sensitivity fetal serology was so high and thus in good agreement with PCR.

Based on our results, we conclude that *N. caninum* appears as an important abortive agent in dairy farms of our study area, and that a comprehensive diagnostic approach can be optimized when combining serological, immunohistochemical, and molecular (PCR) tools to investigate aborted fetuses.

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