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SHORT COMMUNICATION

First identification of *Neospora caninum* by PCR in aborted bovine foetuses in Romania

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Abstract The first identification of *Neospora caninum* infection in aborted bovine foetuses in Romania is reported. Nine aborted foetuses were collected from a dairy farm. The foetal age of foetuses was between 3 and 7 months. A 7-month-old foetus was mummified. *N. caninum* DNA Nc-5 region was amplified from samples extracted from brain tissues of three (33.3%) aborted foetuses. The foetal ages of PCR positive foetuses were 3, 4 and 7 months (mummified). No specific lesions or tissue cysts were found to the histological examination, because of advanced autolysis of brains.

Introduction

Neospora caninum is an apicomplexan parasite first described by Bjerkås et al. (1984) and Dubey et al. (1988) in dogs with meningoencephalomyelitis and myositis. It is one of the most commonly diagnosed causes of abortions in cattle worldwide and produces important economic losses (Dubey 1999; Trees et al. 1999; Anderson et al. 2000). The annual losses due to *N. caninum* in the Swiss dairy cow population were estimated to be 9.7 million euros (Häsler et al. 2006).

The life-cycle is heteroxenous, and the infectious stages are considered to be oocysts, tachyzoites and tissue cysts (Dubey 2003). Dogs and coyotes are definitive hosts (McAllister et al. 1998; Gondim et al. 2004) that shed oocysts in the environment. Also, foxes have been suspected to be a possible definitive host since oocysts of *N. caninum* were find in fox faeces (Wapenaar et al. 2006). The intermediate host is represented by a wide range of animals, including dogs and cattle (Dubey 2003). The seroprevalence of *N. caninum* infection in cattle vary from 2.8% in beef cattle (Loobuyck et al. 2009) to 91.2% in dairy cattle (Guedes et al. 2008).

Infection of cattle produces postnatally after ingestion of oocysts shed by definitive hosts (Dijkstra et al. 2001; Dijkstra et al. 2002) and more frequently by transplacental transmission of tachyzoites from dams to foetuses (Davison et al. 1999; Hietala and Thurmond 1999; Anderson et al. 2000). Moreover, epidemiological works established the association between the presence of dogs and the disease in cattle (Wouda et al. 1999). The main clinical sign of infection in cattle is the abortion from 3 months of gestation to term and most of them occur at 5–6 months of gestation (Dubey 2003). The first report of foetal abortions due to *N. caninum* was in dairy cattle from New Mexico (Thilsted and Dubey 1989).

The diagnosis of bovine neosporosis is based on detection of characteristic lesions (non-suppurative encephalitis) in foetal tissues, combined with parasite detection by immunoperoxidase staining or polymerase chain reaction (PCR) (Lindsay and Dubey 1989; Wouda et al. 1997; Dubey 2003; Wouda and Buxton 2007).

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In Romania, the reports regarding the *N. caninum* are limited to three epidemiological studies in dogs and cattle (Ionescu et al. 2003; Şuteu et al. 2005; Gavrea et al 2008).

Materials and methods

Samples

In 2006, nine aborted bovine foetuses (with 3 to 7 months of age) were obtained from a dairy farm located in the centre of Romania. The foetuses were necropsied and samples of brain were collected in double. The former sets of samples were fixed in 10% neutral buffered formaldehyde. Fixed tissues were embedded in paraffin, sectioned, stained with haematoxylin and eosin and examined by light microscopy for the presence of lesions and parasites. The latter sets of brain samples were stored at −80°C for PCR analysis.

DNA extraction

DNA extraction was performed with a NucleoSpin tissue kit (Macherey-Nagel). After thawing, 25 mg of brain tissue were homogenised and suspended in 180 µl of Buffer T1 and 25 µl proteinase K, mixed by vortexing followed by incubation at 56°C for 3 h. After incubation, it was added 200 µl of Buffer B3 solution, mixed by vortexing and incubated for 10 min at 70°C. After the second incubation to the lysate was added 210 µl ethanol, mixed by vortexing and transferred into the NucleoSpin column. The column was centrifuged at 11.000 rpm for 1 min at room temperature. The NucleoSpin column was transferred in a new collection tube and added 500 µl of Buffer BW, followed by a new centrifugation, addition of 600 µl buffer B5 and centrifugation. Finally, the DNA was eluted from column by centrifugation with 150 µl of elution buffer BE prewarmed at 70°C. The DNA was stored at −20°C till using.

PCR

PCR was performed with the *N. caninum*-specific primers Np6+/Np21+ amplifying Nc-5 region (Müller et al. 1996). Nc-1 strain was used as positive and brain tissue from a free mouse as negative controls. Each reaction mixture of 25 µl contained 25 pM of each primer and 1 µl of DNA. The amplification was performed in Tpersonal-Biometra termocycler. The cycling parameters for the amplification consisted of an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation (94°C, 1 min.), annealing (63°C, 1 min.) and extension (74°C, 3.5 min), with a final extension at 74°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel and observed for the presence of the specific fragment (337 bp) under UV light.

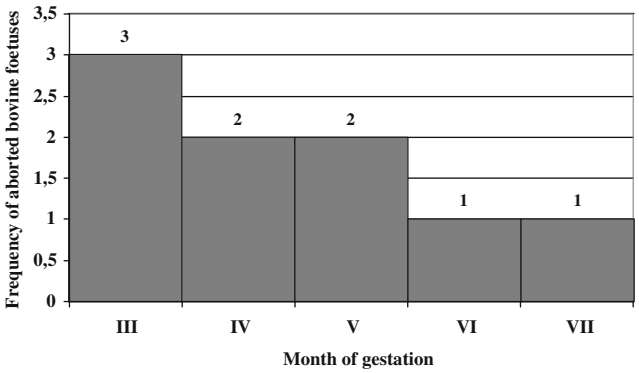

Fig. 1 Frequency distribution of aborted bovine foetuses according to the month of gestation

Results

The frequency distribution of aborted bovine foetuses is presented in histogram 1. The foetal age of foetuses was between 3 and 7 months, and most of the foetuses were 3- to 5-month-old. 7-month-old foetus was mummified (Fig. 1). Because of advanced autolysis of brains, no specific lesions or tissue cysts were found to the histological examination.

Nc-5 fragments of the expected size (about 337 bp) were amplified from the brain tissue of three foetuses (Fig. 2). The foetal ages of PCR positive foetuses were 3, 4 and 7 months (mummified).

Discussions

This is the first report of *N. caninum* indentification by PCR in brain tissues of aborted bovine foetuses in Romania. At the VIIIth International Coccidiosis Conference have been establish that histological, immunohistochemical and molecular detection assays are of outstanding

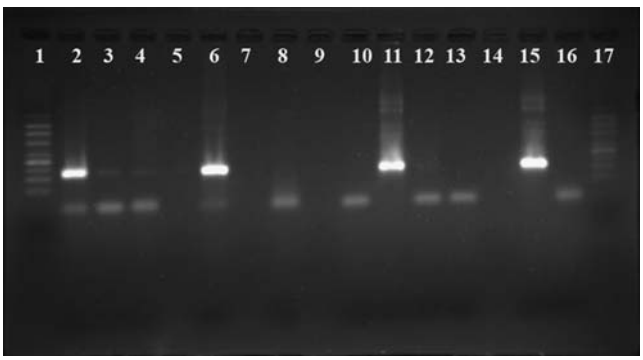

Fig. 2 PCR product of Nc-5 fragment amplified with primer pair Np21+ and Np6+. *Lanes 1* and *17*, 1 kb DNA ladder; *lane 16*, negative control (brain tissue from a free mouse); *lane 15*, positive control (tachyzoites of Nc-1 strain); *lanes 2–4*, *6*, *8* and *10–13*, brain tissues collected from aborted bovine foetuses

importance for the examination of tissues of aborted foetuses. While histology and immunohistochemistry allow direct assessment of pathomorphological changes caused by infection, molecular detection assays such as PCR are superior because of higher sensitivity and specificity in identifying *N. caninum* in foetal tissues (Jenkins et al. 2002). Baszler et al. (1999) indicated a detection limit in PCR, in the range of 20 to 40 tachyzoites in 20 mg of bovine brain tissue, a sensitivities of 100% for formalin-fixed, paraffin-embedded brain tissue (13 of 13 true-positive foetuses were PCR positive) and 77% for fresh brain (ten of 13 true-positive foetuses were PCR positive) and a specificities of 94% for formalin-fixed paraffin-embedded brain tissue (one to 16 true-negative foetuses were PCR positive) and 100% for fresh brain (0 of 13 true-negative foetuses were PCR positive). The same authors concluded that the PCR is more sensitivity than IHC. Also, it was reported a fair agreement between PCR and histopathology (k=0.312), PCR being more sensitive (Medina et al. 2006).

In our study, in 33.3% of aborted foetuses (3/9) was identified *N. caninum* DNA in brain tissues by PCR. The prevalence obtained by us was the same as reported in Iran (Sadrebazzaz et al. 2007; Razmi et al. 2007). A lower prevalence by PCR was reported in China (25%) (Zhang et al. 2007), in Spain (15.3%) (Pereira-Bueno et al. 2003), in Swiss (21%) (Sager et al. 2001) and in Brasil (23%) (Corbellini et al. 2006). A different prevalence was related in Mexico, where 35 (80%) aborted foetuses among 44 were detected infected by *N. caninum* using PCR (Medina et al. 2006). Abortions in cattle caused by *N. caninum* occur at 5–6 months of gestation, and cows may abort from 3 months of gestation to term. Foetuses may die in utero, resorbed, mummified, autolysed or stillborn (Dubey 2003). In our case, the foetal age of foetuses was between 3 and 7 months, and the positive ones were 3, 4 and 7 months. Also, the 7-month-old foetus positive by PCR was mummified.

Foetal brain is the most affected organ (Dubey 2003), and the lesions are represented by focal encephalitis with necrosis and non-supurative inflammation (Barr et al. 1991). In our study, any specific lesions or tissue cysts were found to the histological examination because of advanced lyses. Pescador et al. (2007) indicated that when foetal brain is autolysed, the lung may be used for the presumptive diagnosis of *N. caninum* infection.

In Romania, the seroprevalence of *N. caninum* infection in cattle range between 33.7% (Ionescu et al. 2003) and 56.2% (Gavrea et al 2008) and in shelter dogs, 12.3% (Şuteu et al. 2005). Further investigation is needed for epidemiological status of bovine neosporosis in dairy cattle in our country.

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References

Anderson ML, Andrianarivo AG, Conrad PA (2000) Neosporosis in cattle. Anim Reprod Sci 60–61:417–431. doi:10.1016/S0378-4320(00)00117-2

Barr BC, Conrad PA, Dubey JP, Anderson ML (1991) *Neospora*-like encephalomyelitis in a calf: pathology, ultrastructure, and immunoreactivity. J Vet Diagn Invest 3:39–46

Baszler TV, Gay LJC, Long MT, Mathison BA (1999) Detection by PCR of *Neospora caninum* in fetal tissues from spontaneous bovine abortions. J Clin Microbiol 37(12):4059–4064

Bjerkås I, Mohn SF, Presthus J (1984) Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Z Parasitenk 70:271–274

Corbellini LG, Pescador CA, Frantz F, Wunder E, Steffen D, Smith DR, Driemeier D (2006) Diagnostic survey of bovine abortion with special reference to *Neospora caninum* infection: importance, repeated abortion and concurrent infection in aborted fetuses in Southern Brazil. Vet J 172:114–120

Davison HC, Otter A, Trees AJ (1999) Estimation of vertical and horizontal transmission parameters of *Neospora caninum* infections in dairy cattle. Int J Parasitol 29:1683–1689

Dijkstra T, Barkema HW, Eysker M, Wouda W (2001) Evidence of post-natal transmission of *Neospora caninum* in Dutch dairy herds. Int J Parasitol 31:209–215

Dijkstra T, Barkema HW, Eysker M, Hesselink JW, Wouda W (2002) Natural transmission routes of *Neospora caninum* between farm dogs and cattle. Vet Parasitol 105:99–104

Dubey JP (1999) Recent advances in *Neospora* and neosporosis. Vet Parasitol 84:349–367

Dubey JP (2003) Review of *Neospora caninum* and neosporosis in animals. Korean J Parasitol 41:1–16

Dubey JP, Carpenter JL, Speer CA, Topper MJ, Uggla A (1988) Newly recognized fatal protozoan disease of dogs. J Am Vet Med Assoc 192:1269–1285

Gavrea R, Titilincu A, Mircean V, Cozma V (2008) Seroprevalence of neosporosis in cattle from a dairy farm in center of Romania. Scient Parasitol 2:26–30

Gondim LFP, Mc Allister MM, Pitt WC, Zemlicka DE (2004) Coyotes are definitive hosts of *Neospora caninum*. Int J Parasitol 34:159–161

Guedes MH, Guimarães AM, Rocha CM, Hirsch C (2008) Frequency of anti-*Neospora caninum* antibodies in cows and fetuses from Municipalities of southern Minas Gerais. Rev Bras Parasitol Vet 17(4):189–194

Häsler B, Regula G, Stärk KD, Sager H, Gottstein B, Reist M (2006) Financial analysis of various strategies for the control of *Neospora caninum* in dairy cattle in Switzerland. Prev Vet Med 77(3–4):230–253

Hietala SK, Thurmond MC (1999) Postnatal *Neospora caninum* transmission and transient serologic responses in two dairies. Int J Parasitol 29:1669–1676

Ionescu V, Popa E, Moldoveanu D (2003) Neosporoza la bovine. Rev Rom Parazitol XIII(1):125–126

Jenkins M, Baszler T, Björkman C, Schares G, Williams D (2002) Diagnosis and seroepidemiology of *Neospora caninum*-associated bovine abortion. Int J Parasitol 32(5):631–636

Lindsay DS, Dubey JP (1989) Immunohistochemical diagnosis of *Neospora caninum* in tissue sections. Am J Vet Res 50:1981–1983

Loobuyck M, Frössling J, Lindberg A, Björkman C (2009) Seroprevalence and spatial distribution of *Neospora caninum* in a population of beef cattle. Prev Vet Med 92(1–2):116–122

McAllister MM, Dubey JP, Linsday DS, Jolley WR, Wills RA, McGuire AM (1998) Dogs are definitive hosts of *Neospora caninum*. Int J Parasitol 28:1473–1478

Medina L, Cruz-Vázquez C, Quezada T, Morales E, García-Vázquez Z (2006) Survey of *Neospora caninum* infection by nested PCR in aborted fetuses from dairy farms in Aguascalientes, Mexico. Vet Parasitol 136:187–191

Müller N, Zimmermann V, Hentrich B, Gottstein B (1996) Diagnosis of *Neospora caninum* and *Toxoplasma gondii* infection by PCR and DNA hybridization immunoassay. J Clin Microbiol 34:2850–2852

Pereira-Bueno J, Quintanilla-Gonzalo A, Pérez-Pérez V, Espi-Felgueroso A, Álvarez-Gareía G, Collantes-Fernández E, Ortega-Mora LM (2003) Evaluation by different diagnostic techniques of bovine abortion associated with *Neospora caninum* in Spain. Vet Parasitol 111:413–452

Pescador CA, Corbellini LG, Oliveira EC, Raymundo DL, Driemeier D (2007) Histopathological and immunohistochemical aspects of *Neospora caninum* diagnosis in bovine aborted fetuses. Vet Parasitol 150:159–163

Razmi GR, Maleki M, Farzaneh N, Talebkhan Garoussi M, Fallah AH (2007) First report of *Neospora caninum*-associated bovine abortion in Mashhad area, Iran. Parasitol Res 100:755–757. doi:10.1007/s00436-006-0325-6

Sadrebazzaz A, Habibi G, Haddadzadeh H, Ashrafi J (2007) Evaluation of bovine abortion associated with *Neospora caninum* by different diagnostic techniques in Mashhad, Iran. Parasitol Res 100:1257–1260. doi:10.1007/s00436-006-0417-3

Sager H, Fischer I, Furrer K, Strasser M, Waldvogel A, Boerlin P, Audigé J, 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

Şuteu O, Losson B, Oltean M, Cozma V (2005) First serological survey for canine neosporosis in Romania. Bul Univ Stiint Agric Med 62:591–592

Thilsted JP, Dubey JP (1989) *Neosporosis*-like abortions in a herd of dairy cattle. J Vet Diagn Invest 1:205–209

Trees AJ, Davison HC, Innes EA, Wastling JM (1999) Towards evaluating the economic impact of bovine neosporosis. Int J Parasitol 29:1195–1200

Wapenaar W, Jenkins MC, O'Handley RM, Barkema HW (2006) *Neospora caninum*-like oocysts observed in feces of free-ranging red foxes (*Vulpes vulpes*) and coyotes (*Canis latrans*). J Parasitol 92(6):1270–1274

Wouda W, Buxton D (2007) Immunohistochemistry. In: Ortega-Mora LM, Gottstein B, Conraths FJ, Buxton D (eds) Protozoal abortion in farm ruminants: guidelines for diagnosis and control. CAB International, Athenaeum Press, Gateshead, pp 57–58

Wouda W, Moen AR, Visser IJR, Van Knapen F (1997) Bovine fetal neosporosis: a comparison of epizootic and sporadic abortion cases and different age classes with regard to lesion severity and immunohistochemical identification of organisms in brain, heart, and liver. J Vet Diag Invest 9:180–185

Wouda W, Dijkstra T, Kramer AMH, Van Maanen C, Brinkhof MA (1999) Seroepidemiological evidence for a relationship between *Neospora caninum* infections in dogs and cattle. Int J Parasitol 29:1677–1682

Zhang W, Deng C, Liu Q, Liu J, Wang M, Tian KG, Yu XL, Hu DM (2007) First identification of *Neospora caninum* infection in aborted bovine fetuses in China. Vet Parasitol 149:72–76