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RESEARCH NOTE

Characterization of the first Polish isolate of *Neospora caninum* from cattle

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Abstract

Neospora caninum was isolated from the brain of an apparently healthy calf born to a seropositive cow. The calf was killed 12 hrs after birth and homogenate of its brain was inoculated into Vero cells. *Neospora*-like tachyzoites were detected 66 days later. The identity of the parasite was confirmed by polymerase chain reaction amplification of *N. caninum*-specific fragments using Np21 and Np6 primers. This first Polish isolate of *N. caninum* was designated NC-PolB1.

Key words

Neospora caninum, isolation, PCR, ELISA, Western blot, calf

Neospora caninum is an important causative agent of abortion in cattle worldwide (Dubey and Lindsay 1996, Dubey 2003). Diagnosis of neosporosis is difficult and is aided by serological examination and demonstration of *N. caninum* parasite or its DNA in fetal tissues. Seldom it is possible to isolate viable *N. caninum* from cattle tissues because most *N. caninum* die with the host and the concentration of *N. caninum* in tissues of asymptomatic cattle is low (Dubey and Schares 2006). Currently, there are only few isolates of *N. caninum* worldwide (Dubey and Schares 2006, Dubey *et al*. 2007). We report the first isolate of *N. caninum* from a calf in Poland.

Blood samples were collected from calves born to seropositive cows. The presence of antibodies against *N. caninum* antigens in sera of cows and calves were determined by an enzyme linked immunoassay (ELISA), according to the manufacturer's instruction (IDEXX Laboratories, Inc., Westbrook, Maine, USA) as previously described (Cabaj *et al*. 2005). Sera from calves were found to be highly positive for *Neospora*-specific antibodies by ELISA.

In the present study, 10 calves born to seropositive cows from herds with a history of *Neospora*-associated abortions, were used to isolate the parasite. The calves were asymptomatic and were euthanized at the age of 12 months (1 calf), 9 months (1 calf), 7 months (1 calf), 3 months (4 calves), 1.5 months (2 calves) and 12 hours (1 calf). Brains were removed aseptically and immersed in phosphate-buffered saline at pH 7.2. The cortical layer of the hemispheres was homogenised in 200 ml of PBS and the homogenate was incubated in PBS with 0.25% trypsin at 37°C for 1 h, with constant shaking. The suspension was filtered through sterilised gauze and centrifuged at 400 *g* for 10 min. The supernatant was discarded and the pellet was washed four times with sterile PBS. The sediment was suspended in approximately 10 ml of PBS and inoculated in monolayer Vero (African green monkey kidney) cell lines. Vero cells were cultivated in RPMI 1640 medium supplemented with 1% horse serum and 50 U/ml of antibiotics (streptomycin and penicillin). Cell cultures were incubated at 37°C in a 5% CO_2.

During the first 9 weeks after introduction, the medium was changed every two weeks. The cell cultures were examined daily with an inverted microscope for the presence of tachyzoites. After detection of the parasites, cultivation on Vero cells was continued using RPMI 1640 medium supplemented as described above, the medium was changed every 7–10 days and the parasites were passaged every 7–10 days. Extracellular, *N. caninum*-like tachyzoites were first observed in Vero cell culture 66 days post inoculation of brain homogenate from one newborn calf (12 h after birth). Many tachyzoites were seen after several passages in cell culture.

The Polish isolate was compared with the NC-1 reference isolate of *N. caninum* (Dubey *et al*. 1988) maintained in Vero cells in the same way.

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Protozoan tachyzoites were passed through a 23 gauge needle and 5 µm filter (Millex, 25 mm diam., 5 µm, Millipore, Billerica, MA, USA) three times in sterile PBS by centrifugation at 400 *g* for 10 min. Tachyzoites were suspended in distilled water, freeze-thawed three times and sonicated for 3 min. The protein content of the crude antigen preparations was determined according to Lowry using Folin method, with bovine serum albumin as standard. The antigen preparations were stored at –80°C until use.

SDS-polyacrylamide gel electrophoresis and Western blot analysis were performed under reducing conditions essentially as described by Björkman *et al*. (1994) and Björkman and Hemphill (1998). For analysis under these conditions, mercaptoethanol was added to the antigen preparation and the mixture was boiled for 5 min. The samples were electrophoresed on a 5% stacking gel (pH 6.8) and 12% resolving gel (pH 8.8) SDS-PAGE and separated polypeptides were transferred to nitrocellulose paper (pore size 0.2 µm, Bio-Rad Laboratories, California, USA). The membranes were blocked for 1 h in Quench buffer (20 mM Tris, 0.9% NaCl, pH 9.0) containing 5% non-fat dry milk. Then the blotted polypeptides were exposed to sera diluted 1:20 in PBS-Tween 20 buffer containing 5% non-fat dry milk for 2 h at 37°C. After washing (PBS-Tween 20, pH 7.2, the membranes were incubated for 1 h with anti-bovine IgG whole molecule peroxidase conjugate (Sigma, Missouri, USA) diluted 1:5000. After another wash, immunoreactive proteins were detected with 0.02% DAB (3'3-diaminobenzidinetetrahydrochloride) (Sigma, Missouri, USA). Sera were obtained from 10 naturally infected calves and their mothers. As the negative control serum from a cow found to be seronegative to these parasites was used.

Western blot analysis revealed no major differences between the two isolates NC-PolB1 and NC-1. Sera from cows and calves showed similarities in antigen profiles between *N. caninum* isolates. Antibodies reacted with proteins at: 9, 16, 21, 27, 31, 34, 36, 38, 40, 43, 47, 48, 53 and 58 kDa. No bands were seen when the *Neospora* antigens were incubated with sera from uninfected cow and when antibodies were probed with antigens obtained from uninfected Vero cells.

DNA was extracted from tissues of infected calves, from parasites of the reference strain NC-1 and cell cultures. Genomic DNA was isolated using Nucleospin Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions.

Polymerase chain reaction (PCR) was performed with the *N. caninum* – specific oligonucleotide primers Np21 and Np6 amplifying a portion of the gene5 region including a positive (NC-1) and a negative controls. Primers Np21 and Np6 for PCR were synthesised according to Yamage *et al*. (1996). The reagents used for PCR were purchased from Fermentas (MBI Fermentas, USA), and reaction mixtures were prepared following manufacture's instructions. The amplification reactions were carried out in thermal cycler (Genius, Techne New Jersey, USA), under the following conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles with denaturation at 94°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 25 s and with a final extension of 72°C for 10 min. A negative control (water) and a positive control DNA from the NC-1 isolate were included in each reaction. The PCR products were loaded on a 1% agarose gel containing ethidium bromide and visualised after run, for the presence of the specific DNA fragment using the Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Rochester, NY, USA).

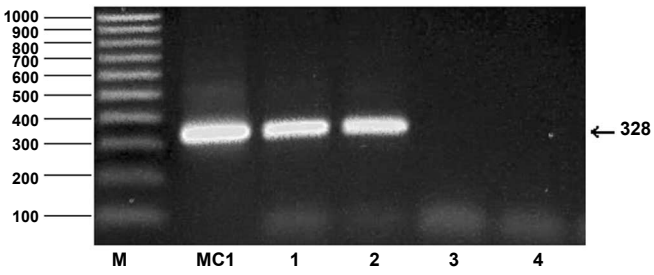

Fig. 1. *Neospora caninum* DNA detected in nervous tissues of infected calves (line 1), infected Vero cells from cell cultures (line 2). The size of the bands matched the positive control (NC-1 isolate). No DNA was amplified from the negative controls (line 3 and 4). M – 100 bp ladder

The specific amplicon of 328 bp was present in the examined samples which confirmed the presence of *N. caninum* DNA in tested samples (Fig. 1).

The low isolation rate of *N. caninum* (1 out of 10 calves) is probably due to low numbers of parasites in tissues of asymptomatic cattle. Most of the isolations in the past were made from aborted or sick calves (Dubey and Schares 2006, Dubey *et al*. 2007). *N. caninum* was isolated from an eight month old calf only once in Italy by Fioretti *et al*. (2000) and from 2 adult cows that had aborted from confirmed neosporosis, from Japan (Sawada *et al*. 2000) and from New Zealand (Okeoma *et al*. 2004).

The present study is the first report describing *Neospora* isolation from healthy, congenitally-infected calf. The Polish isolate of the *N. caninum* has been successfully maintained in cell culture for more than 1 year.

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References

Björkman C., Hemphill A. 1998. Characterization of *Neospora caninum* iscom antigens using monoclonal antibodies. *Parasite Immunology*, 20, 73–80. DOI:10.1046/j.1365-3024.1998.00127.x.

Björkman C., Lunden A., Holmdahl J., Barber J., Trees A.J., Uggla A. 1994. *Neospora caninum* in dogs: detection of antibodies by ELISA using an iscom antigen. *Parasite Immunology*, 16, 643–648.

Cabaj W., Moskwa B., Pastusiak K., Gill J. 2005. Antibodies to *Neospora caninum* in the blood of European bison (*Bison bo-*

nasus bonasus L.) living in Poland. *Veterinary Parasitology*, 128, 163–168. DOI: 10.1016/J.VETPAR.2004.09.033.

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

Dubey J.P., Hattel A.L., Lindsay D.S., Topper M.J. 1988. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. *Journal of the American Veterinary Medicine Association*, 193, 1259–1263.

Dubey J.P., Lindsay D.S. 1996. A review of *Neospora caninum* and neosporosis. *Veterinary Parasitology*, 67, 1–59. DOI: 10.1016/S0304-4017(96)01035-7.

Dubey J.P., Schares G. 2006. Diagnosis of bovine neosporosis. *Veterinary Parasitology*, 140, 1–34. DOI:10.1016/j.vetar.2006.03.035.

Dubey J.P., Schares G., Ortega-Mora L.M. 2007. Epidemiology and control of neosporosis and *Neospora caninum*. *Clinica Microbiology Reviews*, 323–367. DOI:10.1128/CMR.00031-06.

Fioretti D.P., Rosingoli L., Ricci G., Moretti A., Pasquali P., Polidori A. 2000. *Neospora caninum* infection in a clinically healthy calf: parasitological study and serological follow-up. *Journal of Veterinary Medicine*, *Ser. B*, 47, 47–53. DOI: 10.1046/j.1439-0450.2000.00304.x.

Okeoma C.M., Williamson N.B., Pomroy W.E., Stowell K.M., Gillespie L.M. 2004. Isolation and molecular characterisation of *Neospora caninum* in cattle in New Zealand. *New Zealand Veterinary Journal*, 52, 364–370.

Sawada M., Kondo H., Tomioka Y., Park C.-H., Morita T., Shimada A., Umemura T. 2000. Isolation of *Neospora caninum* from the brain a naturally infected adult dairy cow. *Veterinary Parasitology*, 90, 247–252. DOI: 10.1016/S0304-0417(00)00248-X.

Yamage M., Flechtner O., 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. DOI:10.2307/3284160.

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