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First *in vitro* isolation of *Neospora caninum* from a naturally infected adult dairy cow in Slovakia

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Abstract

The impact of *in vitro* isolation and molecular characterisation of *Neospora caninum* as well as sequence analyses was studied. The brain homogenate of a naturally *Neospora*-infected dairy cow (positive in ELISA and Western blot) was intraperitoneally inoculated into Mongolian gerbils (*Meriones unguiculatus*). The brain of gerbils on day 60 post-inoculation was homogenized, and, after trypsin-digestion, cultured on Vero cells. *Neospora*-like tachyzoites were first observed after 77 days of cultivation. The parasite was confirmed by polymerase chain reaction (PCR) using *Neospora*-specific primers Np21 and Np6. The PCR product of the first Slovak isolate (NC-SKB1) was subsequently sequenced and published in GenBank under accession number GU300774. Sequencing and BLAST search identified the isolate as *N. caninum*.

Keywords

Neospora caninum, cow, isolation, cELISA, Western blot, PCR

Introduction

The worldwide distributed protozoan intracellular parasite, *Neospora caninum*, is considered as a major cause for repeated abortions in dairy cattle (Thilsted and Dubey 1989, Anderson *et al*. 1991, Paré *et al*. 1998) and has negative economical impact for breeding. The adult livestock usually do not manifest clinical signs; abortions are often the only sign of infection (Dubey and Lindsay 1996, Hemphill 1999, Dubey 2003). The first serological study carried out by Reiterová *et al*. (2009) in Slovakia revealed a high prevalence of *N. caninum*, mainly in farms located in north-eastern part of the country. The mean seroprevalence in Slovak spotted dairy cattle post-abortion was 20% and in cows without any reproduction problems only 2% have been recorded.

Since the discovery of *N. caninum*, several successful isolations of this parasite have been reported. The first one was from a dog brain following *in vitro* cultivation in Vero cells (Dubey *et al*. 1988). Since then, several *in vitro* isolations of *N. caninum* from dogs or aborted bovine foetuses have been reported (Conrad *et al*. 1993, Barber *et al*. 1995, Yamane *et al*. 1997, Dubey *et al*. 2007). From adult cows, *N. caninum* was isolated only twice, in Japan (Sawada *et al*. 2000) and New Zealand (Okeoma *et al*. 2004).

The aim of the present study was to report the first successful *in vitro* isolation of *N. caninum* from naturally infected dairy cow and its subsequent characterization by molecular techniques comparing it with the *N. caninum* reference isolate NC-1 and sequencing.

Materials and methods

Animals

A culled dairy cow (*Bos taurus*) of the Slovak spotted breed from a herd with a history of *Neospora*-associated abortions with up to 68% prevalence of anti-*Neospora* antibodies in the farm was used for isolation of the parasite.

Mongolian gerbils (*Meriones unguiculatus*) weighing 48 ± 2 g kept under a 12 h light/dark regime at a room temperature (21° ± 3°C) and 50–60% humidity on commercial diet and water were used for passage of tachyzoites of *N. caninum* from the brain homogenate of the infected cow. The experimental protocol was approved by the Parasitological Institute Animal Care Committee.

Serological analysis

The presence of *Neospora*-antibodies in sera of a cow (two blood samples) was detected using competitive ELISA (VMRD, Inc. U.S. Veterinary License, the U.S.A.) according

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to the manufacturer's instructions as previously described (Reiterová *et al*. 2009).

Western blot

Sera obtained from cow were also examined by Western blot. Electrophoresis was performed using a Bio-Rad Mini Protein Slab Cell on a 12% SDS-polyacrylamide gel and 4% stacking gel under reducing conditions with modifications according to Björkman and Hemphill (1998). Purified proteins of NC-1 were electrophoresed and transferred onto nitrocellulose membrane (Bio-Rad Laboratories, California, USA). Membrane strips were blocked for 1 h in phosphate buffered saline (PBS) at pH 7.2 with 5% non-fat dry milk. Then the strips were exposed to sera diluted 1:50 in PBS-Tween 20 with 5% non-fat dry milk at +4°C over night. After washing three times with PBS-Tween 20, the membrane strips were incubated for 1 h with rabbit anti-bovine IgG peroxidase conjugated (A5295, Sigma-Aldrich, Germany) diluted at 1:200. The strips were washed again and bands were developed using 4-chloro-1-naphthol and 0.03% hydrogen peroxide. Results were interpreted according to the present bands.

Bioassay for Neospora caninum *in gerbils and cultivation on cell cultures*

The brain of the cow was removed aseptically and the cortical layer of the hemispheres (50 g of cortex) was homogenized in PBS at pH 7.2 and digested with 0.2% trypsin at 37°C for 45 min with constant shaking. The suspension was filtered through sterilised gauze and centrifuged at 600 *g* for 10 min. The sediment was washed twice in sterile PBS with 500 U of penicillin and 500 mg streptomycin/ml.

1. Part of the sediment at 500 µl was retained for molecular analyses and was preserved at –20°C until further use.

2. The next part of homogenate was cultivated on a monolayer of Vero (African green monkey kidney of species *Cercopithecus aethiops*) cells with RPMI 1640 medium supplemented with 50 U penicillin/ml, 50 mg streptomycin/ml, 4 mM L-glutamine, $NaHCO_3$ 2 mg/L and 2% foetal calf serum (FCS); and incubated at 37°C in a 5% CO_2/95% air environment under sterile conditions. After 1 hour cultivation flasks were washed with fresh medium. Vero cells were examined under an inverted microscope (Olympus, Germany) to detect the presence of tachyzoites.

3. The rest of homogenate was intraperitoneally inoculated into two gerbils (0.5 ml/animal). Infected gerbils were after inoculation regularly monitored. Gerbils were killed by cervical dislocation on day 60 p.i. Prior to necropsy they were treated with 10% ajatine solution for 10 min (skin disinfectant). Parts of their brains were fixed and used for histology. The rest of brain tissues (2.1 g) after homogenisation and trypsin-digestion described above was cultured on Vero cells under the same condition as noticed above.

In vitro *growth and proliferation characteristics of isolates*

An NC-1 strain of *N. caninum* tachyzoites have been maintained on Vero cells in defined RPMI medium described above. The tachyzoites were collected for subsequent antigen preparation. Both isolates, NC-1 and NC-SKB1 were in parallel cultivated on Vero cells in RPMI medium with 2% or 5% FCS, respectively. Circadian microscopic monitoring (under inverted microscope Olympus, Germany) of inoculated cell cultures was made for control of *in vitro* growth and proliferation of cultured isolates for more than 60 days due to the slow development of tachyzoites. Medium was changed weekly. To keep the total amount of cultured cells to a minimum, subcultures were made at three week intervals.

PCR amplification of Neospora caninum

One step PCR was performed with the *N. caninum*-specific primer pairs Np6 and Np21 amplifying a 328 bp amplicon of the Nc-5 gene (Yamage *et al*. 1996). The NC-1 strain was used as the positive and sterile water as negative controls. DNA template of *Toxoplasma gondii*, PCz(RH) strain (obtained from National Reference Laboratory for Toxoplasmosis, Prague, Czech Republic) as other control and marker 100 bp ladder (Promega, Madison, USA) was used. The reagents used for PCR were purchased from Fermentas (MBI Fermentas, USA). Each PCR reaction was performed in a final volume of 25 µl mixture containing 10 pM of each primer, 5 U Taq polymerase, 10 × PCR buffer, 10 mM dNTPs and 1 µl of the sample. The amplifications were carried out in thermal cycler (Bioer, China) under the following conditions: initial denaturation of templates at 95°C for 7 min, followed by 35 cycles of denaturation (95°C, 30 sec.), annealing (55°C, 30 sec.) and extension (72°C, 1 min), with a final extension at 74°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and examined for the presence of the specific fragment under UV light.

Sequencing of Nc5 gene of Neospora caninum

PCR products were after electrophoresis (130 V during 90 min) cut out from 1% agarose gel and subsequently purified using Qiagen DNA purification kit (QIAamp®DNA Mini Kit; QIAGEN, Gmb., Hilden, Germany). The gel-cleaned PCR products were sequenced on DNA sequenator (Avant3100, Applied Biosystems) and submitted to GenBank (USA). Afterward, the BLAST search analysis was performed.

Results

Case report and serology of the cow

A culled 4-year-old dairy cow was born from a *Neospora*-seropositive mother. At an age of two years she gave birth to

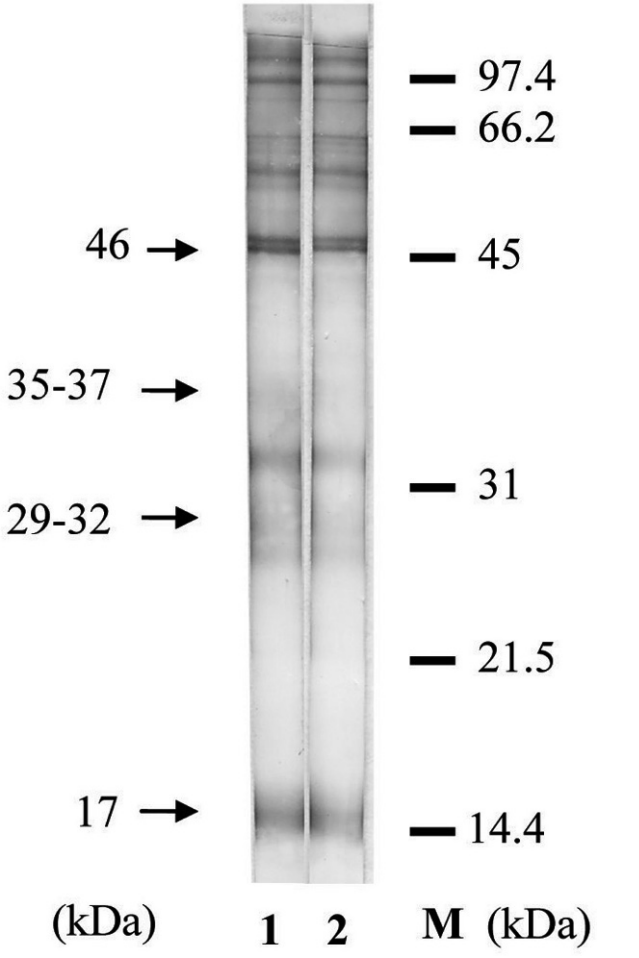

Fig. 1. Reactivity of a *Neospora caninum* positive bovine serum against *N. caninum* tachyzoite antigens separated under reducing conditions of disulfide bonds with 2-mercaptoethanol. **M** – low molecular weight marker; lines **1**, **2** – ELISA-positive bovine serum samples

a healthy bull calf, but throughout the production period her milk yield was reduced and, after the first calving, she exhibited fertility problems. After being mated three times she finally conceived aged three years, but aborted in the second half of gestation. Subsequently she developed partial loss of movement, with a right hind leg limp without a confirmed aetiology. After unsuccessful treatment she was slaughtered.

The serology with competitive ELISA technique revealed moderate levels of specific antibodies. In the first serum sample from January 15, 2009 the percentage of inhibition was 74%. The second sample from March 30, 2009 gave a percentage of inhibition at 54%.

The antigenic profiles detected by Western blotting were similar in both sera of the culled cow. The pattern of antigen recognition corresponded to the *N. caninum* immunodominant antigens with molecular weights of 17; 29–32; 35–37 a 46 kDa (Fig. 1). No antigens were detected when negative sera were analysed.

In vitro *growth and proliferation characteristics of NC-SKB1*

Vero cells inoculated with both, bovine brain and gerbil brain homogenates, respectively, were sub-cultured at 14 day intervals to keep down the expansion rate of Vero cells. In parallel, Sweden NC-1 isolate was maintained according to the same conditions. In cell culture, directly inoculated with bovine brain homogenate, no tachyzoites were noticed. Parasitic growth of *N. caninum* designated as NC-SKB1 was first detected in the cell culture flasks on day 77 post inoculation with a brain homogenate of gerbil. This implies that the parasites isolated from fresh gerbil brain were successfully transferred from the naturally infected adult cow. Parasites were sub-cultured and have been maintained in Vero cell culture for more than 9 months. In case with higher content of foetal calf serum (5% FCS) the growth of cells and the proliferation of tachyzoites were more expansive in comparison with the lower concentration (2% FCS).

PCR

The Slovak isolate NC-SKB1 was compared with the NC-1 reference isolate of *N. caninum*. The presence of specific *N. cani-*

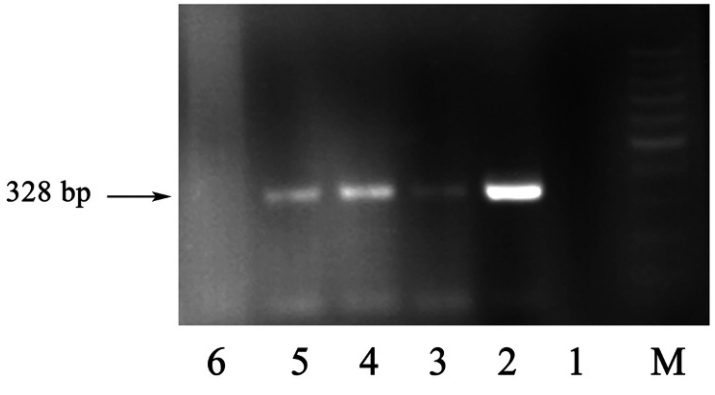

Fig. 2. Standard polymerase chain reaction – molecular detection of Nc5 gene of *Neospora caninum* (**M** – marker, 100 bp ladder; **1** – negative control; **2** – positive control NC-1 isolate; **3** – brain of infected cow; **4** – NC-SKB1 isolate from Vero cells; **5** – brain of inoculated gerbil; **6** – *Toxoplasma gondii*)

num DNA sequence of 328 bp in both, examined samples and positive control (NC-1 isolate) was confirmed (Fig. 2).

Sequencing and BLAST search analysis

The BLAST search provided a definitive genetic characterisation of the parasite. The sequence of the PCR product (GenBank Accession Number GU300774) gave the best score with the *N. caninum* NC5 gene partial sequence (EU073600 isolate 152 from chicken brain, similarity 99%) where was found nucleotide substitution G to C in position 585; FJ464412 Nc Kr2 NC5 gene, similarity 98%) with substitution G482A; EU073599 isolate 197 from chicken brain, similarity 98% substituted C542G.

Discussion

In the past, most of the isolations were made from aborted or sick calves (Dubey and Schares 2006). Until now successful *in vitro* isolation of *N. caninum* from various hosts has been achieved in approximately 80 occasions (Dubey *et al*. 2007). However, from the brain tissue of adult dairy cows the parasite was isolated only twice, in Japan (Sawada *et al*. 2000) and in New Zealand (Okeoma *et al*. 2004).

The present study described an *in vitro* isolation of *N. caninum* which was successfully performed from the brain tissue of naturally infected dairy cattle of the Slovak spotted breed. Anti-*Neospora* antibodies were detected in two serum samples by capture ELISA and clearly accompanied by the clinical feature of paresis. Western blot analyses revealed immuno-dominant pattern of 17; 29–32; 35–37 a 46 kDa with minor differences in two serum samples. Similar profiles were described by Álvarez-García *et al*. (2002) and Atkinson *et al*. (1999) who identified six major proteins in the range of 17–62 kDa. Cabaj *et al*. (2005) detected specific antibodies against a wide range of NC-1 tachyzoite antigens from 9.5 to 58 kDa in the blood of European bison.

Our isolate (NC-SKB1) had long incubation time from the moment of the inoculation of gerbil brain homogenate onto Vero cells to the first observation of free tachyzoites (77 days p.i.). On the other hand, isolation made directly from the brain homogenate of naturally infected cow *in vitro* was ineffective.

The first *in vitro N. caninum* tachyzoites originated from a naturally infected adult cow and were isolated in Japan from the brain tissues of a 2-year old Holstein cow. Nevertheless, histology did not reveal the presence of parasite but a mild perivascular cuffing and glial nodules in the central nervous system were observed. Tachyzoites of *N. caninum* (BT-3 strain) were detected in the Vero cell culture 39 days after inoculation with a brain homogenate of nude mice inoculated with tissue homogenate of cow's brain (Sawada *et al*. 2000). The second isolate of *N. caninum* was obtained from a 2 year-old Friesian cow in New Zealand (Okeoma *et al*. 2004), which was seropositive from day 150 of pregnancy until the birth. In the other cases, a bovine isolate was obtained from the brain tissue of aborted foetuses or calves. In the neighboring countries, the parasite was *in vitro* isolated only in Poland (NC-PolB1). Its brain homogenate was inoculated into Vero cells and *Neospora*-like tachyzoites were detected 66 days later (Goździk and Cabaj 2007).

In general, the parasite was detected mostly at the molecular level because *in vitro* isolation is more demanding. In aborted foetuses the risk problems are rapid tissue autolysis and a small account of tissue cysts in brain of adults. Tachyzoites may be destroyed during a long digestion procedure (Dubey and Schares 2006). Other factors influencing *in vitro* isolation are concentration and type of enzyme used and the duration of digestion. To date there is no standard protocol for this procedure. In our case a lower concentration of trypsin and shorted time of the digestion was used.

PCR tests allow a fast and highly sensitive identification of the parasite in naturally and experimentally infected animals (Müller *et al*. 1996, Collantes-Fernández *et al*. 2006). The single-step PCR using Np6 and Np21 primers targeted to the genomic region Nc5 was used to confirm *N. caninum* in our isolate NC-SKB1.

The sequence analyses allowed us to detect similarity between different individual isolates. Our first *N. caninum* isolate (NC-SKB1) have shown highly similar sequences with EU073600 isolate from outdoor chicken brains from Brazil. The first finding of *N. caninum* in chickens points to the important role of birds as intermediate hosts with serious epidemiological consequences (Costa *et al*. 2008).

In conclusion, we may assume that the acquisition of *in vitro* isolate provides broader potentialities for further studies. A better knowledge of this interesting topic, and particularly of the sequence analyses of isolates underlying the meaning of *in vitro* cultivation, could have considerable implications in molecular epidemiology of *Neospora caninum*.

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