Veterinary Parasitology 197 (2013) 346–349

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Short communication

First molecular detection of *Neospora caninum* in European brown bear (*Ursus arctos*)

A. Čobádiová, B. Víchová, V. Majláthová, K. Reiterová *

Institute of Parasitology of the Slovak Academy of Sciences, Košice, Slovakia

ARTICLE INFO

Article history:
Received 20 November 2012
Received in revised form 30 April 2013
Accepted 7 May 2013

Keywords:
Neospora caninum
Brown bear
Ursus arctos
PCR
Sequencing
Slovakia

ABSTRACT

Neospora caninum is an important intracellular protozoan parasite with an affinity to the central nervous system of many animals and a major causative agent of repeated abortions in cattle. In total, 45 muscle, liver, or spleen samples of brown bear sampled in different locations of Central Slovakia were examined by PCR. Genotyping of *N. caninum* was based on amplification of *Nc-5* gene and ITS1 rRNA fragments and subsequent sequencing. Presence of *N. caninum* DNA was confirmed in 24.4% (11/45) of tested animals. The results obtained in study confirm the first molecular evidence of *N. caninum* DNA in European brown bear (*Ursus arctos*) in Slovakia.

1. Introduction

Neospora caninum is a worldwide distributed protozoan intracellular parasite. It is considered a major causative agent of repeated abortions in dairy cattle (Dubey, 2003) and has got negative economic impact for their breeding. Its life cycle involves canines as definitive hosts and ruminants and many other species as intermediate hosts. Dogs (*Canis familiaris*), coyotes (*Canis latrans*) and grey wolves (*Canis lupus*) are natural definitive hosts of the parasite (McAllister et al., 1998; Gondim et al., 2004; Dubey and Schares, 2011). The oocysts shed in faeces of the definitive host may contaminate the environment and serve as a source of infection. However, the important way by which an animal can acquire the infection is transplacental (vertical) transmissions of the parasite from the dam to the foetus from mid gestation phase, resulting in persistently infected offspring which appear clinically normal, or post-natally via colostrum or milk (Dubey and Lindsay, 1996; Davison et al., 1999; Moskwa and Cabaj, 2007; Williams et al., 2009). Expelled foetal membranes after uncontrolled abortion, e.g. on the pasture, can be eaten by dogs or wild canines, and thus promote spreading of the infection. *N. caninum* has a wide range of intermediate hosts, including farm and free-living animals. The parasite has not previously been diagnosed in bears; according to Dubey and Thulliez (2005) antibodies to *N. caninum* were not found in 197 examined black bears (*Ursus americanus*) what indicated, that bears are not the hosts for *N. caninum*. From 29 brown bear (*Ursus arctos*) sera samples collected during the hunting season of 2001 in Scandinavia two sera tested positive, but the results were dubious since both sera were of bad quality and nonspecific cross-reactions could not be excluded (Åsbrink, 2002 ex. Björkman et al., 2010). European brown bear (*Ursus arctos*) is a large bear distributed across much of northern Eurasia and North America. Their principal range includes parts of the Carpathian region. The brown bears are highly omnivorous and they have been recorded to consume the greatest variety of food.

The aim of the present study was to confirm *N. caninum* infection in brown bears – exclusive free-living hosts in Slovakia.

* Corresponding author. Present address: Institute of Parasitology of the Slovak Academy of Sciences Hlinkova 3, 040 01 Košice, Slovakia. Tel.: +421 55 633 44 55; fax: +421 55 633 14 14.
E-mail address: reiter@saske.sk (K. Reiterová).

0304-4017/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.vetpar.2013.05.005

2. Materials and methods

2.1. Sample collection

Tissue samples (muscle, liver, or spleen) from 45 brown bears (*Ursus arctos*) shot within protective regulated shooting in the different localities in geographically dispersed montane and sub-montane areas of Central Slovakia, were collected by the professional hunters from May to December 2009 and delivered to our lab. Samples were stored in plastic vials with 70% ethanol until the isolation of genomic DNA.

2.2. DNA isolation

Isolation of genomic DNA was performed using NucleoSpin® Tissue kit (Macherey-Nagel, Germany) according to manufacturer's instructions with a previous tissue digestion with proteinase K (Promega, Madison, WI) at 56 °C, overnight.

2.3. PCR amplification

Detection of *N. caninum* was based on PCR amplification of 328 bp long portion of *Nc-5* gene performed with specific primer pairs Np6 and Np21 (Yamage et al., 1996). Additionally, positive samples were further analysed for the presence of internal transcribed spacer1 (ITS1) rRNA of *N. caninum* according to Bartley et al. (2013). The NC-1 strain of tachyzoites kindly provided by Prof. Jens G. Mattsson, Ph.D. (Department of Virology, Immunobiology and Parasitology, National Veterinary Institute, Uppsala, Sweden) was used as the positive control. Sterile water was added instead of tested DNA as negative control in each PCR assay. The reagents used for the preparation of PCR mix 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 2 μl of the sample. The amplification of Nc-5 gene was carried out under the following conditions: initial denaturation of templates at 95 °C for 7 min, followed by 35 cycles of denaturation (95 °C, 30 s), annealing (55 °C, 30 s) and extension (72 °C, 1 min), with a final extension at 74 °C for 10 min. Nested PCR amplification of ITS1 rRNA fragment ran under reaction conditions published by Bartley et al. (2013). The PCR products were electrophoresed on a 1% agarose gel stained with GelRed stain (Roche Diagnostics) and further visualized under the UV light.

Two, randomly chosen positive *Nc-5* amplicons and four ITS1 rRNA amplicons were purified using Qiagen DNA purification kit (QIAamp®DNA Mini Kit; QIAGEN, Gmb., Hilden, Germany) and sequenced at the Laboratory of Biomedical Microbiology and Immunology, (University of Veterinary Medicine and Pharmacy, Košice, Slovakia). The complementary strands of sequenced products were manually assembled. Sequences were compared with the GenBank entries by Blast N2.2.13. Identities of obtained nucleotide sequences were computed using EMBOOS pairwise alignment (needle method) after manual removing of gaps. Phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004). Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

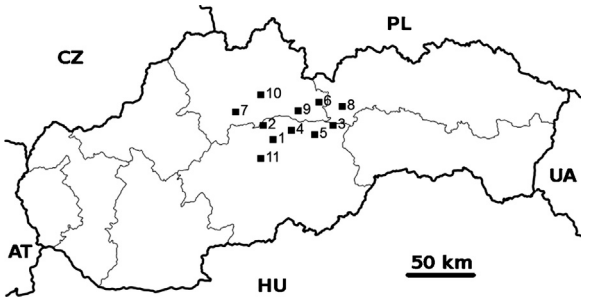

Fig. 1. The occurrence of *Neospora caninum* in European brown bears in Central Slovakia, (■) represents the localities where positive bears were shot. Abbreviations: AT – Austria; CZ – Czech Republic; PL – Poland; UA – Ukraine; HU – Hungary.

3. Results and discussion

Out of 45 examined bears, *Nc-5* gene of *N. caninum* was detected in 11 animals. Nine positive samples were detected in genomic DNA isolated from the liver and two from the spleen of tested bears shot in localities marked in Fig. 1.

Two nucleotide sequences of *Nc-5* gene fragments with a length of 287 and 295 bp, respectively were submitted to GenBank database (Accession Nos. KC143079; KC143080). They were nearly identical with each other within overlapped region as well as with the partial sequence of *Nc-5* gene previously obtained from brain of naturally infected adult female dairy cow in Slovakia (Reiterová et al., 2011). Blast analyses of obtained isolates revealed 94% identity with nucleotide sequences isolated previously from brain of cattle (FJ464412), chicken (EU735991) or whole blood of naturally infected European bison from Poland (HM031965).

Due to the lack of genomic DNA samples, we were able to further screen only 6 out of 11 *Nc-5* positive samples for the presence of ITS1 rRNA of *N. caninum*. Four tested samples provided positive ITS1 result. All these samples were purified as previously described and sequenced. Representative ITS1 rRNA sequence was added in GenBank under accession No. KC985243. All obtained sequences were 100% identical in overlapped region and within the 250 bp long aligned portion showed the identity with *N. caninum* ITS1 sequences from cow (JN634857), sheep (DQ832318), dog (AY665718) or dingo (GU194959) isolated previously elsewhere in the world. In order to show the phylogenetic relationships between *N. caninum* sequence obtained in this study and some other representatives from the family Toxoplasmatidae, we have constructed the phylogenetic tree based on the portion of ITS1 rRNA. *N. caninum* ITS1 rRNA sequence from European brown bear lied within the *N. caninum*-clade (Fig. 2).

To our knowledge this is the first molecular evidence of *N. caninum* DNA in European brown bear. The current