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# Isolation of *Neospora caninum* from kidney and brain of a bovine foetus and molecular characterization in Brazil

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## HIGHLIGHTS

- *N. caninum* can be isolated from kidney of a foetus.
- The new isolate, BNC-PR4 is a bovine isolate with low virulence.
- Multilocus-microsatellite genotyping revealed a unique genetic profile that differed from previously reported isolates.
- We report the first bovine isolate of *N. caninum* from kidney.

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## ABSTRACT

Bovine neosporosis has become a disease of international concern as it is among the main causes of abortion in cattle. Viable *N. caninum* has been isolated from brains of fetuses and neonatal calves, and there is no report of isolation of tachyzoites from kidney. Also, detailed information about the genetic diversity of *N. caninum* is scarce. *N. caninum* tachyzoites were isolated from the kidney and the brain of an aborted 4-month-old bovine foetus. The parasite was confirmed to be *N. caninum* by PCR. The tachyzoites of the new isolate, named BNC-PR4, were propagated in Vero cell cultures. Pathogenicity of the parasite was examined in BALB/c mice. Mice inoculated intraperitoneally with BNC-PR4 failed to yield clinical signs of disease and did not induce severe brain lesions, suggesting a bovine isolate with low virulence. The *N. caninum*-positive DNA sample was further analyzed by multilocus microsatellite (MS) genotyping for MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21. Multilocus-microsatellite genotyping revealed a unique genetic profile that differed from previously reported isolates.

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## 1. Introduction

*Neospora caninum* is a protozoan parasite of the phylum Apicomplexa, and has been recognized as a major cause of abortion and reproductive failure in cattle, in many countries (Dubey and Schares, 2011; Campero et al., 2015). Congenital infection has been shown to be important in the spread of *N. caninum* and it appears to be the major mode of transmission in cattle (Hall et al., 2005). The efficiency of vertical transmission is important for the persistence of *N. caninum* within herds and can result in abortion, weak and underweight calves, or as in most cases, asymptomatic congenitally infected newborns (Dubey et al., 2007).

Infection in a pregnant animal may result in abortion. Worldwide, these abortions are a major root cause of economic loss to both the dairy and beef industries (Larson et al., 2004; Dubey and Schares, 2011; Reichel et al., 2013). In addition to abortions, *N. caninum* causes neurological syndrome in calves (Locatelli-Dittrich et al., 2003; Santos et al., 2006). In Brazil, *N. caninum* DNA was detected in the brain of adult cattle with neurological

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signs, and the positive animals were from 20 municipalities in Parana state (Carvalho-Patrício et al., 2013), showing the wide distribution of the parasite.

*N. caninum* has been isolated in cell cultures inoculated with homogenates of brain and spinal cord of fetuses (Dubey and Schares, 2011), and there is no report of isolation of *N. caninum* from kidney.

Four studies report parasite isolation from bovine samples in Brazil. The parasite was isolated from a blind calf and from a 7-month-old aborted bovine foetus (Locatelli-Dittrich et al., 2003, 2004), from an asymptomatic calf (Nc-Goiás1) (García-Melo et al., 2009), and from an adult naturally infected cattle (NC-SP1) (Oliveira et al., 2017), in Parana, Goiás and São Paulo State, respectively. However, there are two reports of the isolation of the parasite from bovine samples and genetic study using polymorphic microsatellite markers (García-Melo et al., 2009; Oliveira et al., 2017). Recently, Brom et al. (2014) reported that there are genotypic differences in the strains of *N. caninum* that are responsible for foetal transmission in zebuine fetuses, in Goiás, Brazil.

Biological characterization of different laboratory-isolates of *N. caninum* has shown extensive genetic diversity (Schock et al., 2001; Regidor-Cerrillo et al., 2006, 2008) and significant variations in their *in vivo* pathogenicity and *in vitro* growth characteristics (Schock et al., 2001; Regidor-Cerrillo et al., 2006; García-Melo et al., 2009). Little is known of the population genetic structure of *N. caninum* (Goodswen et al., 2013).

In this study we describe the isolation of a new *N. caninum* isolate (BNC-PR4), obtained from an aborted foetus in Parana state (southern Brazil). The parasite was confirmed to be *N. caninum* by PCR. Pathogenicity of this new isolate, BNC-PR4, was also determined in BALB/c mice. The *N. caninum*-positive DNA sample was further analyzed by multilocus microsatellite (MS) genotyping for MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21.

## 2. Materials and methods

Procedures were conducted in accordance with the guidelines of the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Federal University of the State of Paraná, Brazil (protocol number 048/2014).

### 2.1. Herd, animals and sampling

In 2012 a study was conducted in a dairy herd (Holstein) located in Quatro Barras, Parana State, southern region of Brazil. The cows were milked two times per day, and were fed total mixed rations, corn silage and straw. Breeding was exclusively by artificial insemination with frozen semen, and the herd was free of brucellosis and tuberculosis. The dairy herd had a history of abortion. In 2012 blood was obtained from 52 adult cows by tail venipuncture. Sera were separated and stored at - 20 °C until assayed. An indirect fluorescent antibody test (IFAT) was used to detect *N. caninum* and *Toxoplasma gondii* antibodies.

### 2.2. Samples for isolation of Neospora caninum

Four aborted foetuses (1, 2, 3 and 4), from *N. caninum* seropositive cows, were sent to the Veterinary Hospital of the Federal University of Paraná in 2012. Samples of brain, heart, kidney and liver were obtained aseptically for tissue culture, and PCR.

### 2.3. Cell culture

Tissue samples collected from brain, heart, kidney and liver were processed for *Neospora* isolation. The isolation of *Neospora* was according to the methods described by Conrad et al. (1993). For *in vitro* cultivation, the samples were inoculated onto a 24-h cell monolayer culture of Vero cells. The cultures were maintained in DMEM medium supplemented with 2% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37 °C with 5% $CO_2$. Flasks containing cells were examined daily using an inverted microscope (Olympus) and every 2 days, the culture medium was changed.

Tachyzoites were first observed in Vero cells 20 and 10 days after inoculation with the brains and kidney homogenates from the foetus 2, respectively. The tachyzoites obtained from the cell culture were sub-passaged on Vero cells, and the number of parasites gradually increased in subsequent passages.

### 2.4. PCR of the isolate

After 75 days of isolation, a portion of the infected cells was scraped from the flasks (flasks with parasites isolated from brain and kidney) and subjected to polymerase chain reaction (PCR) examination. PCR was carried out using genomic DNA extracted from parasites grown in cell culture. DNA was extracted using a commercial kit (PureLink™ Genomic DNA Mini Kit; Invitrogen®) according to the manufacter's protocol. The primers Np6 and Np21 were used to amplify a 328 bp product of the Nc5 region of *N. caninum* DNA (Yamage et al., 1996). DNA from *N. caninum* NC-1 strain was used as positive control and DNA from Vero cells as negative control. PCR results were analyzed by 1% agarose gel electrophoresis stained with SYBR safe (Invitrogen, USA) to confirm product size. DNA extracted from tachyzoites harvested in cell cultures was positive by Np6/Np21 - PCR. This new isolate was designed BNC-PR4. Protozoa were maintained in Vero cell culture.

### 2.5. DNA extraction and genotyping

Genomic DNA was extracted from 20 to 50 mg of foetal brains using the commercial kit Maxwell® 16 Mouse Tail DNA Purification Kit, developed for automated Maxwell® 16 System (Promega, Wisconsin, USA) following the manufacturer's recommendations. The concentration of DNA for all samples was adjusted to 50–100 ng/μl. Detection of parasite DNA was previously tested by a nested PCR specific for the ITS-1 region of *N. caninum* as described by Buxton et al. (1998), using the amplification conditions and analytic procedures previously described (Regidor-Cerrillo et al., 2014).

Microsatellite analysis was performed on *N. caninum* PCR-positive DNA samples extracted from foetus 2. Specifically, MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12 and MS21 markers were amplified using specific primers and nested-PCR conditions previously described by Regidor-Cerrillo et al. (2013). The sizes of the PCR products for all microsatellites were determined using a 48-capillary 3730 DNA Analyser (Applied Biosystems, Foster City, CA) with GeneScan-500 (LIZ) Size Standars (Applied Biosystems) at the Unidad Genómica del Parque Científico de Madrid, and the results were analyzed with GeneMapper1 v3.5 software. The microsatellites MS7 and MS10 need also to be analyzed by sequencing and both markers were sequenced using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and a 3730 DNA Analyser (Applied Biosystems) at the Unidad Genómica del Parque Cientifico de Madrid for complete allele identification. Sequences were analyzed using BioEdit Sequence Alignment Editor v.7.0.1 software (Copyright, 1997–2004 Tom Hall, Ibis Therapeutics, Carlsbad, CA 92008, USA). Allele assignment was performed as described previously (Regidor-Cerrillo et al., 2013).

The eBURST software was used to explore the closest genetically related genotypes of Brazilian genotypes in a dataset involving 82