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Short communication

Genetic characterisation of *Neospora caninum* strains from clinical samples of zebuine foetuses obtained in abattoirs in Goiás, Brazil

Paula Rogério Fernandes Brom[a], Javier Regidor-Cerrillo[c], Esther Collantes-Fernández[c], Luis Miguel Ortega-Mora[c], Marcelo Sales Guimarães[a,*], Andréa Caetano da Silva[b,*]

[a] *Veterinary Parasitology Center/Veterinary and Zootechnics School, Federal University of Goiás, Caixa postal 131, CEP: 74001-970, Goiânia, Goiás, Brazil*
[b] *Veterinary Parasitology Laboratory/Department of Microbiology, Immunology, Parasitology and Pathology, Institute of Public Health and Tropical Pathology, Federal University of Goiás, Rua 235, s/n, Setor Universitário, CEP 74605-050, Goiânia, Goiás, Brazil*
[c] *SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain*

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ABSTRACT

The *Neospora caninum* microsatellite markers were applied to clinical samples. Genotyping technology involving fluorescently labelled DNA fragment analysis was used in combination with DNA sequencing for markers with complex repetitive sequences. Nineteen DNA samples from 15 brains and four hearts of naturally infected non-aborted zebuine foetuses from abattoirs in Goiás, Brazil. *N. caninum* had been detected in these foetuses by nested-PCR of the internal transcribed spacer-1 rRNA region, and the samples were analysed using these microsatellites. Seven complete or nearly complete allele profiles were obtained from six foetuses. Three distinct profiles of *N. caninum* were identified in a unique microregion (Meia Ponte) of Goiás. Two alleles for the same marker were detected in a unique foetus that was probably infected with two different strains. A new allele for one of the microsatellites is described. The multilocus analysis performed here revealed a preliminary means of discriminating between individual strains according to their geographical origins. These are the first results that have been obtained regarding the molecular characterisation of strains of *N. caninum* from infected zebuine foetuses in South America and reveal for the first time that there are genotypic differences in the strains that are responsible for foetal transmission in zebuine foetuses.

1. Introduction

Neospora caninum is a protozoan of the phylum Apicomplexa. It is considered to be the major cause of abortion in cattle (Dubey et al., 2007). The global distribution and broad host range of these parasites, in addition to their capacity for sexual reproduction, suggest that significant biological and genetic diversity exist in *N. caninum*. Indeed, biological diversity has been reported among some isolates

* Corresponding authors at: Center for Veterinary Parasitology, Veterinary and Zootechnics School, Federal University of Goiás, Campus Samambaia, Campus II, PO Box 131, 74001-970 Goiânia, GO, Brazil. Tel.: +55 62 3521 1574; fax: +55 62 3521 1839.
E-mail addresses: marsalvet@gmail.com (M.S. Guimarães), acaetanos@hotmail.com (A.C.d. Silva).

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regarding their capacities to produce pathology in experimental murine (Collantes-Fernández et al., 2006; Pereira García-Melo et al., 2010) and bovine infections (Rojo-Montejo et al., 2009a) and in *in vitro* studies (Pérez-Zaballos et al., 2005; Rojo-Montejo et al., 2009b).

Genetic diversity in *N. caninum* has also been shown with a variety of molecular techniques and genetic markers (Gondim et al., 2004; Regidor-Cerrillo et al., 2006; Pedraza-Díaz et al., 2009). Microsatellite sequences, also known as simple sequence repeats (SSRs), have been shown to be the most suitable polymorphic markers for the typing of *N. caninum* isolates and have been applied to genetically characterise sets of isolates that have been obtained from cattle and canines worldwide. Microsatellite sequences have allowed for the detection of extensive intra-species diversity within healthy and clinically infected animals (Regidor-Cerrillo et al., 2006, 2008; Al-Qassab et al., 2009; Basso et al., 2009; García-Melo et al., 2009; Pedraza-Díaz et al., 2009; Rojo-Montejo et al., 2009b). Microsatellite analysis has improved in recent years, and 12 polymorphic microsatellite loci for *N. caninum* that exhibit three to nine separate alleles have been described in isolates grown *in vitro* (Regidor-Cerrillo et al., 2006).

Nevertheless, detailed information about the genetic diversity of isolates of *N. caninum* from different geographical locations is scarce (Al-Qassab et al., 2009). The actual genetic diversity of *N. caninum* might be determined by the analyses of *N. caninum* isolates from infected but non-aborted foetuses as has been proposed for aborted foetuses by Pedraza-Díaz et al. (2009). If so, microsatellite typing could be used to identify infection sources in molecular epidemiological studies and to determine the sources of the isolates circulating in a delimited region (Basso et al., 2010). If applied to *N. caninum* isolates, this technique may aid the genetic characterisation and discrimination of different *N. caninum* isolates, which in turn, will be central to prevention, surveillance, and the application of suitable control measures for bovine neosporosis. Here, we report on the genetic analyses of *N. caninum* strains from clinical samples of zebuine (*Bos indicus*) foetuses that were naturally infected by vertical transmission.

2. Materials and methods

2.1. Sample collection, DNA extraction, and nested-PCR ITS-1 amplification

Tissue samples were obtained randomly from dead zebuine (*B. indicus*) foetuses at a local commercial slaughterhouse in Goiás. Data about the locations at which the foetuses were collected were recorded. A total of 195 foetuses were obtained during the slaughtering and used to collect 585 different fresh sections of brain, heart, and liver, which were stored at −80 °C until DNA extraction. DNA was prepared from 20 mg of tissue using a commercially available kit (Real Pure, Durviz, Patema, Spain) according to the manufacturer's instructions. The concentrations of DNA were determined by spectrophotometric analyses at A260/280, and all DNA samples were adjusted to a final concentration of 60 ng/mL. The DNA samples were stored at −20 °C until PCR analysis. All foetal DNA samples were subjected to an *N. caninum*-specific nested-PCR of the internal transcribed spacer 1 (ITS-1) sequence with the primers pairs NN1/NN2 and NP1/NP2 (Buxton et al., 1998). To avoid false positive reactions, DNA extraction, PCR sample preparation, and electrophoresis were performed in separate rooms using different sets of equipment and aerosol barrier tips. DNA from the cultured *N. caninum* tachyzoites was used as a positive control, and pure water was used as a negative control. The controls were included at all stages and for all batches.

2.2. Evaluation of parasite burden

Parasite loads were determined using real-time PCR on the brain, heart and the tissues that had previously been found to be positive by nested-PCR ITS-1. We used primer pairs from the *N. caninum* Nc-5 sequence to quantify the parasites and primers from the 28S rRNA gene to quantify the host DNA. Reactions for *Neospora* Nc5 and 28S rRNA were performed as described by Collantes-Fernández et al. (2002). The data were analysed with Sequence Detection System Software v.1.6 (PE Applied Biosystems, Foster City, CA), and the results were exported to Microsoft Excel for statistical analyses. The parasite numbers in the tissue samples (i.e., the parasite loads) are expressed as parasite number/μg host DNA. After amplification of the *N. caninum* Nc5 sequence, PCR product melting curves were acquired by a stepwise temperature increase from 55 to 95 °C over 20 min. The data were analysed using the Dissociation Curves 1.0f software (PE Applied Biosystems, Foster City, CA).

2.3. PCR amplification of microsatellites

The specimens that tested positive in the nested-PCR ITS-1 assays were further analysed with multilocus microsatellite typing for nine markers (MS4, MS5, MS6A, MS6B, MS7, MS8, MS10, MS12, and MS21) of *N. caninum* as described by Regidor-Cerrillo et al. (2006) and Pedraza-Díaz et al. (2009) to determine the genetic profiles of the *N. caninum* strains that infected the population analysed here. The primers were specific for the parasite and amplified a fragment of approximately 300 bp.

2.4. Automated allele sizing

Allele determination was performed for all microsatellite markers according to the sizes obtained by capillary electrophoresis. The allele sizes amplified in each sample were determined by fragment analysis using reverse primers that were fluorescent end-labelled with 6-FAM in the secondary PCR. The dilutions of PCR products were prepared in sterile distilled water to a concentration of approximately 1/10–1/20 ng/μL. Next, 13.75 μL of HiDi formamide and 0.25 μL of Gene Scan-500 (LIZ) Size Standards (Applied Biosystems, Foster City, CA) were added to 1 μL of each of the diluted PCR products. The sizes of the fluorescent PCR products were determined using a 48-capillary 3730 DNA analyser (Applied Biosystems) and the GeneScanTM 500 LIZ® Size Standard (Applied