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First genetic identification of *Cryptosporidium parvum* subtype IIaA14G2R1in beef cattle in Brazil

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ABSTRACT

The presence of *Cryptosporidium* spp. in a cattle herd registered with an outbreak of diarrhea was investigated and the the molecular subtyping of *Cryptosporidium parvum* was characterized. Fecal samples from 85 Nellore beef cattle (*Bos indicus*) were collected and examined with Ziehl–Neelsen modified staining method. Fifty-four cattle (63.52%) had *Cryptosporidium* spp. oocysts in their feces. Fragments of genes encoding the 18S ribosomal RNA subunit and a 60-kDa glycoprotein (*gp60*) were amplified by nested PCR accomplished in the 11 most heavily parasitized samples, and the amplicons were sequenced. Eight of the 11 analyzed samples were positive for 18S rRNA sequences and identified monospecific infections with *C. parvum*. Seven samples were positive for *gp60* and identified subtypes IIaA15G2R1 (6/11) and IIaA14G2R1 (1/11). This report is the first for *C. parvum* subtype IIaA14G2R1 in beef cattle in Brazil.

1. Introduction

Some species of *Cryptosporidium*, such as *Cryptosporidium parvum*, cause varying degrees of pathogenicity. *C. parvum* was first identified in mice by Tyzzer, 1912 and has since been recognized as the most common and pathogenic specie in cattle (Fayer et al., 2007; Plutzer and Karanis, 2009). Its presence has been reported in several regions around the world. Young cattle are the main reservoirs of zoonotic subtypes (Wang et al., 2011) contribute to the spread of the disease.

Sequences of genes encoding 18S ribosomal RNA (rRNA) enable the identification of species and genotypes of *Cryptosporidium*, and sequences of the DNA encoding the GP60 glycoprotein allow the discrimination between different zoonotic (IIa and IId) and non-zoonotic (IIb, IIc, IIe, and III) genotypes and subgenotypes of *C. parvum* (Plutzer and Karanis, 2009; Xiao, 2010). These diagnostic tools have identified *C. parvum* subtype IIa as the most prevalent subtype worldwide (Wang et al., 2011), especially in dairy cattle. Young cattle are the main reservoir for the highly zoonotic subtype IIaA15G2R1 (Xiao, 2010; Imre and Dărăbus, 2011). In Brazil, there are studies only in dairy cattle (Meireles et al., 2011; Couto et al., 2014), however, none in beef cattle raised on pasture.

The aim of this study was to investigate the presence of *Cryptosporidium* spp. in a cattle herd where an outbreak of diarrhea had been registered and to characterize the parasite population.

2. Material and methods

2.1. Study site and the diarrheal outbreak

A diarrheal outbreak occurred in November 2010 on a farm in the municipality of Ribas do Rio Pardo, Mato Grosso do Sul, Brazil, in a herd of Nellore beef cattle (*Bos indicus*) reared under intensive conditions. The herd consisted of 4000 breeding stock, 1500 of which were breeding cows. Cases of diarrhea began in calves 30–60 days of age. Sporadic cases were also reported in calves under 30 days old. The animals became prostrate and severely dehydrated, with liquid, fetid, dark stools. Blood and mucus were also observed in the stools in some cases. Some calves, especially those between 0 and 30 days old, were treated with gentamicin (4 mg/kg), intramuscular injection, for three consecutive days. This treatment, however,

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failed to prevent 25 deaths in the herd. Intermittent diarrhea was prominent in other groups, and fecal consistency improved in older animals.

Frequency tables were constructed and associations between *Cryptosporidium* spp. occurrence, age, sex, and symptoms of diarrhea were verified using a chi-square test ($P < 0.05$). Sex of the animal did not affect the rate of *Cryptosporidium* spp. infection ($P = 0.62$). Diarrhea was also not associated with parasitism ($P = 0.62$), demonstrating that *Cryptosporidium* spp. was not the sole or primary cause of the diarrhea outbreak. Age was the only variable found to be associated with *Cryptosporidium* spp. Infection: animals less than one month of age showed higher frequency of *Cryptosporidium* spp. infection (77.77%) ($P = 0.0285$) than animals from 1 to 4 months (55.26%); 4–12 months (70%), and 12–24 (50%) months of age, as determined by Fisher's exact test.

2.2. Fecal samples

Prior to the main investigation, 27 fecal samples had been sent to the microbiology laboratory of the School of Veterinary Medicine and Animal Science of the Federal University of Mato Grosso do Sul for culturing and antibiograms. These samples, however, were not evaluated for the presence of *Cryptosporidium* spp. *Escherichia coli* was detected in 11 of the samples (40.74%), and the antibiograms indicated the presence of *E. coli* sensitive to florfenicol, gentamicin, and amoxicillin + clavulanic acid. For the *Cryptosporidium* investigation, a second fecal collection was carried out directly from the rectums of 85 randomly selected animals in various age groups and stored in refrigerated boxes until their examination.

2.3. Laboratory tests

2.3.1. Parasitological diagnosis (modified Ziehl–Neelsen technique)

Duplicate microscopic smears were prepared for each fecal sample and stained using a modified Ziehl–Neelsen technique for the detection of *Cryptosporidium* spp. oocysts (Casemore et al., 1985). Oocysts were counted in 40 fields of view on each slide. The mean number of oocysts per field of view was ranked using the classification system described by Guimarães et al. (2009): zero (no oocysts), 1–5 (light infection), 6–10 (mild infection), 11–15 (moderate infection), 16–20 (high infection), and >20 (severe infection). Eleven of the samples from the most heavily (4 severe, 2 high and 1 mild infection) parasitized animals were selected for purification and subsequent molecular determination of the species and subtypes responsible for the infection. These aliquots were individually stored at 4 °C in 50-mL Falcon tubes containing a 5% solution of potassium dichromate.

2.3.2. Genomic DNA extraction

Oocysts were disrupted following the methodology described by Xiao et al. (2004); Coupe et al. (2005), with slight modifications. Briefly, pellets containing purified oocysts were resuspended in 200 μL of lysis buffer containing 12.5% Chelex-100 (Bio-Rad, Hercules, USA), 1% polyvinylpyrrolidone K-90 (PVP) (USB, Cleveland, USA), 10 mM Tris, 10 mM EDTA, and 10 μL of 10% sodium dodecyl sulfate. The samples were incubated at 99 °C for 20 min on a rotary shaker at 900 rpm followed by the addition of 30 μL of PVP-TE (10% PVP in Tris-EDTA buffer), 66.6 μL of 1 M KOH, and 18.6 μL of 1 M dithiothreitol and incubated at 65 °C for 20 min on the rotary shaker at 900 rpm. The solution was then neutralized with 8.6 μL of 25% hydrochloric acid. DNA was extracted using silica and guanidine thiocyanate (Sigma, St. Louis, USA) as described by McLauchlin et al. (2000).

2.3.3. Nested PCR

The amplification of a fragment of the 18S rRNA gene (Xiao et al., 2001) and GP60 gene (Peng et al., 2003) of *Cryptosporidium* by nested PCR was performed in a volume of 25 μl containing 1 × PCR buffer, 2.5 μl of DNA template, 1.0 unit of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, USA), 2.5 mM MgCl2, 200 μM each dNTP, 0.6 μg/μl of non-acetylated bovine serum albumin (BSA) (Sigma, St. Louis, USA), and 200 nM each primer. The conditions for amplification were as following: 2 min at 94 °C, followed by 40 cycles of 45 s at 94 °C, 45 s at 55 °C (18S rRNA gene) or 50 °C (GP60 gene) and 60 s at 72 °C, followed by 72 °C for 7 min, using the Mastercycler® ep realplex (Eppendorf, Hamburg,Germany). The secondary PCR conditions were the same as the conditions of the primary reaction except for the absence of BSA. Ultrapure autoclaved water and bovine *C. parvum* DNA, previously identified as type B 18S rRNA (GenBank EF175936) and as subtype IIaA15G2R1 (GenBank EF175937), were used as negative and positive controls, respectively.

2.3.4. DNA sequencing

Sequencing accuracy was confirmed by sequencing secondary PCR products from at least two reactions in both directions on an ABI377 automated sequencer using an ABI Big Dye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The sequencing reactions used the same primers as those for the nested PCRs (Peng et al., 2003). The amplicon sequences were aligned with reference sequences for *Cryptosporidium* spp. using Clustal W (Thompson et al., 1997). The degree of sequence similarity was determined using the BioEdit program (Hall, 1999) to identify *Cryptosporidium* species, alleles, and subtypes.

3. Results

Of the 85 animals examined, 54 (63.52%) had *Cryptosporidium* spp. oocysts in their feces. Of these, 81.48, 12.96, 0.00, 1.85, and 3.70% had light, mild, moderate, high, and severe infections, respectively (Table 1). The 18S rRNA sequences indicated monospecific infections with *C. parvum*. Attempts to amplify this gene fragment failed in three microscopically positive samples (animals I, J, and K) collected from calves aged between 4 and 24 months (Table 2).

Of the 11 samples analyzed by nested PCR, seven (63.63%) were positive for *gp60* amplification, all from animals 0–4 months old (Table 2). The sample from animal B was positive following microscopic examination and nested PCR of the 18S rRNA gene, but *gp60* could not be amplified. All samples from animals older than four months (I, J, and K) and a sample from an animal younger than one month (B) that were positive in the microscopic examination were negative for PCR amplification of the *gp60* gene. PCR-positive samples were sequenced, and infections with the *C. parvum* subtypes IIaA15G2R1 (85.71%) and IIaA14G2R1 (14.28%) were identified (Table 2).

4. Discussion

Protozoa of the genus *Cryptosporidium* have been a subject of numerous taxonomic studies, in which new species or genotypes in a variety of hosts and reservoirs have been identified. Human infections have been reported, increasing the epidemiologic importance of the parasite (Navarro-i-Martinez et al., 2011). The identification and molecular characterization of *Cryptosporidium* spp. in intensively farmed beef cattle in Brazil, however, have received little attention.

The analysis of 18S rRNA gene sequences demonstrated monospecific infection by *C. parvum*. Our analysis cannot be extrapolated to all cattle on the property, so other species of the genus may