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Research paper

First description of *Cryptosporidium hominis* GP60 genotype IkA20G1 and *Cryptosporidium parvum* GP60 genotypes IIaA18G3R1 and IIaA15G2R1 in foals in Brazil

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ABSTRACT

The present study focuses on *Cryptosporidium* infections of foals in Brazil. A total of 92 animals of different breeds from 11 farms in the vicinity of Araçatuba in the state of São Paulo, were examined. According to PCR targeting the 18S rRNA gene, *Cryptosporidium* sp. DNA was detected in 21.7% (20/92) of foals. Good quality 18S rRNA, actin, HSP70 and gp60 genes nPCR amplicons were obtained from five fecal samples. PCR amplification and sequencing of a fragment of the GP60 sporozoite surface glycoprotein gene revealed *C. parvum* genotypes IIaA18G3R1, IIaA15G2R1. Interestingly, we also detected in two foals a GP60 genotype related to the human parasite *C. hominis*.

1. Introduction

Protozoa in the genus *Cryptosporidium* are important pathogens that cause diarrhea in humans, bovines and others species of animals worldwide (Thompson et al., 2005).

Cryptosporidiosis in immunocompetent individual is a self-limiting disease, but in infants, young animals and immunocompromised individulals it may cause a debilitating infection. Cryptosporidiosis typically causes aqueous diarrhea and weight loss (Johnson et al., 1997). In equines, clinical manifestations are rare (Olson et al., 1997).

There are currently 26 named species of *Cryptosporidium* (Ryan et al., 2014). About 61 genotypes, defined based on host specificity and the sequence of genetic markers (Plutzer and Karanis, 2009) have been reported. Cryptosporidiosis in equines was first described in Arab foals with diarrhea (Snyder et al., 1978). In Brazil, a previous study using nested PCR for detecting *Cryptosporidium* in equines found no positive animals (Sevá et al., 2010). Equine infections are attributed to *C. parvum* and to what has been named "horse genotype" (Santin, 2013). A recent report described a *C. hominis* infection in a horse from Algeria (Laatamna et al., 2015). The

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detection of *Cryptosporidium* parasites taxonomically related to *C. hominis* in equines raises new questions about the epidemiology of equine cryptosporidiosis and the zoonotic potential of *Cryptosporidium* parasites infecting equines. The present study aimed to assess the prevalence of *Cryptosporidium* infections in foals in Brazil and investigate the taxonomic status of equine isolates.

2. Materials and methods

2.1. Study description

Fecal samples were collected from all foals one year of age or younger raised on 11 randomly selected farms in the municipalities of Araçatuba, Birigui, Guararapes and Santo Antônio do Aracanguá in the northwestern region of São Paulo State, Brazil (Fig. 1), from November 2010 to March 2011. A total of 92 animals of different breeds were examined. Fifty six foals were male and 36 female, aged from three to 330 days. Foals were allocated to two age groups: ≤60 days and >60 days. This study was approved by the Animal Experimentation Ethics Committee of Araçatuba School of Dentistry (FOA), UNESP- Universidade Estadual Paulista Julio de Mesquita Filho (approval n°. 2009-002165).

Fecal samples were collected directly from the rectal ampulla and stored at −20 °C for subsequent DNA extraction and PCR analysis. No microscopic examination or immunofluorescence analyses were performed. To describe the extent of hydration of fecal samples, the following classification was adopted: solid, semi-solid, pasty or liquid. Information related to fecal conditions was inserted into a data bank correlating fecal consistency, age and presence of *Cryptosporidium* DNA. Fecal samples were concentrated by centrifugal sedimentation in water/ether (Meloni and Thompson, 1996) and stored in 5% dichromate for subsequent DNA extraction. To remove the dichromate prior to DNA extraction, approximately 5% of the samples was mixed by vortexing and washed multiple times by centrifugation at 2000*g* for 2 min and resuspending in water.

2.2. Fecal DNA extraction

DNA was extracted from 92 foal fecal samples processed as described above using the QIAamp® DNA Stool Mini Kit (Qiagen). Prior to extraction, samples in ASL buffer were frozen in liquid nitrogen and thawed at 95 °C five times. DNA was eluted in 50 μl of AE buffer and stored at −20 °C.

2.3. Molecular characterization

A fragment of the 18S rRNA gene was amplified by nested PCR (nPCR) as described (Xiao et al., 2000). *Cryptosporidium* species were identified by sequencing the rRNA PCR products. Samples initially classified as *Cryptosporidium* based on the 18S rRNA sequence were subjected to nPCR analysis targeting the gp60 gene (Glaberman et al., 2002), the actin gene (Sulaiman et al., 2002), and the HSP70 gene (Morgan et al., 2001). *C. parvum* DNA was amplified in parallel as a positive nPCR control. Negative control PCRs without added DNA were included throughout.

Good quality 18S rRNA, actin, HSP70 and gp60 genes nPCR amplicons were obtained from five fecal samples. These were purified using a QIAquick® Gel Extraction kit (Qiagen) and sequenced bidirectionaly at the Center for Sequencing and Functional Genomics of UNESP, Jaboticabal Campus. The amplicons were sequenced using the "ABI Prism® Dye Terminator 3.1" protocol (Applied Biosystems) using a ABI 3730XL sequencer (Applied Biosystems). The primers used for sequencing were the same as used for the secondary nPCR. To identify the species and genotypes, the amplicon nucleotide sequences were aligned using Clustal X (Thompson et al., 1997) with sequences of *Cryptosporidium* spp. downloaded from GenBank. The phylogenetic analysis was performed using MEGA version 5.1 (Kumar et al., 2004). Phylogenetic trees (Fig. 2) were generated using the Neighbor-Joining method based on Kimura's two-parameter model with bootstrap values obtained from 1000 replicates.

Table 1
Origin and genotype of PCR positive foals.

Location	18S	gp60	actin	HSP70
Araçatuba	*C. parvum*	IIaA18G3R1	*C. parvum*	*C. parvum*
Birigui	*C. parvum*	IIaA15G2R1	*C. parvum*	*C. parvum*
Birigui	*C. parvum*	IIaA15G2R1	*C. parvum*	*C. parvum*
Araçatuba	*C. hominis*	IkA20G1	*C. hominis*	*C. hominis*
Araçatuba	*C. hominis*	IkA20G1	*C. hominis*	*C. hominis*

2.4. Nucleotide sequence accession number

The nucleotide sequences generated in this study were deposited in GenBank ™ under accession numbers KT948746, KT948747, KT948748, KT948749, KT948750, KT948751 and KT948752.

2.5. Statistics

The association between occurrence of *Cryptosporidium* infection and the variables sex, age and fecal consistency were analyzed with the Chi-square (χ^2) test or Fisher's Exact test, employing the software SAS and adopting 5% as significance level.

3. Results

According to PCR targeting the 18S rRNA gene, 21.7% (20/92) of the foals were *Cryptosporidium* positive. An 18S rRNA amplicon sequence was obtained from 5 of 20 nPCR positive samples. No association between *Cryptosporidium* infection and sex, age and fecal consistency was found ($P \geq 0.05$). The actin and HSP70 sequence from a fecal sample of a 10 day old filly was characterisitic of *C. parvum.* Sequencing of a portion of the sporozoite surface glycoprotein GP60 revealed the presence of genotype IIaA18G3R1 in this animal (Fig. 2). The actin and HSP70 sequence from two foals 35 and 75 days of age, respectively, were identified as *C. parvum.* Sequencing of a portion of the sporozoite surface glycoprotein GP60 revealed the presence of genotype IIaA15G2R1 (Fig. 2). The actin and HSP70 from two samples from foals four and five months of age, respectively, were identified as *C. hominis.* Sequencing of a portion of the sporozoite surface glycoprotein GP60 revealed the presence of genotype IkA20G1 (Fig. 2). The results are summarized in Table 1.

4. Discussion

The PCR analysis of fecal DNA originating from 92 foals indicates that *Cryptosporidium* parasites are present in the horse population we surveyed. The GP60 genotypes IIa (Fig. 2) we found in our study is typically found in zoonotic *C. parvum* (Nazemalhosseini-Mojarad et al., 2012). The same IIa genotype was also found foals in New Zealand (Grinberg et al., 2008) indicating that it is geographically widespread in equines. In *C. hominis*, the most common GP60 genotypes are designated Ia, Ib, Id, Ie and If (Xiao and Ryan, 2007). The GP60 Ik genotype was previously found in equines in Algeria (Laatamna et al., 2015). Because *C. hominis* typically does not infect animals, it is unclear whether the genotype we and Laatamna et al., 2015 detected is infectious to humans.

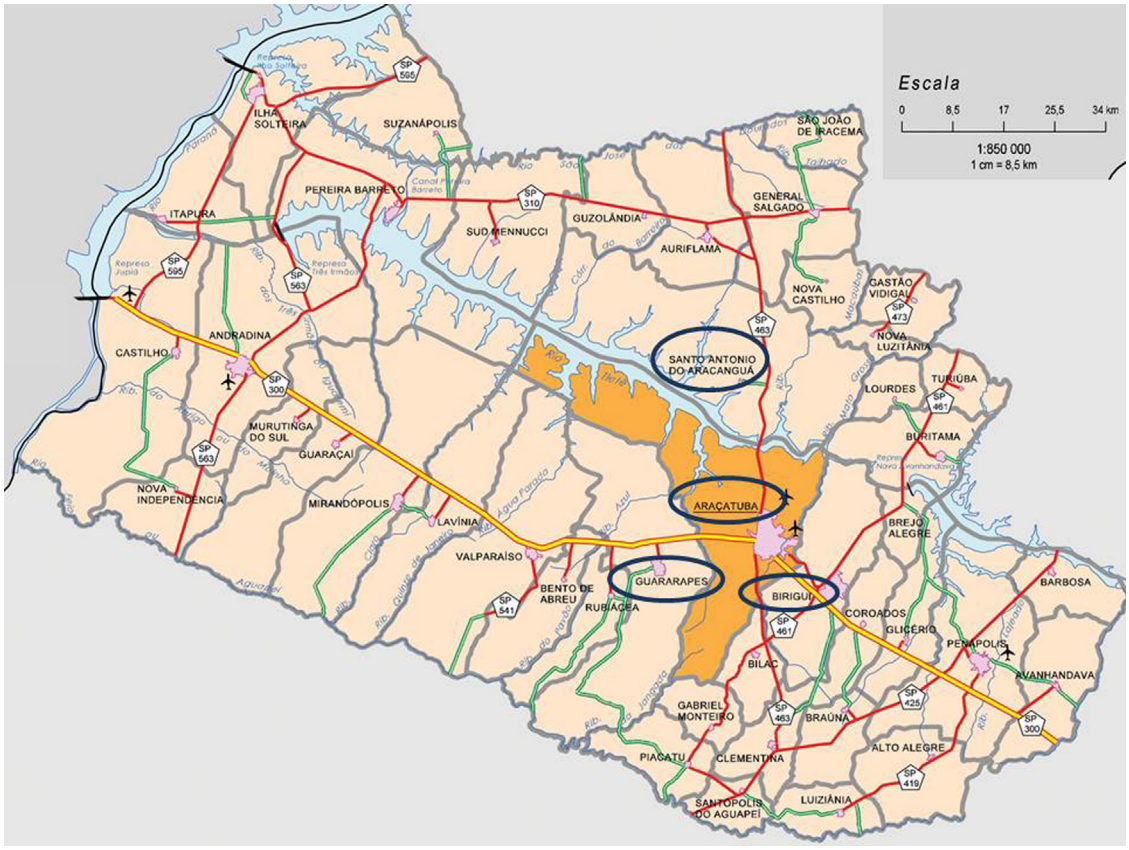

Fig. 1. Map of the District of Aracatuba in Sao Paulo state showing collection sites.

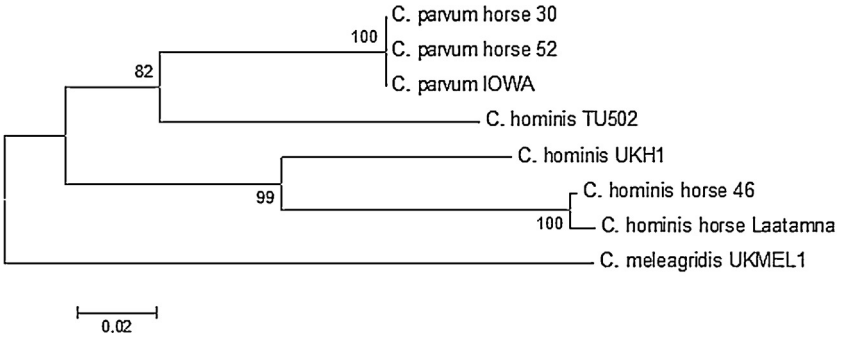

Fig. 2. Neighbor-joining phylogenetic tree based on a 173-nucleotide sequence from the GP60 gene shows the similarity between horse isolate 46 from this study and a recently described equine isolate from Algeria (Laatamna et al., 2015). Bootstrap values based on 1000 replicates are shown.

The three *C. parvum* positive samples we found were from different farms, however the two samples infected with what appears to be *C. hominis* (Table 1) originate from the same property. It is interesting to note that adult horses from the property where *C.hominis* infected foals were identified drank water from the Baguaçu River in the city of Araçatuba, São Paulo. The river receives untreated urban wastewater (Milani, 2007). Therefore, adult horses may acquire *C. parvum* and possibly *C. hominis* from the river and represent a possible source of infection for foals on this property through direct fecal-oral transmission as *Cryptosporidium* positive foals drank water from artesian wells. The possibility that untreated urban wastewater can be the source of equine cryptosporidiosis remains to be investigated. Also remains to be elucidated is whether the same *C. hominis* genotype is found in surface water and whether oocysts excreted by there animals are potentially infectious to humans.

C. parvum gp60 genotype IIaA18G3R1 was observed in a 10 days old foal that showed no evidence of diarrhea (Table 1). This genotype was also found in foals with diarrhea in New Zealand (Grinberg et al., 2008). The IIaA15G2R1 genotype was identified in the Czech Republic and in Poland in a 3 year old asymptomatic equine (Wagnerová et al., 2015) and also in fecal samples from the environment in Italy (Galuppi et al., 2015).

The epidemiology of equine cryptosporidiosis is not well studied. Routes of transmission, risk factors, clinical signs, prevalence and economic impact (Veronesi et al., 2010) remain to be characterized. However neonatal infections are frequent and according to two studies occur in about 50% of the cases of foal diarrhea in (Grinberg et al., 2009; Perrucci et al., 2011).

The identification of a GP60 genotype IkA20G1 (Fig. 2). in our survey may indicate the presence of *C. hominis* or *C. hominis*-like parasites in horses. Interestingly, a very similar GP60 sequence was recently identified in a horse in Algeria (Laatamna et al., 2015)

(Fig. 2). As found by these authors, in our study the HSP70 sequence of horse isolate 46 was identical to a sequence found in *C. hominis*. In the absence of any biological characterization of *Cryptosporidium* parasites from horses related to *C. hominis*, it is unknown whether the horse plays a role in the epidemiology of *C. hominis*. The taxonomic significance of this observation remains to be investigated.

5. Conclusion

The identification of *C. hominis*-like parasites in foals confirms a previous case from Algeria and indicates that this genotype is widely distributed. This is the first report of infections of foals with *C. hominis*-like parasites and with *C. parvum* GP60 genotypes IIaA18G3R1 and IIaA15G2R1 from Brazil.

Conflict of interest

The authors declare no conflict of interest.

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