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Research paper

Common occurrence of *Cryptosporidium hominis* in horses and donkeys

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ABSTRACT

Extensive genetic variation is observed within the genus *Cryptosporidium* and the distribution of *Cryptosporidium* species/genotypes in humans and animals appears to vary by geography and host species. To better understand the genetic diversity of *Cryptosporidium* spp. in horses and donkeys, we characterized five horse-derived and 82 donkey-derived *Cryptosporidium* isolates from five provinces or autonomous regions (Sichuan, Gansu, Henan, Inner Mongolia and Shandong) in China at the species/genotype and subtype levels. Three *Cryptosporidium* species/genotypes were identified based on the analysis of the SSU rRNA gene, including *Cryptosporidium parvum* (n = 22), the *Cryptosporidium* horse genotype (n = 4), and *Cryptosporidium hominis* (n = 61). The identification of *C. hominis* was confirmed by sequence analysis of the HSP70 and actin genes. Subtyping using sequence analysis of the 60 kDa glycoprotein gene identified 21 *C. parvum* isolates as subtype IIdA19G1, the four horse genotype isolates as subtypes VIaA15G4 (n = 2) and VIaA11G3 (n = 2), and the 61 *C. hominis* isolates as IkA16G1 (n = 59) and IkA16 (n = 2). The common finding of *C. hominis* reaffirms the heterogeneity of *Cryptosporidium* spp. in horses and donkeys and is possibly a reflection of endemic transmission of *C. hominis* in these animals. Data of the study suggest that horses and donkeys as companion animals may potentially transmit *Cryptosporidium* infections to humans.

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1. Introduction

Cryptosporidium spp. are important zoonotic parasites responsible for diarrhea in humans and animals. Significant morbidity and mortality caused by cryptosporidiosis have been reported in HIV/AIDS patients and children (Sow et al., 2016). The infective oocysts of *Cryptosporidium* spp. from humans and animals are ubiquitous in the environment, and cryptosporidiosis can be acquired via the fecal–oral route directly from infected persons or animals or indirectly from food or water contaminated by feces of infected hosts (Fayer et al., 2013). A single *Cryptosporidium* oocyst was reported to have the ability to initiate infection in immunosuppressed persons while as few as 10 oocysts caused clinical symptoms in immunocompetent persons (Graczyk et al., 1997).

Extensive genetic variation exists within the genus *Cryptosporidium*. Currently, 30 *Cryptosporidium* species and more than 40 genotypes have been described. Among them, >20 have been reported in humans, with *Cryptosporidium hominis* and *Cryptosporidium parvum* responsible for the majority of infections (Ryan et al., 2014, 2015; Li et al., 2015; Kváč et al., 2016; Holubová et al., 2016). The distribution of *Cryptosporidium* species/genotypes in humans and animals appears to vary by geography and host species. For example, in humans, both *C. parvum* and *C. hominis* are common in Europe and New Zealand while *C. parvum* predominates in the Middle East and *C. hominis* predominates in developing countries (Xiao, 2010). To track the infection sources, assess transmission dynamics of *Cryptosporidium* spp., and explore the association between subtypes and virulence or clinical presentations, sequence analysis of the 60-kDa glycoprotein (gp60) gene has been used extensively in subtyping *C. hominis*, *C. parvum*, and related species (Ryan et al., 2014). At present, 16 *Cryptosporidium* species/genotypes have been successfully subtyped at the gp60 locus and named according to the terminology initially developed by Sulaiman et al. (2005). The Roman numeral letters I, II, III, IV, V, VI, VII, VIII, IX, X, XI, XII, XIII, XIV, XV, and XVI (KP099095) have been used in referring to subtype families of *C. hominis*, *C. parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium fayeri*, *Cryptosporidium cuniculus*, *Cryptosporidium* horse genotype, *Cryptosporidium wrairi*, *Cryptosporidium* ferret genotype, *Cryptosporidium tyzzeri*, *Cryptosporidium* mink genotype, *Cryptosporidium* opossum genotype I, *Cryptosporidium ubiquitum*, *Cryptosporidium erinacei*, *Cryptosporidium* chipmunk genotype I, *Cryptosporidium viatorum*, and *Cryptosporidium*

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skunk genotype, respectively (Ryan et al., 2014; Guo et al., 2015; Stensvold et al., 2015).

Domestic horses and donkeys are common worldwide. They are usually used for draft, food and pets. Horses and donkeys can be infected with host-specific and zoonotic parasites including *Cryptosporidium* spp. To date, nine *Cryptosporidium* species/genotypes have been identified in horses, including *C. parvum*, *Cryptosporidium* horse genotypes, *C. erinacei*, *Cryptosporidium muris*, *C. hominis*, *C. tyzzeri*, *Cryptosporidium felis*, *C. ubiquitum* and *Cryptosporidium andersoni* (Cohen and Snowden, 1996; Majewska et al., 1999; Liu et al., 2015). Compared to cryptosporidiosis in horses, our understanding of cryptosporidiosis in donkeys is poor, especially the identity of species that may be present; only two *Cryptosporidium* species (*C. parvum* and *C. muris*) have been identified thus far (Laatamna et al., 2015).

In mainland China, only two studies characterized a small number of *Cryptosporidium* isolates from horses (Liu et al., 2015; Qi et al., 2015a). To understand the distribution and genetic diversity of *Cryptosporidium* species/genotypes in horses and donkeys in China, we genotyped and subtyped 87 *Cryptosporidium* isolates from multiple areas.

2. Materials and methods

2.1. Ethics statement

This study was carried out according to the Chinese Laboratory Animal Administration Act of 1988. The research protocol was reviewed and approved by the Research Ethics Committee of the Henan Agricultural University. Prior to conducting this study, we contacted the owners or managers of these animals and obtained their permission. No specific permits were required for the described field studies based on the fact that all the animals involved in this study did not belong to endangered or protected species.

2.2. Source of Cryptosporidium isolates

During the period of March 2008 to November 2013, 87 *Cryptosporidium*-positive specimens identified by microscopy (5 from horses and 82 from donkeys) were obtained from five provinces or autonomous regions (Sichuan, Gansu, Henan, Inner Mongolia and Shandong) in China. The isolates were from intensive farms, trade markets, grazing farms, stud farms and individual owners as well as one zoo. At the time of sampling, information on age and health status of each animal was collected. The host and geographical distribution of *Cryptosporidium* isolates is shown in Table 1.

Table 1
Distribution of *Cryptosporidium* species/genotypes and subtypes in horses and donkeys in China in this study.

Host and collection site	Species/genotypes (n)	Subtype (n)
Horse		
Sichuan	*C. parvum* (3)	IIdA19G1 (3)
Inner Mongolia	*C. parvum* (1)	–
Gansu	*C. hominis* (1)	IkA16G1 (1)
Subtotal	*C. hominis* (1); *C. parvum* (4)	IkA16G1 (1); IIdA19G1 (3)
Donkey		
Henan	*C. parvum* (4)	IIdA19G1 (4)
	C. hominis (7)	IkA16G1 (5); IkA16 (2)
	Horse genotype (2)	IkA11G3 (2)
Shandong	*C. parvum* (14)	IIdA19G1 (14)
	C. hominis (53)	IkA16G1 (53)
	Horse genotype (2)	VIaA15G4 (2)
Subtotal	*C. hominis* (60); *C. parvum* (18); Horse genotype (4)	IkA16G1 (58); IkA16 (2); IIdA19G1 (18); VIaA15G4 (2); VIaA11G3 (2)

The bars denote that subtyping was unsuccessful.

All fecal specimens positive for *Cryptosporidium* oocysts were stored in 2.5% potassium dichromate at 4 °C, after diluted fecal materials were filtrated through a sieve and centrifuged at 1500*g* for 10 min. *Cryptosporidium* oocysts in fecal suspension were concentrated by using the Sheather's sucrose flotation method.

2.3. Genomic DNA extraction from Cryptosporidium oocysts

Fecal specimens were washed off with potassium dichromate with distilled water by centrifugation. Genomic DNA was extracted from 200 mg specimen using an E.Z.N.A.R® Stool DNA Kit (Omega Biotek Inc., Norcross, GA, USA) and manufacturer-recommended procedures. Eluted DNA was stored in the freezer at −20 °C until PCR analysis.

2.4. Cryptosporidium genotyping and subtyping

Cryptosporidium species/genotypes in extracted DNA were identified by PCR amplification of an approximately 830 bp fragment of the SSU rRNA gene and restriction fragment length polymorphism (RFLP) analysis using restriction enzymes *Ssp*I and *Vsp*I (Xiao et al., 2001). The diagnosis was confirmed by DNA sequencing of the secondary PCR products. The identification of *C. hominis* was confirmed by PCR and sequence analysis of the partial (approximately 1950 bp) 70 kDa heat shock protein (HSP70) and actin (approximate 1066 bp) genes (Sulaiman et al., 2000, 2002). In some analysis, two new sets of primers for nested PCR amplification of 1005–1033 bp of the HSP70 gene were used, including F1 (TCTGCGCTGATTACTTCCG) and R1 (CATACCGCC TGGCATACC) in primary PCR and F2 (CGTGCACTTTAGCTCCAGT) and R2 (GCATACCACCTGGCATTC) in secondary PCR. All *Cryptosporidium* isolates were further subtyped by PCR analysis of an approximately 850 bp fragment of the gp60 gene as described previously (Alves et al., 2003).

2.5. Nucleotide sequence analysis

All secondary PCR products were sequenced directly on an ABI PRISM™ 3730 XL DNA Analyzer (Applied Biosystems, USA) by using the secondary PCR primers and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence accuracy was confirmed by bi-directional sequencing and sequencing of a new PCR product if necessary. Nucleotide sequences obtained in the present study were aligned with each other and reference sequences downloaded from GenBank database using the ClustalX 1.83 (http://www.clustal.org/) to determine *Cryptosporidium* species/genotypes and subtypes. The terminology initially developed by Sulaiman et al. (2005) and recently updated by Ryan et al. (2014) was used in naming gp60 subtype families and subtypes.

2.6. Phylogenetic analysis

To assess the genetic relationships of nucleotide sequences of *Cryptosporidium* obtained here to known species/genotypes/subtypes, phylogenetic analysis was performed by constructing neighboring-joining trees of the SSU rRNA and gp60 genes using the program Mega 5 (http://www.megasoftware.net/) based on the evolutionary distances calculated by Kimura-2-parameter model. The reliability of the trees was assessed using the bootstrap analysis with 1000 replicates.

3. Results

3.1. Cryptosporidium species/genotypes

PCR amplification was successful for all 87 specimens at the SSU rRNA locus. By DNA sequencing of the PCR products, four types of nucleotide sequences were obtained, with 13 polymorphic sites among them. Two types (KU200953 and KU200956) of sequences from 22 isolates (25.3%, 22/87) belonged to *C. parvum* with one nucleotide difference

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