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SHORT COMMUNICATION

Occurrence and Genetic Characteristics of *Cryptosporidium hominis* and *Cryptosporidium andersoni* in Horses from Southwestern China

Lei Deng[a], Wei Li[a], Zhijun Zhong[a], Chao Gong[a], Xuefeng Cao[a], Yuan Song[a], Wuyou Wang[a], Xiangming Huang[b], Xuehan Liu[a], Yanchun Hu[a], Hualin Fu[a], Min He[a], Ya Wang[a], Yue Zhang[b], Kongju Wu[b] & Guangneng Peng[a]

a The Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan Province 611130, China

b Chengdu Giant Panda Breeding Research Base, Chengdu, Sichuan Province 625001, China

Keywords

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Correspondence

K. Wu, Chengdu Giant Panda Breeding Research Base, Chengdu, Sichuan Province 625001, China
Telephone number: 13568988237;
FAX number: 86-028-83510011;
e-mail: 646401864@qq.com

G. Peng, The Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan Province 611130, China
Telephone number: 13981606712;
FAX number: 86-028-86291162;
e-mail: pgn.sicau@163.com

Lei Deng, Wei Li, Zhijun Zhong and Chao Gong contributed equally to this work.

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ABSTRACT

A total of 333 fecal specimens from horses in southwestern China were genotyped based on analysis of the small subunit rRNA (*SSU* rRNA) gene. *Cryptosporidium hominis* and *Cryptosporidium andersoni* were identified in 2 and 4 stool specimens, respectively. The identification of *C. hominis* was confirmed by sequence analysis of the 70-kDa heat shock protein (*HSP70*) and oocyst wall protein (*COWP*) genes. Subtyping analysis of the 60-kDa glycoprotein (*GP60*) gene sequence of *C. hominis* revealed a new rare subtype Id, named IdA15; only three Id isolates have been reported in humans to date. Multilocus sequence typing (MLST) analysis indicated that the *C. andersoni* subtype was A6, A5, A2, and A1 at the four minisatellite loci (MS1, MS2, MS3, and MS16, respectively). This is the first report to identify the presence of *C. andersoni* and *C. hominis* in horses in southwestern China and the first to identify a rare zoonotic subtype Id of *C. hominis* in horses. These findings suggest that infected horses may act as potential reservoirs of *Cryptosporidium* to transmit infections to humans.

CRYPTOSPORIDIUM spp. are common protozoan parasites that cause diarrhea in a wide variety of vertebrate animals. Although *Cryptosporidium* was initially discovered by Tyzzer in 1907, it was not recognized as an important parasite for the next 50 yr (Tyzzer 1907). Subsequently, *Cryptosporidium* was also identified in turkeys, calves and humans with diarrhea, and received more attention around the world (Current et al. 1983; Panciera et al. 1970; Slavin 1955). *Cryptosporidium* attracted great public health attention in the large waterborne outbreak in Milwaukee (U.S.A.) in 1993, where over 400,000 people were infected (Kenzie et al. 1995). In young calves, high morbidity caused by *Cryptosporidium* can lead to substantial production losses to the livestock industry (Graaf et al. 1999).

Most animals become infected through direct contact with an infected host, or through indirect ingestion of oocyst-contaminated water or foods (Cacciò 2005; Fayer et al. 2000). Although clinical presentations of cryptosporidiosis are often self-limiting or sometimes asymptomatic in healthy individuals, fatal and chronic diarrhea

can occur in immunocompromised or immunosuppressed individuals (Petri et al. 2008). Recent molecular epidemiologic surveys of cryptosporidiosis from different host species have identified at least 30 valid species and more than 70 genotypes, with new genotypes continually being found (Ryan et al. 2014, 2015). Of these, *Cryptosporidium hominis* is primarily a human pathogen (Xiao et al. 2001), and *Cryptosporidium andersoni* is the major species responsible for bovine cryptosporidiosis (Liu et al. 2015).

In China, horses are commonly used for work, entertainment, and competitions, usually living in close association with humans. Cryptosporidiosis was initially described in immune-deficient Arabian foals by Snyder et al. (1978). To date, nine *Cryptosporidium* species/genotypes have been identified in equines in more than eight countries (Liu et al. 2015). However, only limited reports about horse cryptosporidiosis are available in China, and the zoonotic transmission of *Cryptosporidium* between humans and horses remains unknown. Thus, the present study was conducted to identify *Cryptosporidium* species/genotypes in horses from southwestern China, and to assess the zoonotic potential of cryptosporidiosis from horses to humans.

MATERIALS AND METHODS

Ethics statement

The present study protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Sichuan Agricultural University. Permission was obtained from farm managers before the collection of fecal specimens from horses.

Specimen collection

In total, 333 fecal samples from horses of different ages (3 mo to 16 yr) were randomly collected on five farms with different management systems in the Sichuan and Yunnan provinces of southwestern China between August 2015 and April 2016. In total, 100 horses from Farm 1 (48 horses) and Farm 2 (52 horses), which are both equestrian clubs, were kept in stables; 56 horses from Farm 3 were kept in pastures; 12 horses from Farm 4, mainly used for experimental research, were kept in stables; and 165 horses from Farm 5 were used for breeding in pastures and stables. Each sample was collected using a sterile disposable latex glove immediately after the horse defecated onto the ground, and was then placed into an individual sterile plastic container. No obvious clinical signs were observed in any of the sampled horses. Samples were maintained at 4 °C until DNA extraction.

DNA extraction

Approximately 0.5 g of each of the stored fecal specimens was washed three times with distilled water by centrifugation at 2,000 *g* for 10 min. DNA was extracted from the washed fecal samples, using an EZNA® Stool DNA kit (Omega Biotek, Norcross, GA) according to the manufacturer's recommended protocol. DNA was eluted in 200 μl of the Solution Buffer of the kit and stored at −20 °C until used for polymerase chain reaction (PCR) analysis.

Cryptosporidium genotyping and subtyping

Cryptosporidium species/genotypes were identified in all of the extracted DNA samples by nested PCR amplification of an ~830-bp fragment of the small subunit rRNA (*SSU* rRNA) gene (Xiao et al. 1999) (Table S1). The identification of *C. hominis* was confirmed by PCR and sequence analysis of the approximately 1,950- and 550-bp fragments for the *Cryptosporidium* oocyst wall protein (*COWP*) and 70-kDa heat shock protein (*HSP70*) genes, respectively (Sulaiman et al. 2000; Xiao et al. 2000) (Table S1). Subtype identification of *C. hominis* isolates was performed by sequence analysis of an approximately 800-bp fragment of the 60-kDa glycoprotein (*GP60*) gene, and *C. andersoni* isolates were determined by amplification at four loci MS1, MS2, MS3, MS16 (fragment lengths of approximately 550, 450, 530, and 590 bp, respectively). Primers and amplification conditions were adopted as previously described (Sulaiman et al. 2005; Feng et al. 2011) (Table S1).

Nucleotide sequencing and analysis

All secondary PCR products were directly sequenced at Life Technologies (Guangzhou, China) using an ABI Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). The accuracy of the sequences was confirmed by bidirectional sequencing, and a new PCR secondary product was re-sequenced if necessary. The nucleotide sequences obtained in this study were aligned with the corresponding *Cryptosporidium* reference sequences downloaded from the GenBank database, using BLAST (http://blast.ncbi.nlm.nih.gov) and ClustalX 1.83 (http://www.clustal.org/) to determine *Cryptosporidium* species/genotypes and subtypes.

Phylogenetic analysis

Phylogenetic analysis was performed by constructing neighboring-joining trees of the *GP60* genes, using the program Mega 6 (http://www.megasoftware.net/) based on the evolutionary distances calculated by the Kimura-2-parameter model. The reliability of the trees was assessed by bootstrap analysis with 1,000 replicates.

Nucleotide sequence accession numbers

The unique partial nucleotide sequences of the *SSU* rRNA, *HSP70, COWP, GP60* genes and the four minisatellite loci (MS1, MS2, MS3, and MS16) obtained in the present study were deposited in the GenBank database under accession numbers KX926452–KX926463 and KY210530–KY210545, respectively.

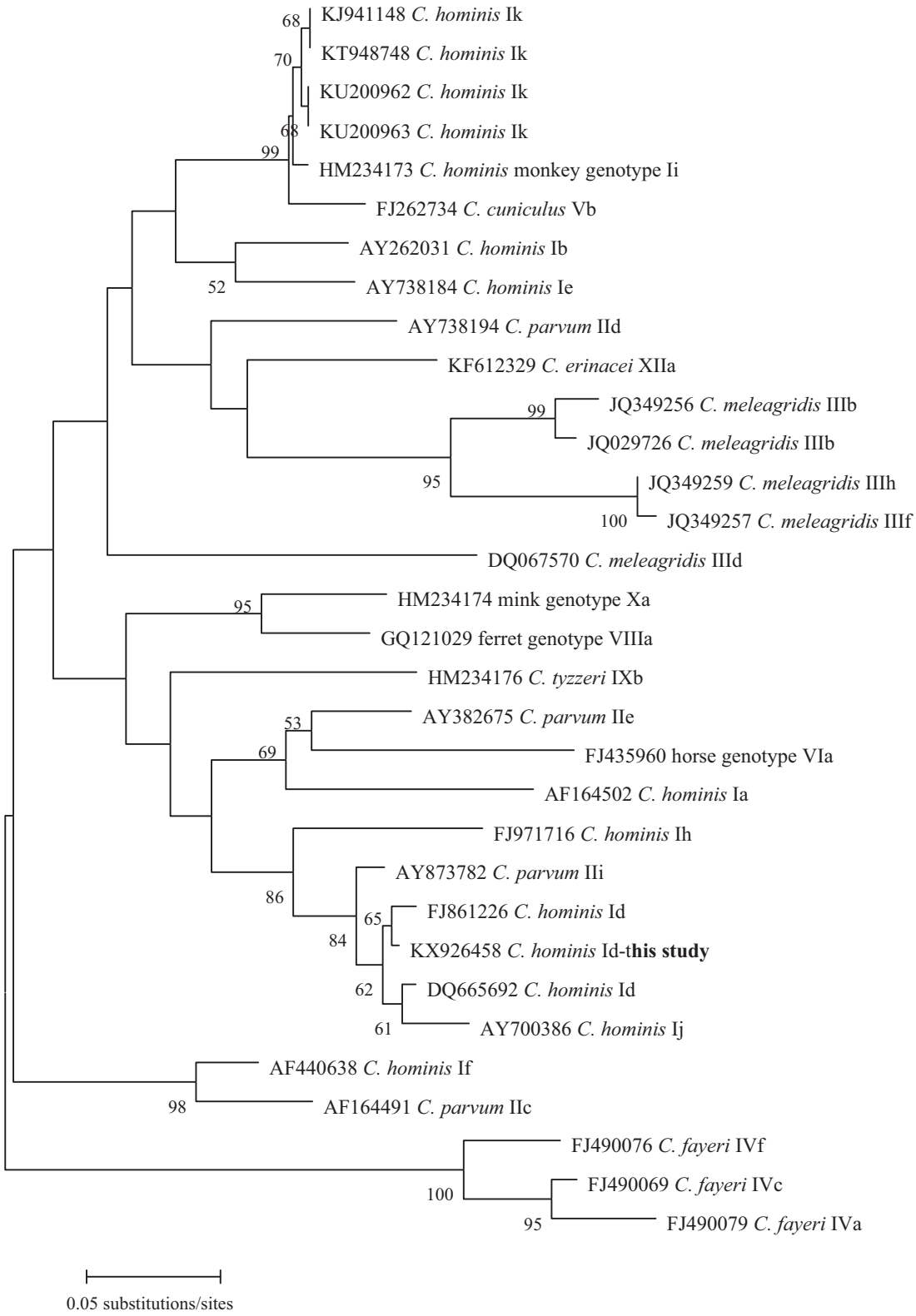

Figure 1 Phylogenetic relationship of 60-kDa glycoprotein (*GP60*) nucleotide sequences of the *Cryptosporidium* equine isolate in this study to multiple subtype families in *C. hominis*, as inferred by a neighbor-joining analysis based on evolutionary distances calculated using the Kimura two-parameter model. The tree was rooted with partial subtypes of *C. fayeri*. Bootstrap values were obtained using 1,000 pseudo-replicates, with only values above 50% reported.

RESULTS AND DISCUSSION

In the present study, six of the 333 (1.8%) fecal specimens were detected to be positive for *Cryptosporidium* by PCR amplification of the partial *SSU* rRNA gene, which is consistent with the previously reported low infection rate of *Cryptosporidium* in horses in Algeria (2.3%), Poland (3.4%), and New York state (5.1%) (Burton et al. 2010; Laatamna et al. 2015; Wagnerová et al. 2015). The age of the *Cryptosporidium*-positive horses was between 2 and 3 yr. Analysis of the sequences of the *SSU* rRNA gene of the six *Cryptosporidium*-positive samples revealed the presence of two *C. hominis* and four *C. andersoni* isolates. Comparison of the *SSU* rRNA gene sequences revealed that the two *C. hominis* isolates were identical to the *C. hominis* (KF679723) isolate derived from a rhesus macaque in China (Karim et al. 2014), and the three *C. andersoni* isolates were identical to the *C. andersoni* (KT175425) isolate derived from wastewater in Iran. However, the remaining sequence of *C. andersoni* had one nucleotide substitution (T/C at nucleotide 595) when compared to the *C. andersoni* (KF271453) isolate derived from a human in China. The *HSP70* sequence of one isolate had two nucleotide substitutions (T/G and A/T at positions 41 and 879, respectively) compared to the sequence (KF679727) derived from a rhesus macaque in China, but the other isolate was identical to the published *C. hominis* sequence KM116517 derived from a human in China. Interestingly, the two *C. hominis COWP* gene sequences obtained in this study were identical to each other, and were also identical to *C. hominis* isolates obtained from a human (DQ388389) and a monkey (AF266272).

In a previous study, *C. hominis* was first identified in only one horse in Algeria, and a novel *GP60* subtype family, Ik, was identified (Laatamna et al. 2015). In Brazil, *C. hominis* was reported in two foals of 4 and 5 mo of age, respectively, and one subtype IkA20G1 was found (Inácio et al. 2017). In contrast, *C. hominis* was identified as the dominant parasite (61/87, 70.1%) in horses in China, and two subtypes were found: IkA16G1 and IkA16 (Jian et al. 2016). In the present study, two isolates of *C. hominis* were successfully amplified at the *GP60* locus and were identified for subtypes. DNA sequence analysis showed that the two isolates have a maximum nucleotide identity of 99% to a previously characterized Australian *C. hominis* subtype, IdA26 (FJ861226). These subtypes have less than 11 nucleotide TCA repeats and five nucleotide substitutions: A/G, G/A, T/C, C/A, and T/C at nucleotides 182, 258, 316, 484, and 835, respectively. The two isolates were determined to belong to the Id subtype based on phylogenetic analysis, and were named IdA15 in accordance with current *Cryptosporidium* nomenclature conventions (Fig. 1) (Sulaiman et al. 2005). Human infections with subtype Id *C. hominis* have previously been reported in China, Canada (Ontario and British Columbia), and the Arctic, and no animal-derived subtype Id isolates have been described to date (Karine et al. 2016; Ong et al. 2008; Trotzwilliams et al. 2006; Wang et al. 2011). The subtype Id of *C. hominis* has been detected in humans in the area of Tianjin and Henan province in China (Wang et al. 2011). The two isolates found in horses were from Farm 2 in this area. On this farm close contact between people and horses may be responsible for infection of the horses with *C. hominis*. Whether these horses are truly infected or merely act as mechanical carriers of accidentally ingested oocysts of anthroponotic origin remains to be determined.

Cryptosporidium andersoni was considered the major species causing bovine cryptosporidiosis in previous studies (Liu et al. 2015; Zhao et al. 2015), but it has been identified in various hosts in China, including humans, golden takins, bactrian camels, cattle, sheep, goats, and yaks (Liu et al. 2014a,b; Qi et al. 2015; Zhao et al. 2015). Each *C. andersoni*-positive isolate was successfully amplified and sequenced at the four minisatellite loci: MS1, MS2, MS3, and MS16. Multiple-sequence alignment analysis of the four *C. andersoni*-positive sequences showed A6, A5, A2, and A1 at the four loci, respectively. This MLST subtype was first reported in Bactrian camels in the Henan province, and has also been reported in Bactrian camels in the Sichuan province in China (Wang et al. 2012).

In summary, this is the first report of *C. hominis* and *C. andersoni* in horses in southwestern China, as well as the first report of the rare zoonotic subtype Id of *C. hominis* and the MLST subtypes A6, A5, A2, and A1 of *C. andersoni* in horses overall. *Cryptosporidium andersoni* has been identified in other animals, and the MLST subtypes A6, A5, A2, and A1 have also been reported in other animals in southwestern China. It is therefore likely that the horse-derived *C. andersoni* resulted from cross-species transmission. Importantly, the presence of the *C. hominis* Id subtype in both humans and horses indicates that it may present a threat to public health.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Gene locus and primer sequences used in this study, annealing temperatures used in the PCR and expected sizes of the PCR products.