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Research paper

Different distribution of *Cryptosporidium* species between horses and donkeys

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ABSTRACT

Few studies have been conducted on the distribution of *Cryptosporidium* species and subtypes in equine animals. In this study, 878 stool specimens were collected during 2015–2019 from 551 donkeys and 327 horses in Shandong, Xinjiang, and Inner Mongolia, China and screened for *Cryptosporidium* spp. by PCR analysis of the small subunit rRNA gene. The *Cryptosporidium* species presented were identified by sequence analysis of the PCR products and subtyped by sequence analysis of the 60 kDa glycoprotein gene. The infection rates of *Cryptosporidium* spp. in horses and donkeys were 3.1% (10/327) and 14.5% (80/551), respectively. Four *Cryptosporidium* species/genotypes were identified, including *C. parvum* (in 5 horses), *C. hominis* (in 75 donkeys), *Cryptosporidium* horse genotype (in 5 horses and 4 donkeys) and a new genotype that is genetically related to *Cryptosporidium* mink genotype (in 1 donkey). All *C. parvum* isolates were subtyped as IIdA19G1, *C. hominis* as IkA16G1, and horse genotype as VIaA15G4. Data from this study indicate that four *Cryptosporidium* species are circulating in horses and donkeys in the study areas, with *C. hominis* as a dominant *Cryptosporidium* species in only donkeys. Attention should be paid to reduce the transmission of these zoonotic *Cryptosporidium* spp.

1. Introduction

Cryptosporidium spp. are protozoan parasites inhabiting the gastrointestinal epithelium of vertebrates (Checkley et al., 2015). They are major causes of moderate-to-severe diarrhea and death in young children (Collaborators, G.B.D.D.D, 2017). Nearly 40 *Cryptosporidium* species and about the same number of genotypes have been established (Feng et al., 2018). Among them, *C. hominis* and *C. parvum* are the most common human-pathogenic ones, with *C. parvum* being the most important zoonotic species (Xiao, 2010).

Horses and donkeys are common hosts of *Cryptosporidium* spp. (Jian et al., 2016). Few studies, however, have been conducted on the identification of *Cryptosporidium* species in equine animals (Burton et al., 2010; Galuppi et al., 2015; Laatamna et al., 2015). Several studies, including four from China, have reported the occurrence of *C. hominis*, *C. parvum* and *Cryptosporidium* horse genotype in these animals (Deng et al., 2017; Jian et al., 2016; Liu et al., 2015; Qi et al., 2015b). Other less common *Cryptosporidium* spp. identified include *C. muris*, *C. andersoni* and *C. tyzzeri* (Deng et al., 2017; Wagnerova et al., 2015). The clinical significance of these *Cryptosporidium* species in equine animal is not yet clear.

Horses and donkeys are important farm animals in Xinjiang, Shandong and Inner Mongolia, China. In recent years, the production of donkey-hide gelatin (Dong-E *E*-Jiao, a traditional Chinese medicine) has been an important part of the poverty-alleviation program in these areas. More than 20,000 households and at least 60,000 population in over 1000 townships have increased their income by ¥18 billion (http://www.xinhuanet.com//health/2017-08/21). As a result, there is a significant increase in the number of donkeys in China (http://news.wugu.com.cn/article/1397471.html).

This study examines the prevalence of *Cryptosporidium* spp. in domestic horses and donkeys in Shandong, Inner Mongolia and Xinjiang. Data generated improve our understanding of the transmission of *Cryptosporidium* spp. in equine animals.

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2. Materials and methods

2.1. Ethics statement

Specimens were collected from horses and donkeys with the permission of the owners or farm managers. Animals sampled were handled following established procedures of the Chinese Laboratory Animal Administration Act of 2017. The research protocol was reviewed and approved by the Research Ethics Committee of the South China Agricultural University.

2.2. Specimens

During 2015–2019, 878 fecal specimens were collected from equine animals in Shandong, Inner Mongolia and Xinjiang, China. Among them, 551 were from donkeys in Shandong (155), Inner Mongolia (176) and Xinjiang (220), while 327 from horses in Inner Mongolia (81) and Xinjiang (246). These farms were selected based on the willingness of participation in the study. Most specimens from donkeys were collected from the rectum of animals under 6 months in age, while those from horses were collected from fresh droppings on pasture of animals of 1 to 27 months in age (Fig. 1). Information on the age and geographical location of animals sampled was collected. No animals sampled during the study had any clinical signs such as diarrhea. The specimens were kept in 2.5% potassium dichromate at 4 degree for < 6 months prior to DNA extraction.

2.3. DNA extraction

Genomic DNA was extracted from approximately 500 mg of each fecal specimen, after it was washed off potassium dichromate three times with distilled water by centrifugation. The Fast DNA Spin Kit for Soil (MP Biomedical, Santa Ana, CA, USA) was used in DNA extraction (Jiang et al., 2005). The DNA harvested was stored at −20 °C before PCR analysis.

2.4. PCR analysis

The occurrence of *Cryptosporidium* spp. was determined by PCR analyses of a ∼830-bp fragment of the small subunit (SSU) rRNA gene (Xiao et al., 1999). DNA sequence analysis of all positive PCR products was used in the identification of *Cryptosporidium* species involved. Subtypes of the *C. parvum*, *C. hominis* and *Cryptosporidium* horse genotype present were determined by PCR-sequence analysis of the 60 kDa glycoprotein (*gp60*) gene (Alves et al., 2003).

2.5. Sequence analysis

The secondary PCR products of the SSU rRNA and *gp60* gene were sequenced bi-directionally on an ABI 3730 Autosequencer to identify *Cryptosporidium* species and subtypes, respectively. The nucleotide sequences generated were assembled by using ChromasPro 2.1.5.0 (http://technelysium.com.au/ChromasPro.html), edited by using BioEdit 7.1.3.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and aligned with each other and reference sequences from GenBank (https://www.ncbi.nlm.nih.gov) by using ClustalX 2.0.11 (http://clustal.org). The established nomenclature system was used in naming *Cryptosporidium* subtypes (Xiao and Feng, 2017). Representative nucleotide sequences generated from the study were submitted to GenBank under accession numbers MK761058-MK761063 and MK775038-MK775045.

2.6. Statistical analysis

Differences in *Cryptosporidium* infection rates between hosts and age groups were analyzed by using the χ^2 test implemented in SPSS v.20.0 (IBM Corp., New York, NY, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Occurrence of *Cryptosporidium* spp. in donkeys and horses

Among the 551 specimens collected from donkeys, 80 (14.5%) were positive for *Cryptosporidium* spp. in PCR analysis of the SSU rRNA gene. The infection rates for animals in Dezhou, Qingdao, Aohan, Hohhot, Hotan, and Aksu were 6.8% (10/148), 0% (0/7), 61.3% (49/80), 19.8% (19/96), 1.7% (2/115), and 0% (0/105), respectively (Table 1). Among the 327 fecal specimens collected from horses, 10 (3.1%) were positive for *Cryptosporidium* spp.; the difference in infection rates between donkeys and horses was significant ($\chi^2 = 29.3$, $df = 1$, $P < 0.0001$). *Cryptosporidium* infection rates in horses ranged from 0% to 14.3%

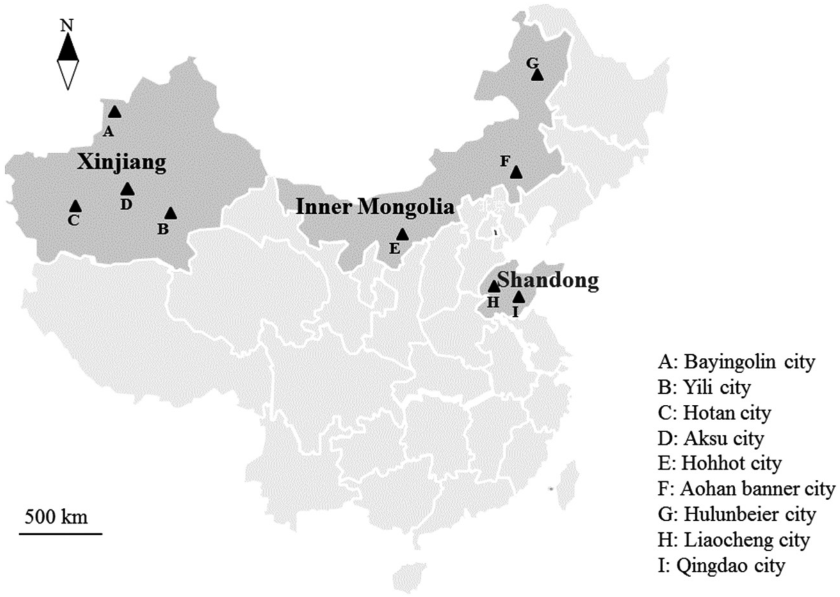

Fig. 1. Map of districts in Xinjiang, Shandong and Inner Mongolia in China showing the specimen collection sites with triangles.

Table 1
Occurrence of *Cryptosporidium* spp. in donkeys and horses in Shandong, Inner Mongolia and Xinjiang.

Host	Location (city)	Total no. of specimens	No. positive for *Cryptosporidium* (%)	*Cryptosporidium* species (no. of specimens)	Subtype (no. of specimens)
Donkey	Liaocheng	148	10 (6.8%)	*C. hominis* (6) Horse genotype (4)	IkA16G1 (4) VIaA15G4 (1)
	Qingdao	7	0 (0%)	–	–
	Aohan banner	80	49 (61.3%)	*C. hominis* (49)	IkA16G1 (49)
	Hohhot	96	19 (19.8%)	*C. hominis* (19)	IkA16G1 (19)
	Hotan	115	2 (1.7%)	*C. hominis* (1), Mink genotype-like (1)	–
	Aksu	105	0 (0%)	–	–
	Total	551	80 (14.5%)	*C. hominis* (75) Horse genotype (4) Mink genotype-like (1)	IkA16G1 (72) VIaA15G4 (1)
Horse					
	Hulunbeier	49	7 (14.3%)	*C. parvum* (2) Horse genotype (5)	IIdA19G1 (1) VIaA15G4 (4)
	Hohhot	32	3 (9.4%)	*C. parvum* (3)	IIdA19G1 (2)
	Yili	55	0 (0%)	–	–
	Bayingolin	191	0 (0%)	–	–
	Total	327	10 (3.1%)	*C. parvum* (5) Horse genotype (5)	IIdA19G1 (3) VIaA15G4 (4)

Table 2
Infection rates and subtype occurrence of *Cryptosporidium* spp. in equine animals in Xinjiang, Shandong and Inner Mongolia by age.

Host	Age (months)	Total no. of specimens	No. positive for *Cryptosporidium* (%)	Species/genotypes (no. of specimens)	Subtype (no. of specimens)
Donkeys	< 6	379	80 (21.1%)	*C. hominis* (75); Horse genotype (4) Mink genotype-like (1)	IkA16G1 (72) VIaA15G4 (1)
	6–12	39	0 (0%)	–	–
	> 12	133	0 (0%)	–	–
	Total	551	80 (14.5%)	*C. hominis* (75); Horse genotype (4) Mink genotype-like (1)	IkA16G1 (72) VIaA15G4 (1)
Horses	< 6	147	2 (1.4%)	*C. parvum* (2);	–
	6–12	164	6 (3.7%)	*C. parvum* (1); Horse genotype (5)	IIdA19G1 (1) VIaA15G4 (4)
	> 12	16	2 (12.5%)	*C. parvum* (2)	IIdA19G1 (2)
	Total	327	10 (3.1%)	*C. parvum* (5); Horse genotype (5)	IIdA19G1 (3) VIaA15G4 (4)

Table 3
Differences in infection rates of *Cryptosporidium* spp. in horses between foals and adult horses in various studies.

Location	Total no. of specimens	Age	No. positive for *Cryptosporidium* (%)	Reference
Canada	24	< 6 months	5 (21%)	(Olson et al., 1997)
	10	Adults	1 (10%)	–
Ohio, USA	55	Foals and weanlings	6 (10.9%)	(Xiao and Herd, 1994)
	42	Adults	0 (0%)	(Xiao and Herd, 1994)
Italy	120	> 8 weeks	11 (9.2%)	(Veronesi et al., 2010)
	30	Adults	1 (3.3%)	–
Inner Mongolia, China	9	< 6 months	2 (22.2%)	This study
	72	> 6 months	8 (11.1%)	–

among the four study sites. The infection rates were 14.3%, (7/49) and 9.4% (3/32) in Hulunbeier and Hohhot of Inner Mongolia ($\chi^2 = 0.097$, $df = 1$, $P = 0.51$), respectively. None of the 246 specimens from Bayingolin and Yili of Xinjiang were positive for *Cryptosporidium* spp. (Table 1). *Cryptosporidium* spp. were identified in all three age groups: < 6, 6–12 and > 12 months (Table 2). Excluding horse specimens from Xinjiang, where none of them were positive for *Cryptosporidium* spp., horses under 6 months had higher *Cryptosporidium* infection rate than those above 6 months in Inner Mongolia (22.2% and 11.1%, respectively; $\chi^2 = 0.017$, $df = 1$, $P = 0.34$; Table 3).

3.2. *Cryptosporidium* species/genotypes in donkeys and horses

Sequence analysis of the SSU rRNA PCR products identified the presence of *C. parvum*, *C. hominis*, *Cryptosporidium* horse genotype, and a *Cryptosporidium* mink genotype-like parasite in specimens from this study. Among the four *Cryptosporidium* species/genotypes, *C. hominis* (n = 75), *Cryptosporidium* horse genotype (n = 4) and *Cryptosporidium* mink genotype-like (n = 1) were seen in specimens from donkeys, while *C. parvum* (n = 5) and *Cryptosporidium* horse genotype (n = 5) were seen in specimens from horses. By geographic location, *C. hominis* was found in donkeys in all three provinces or autonomous regions (Shandong, Inner Mongolia, and Xinjiang), while *Cryptosporidium* horse genotype was only found in donkeys in Shandong Province, and *Cryptosporidium* mink genotype-like was found in donkeys in Xinjiang. In contrast, both *C. parvum*, and *Cryptosporidium* horse genotype were found in horses in Inner Mongolia.

3.3. *Cryptosporidium* subtypes in donkeys and horses

DNA sequence analysis of the *gp60* gene indicated that the *C. parvum*, *C. hominis* and *Cryptosporidium* horse genotype detected belonged to the IIdA19G1 (n = 3), IkA16G1 (n = 72) and VIaA15G4 (n = 5) subtypes, respectively (Table 1). The nucleotide sequence generated were identical to KJ802724, KU200963, and KU200961 in GenBank, respectively.

4. Discussion

Donkeys and horses were shown to have different *Cryptosporidium* infection rates in this study. The significantly higher *Cryptosporidium* infection rate in donkeys could be attributed to the intensive nature of animal management in this area, as a large number of animals are farmed for Dong-E *E*-Jiao production, with animals being housed in confined areas instead of free ranging. Thus, the 14.5% (80/551) infection rate of *Cryptosporidium* spp. in donkeys in this study is higher than the infection rate of 1.6% in Algeria, where the animal production is much less intensive (Laatamna et al., 2015). The young age of animals studied (under 6 months) might have further contributed to the high *Cryptosporidium* infection rate observed in the present study. In contrast, the 3.1% (10/327) infection rate of *Cryptosporidium* spp. in horses is consistent with previous reports of *Cryptosporidium* spp. in the United States (7.4%), Czech Republic (3.4%), Algeria (2.3%), and Taiwan (7.6%), Xinjiang (2.7%) and the southwest (1.8%) of China (Burton et al., 2010; Deng et al., 2017; Laatamna et al., 2015; Qi et al., 2015b; Wagnerova et al., 2015). Horses under 6 months also had a higher *Cryptosporidium* infection rate than those above 6 months in Inner Mongolia in this study (22.2% versus 11.1%). The number of animals involved, however, was too small to make concrete conclusion, although, similar observations were made in the United States, Canada and Italy (Table 3).

In this study, there were differences in the occurrence of *Cryptosporidium* species/genotypes between horses and donkeys. The *Cryptosporidium* horse genotype was identified in both horses and donkeys, while *C. hominis* was only found in donkeys; the number of infections with *C. parvum* or *Cryptosporidium* mink genotype-like were too small to make any definitive conclusion. Similar trends in the distribution of *Cryptosporidium* spp. were obtained in previous studies in China (Jian et al., 2016; Qi et al., 2015b). The *Cryptosporidium* mink genotype-like was first found in one donkey, and differs from the *Cryptosporidium* mink genotype by four single nucleotides. Although *Cryptosporidium* mink genotype is a minor human pathogen (Ng-Hublin et al., 2013), the human infectivity of the *Cryptosporidium* mink genotype-like is unclear. Differences in the distribution of *Cryptosporidium* species/genotypes have been found among studies conducted in horses and donkeys in Czech Republic, New Zealand, UK, USA, Brazil and, Jordan (Chalmers et al., 2005; Grinberg et al., 2009; Hajdusek et al., 2004; Hijjawi et al., 2016; Inacio et al., 2017; Wagnerova et al., 2016). They, however, could be attributed to variations in animal management, as indicated in the present study between donkeys and horses. Donkeys in this study were mainly raised indoor on farms, thus had no contact with other farm animals. In contrast, horses shared pastures with cattle and sheep, which might favor cross-species transmission of *C. parvum*.

The *Cryptosporidium* horse genotype found in this study is probably an equine adapted one. It was initially reported from a Prezewalski wild horse in the Czech Republic (Ryan et al., 2003). Subsequently, it had been commonly found in horses and donkeys in America, Italy and China (Burton et al., 2010; Caffara et al., 2013; Jian et al., 2016). Nevertheless, this *Cryptosporidium* genotype has been occasionally detected neonatal calves and hedgehogs (Abe and Matsubara, 2015; Burton et al., 2010; Caffara et al., 2013; Thompson et al., 2007). Two human patients with diarrhea were found positive for the *Cryptosporidium* horse genotype in the United Kingdom and United States (Robinson et al., 2008; Xiao et al., 2009). More studies are needed to better understand the host range of the *Cryptosporidium* horse genotype.

The *C. hominis* found in this study also appears to be a host-adapted subtype family. All *C. hominis* isolates identified in the study belong to the subtype family Ik. Previously, *C. hominis* was considered a human-adapted species and was only occasionally diagnosed in animals (Feng et al., 2018; Ryan et al., 2014). The Ik subtype family of *C. hominis* was originally described in an Algerian horse (Laatamna et al., 2015). Subsequently, it had been reported in equine animals and two human patients (Inacio et al., 2017; Jian et al., 2016; Lebbad et al., 2018). In addition to the sequence differences at the *gp60* locus, there are some minor sequence differences in the SSU rRNA gene between common *C. hominis* subtype families and Ik (Feng et al., 2018). This was supported by recent identification of the genetic uniqueness of Ik at the whole genome level (Sikora et al., 2017). Further studies are needed to understand the public health significance of this equine-adapted *C. hominis* subtype family.

The IId *C. parvum* subtype family identified in this study has significant public health implications. Subtype family IId was previously identified in horses and donkeys in multiple locations in China (Jian et al., 2016). It is the dominant *C. parvum* in dairy calves in this country (Feng and Xiao, 2017). Other hosts of the IId subtype family include various rodents, golden takins, yaks, sheep and goats in China (Lv et al., 2009; Mi et al., 2014; Qi et al., 2015a; Zhao et al., 2015a; Zhao et al., 2015b). It has also been found in human patients in Henan (Wang et al., 2013). Elsewhere, humans in Mideast countries are commonly infected with IId subtypes (Sulaiman et al., 2005). Similarly, people in Egypt, Ethiopia, Iran, Malaysia and some European countries are sometimes infected with IId subtypes (Adamu et al., 2014; Ibrahim et al., 2016; Insulander et al., 2013; Iqbal et al., 2012; Kiani et al., 2017; Lim et al., 2011). More in-depth studies are needed to understand the transmission of this *C. parvum* subtype family in equine animals.

5. Conclusion

Cryptosporidium hominis appears to be common in donkeys but not horses in China. The subtypes of *C. hominis*, *C. parvum* and *Cryptosporidium* horse genotype identified are known human pathogens, thus have public health implications. Further studies are needed to assess the host ranges and transmission dynamics of these pathogens within the country, including characterization of *Cryptosporidium* spp. from humans living in rural areas, who have frequent contact with equine animals.

Declaration of Competing Interest

We have no conflict of interest to declare.

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