Molecular prevalence and subtyping of *Cryptosporidium hominis* among captive long-tailed macaques (*Macaca fascicularis*) and rhesus macaques (*Macaca mulatta*) from Hainan Island, southern China

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**Abstract**

**Background:** *Cryptosporidium* is an important zoonotic parasite that is commonly found in non-human primates (NHPs). Consequently, there is the potential for transmission of this pathogen from NHPs to humans. However, molecular characterization of the isolates of *Cryptosporidium* from NHPs remains relatively poor. The aim of the present work was to (i) determine the prevalence; and (ii) perform a genetic characterization of the *Cryptosporidium* isolated from captive *Macaca fascicularis* and *M. mulatta* on Hainan Island in southern China.

**Methods:** A total of 223 fresh fecal samples were collected from captive *M. fascicularis* (*n* = 193) and *M. mulatta* (*n* = 30). The fecal specimens were examined for the presence of *Cryptosporidium* spp. by polymerase chain reaction (PCR) and sequencing of the partial small subunit (SSU) rRNA gene. The *Cryptosporidium*-positive specimens were subtyped by analyzing the 60-kDa glycoprotein (*gp60*) gene sequence.

**Results:** *Cryptosporidium* spp. were detected in 5.7% (11/193) of *M. fascicularis*. All of the 11 *Cryptosporidium* isolates were identified as *C. hominis*. Subtyping of nine of these isolates identified four unique *gp60* subtypes of *C. hominis*. These included IaA20R3a (*n* = 1), IoA17a (*n* = 1), IoA17b (*n* = 1), and IiA17 (*n* = 6). Notably, subtypes IaA20R3a, IoA17a, and IoA17b were novel subtypes which have not been reported previously.

**Conclusions:** To our knowledge, this is the first reported detection of *Cryptosporidium* in captive *M. fascicularis* from Hainan Island. The molecular characteristics and subtypes of the isolates here provide novel insights into the genotypic variation in *C. hominis*.

**Keywords:** *Cryptosporidium*, *Macaca fascicularis*, *Macaca mulatta*, Gp60, SSU rRNA

**Background**

*Cryptosporidium* is a protozoan belonging to the phylum Apicomplexa. The parasite is the causative agent of *Cryptosporidiosis*, the clinical signs of which include diarrhea, malabsorption and wasting in humans [1].

Cryptosporidiosis is a significant threat to immunocompromised patients, especially among patients with human immunodeficiency virus (HIV)/ Acquired Immunodeficiency Syndrome (AIDS) in whom the mortality rate is high [2]. Cryptosporidiosis in children is associated with malnutrition and poor growth and is one of the most important causes of diarrhea-associated death among young children in developing countries [3]. In addition to humans, epidemiological evidence showed that *Cryptosporidium* is capable of infecting more than 260...
vertebrate species, including mammals, birds, reptiles, fish and amphibians [4, 5]. Cryptosporidium oocysts are ubiquitous in the environment and more than 550 water-borne and food-borne outbreaks of cryptosporidiosis have been reported globally, with the sources of infection linked to drinking or recreational water, fruits, vegetables, or cow’s milk [6, 7]. Because of the clinical and public health importance of Cryptosporidium, this protozoan is considered as a category B list priority pathogen by the National Institutes of Health (NIH) of the USA and the fifth most important food-borne parasite by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee [8, 9].

Extensive genetic variations have been reported within the genus Cryptosporidium. Thus far, 38 species and more than 40 genotypes have been identified [10]. Over 20 species or genotypes of Cryptosporidium have been found in humans [10]. Most human-pathogenic Cryptosporidium species and genotypes have also been found in animals [10]. The accurate identification of Cryptosporidium in animals at the species and/or genotype level is essential for the assessment of the potential zoonotic sources of infection among humans [10]. The two most common species detected in humans, C. parvum and C. hominis, are responsible for >90% of human cases of cryptosporidiosis worldwide, and are responsible for almost all outbreaks of cryptosporidiosis [11]. While C. parvum is generally accepted to be a zoonotic pathogen and it was widely accepted that cases of C. hominis were transmitted from human-to-human, but recent reports have shown that C. hominis are more commonly found in animals (including NHPs, horses, and donkeys) [10]. Molecular subtyping has been increasingly used to study the transmission of Cryptosporidium in human and animals. Several subtypes of C. hominis and C. parvum have been identified based on the 60 kDa glycoprotein (gp60) gene sequence, which is the most commonly used genetic locus for the subtyping of Cryptosporidium [12]. These subtyping results have shown that the same subtypes of C. parvum may be found in humans and their epidemiologically-linked animals, suggesting that infected animals are a major source of human infection [13, 14].

Among animals, NHPs, due to their high level of genetic homology to humans, are invaluable experimental models for biomedical research. In addition, they may be susceptible to infection with numerous human pathogens including Cryptosporidium [15]. More than 40 studies from 12 countries have been published describing infection of NHPs with Cryptosporidium. However, few studies have included data on genotyping [16]. Eight species of Cryptosporidium have been reported in non-human primates including C. hominis, C. parvum, C. felis, C. muris, C. ubiquitum, C. meleagridis, C. bovis and C. andersoni [15, 17–29]. Interestingly, all of these species have been detected in humans as well. Macaca fascicularis (long-tailed macaque) and M. mulatta (rhesus macaque) are two common species of NHPs which live in close proximity to many humans, and frequently interact with human communities in many locations, including China. The health of M. fascicularis and M. mulatta are therefore an important public health issue. The aim of the present study was to determine the prevalence of natural Cryptosporidium infection in captive M. fascicularis and M. mulatta. The sampled animals were from a facility that breeds NHPs for research purposes in Hainan Island, China. The second aim of this study was to subtype the C. hominis isolates sequencing the gp60 gene.

Methods

Collection of fecal specimens

A total of 223 fresh fecal samples were collected from 193 M. fascicularis and 30 M. mulatta between July and August 2018 at the breeding base of experimental primates of Hainan Jingang Biological Technology Co., Ltd., at Haikou, Hainan, China. This breeding base of experimental primate was established in 2003. At the time of sample collection, the facility housed over 10,000 animals. All M. fascicularis in the facility were reared in groups, with the exception of infants, who were housed alone with their mothers until weaning (at approximately 8 months of age). Young animals aged 1–2 years were kept in individual cages for a quarantine period of 30 days before being sold to research laboratories. Two groups of M. fascicularis were sampled in this study: one group contained 125 weaned (one year-old) M. fascicularis who were housed individually, and the other group contained 68 adult M. fascicularis (>five years of age) who were housed in groups of 20–30 animals per cage for breeding purposes. For singly housed animals, fresh feces were collected directly after defecation. For animals housed in groups, fresh fecal deposits were collected from the ground in the early morning, as the floors of animal houses were cleaned every evening. To minimize the chance of duplicate sampling of animals, only one fecal specimen was collected at one location of the ground in each animal pen within any house each time. The M. mulatta sampled in this study were all >10 years-old and were maintained in individual cages for research laboratories. All the fecal samples were put into individual plastic bags marked with the age and health status of each animal. All samples were transported to our laboratory in a cooler with ice packs within 24 h and were stored at 4 °C until the time of processing.
DNA extraction
Prior to DNA extraction, all fecal specimens were processed by filtering through mesh to remove large solids, concentrating, and washing three times with distilled water by centrifugation for 10 min at 1500×g. Genomic DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. DNA was eluted in 200 ml of Buffer AE and stored at −20 °C prior to use in PCR analysis.

Cryptosporidium genotyping and subtyping
All DNA preparations were tested for the presence of Cryptosporidium spp. by nested PCR amplification of an 830 bp nucleotide fragment of the SSU rRNA gene, using the primers previously described by Xiao et al. [30]. The cycling parameters for the PCR reactions were optimized and used as follows: 94 °C for 3 min and 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 90 s, followed by a final extension step at 72 °C for 7 min. TaKaRa TaqDNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all PCR reactions. A negative control with no DNA was included in all PCR tests. Subtyping of Cryptosporidium-positive samples was conducted by nested PCR amplification of fragments of approximately 800–850 bp of the gp60 gene [31].

Sequence analysis
All PCR products were sequenced using PCR primers for each locus on an ABI PRISM™ 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA), in conjunction with the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA). The accuracy of the sequencing data was confirmed by sequencing in both directions. Nucleotide sequences obtained in the present study were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/blast/) and then analyzed and aligned with each other and the published reference sequences of Cryptosporidium in GenBank, using ClustalX 1.81 (http://www.clustal.org/).

Statistical analysis
Differences in infection rates among different groups of animals were compared using the Chi-square test or Fisher’s exact test in SPSS 17.0. P<0.05 was considered to be statistically significant.

Results
Prevalence rate of Cryptosporidium spp. in M. fascicularis and M. mulatta
Of the 223 fecal samples tested, 11 (4.9%) were found to be positive for Cryptosporidium by PCR (Table 1). All 11 Cryptosporidium-positive samples were from M. fascicularis (11/193, 5.7%) and none was found in M. mulatta (0/30, 0%), although this did not reach statistical significance (P=0.37). It was also observed that only young M. fascicularis were infected with Cryptosporidium. Furthermore, infection was observed in both males and females, as well as in both diarrhea and non-diarrhea groups. Whereas differences in the Cryptosporidium infection rates between female (6.5%; 6/92) and male (5.0%; 5/101) M. fascicularis were not significant (P>0.05), positive animals with diarrhea (13.8%; 4/29) were significantly higher than without diarrhea (4.3%; 7/164) (P=0.04).

Genetic characterization of the Cryptosporidium spp. at the SSU rRNA and gp60 loci
Sequence analysis of 11 PCR products of the SSU rRNA gene of Cryptosporidium indicated that all 11 samples were C. hominis. Furthermore, the obtained sequences represented five types with six polymorphic sites (Fig. 1). One SSU rDNA sequence representing seven Cryptosporidium isolates was identical to that of the C. hominis isolate derived from a rhesus macaque in Henan Province of China (GenBank: KF679722). The other four sequences (GenBank: MK270514-MK270517) had not been reported previously. These sequences had either one (position 528 or 760) or three (positions 475, 609 and 661) base variations compared with the sequence KF679722, which had the greatest similarity to the novel sequences obtained here. The first nucleotide of the reference sequence (KF679722) was considered to be position number one.

Cryptosporidium hominis-positive specimens were subtyped by sequence analysis of the gp60 gene. Of the 11 C. hominis isolates obtained, nine were successfully amplified. Sequence analysis of the nine gp60 gene sequences suggested that they belong to three subtype families (Ia, Li and Io). Four subtypes, IaA20R3a (n=1), IoA17a (n=1), IoA17b (n=1) and IiA17 (n=6), were

| Group          | No. examined | No. positive (%) | Cryptosporidium species (n) | Subtype (n) |
|---------------|-------------|------------------|------------------------------|-------------|
| Age Young     | 125         | 11 (8.8)         | C. hominis (11)              | IaA20R3a (1); IoA17a (1); IoA17b (1); IiA17 (6) |
| Adult         | 68          | 0                | –                            | –           |
| Sex Female    | 92          | 6 (6.5)          | C. hominis (6)               | IiA17 (4)   |
| Male          | 101         | 5 (5.0)          | C. hominis (5)               | IaA20R3a (1); IoA17a (1); IoA17b (1); IiA17 (2) |
| Symptom Diarrhea | 29         | 4 (13.8)         | C. hominis (4)               | IaA20R3a (1); IoA17a (1); IoA17b (1); IiA17 (1) |
| Non-diarrhea  | 164         | 7 (4.3)          | C. hominis (7)               | IiA17 (5)   |
detected in the present study based on the established nomenclature system of the gp60 subtypes. Among them, the novel subtypes IaA20R3a, IoA17a and IoA17b have not been reported previously. The sequence of subtype IaA20R3a (MK270518) has a maximum homology of 97% to isolate JF927196 which was isolated from Bangladeshi children. There were three nucleotide differences observed between IoA17a (MK270519) and IoA17b (MK270520). In addition, these isolates shared 98% identity with the sequence (KX926459) of subtype IdA15 which was initially misdiagnosed, and has subsequently been renamed IoA15 [10]. The novel gp60 subtypes IaA20R3a, IoA17a and IoA17b showed the same rRNA type KF679722. Isolate liA17 was identical to the sequence of a C. hominis gp60 subtype isolated from a rhesus macaque in Henan, China (HQ397716). The four novel rRNA types (MK270514-MK270517) showed the same gp60 subtype liA17. Subtype liA17 was isolated from feces in monkeys with diarrhea as well as the non-diarrhea group. In contrast, subtypes IaA20R3a, IoA17a and IoA17b were only detected in the diarrhea group.

Discussion
In the present study, Cryptosporidium hominis was only identified in M. fascicularis, with an overall prevalence of 5.7% (11/193). This observation is consistent with the report in captive M. mulatta from the Qinling Mountains (7.0%) and lower than that reported in wild M. mulatta (10.9%) from Guizhou, China [19, 21]. While the prevalence of Cryptosporidium in M. fascicularis observed here is higher than in other Chinese primates, another study of 26 NHP species by Karim et al. [20] observed Cryptosporidium was detected in 19 of the 2660 (0.7%) faecal specimens tested from China. Interestingly, only four NHP species were infected with Cryptosporidium (0.7% of rhesus macaques, 1.0% of cynomolgus monkeys, 10.0% of slow lorises and 6.7% of Francois’ leaf monkeys). Low prevalence of Cryptosporidium was also reported in laboratory-reared cynomolgus monkeys (0.5%) in Guangxi, China [22]. In addition, the prevalence of Cryptosporidium in M. fascicularis in the present study was higher than that reported for M. fascicularis in Thailand (1.0%), mountain gorillas in Rwanda (4.0%) and Uganda (4.0%), in orangutans from Indonesia (2.7%), in olive baboons from Kenya (2.6%), and in western lowland gorillas from the Central African Republic (0.5%) [16, 17, 23–25, 27]. However, although the reported positivity rates were lower in wild primates conducted in other countries compared to the present study, NHPs from Tanzania exhibited much higher rates of infection than those observed here [26]. The differences in prevalence may be related to factors such as differences in regional environments, the sensitivity and specificity of detection methods, animal the health statuses of the animals at the time of sampling, and the overall sample size. We also observed that the infection rate of Cryptosporidium in M. fascicularis (5.7%) was higher than that in M. mulatta (0%). In consideration of this observation, as well as those mentioned above, the species of NHPs may reflect differences in susceptibility, which could explain the species-to-species variation in prevalence. In fact, 64.8% of the M. fascicularis in the present study were less than one year-old, whereas all of the M. mulatta were over 10 years of age. For M. fascicularis, infection rates of young animals were higher than that of adult animals. These data suggest that age may be a risk factor in NHPs. In support of
this conclusion, another study has reported that infant baboons were infected at a higher rate (15.4%) than that of adults (1.3%) [15].

In the present study, only *C. hominis* was detected in *M. fascicularis*. Although there are other reports of *Cryptosporidium* spp. in non-human primates, few have provided detailed molecular information about these parasites in the great apes (Table 2) [15, 17–29]. Previous studies have indicated that at least eight species of *Cryptosporidium* have been reported in NHPs, including *C. hominis*, *C. parvum*, *C. felis*, *C. muris*, *C. bovis*, *C. ubiquitum*, *C. meleagridis* and *C. andersonii*. Among these, *C. hominis* was the most common *Cryptosporidium* species detected in NHPs (80/120) (Table 2) [15, 17–29]. The potential for zoonotic transmission is evident for the species and subtypes detected. *C. hominis* was the most common species in human cases of cryptosporidiosis in China, accounting for 48.3% (127/263) of all cryptosporidiosis cases [32]. Although *C. hominis* is widely considered a human-specific pathogen, and humans are the only natural host, it is a rather common *Cryptosporidium* species detected in non-human primates, horses and donkeys [10]. Furthermore, *C. hominis* has recently been identified in cattle, yaks, sheep, goats, kangaroos, rodents, hedgehogs, dogs and dugong [10]. Experimental infections with *C. hominis* have been successful in Mongolian gerbils, piglets and mouse [33–35]. The true and natural host range of *C. hominis* needs to be confirmed by subsequent molecular data from epidemiological studies of *Cryptosporidium*.

### Table 2 Prevalence and distribution of *Cryptosporidium* species and subtypes in natural infection of non-human primates worldwide

| Country          | Species of NHPs                          | No. positive/no. examined (%) | Species (n) | Subtype (n)\(^a\) | Ref   |
|------------------|-----------------------------------------|------------------------------|-------------|-------------------|------|
| China            | *Macaca mulatta* (rhesus macaque)       | 6/86 (7.0)                  | *C. andersonii* (5), *C. parvum* (1) | *IkA7G4* (1)\(^b\) | [19] |
| China            | *Macaca mulatta* (rhesus macaque)       | 9/1316 (0.7)                | *C. hominis* (9) | *IbA12G3* (7), *PN* (2) | [20] |
| China            | *Macaca fascicularis* (long-tailed macaque) | 8/778 (1.0)                | *C. muris* (4), *C. hominis* (4) | *IiA7* (1), *PN* (3) | [20] |
| China            | *Nycticebus bengalensis* (Bengal slow loris) | 1/10 (1.0)                 | *C. muris* (1) | –                 | [20] |
| China            | *Pongo abelii*, *Pongo pygmaeus* (orangutans) | 8/298 (2.7)                | *C. parvum* (2), *C. muris* (6) | – | [23] |
| China            | *Papio anubis* (olive baboon)            | 6/235 (2.6)                 | *C. hominis* (6) | – | [15] |
| China            | *Gorilla beringei beringei* (mountain gorilla) | 4/100 (4.0)                | *C. muris* (2), *C. meleagridis* (2) | – | [24] |
| China            | *Macaca fascicularis* (long-tailed macaque) | 1/205 (0.5)                | *C. hominis* (1) | *IdA14* (1) | [22] |
| China            | *Papio anubis* (olive baboon)            | 5/47 (10.6)                 | *C. hominis* (5) | *IaA13R7* (2), *IaA13R8* (6), *IaA14R7* (1), *IaA15G2R1* (1) | [26] |
| China            | *Pan troglodytes* (chimpanzee)           | 12/58 (20.7)                | *C. hominis* (7), *C. suis* (6) | *IaA12G2* (2), *IbA9G3* (2), *IiA14* (1) | [26] |
| China            | *Pan troglodytes* (chimpanzee)           | 4/26 (15.4)                 | *C. hominis* (4) | *IaA12G2* (3/4) | [26] |
| China            | *Gorilla beringei beringei* (mountain gorilla) | 4/100 (4.0)                | *C. parvum* (4) | – | [27] |
| Uganda           | *Propithecus coquereli* (Coquerel’s sifaka) | 1                          | *C. suis* (1) | – | [28] |
| USA              | *Macaca mulatta* (rhesus macaque)       | 1                          | *C. hominis* (1) | *IiA17* | [29] |

\(^a\) The numbers of subtypes are not consistent with the positives of *C. hominis* in some countries because not all isolates were genotyped successfully

\(^b\) Subtype *IkA7G4* was misnamed

Abbreviations: Ref, reference; pn, PCR-negative; –, not subtyping or no subtypes
To date, more than 10 subtype families have been recognized in *C. hominis* based on sequence analysis of the gp60 gene; six of these subtype families (Ia, Ib, Id, Ie, If, and li) have been identified in NHPs (Table 2) [15, 17–29]. Subtype families Ia, Ib, Id and Ie account for the majority of worldwide cases in humans [10]. In the present study, three subtype families (Ia, Io, and li) were identified, and were composed of IaA20R3a, IoA17a, IoA17b and liA17. Among them, subtype liA17 was the dominant subtype (66.7%) which has been reported in a rhesus monkey from the USA, in a crab-eating macaque from China, and two humans from Sweden who had visited a monkey farm in Thailand [20, 29, 36]. The subtypes IaA20R3a, IoA17a and IoA17b identified here have not been reported to the best of our knowledge, thus representing new subtypes. The subtype family Ia is common in humans from China, and IaA14R4 was the cause of a cryptosporidiosis outbreak in a pediatric ward in Shanghai [37]. In fact, *C. hominis* Ia subtype families have also been detected at high frequencies in rhesus monkeys from Guizhou, China. Thus, NHPs can play a potential role in zoonotic transmission of *C. hominis* in China [21]. Also of concern, the gp60 subtype family Io, and the related Id, have been identified in two horses in China [38]. The new subtypes IoA17a and IoA17b found in *M. fascicularis* here, is possibly a reflection of endemic transmission of *C. hominis* in these animals. The true subtype constitutions of *C. hominis* in *M. fascicularis* need to be confirmed by more systematic epidemiological studies of *Cryptosporidium* from these animals in the future.

**Conclusions**

In this study, we determined the prevalence rate of *C. hominis* among *M. fascicularis* in a breeding base of experimental primates in Hainan Island of China. The novel subtypes of *C. hominis* detected in *M. fascicularis* might present the endemic genetic characterization of population structure of *Cryptosporidium*, although the genetic diversity among *C. hominis* subtypes is not well understood. To better evaluate the transmission of *Cryptosporidium* from *M. fascicularis* to humans, more studies investigating the biology, population genetics and transmission dynamics of *Cryptosporidium* in *M. fascicularis* throughout different geographical regions are needed.

**Acknowledgements**

Not applicable.

**Funding**

This work was supported partially by the Innovation Research Team Project of Hainan Natural Science Foundation (2018CXTD340) and the National Natural Science Foundation of China (No. 81672072 and No. 81760378). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**Availability of data and materials**

The novel representative sequences of *C. hominis* identified in this study were submitted to the GenBank database under the accession numbers MK270514–MK270517 (18S rRNA gene), and MK270518–MK270520 (gp60 gene).

**Authors’ contributions**

Designed and conceived the research: WZ and GL. Collected samples: WZ, H-H Z, M-Y Q, M-C L and J-H L. Performed experiments: WZ and H-H Z. Analysis and interpretation: WZ. Contributed reagents, materials and/or analysis tools: H-R J, F-F Y. Wrote the paper: WZ. Revised the paper: JF-W C. Grant funding: GL. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

Prior to the initiation of the study, the farm owners were contacted, and permission to sample the animals was obtained. Rather than handling the animals directly, fecal samples were instead collected in order to minimize discomfort and stress to the animals. No animals were harmed in any way during the course of the study.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Received:** 6 January 2019  **Accepted:** 19 April 2019  **Published online:** 30 April 2019

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