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Cryptosporidium spp. in wild rats (*Rattus* spp.) from the Hainan Province, China: Molecular detection, species/genotype identification and implications for public health

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ABSTRACT

Wild rats (*Rattus* spp.) carry many zoonotic pathogens including *Cryptosporidium*. Due to the close proximity of rats to humans in urban environments, the potential for disease transmission is high. *Cryptosporidium* is a protozoan parasite which when ingested causes serious human illness. Despite its importance, genetic characterization of *Cryptosporidium* in wild rats in the Hainan province of China has not been performed. In this study, we analyzed the occurrence and genetics of *Cryptosporidium* in wild rats from Hainan, China. From December 2017 to October 2018, 150 wild rats were captured and fresh fecal material was collected from intestinal sections. Rat species were identified by PCR-based amplification and analysis of the vertebrate cytochrome *b* (*cytb*) gene. *Cryptosporidium* was examined by PCR amplification of the partial small subunit of ribosomal DNA (SSU rDNA). *C. viatorum* were subtyped by PCR analysis of the gp60 gene. A total of four rat species were identified including Asian house rats (*Rattus tanezumi*) (n = 46), brown rats (*Rattus norvegicus*) (n = 56), Edward's long-tailed rats (*Leopoldamys edwardsi*) (n = 38) and muridae (*Niviventer fulvescens*) (n = 10), with *Cryptosporidium* positive rates of 73.9%, 28.6%, 55.3% and 40.0%, respectively (average infection rate: 50.0%, 75/150. Sequence analysis confirmed the presence of four *Cryptosporidium* species and two genotypes including *C. viatorum* (n = 11); *C. occultus* (n = 2); *C. muris* (n = 1); and *C. erinacei* (n = 1); rat genotypes III (n = 13) and IV (n = 47). Three novel subtypes of *C. viatorum* were identified in 6 of the 11 infected Edward's long-tailed rats: XVcA2G1a (n = 4), XVcA2G1b (n = 1) and XVdA3 (n = 1). The identification of human pathogenic *C. viatorum* and zoonotic *C. occultus*, *C. muris* and *C. erinacei,* suggested that wild rats infected with *Cryptosporidium* pose a threat to human health. Taken together, these findings highlight the need to control the rat population in Hainan, China. The need to improve the public awareness of the risk of disease transmission from wild rats to humans is also highlighted.

1. Introduction

Cryptosporidium is a single-celled eukaryote initially recognized as an opportunistic pathogen in AIDS patients. *Cryptosporidium* was subsequently shown to cause disease in those with fully functional immune systems evidenced by the massive outbreak in 1993 in Milwaukee (Wisconsin) with > 400,000 cases of diarrheal disease recorded (Mac Kenzie et al., 1994). To date, *Cryptosporidium* are recognized as one of the leading diarrhea-associated protozoa. Recent human studies have implicated *Cryptosporidium* as the second leading cause of death in children due to diarrheal disease which is responsible for ~10% of global child mortality (Sow et al., 2016; GBD Diarrhoeal Diseases Collaborators, 2018). In addition to human infections, *Cryptosporidium* has been identified in numerous animal species including domesticated

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livestock, poultry, companion animals, and wildlife, demonstrating its zoonotic nature and threat to public health (Khan et al., 2018; Ryan et al., 2016; Pumipuntu and Piratae, 2018). *Cryptosporidium* has also been identified in some water and food products, indicating the possibility of water-borne and food-borne transmission (Rosado-García et al., 2017; Ryan et al., 2018). Due to both clinical and public health awareness, *Cryptosporidium* has been ranked as a category B bio-defense pathogen by the National Institutes of Health (NIH) and in the Environmental Protection Agency (EPA) microbial contaminant candidate list of concern for waterborne transmission (https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens).

PCR-based techniques that employ suitable gene markers have been widely used for the accurate identification and characterization of microorganism species and strains (Ryan et al., 2017). In recent years, a dramatic increase in human-infective species and animal-specific genotypes have been reported (Ryan et al., 2016). Through amplification and sequencing of the small subunit (SSU) and rRNA gene analysis, at least 38 *Cryptosporidium* species and over 40 genotypes are now recognized (Feng et al., 2018). To date, more than 20 *Cryptosporidium* species/genotypes have been isolated in humans, eight of which are responsible for the majority of human cryptosporidiosis cases (Feng et al., 2018). Amongst them, *C. parvum* is generally accepted to be a zoonotic pathogen, based on genotypic subtyping that identified *C. parvum* subtypes in humans and epidemiologically-linked animals (Chalmers et al., 2011; Feng et al., 2018).

Rodents play a major role in the transmission of emerging pathogens including viruses, bacteria, rickettsia, and protozoa (Meerburg et al., 2009). Amongst them, wild rats are most common and typically reside in human populated areas, particularly rural areas with less than desirable hygiene conditions. The movement of rodents facilitates the transmission and spread of disease. This is largely due to their large numbers, mobile nature, and tolerance to pathogens (Koehler et al., 2018). Recent studies revealed the identity of *Cryptosporidium* spp. in rats in Asia, Australia and Europe, identifying the occurrence of 17 *Cryptosporidium* species or genotypes including *C. parvum*, *C.ubiquitum*, *C. muris*, *C. andersoni*, *C. proliferans*, *C. scrofarum*, *C. meleagridis*, *C. occultus*, *C. viatorum* and *C. tyzzeri*, *C. canis* and *Cryptosporidium* rat genotypes I to IV, *Cryptosporidium* pika genotype and *Cryptosporidium* Qinghai vole genotype. Amongst them, *C. parvum*, *C. muris* and rat genotype III were most frequently observed in rats, suggesting them to be major sources of human infection (Koehler et al., 2018; Zhao et al., 2018; Zhang et al., 2018).

In China, studies on zoonotic protozoa in rats are limited and no studies have been performed in Hainan (Zhao et al., 2015, 2018; Zhang et al., 2018). The aims of this study were to determine the prevalence of *Cryptosporidium* in wild rats captured from different areas of Hainan and to characterize the isolates to assess their zoonotic potential at the species and subtype level.

2. Materials and methods

2.1. Ethical committee approval

The research of protocol was reviewed and approved by the Research Ethics Committee and the Animal Ethical Committee of Hainan Medical University. In the present study, all the wild rats were handled and cared for according to the Chinese Laboratory Animal Administration Act of 1998.

2.2. Study site and rodent collections

From December 2017 to October 2018, a total of 150 fecal specimens were collected from wild rats from six areas of the Hainan Province, China (Fig. 1). All rats were captured in cage traps baited with sunflower seeds and peanut/sesame butter. In each location, 20 cage traps were installed at sunset and gathered before sunrise, with

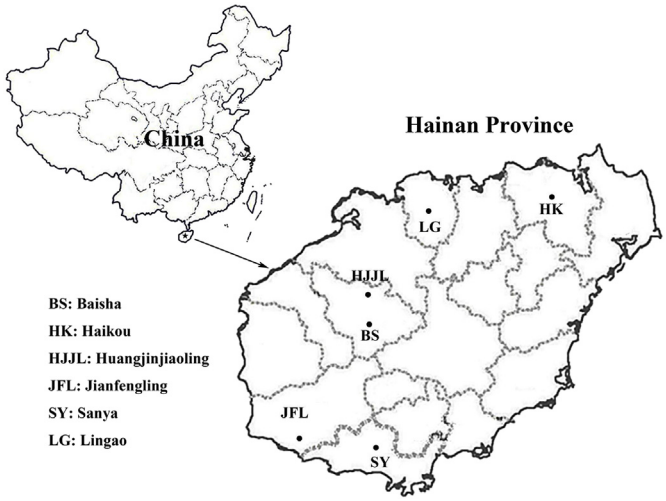

Fig. 1. Geographical locations of rat sources involved in the present study in Hainan Province, China.

traps positioned 5 m apart in transects. All rats were transported to the laboratory within 48 h of capture and sacrificed through CO_2 inhalation.

2.3. Fecal sample collection and DNA extraction

Fresh fecal material (approximately 500 mg) was collected directly from the intestine of each rat. Each fecal specimen was washed with distilled water by centrifugation for 10 min at 1500 *g* at room temperature. Genomic DNA was directly extracted from ~200 mg of each processed specimen using a QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. To obtain high yields of DNA, the lysis temperature was increased to 95 °C. DNA was eluted in 200 mL of AE elution buffer (provided in the kit) and stored at −20 °C prior to PCR analysis.

2.4. Identification of rat species

The molecular identification of the rat species was performed from fecal DNA by PCR-based amplification of a 421 bp region of the universal vertebrate cytochrome *b* (*cytb*) gene. PCR conditions and primer design followed those described by Verma and Singh (2003). Each PCR consisted of 35 cycles of 94 °C for 30 s (denaturation), 51 °C for 30 s (annealing), and 72 °C for 30 s (extension); an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 5 min were also included.

2.5. 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 as previously described (Xiao et al., 1999). All PCR amplifications were performed with positive controls (*C. homini* DNA for *Cryptosporidium*) and negative controls (2μ deionized water) which contained no DNA. Subtyping of *C. viatorum* samples was performed through nested PCR amplification of ~800–850-bp fragments of the gp60 gene as described by Stensvold et al. (2015). TaKaRa TaqDNA Polymerase (TaKaRa Bio Inc., Tokyo, Japan) was used for all PCR reactions. All secondary PCR products were subjected to electrophoresis on 1.5% agarose gels and visualized by DNAGREEN staining (Tiandz, Inc., Beijing, China).

2.6. DNA sequencing and analysis

All secondary PCR products were sequenced using the same secondary PCR primers on an ABI PRISM™ 3730 DNA Analyser (Applied Biosystems, Carlsbad, CA, USA) using a BigDye Terminator v3·1 Cycle Sequencing kit (Applied Biosystems). The accuracy of the sequencing data were confirmed by sequencing the PCR products in both directions. Further PCR products from specific DNA preparations were sequenced as required. 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/).

2.7. Nucleotide sequence accession numbers

Representative gp60 gene sequences of *C. viatorum* observed here were deposited in GenBank under accession numbers MK433560 to MK433562.

3. Results

3.1. Identification of rat species

PCR and sequencing analysis of cytb amplicons showed that 46 of the 150 samples tested were from Asian house rats (*Rattus tanezumi*), 56 were from brown rats (*Rattus norveqicus*), 38 were from Edward's long-tailed rats (*Leopoldamys edwardsi*) and 10 were from muridae (*Niviventer fulvescens*) (Table 1). All cytb gene sequences had 100% identity with the reference sequence: KT808632 for *R. norveqicus*, MG748345 for *R. tanezumi*, KP992477 for *L. edwardsi* and MG748255 for *N. fulvescens*. Rats were captured from six locations; 20 rats from Haikou City were identified as *R. norvegicus*, all 25 rats from Huangjinjiaoling were L. *edwardsi* and all 35 rats from Lingao City were *R. tanezumi*. Rats form Baisha City, Jianfengling, and Sanya City belonged to two, three and four species, respectively (Table S1).

3.2. Frequency of cryptosporidium in rat species

A total of 75/150 (50.0%) rat specimens were positive for *Cryptosporidium* by PCR analysis. *Cryptosporidium* was found in all the four rat species including 34 *R. tanezumi*, 16 *R. norvegicus*, 21 *L. edwardsi* and four *N. fulvescens*. *R. tanezumi* had the highest *Cryptosporidium* infection rates (73.9%), followed by *L. edwardsi* (55.3%), *N. fulvescens* (40.0%) and *R. norvegicus* (28.6%) (Table 1). *Cryptosporidium* was present in all six of the investigated areas with positive rates ranging from 25.0% to 88.6% (Table S1).

3.3. Genetic characterization and distribution of cryptosporidium species/subtypes

A total of 4 species and 2 genotypes of *Cryptosporidium* were identified in the wild rats examined, including *C. viatorum*, *C. occultus*, *C. muris*, *C. erinacei*, and *Cryptosporidium* rat genotypes III and IV. Analysis of the 18S rRNA sequences of *Cryptosporidium* rat genotype IV revealed 47 sequences belonging to four types. Amongst them, a single sequence representing 32 *Cryptosporidium* IV isolates was identical to *Cryptosporidium* IV isolated from *R. norvegicus* in Sweden (JN172970), whilst 12 sequences showed 100% homology to *Cryptosporidium* found in Spanish water (KY483983). Three *Cryptosporidium* IV isolates had 100% similarity to sequences of *R. norvegicus* in Heilongjiang of China (n = 2, MG917670) and storm water in New York of United States of America (n = 1, AY737584). All 13 sequences of rat genotype III had 100% homology to the *Cryptosporidium* isolate in *R. rattus* (wild black rats) from Australia (JX294371). Likewise, all 11 sequences of *C. viatorum* were identical to those reported in the Nigerian population (JX644908). The sequences of the other three *Cryptosporidium* species (*C. occultus*, *C. muris*, *C. erinacei*) have been previously described: for MG699179 *C. occultus*. for AB697054 *C. muris* and for KF612324 *C. erinacei*.

Cryptosporidium viatorum was subtyped by gp60 gene sequence analysis and six of the 11 specimens were successfully amplified. Three subtypes belonged to two subtype families, including XVc (XVcA2G1a and XVcA2G1b in four and one *L. edwardsi*, respectively) and XVd (XVdA3 in one *L. edwardsi*). All three subtypes were novel and have not previously been identified. A single nucleotide change was observed between XVcA2G1a and XVcA2G1b, both of which had 95.3% (875 of 918 bp) identity with the XVaA3e (KP115940) subtype isolated from a human in Sweden. The XVdA3 subtype had 87% homology to that of the XVbA2G1 subtype (MG021319) from *R. lutreolus* (Australian swamp rat).

Cryptosporidium rat genotypes III and IV were found in all four rat species, *C. occultus* in *R. tanezumi* and *R. norvegicus*, *C. viatorum*, *C. muris*, and *C. erinacei* only in *L. edwardsi*, *R. norvegicus* and *R. tanezumi*, respectively (Table 1). Geographical assessment demonstrated that *Cryptosporidium* rat genotype IV was present in all six locations, *Cryptosporidium* rat genotype III was in Jianfengling, Kaikou and Lingao Cities, whilst *C. viatorum*, *C. occultus*, *C. muris*, *C. erinacei* were in Huangjingjiaoling, Sanya, Haikou and Lingao Cities, respectively (Table S1).

4. Discussion

This study is the first report of *Cryptosporidium* in wild rats in Hainan, China. *Cryptosporidium* is commonly found in rodents and variable prevalence rates have been reported, including 8.0–31.4% in mice, 2.1–63.0% in rats and 0.8–73.0% in voles (Koehler et al., 2018; Zhao et al., 2018). Amongst the rodents, rats have been surveyed for *Cryptosporidium* in 25 studies, with brown rats, Asian house rats and black rats the most common species (Koehler et al., 2018). Prior to this study, at least 2828 rat fecal samples were examined from Egypt (20.9%), Nigeria (1.5%), Brazil (16.8%), Sweden (12.0%), Japan (2.1%–38.0%), China (4.0%–9.3%), the Philippines (18.6%), the UK (24.0% and 63.0%) and Iran (17.1%) (Koehler et al., 2018; Zhao et al., 2018). The prevalence of *Cryptosporidium* in wild rats from Hainan was higher than that in any other study worldwide except one conducted in

Table 1
Prevalence and distribution of *Cryptosporidium* species and subtypes in four species of wild rodent.

Rodent species	No. of specimens	*Cryptosporidium species*	
		No. of positive (%)	Species/Genotype(s) (no. of specimens)
Asian house rat (*Rattus tanezumi*)	46	34 (73.9)	Rat genotype IV (24); Rat genotype III (8); *C. occultus* (1); *C. erinacei* (1)
Brown Rat (*Rattus norvegicus*)	56	16 (28.6)	Rat genotype IV (13); Rat genotype III (1); *C. muris* (1); *C. occultus* (1)
Edward's long-tailed rat (*Leopoldamys edwardsi*)	38	21 (55.3)	*C. viatorum* (11); Rat genotype IV (8); Rat genotype III (2)
Muridae (*Niviventer fulvescens*)	10	4 (40.0)	Rat genotype III (2); Rat genotype IV (2)
Total	150	75 (50.0)	Rat genotype IV (47); Rat genotype III (13); *C. viatorum* (11); *C. occultus* (2); *C. muris* (1); *C. erinacei* (1)

the UK, where up to 63.0% (46/73) of brown rats were infected with *Cryptosporidium*. (Koehler et al., 2018; Webster and Macdonald, 1995). These findings highlight the importance of epidemiological investigations of *Cryptosporidium* in these animals. It is difficult to explain the discrepancies in the prevalences of *Cryptosporidium* spp. among different studies because prevalences are affected by many factors, including the host species composition, the geographical distributions in the sample populations, the sample sizes, the seasons, the examination methods and the ecological conditions.

According to previous epidemiological reports, nine species and four genotypes of *Cryptosporidium* have been detected in rats, in which *C. parvum*, *C. muris* and rat genotype III were the most frequent (Koehler et al., 2018). In Hainan, 11/75 (14.7%) isolates were *C. viatorum* which was first described in 2012 from travelers returning to the UK from the Indian subcontinent, and subsequently found in humans from Bangladesh, Barbados, Colombia, Ethiopia, Guatemala, India, Kenya, Nepal, Nigeria and Pakistan (Elwin et al., 2012; Stensvold et al., 2015; Khalil et al., 2018). There were no reports of *C. viatorum* in any animal species other than humans prior to recent studies reporting its occurrence in three Australian swamp rats (*R. lutreolus*) (Koehler et al., 2018). In this study, *C. viatorum* was found for the first time in Edward's long-tailed rats and brown rats, indicating that this genotype has a broader range of reservoir hosts than initially anticipated. *C. viatorum* has been frequently identified in urban wastewater in Shanghai City, China (Huang et al., 2017). The potential therefore exists for *C. viatorum* to spread to the environment and other hosts including humans from infected Edward's long-tailed rats.

C. viatorum-positive specimens were subtyped by gp60 sequence analysis. Of the 11 *C. viatorum* isolates obtained, six were successfully amplified and belonged to three subtypes including XVcA2G1a (n = 4), XVcA2G1b (n = 1) and XVdA3 (n = 1). To our knowledge, these have not been previously characterized and thus represent novel subtypes. To date, only eight subtypes of *C. viatorum* (XVaA3a to XVaA3f, XVaA6 and XVbA2G1) have been identified globally (Koehler et al., 2018). Subtypes XVaA3a to XVaA3f were identified only in humans (Stensvold et al., 2015), XVaA6 was isolated in wastewater (Huang et al., 2017), and XVbA2G1 was identified in three Australian swamp rats (Koehler et al., 2018). The new gp60 subtypes of *C. viatorum* identified in this study highlight its high intraspecific variation that may be host-associated. The true subtype constitution of *C. viatorum* now requires further confirmation through systematic epidemiological studies of *Cryptosporidium* from different hosts.

C. occultus (n = 2), *C. muris* (n = 1) and *C. erinacei* (n = 1) were found in the wild rats examined, all of which have the ability to infect humans and animals. *C. occultus* previously known as the *Cryptosporidium* suis-like genotype, has been identified in humans in Canada, cattle in Denmark, India and China, yaks in China, and in rats from the Philippines and China (Kváč et al., 2018; Zhao et al., 2018). *C. muris* was the most common *Cryptosporidium* species found in rats, and was identified in other animal hosts including mice, cats, marsupials (bilbies), deer, and non-human primates (Karim et al., 2014; Huang et al., 2018). In humans, *C. muris* is most commonly found in children and HIV + individuals from developing countries including Saudi Arabia, Iran, Thailand, Kenya, Slovakia, Chile, Peru and the Slovak Republic (Chappell et al., 2015). *C. erinacei* was previously known as the *Cryptosporidium* Hedgehog genotype, which was first reported in a hedgehog (*Erinaceus europaeus* L.) from Denmark in 2002 (Enemark et al., 2002; Kváč et al., 2014). In addition, *C. erinacei* has been identified in horses from Algeria and in an immunocompetent individual from the Czech Republic (Laatamna et al., 2013; Kváč et al., 2013). This is the first report of *C. erinacei* in Asian house rats, indicating that this species has an extensive host range. In fact, previous research has shown that this species was not infectious for SCID and BALB/c mice (*Mus musculus*), Mongolian gerbils (*Meriones unguiculatus*), and golden hamsters (*Mesocricetus auratus*), thus whether the finding of *C. erinacei* in Asian house rats represented a natural infection needs to be confirmed with more systematic characterization of cryptosporidiosis in those animals. Meanwhile, we are unable to determine the true source of infection and transmission dynamics of *C. erinacei* in Asian house rats due to the lack of *C. erinacei* data from humans and animals in the investigated areas. Taken together, these results suggest that *C. occultus*, *C. muri* and *C. erinacei* have the potential for zoonotic transmission, and must be considered a potential threat to human health, despite accounting for 5.3% (4/75) of all *Cryptosporidium* isolates in the investigated wild rats.

In this study, 60/75 (80.0%) of the infected wild rats had *Cryptosporidium* rat genotype III or IV, which appear to be rat-adapted genotypes. To date, *Cryptosporidium* rat genotype IV was only recorded in *R. norvegicus* (Zhao et al., 2018) whilst *Cryptosporidium* rat genotype III was found in cats, mice and rats (Ng-Hublin et al., 2013; Paparini et al., 2012; Lv et al., 2009; Yang et al., 2015). The potential of rat genotype III and IV to cause disease in humans or livestock is unknown, but both can contaminate water supplies evidenced by their detection in streams in the USA and raw water in the UK and China (Feng et al., 2009; Jiang et al., 2005; Chalmers et al., 2010). To improve our understanding of the extent of host adaptation of the *Cryptosporidium* genotypes isolated from rats, an examination of a larger range of animal and human isolates across larger geographical regions coupled to longitudinal studies are required. This will permit more accurate assessments of the role of wild rats in the transmission of *Cryptosporidium* to humans and other animals.

In conclusion, this study demonstrates the high prevalence and wide distribution of *Cryptosporidium* spp. in wild rats in Hainan, China. Considering the infestation of the sampled rats with human-pathogenic *C. viatorum* and zoonotic *Cryptosporidium* species/genotypes including *C. occultus*, *C. muris* and *C. erinacei*, they are likely to play a role in the transmission of *Cryptosporidium* to humans and may emerge as an important source of water contamination in Hainan. It is thus strongly recommended that measures should be taken to control the rodent populations in Hainan and that the local public are aware of the risk of disease transmission to humans through wild rats.

Declaration of interest

We have no conflict of interest to declare with this work.

Conflicts of interest

The authors declared that they have no conflicts of interest to this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.03.017.

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