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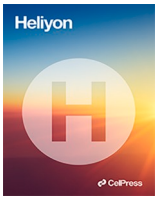

Research article

Molecular detection and genetic variability of *Cryptosporidium* spp. in wild Asian house shrews (*Suncus murinus*) from southern Zhejiang province, China

Jiangfeng Li [a,1], Zhongying Yuan [b,1], Junchen Xu [c,1], Xianming Xin [c], Jiani Liu [c], Xinrui Zhang [c], Shanshan Zhou [b], Zhen Li [b], Shuai Chen [a], Huicong Huang [c], Wei Zhao [c,**], Yanyan Jiang [b,*]

[a] *Wenzhou Center for Disease Control and Prevention, Wenzhou, Zhejiang, 325000, China*
[b] *National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention (Chinese Center for Tropical Diseases Research), National Key Laboratory of Intelligent Tracking and Forecasting for Infectious Diseases, NHC Key Laboratory of Parasite and Vector Biology, National Center for International Research on Tropical Diseases, WHO Collaborating Centre for Tropical Diseases, 200025, Shanghai, China*
[c] *School of Basic Medical Sciences, Wenzhou Medical University, Wenzhou, Zhejiang, 325035, China*

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ABSTRACT

Shrews play a crucial role as repositories for diverse pathogens linked to zoonotic infectious diseases. However, the genetic information regarding *Cryptosporidium* in Chinese shrews remains unexplored. The objectives of this study were twofold: to determine the occurrence rate of *Cryptosporidium* spp. in wild shrews residing in the southern part of Zhejiang Province, China, and to investigate their genetic characteristics. A total of 282 wild shrews were captured between April and October of 2023. The detection of *Cryptosporidium* in fecal samples, collected from each animal's rectum, was performed using PCR and sequencing of the partial small subunit of ribosomal RNA (*SSU rRNA*) gene. The 60-kDa glycoprotein (*gp60*) gene was utilized to further subtype the positive samples of *C. viatorum* and *C. parvum*. All animals were identified as *Suncus murinus*, and a positive result for *Cryptosporidium* was obtained in 14.2 % (40/282) of the samples. The following species and genotypes were identified: *C. ratti* ($n = 19$), *C. parvum* ($n = 2$), *C. viatorum* ($n = 1$), *Cryptosporidium* rat genotype IV ($n = 13$), and *Cryptosporidium* skunk genotype ($n = 5$). Furthermore, the subtypes IIdA15G1 and XVdA3 were detected within *C. parvum* and *C. viatorum*, respectively. Molecular evidence indicates that *S. murinus* is concurrently infected with rodent-adapted and zoonotic species/genotypes, actively contributing to the dissemination of cryptosporidiosis.

1. Introduction

Cryptosporidium, a recently emerged zoonotic intestinal protozoan, has significantly impacted both veterinary and public health.

* Corresponding author.
** Corresponding author.
E-mail addresses: hayidazhaowei@163.com (W. Zhao), jiangyy@nipd.chinacdc.cn (Y. Jiang).
[1] These authors contributed equally.

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Among humans, cryptosporidiosis has become the second leading contributor to diarrhea among children under the age of two [1]. Additionally, immunocompromised individuals are at particularly high risk, often succumbing to fatal diarrhea upon infection with *Cryptosporidium* [2]. Cryptosporidiosis also poses a substantial threat in veterinary medicine, ranging from fatal consequences in neonatal calves and other animals to diarrhea and growth retardation, even in the absence of any apparent clinical signs [3]. The widespread concern among scholars is due to *Cryptosporidium*'s ability to cause large-scale waterborne disease outbreaks. Globally, over 1500 outbreaks have been reported, with a notable instance in Wisconsin, where it affected 403,000 individuals and resulted in an estimated economic loss of 96.2 million US dollars [4,5]. Moreover, *Cryptosporidium* is also widely recognized as a major foodborne disease pathogen, resulting in over 8 million instances of foodborne illness every year [6]. *Cryptosporidium* is generally transmitted via the fecal-oral route, predominantly through the ingestion of ubiquitous infectious oocytes [7]. To effectively prevent *Cryptosporidium* outbreaks, it is imperative to accurately identify their source and mode of transmission, particularly given the current absence of drugs and vaccines. Therefore, precise identification appears to be the linchpin of prevention and control measures.

By employing molecular diagnostic techniques that combine PCR amplification with sequencing, we gained valuable insight into the potential sources of *Cryptosporidium* infection [8]. Up until now, *Cryptosporidium* has been recognized to encompass at least 50 species and more than 120 genotypes, most of which exhibit host adaptability [9]. Nonetheless, 22 species and two genotypes have been definitively shown to infect both humans and animals, creating a potential threat of zoonotic transmission [9,10]. Moreover, several species of *Cryptosporidium*, which were formerly limited to animals, are now frequently encountered in humans, such as *C. andersoni*, whereas species historically confined to humans are increasingly being detected in animals, such as *C. viatorum* [11,12]. This trend indicates a broadening of the host range for certain *Cryptosporidium* species. Consequently, it is crucial to intensify surveillance efforts for *Cryptosporidium* in animals that have close contact with humans, particularly among wild rodents and shrews.

Rodents, despite being extensively studied as hosts of *Cryptosporidium* worldwide, harbor an extensive array of over 26 species and

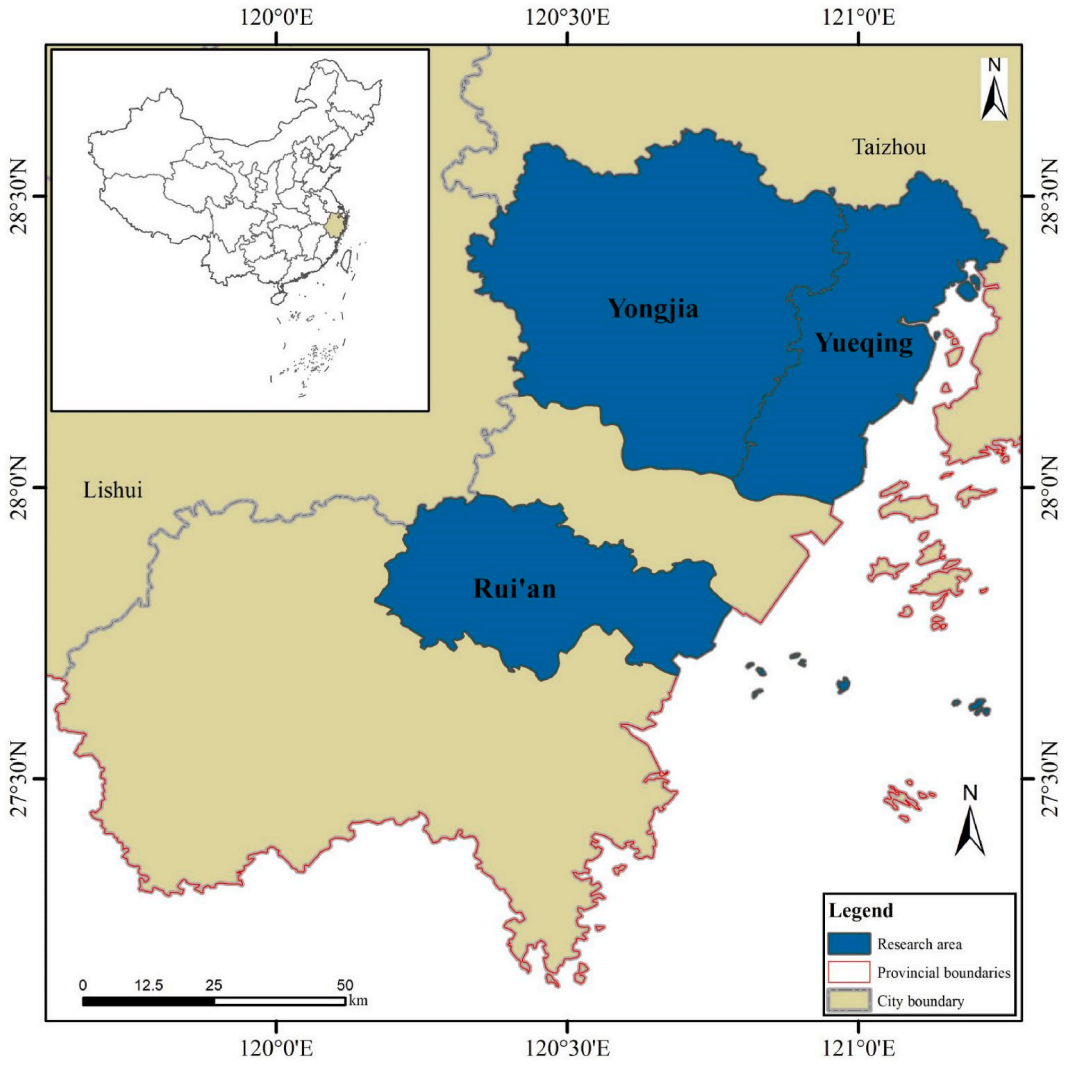

Fig. 1. Map depicting the three sampling locations of shrews in Wenzhou city, Zhejiang Province, China.

59 genotypes of the parasite and are distributed among a range of more than 40 rodent species [9]. While the majority of *Cryptosporidium* species and genotypes found in rodents display a degree of host specificity or limited host ranges, it is noteworthy that rodents can carry 78.3 % (18/23) of the species that infect humans, emphasizing their crucial role in the transmission of *Cryptosporidium* [9]. In contrast, the role of shrews in *Cryptosporidium* carriage remains enigmatic, with limited documentation restricted to *Sorex araneus* (a common shrew) carrying two genotypes: *Cryptosporidium* shrew genotype I and shrew genotype II [13]. This uncertainty underscores the need for further research to elucidate the prevalence and impact of *Cryptosporidium* in shrews.

Analogous to brown rats, shrews are frequently encountered in villages and urban centers throughout China, cohabiting closely with humans. However, the extent of *Cryptosporidium* infection among these animals remains unknown. Zhejiang Province, which is situated on the east coast of China (Fig. 1), has one of the most advanced economies in the nation. Previous epidemiological studies conducted in Zhejiang have suggested the presence of *Cryptosporidium* in humans, some farmed animals (pigs and chickens), racehorses, and wild rodents [14–18]. Nevertheless, no epidemiological surveys have been conducted on *Cryptosporidium* infecting shrews residing in this province. Hence, the objective of the current study was to elucidate the distribution, prevalence, and genetic attributes of *Cryptosporidium* species infecting shrews in the southern region of Zhejiang Province, China. Additionally, we aimed to evaluate zoonotic transmission risk by genotype and subtype comparisons through similarity and phylogenetic analysis.

2. Materials and methods

2.1. Ethical concerns

The Research Ethics Committee of Wenzhou Medical University carried out a review of the protocols pertaining to the present study, ultimately granting its approval with the reference number SCILLSC-2021-01.

2.2. Sample collection

Between April 1st and October 31st, 2023, 282 shrews were successfully trapped in Zhejiang Province, China, across three distinct locations, namely, Yueqing ($n = 26$), Yongjia ($n = 68$) and Rui'an ($n = 188$) (Fig. 1). The successful trapping of all these shrews was attributed to the use of cage traps baited with deep-fried dough sticks. At each designated location, approximately 50 cage traps were deployed at sunset and retrieved before sunrise. These traps were arranged in a linear fashion, with a distance of 5 m between each trap, forming transects. After the animals were trapped, data pertaining to the collection time and region were recorded. This study exclusively focused on shrews, and all the shrews were promptly transported to a controlled laboratory environment within 48 h and humanely euthanized via CO_2 inhalation. Subsequently, a fresh feces sample weighing 500 mg was promptly collected from the rectum of each shrew. Afterwards, the sample was securely stored in a low-temperature safety box and immediately transported to the laboratory for further analysis.

2.3. DNA extraction

Out of each 500 mg sample, 200 mg was specifically reserved for DNA extraction utilizing the QIAamp DNA Mini Stool Kit (Qiagen, Hilden, Germany). The lysis temperature was elevated to 95 °C, while the remaining steps were performed to in accordance with the manufacturer's recommended guidelines. Prior to undergoing PCR analysis, the extracted DNA was reconstituted in 200 μL of AE elution buffer, supplied with the kit, and securely stored at −20 °C for subsequent use.

2.4. Identification of shrew species

To identify the shrew species, we conducted PCR amplification of the vertebrate cytochrome *b* (*cytb*) gene, which specifically targeted a 421-base pair (bp) fragment extracted from fecal DNA. The primer sequences and PCR conditions utilized in this study adhered strictly to the previously described protocol outlined by Verma and Singh [19].

2.5. Cryptosporidium genotyping and subtyping

All DNA samples underwent nested PCR amplification, specifically targeting an 830 bp fragment of the partial small subunit of ribosomal RNA (*SSU rRNA*) gene belonging to *Cryptosporidium*. This amplification process utilized primers that were previously designed by Xiao et al. [20]. Subsequently, the positive *C. parvum* and *C. viatorum* isolates were further subtyped via nested PCR amplification of the 60-kDa glycoprotein (*gp60*) gene. This was achieved by employing primers previously developed by Alves et al. and Stensvold et al. respectively [21,22]. TaKaRa Taq DNA Polymerase was utilized in every PCR amplification process. Each round of PCR amplification included positive controls containing DNA derived from chickens infected with *C. bailey*, as well as negative controls lacking DNA templates. The PCR products were then analyzed via gel electrophoresis in TAE buffer on a 1.5 % agarose gel stained with GelRed (Biotium Inc., Fremont, CA, USA).

2.6. Sequencing analysis

The expected-sized PCR products were subjected to Sanger sequencing by Sangon Biotech (Shanghai) Co., Ltd., employing the

BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) on an ABI Prism 3730 XL DNA Analyzer. To ensure the accuracy of the nucleotide sequence, sequencing was conducted from both termini of the product, and additional PCR products were sequenced whenever mutations were detected. To identify the species and subtype of Cryptosporidium, the obtained sequences were aligned with reference sequences retrieved from the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) using ClustalX 2.0 software (http://www.clustal.org/).

2.7. Statistical analyses

Statistical analysis was conducted using the SPSS software (Windows version 22.0). The chi-square test was employed to assess the disparities in the prevalence of *Cryptosporidium* among various geographical regions, genders, and seasons. A statistically significant difference was considered if $P \leq 0.05$.

2.8. Nucleotide sequence accession numbers

The nucleotide sequences of *Cryptosporidium* obtained in this study have been deposited and registered in GenBank. The SSU rRNA sequences of five species/genotypes were assigned accession numbers PP038021, PP038022, PP038024, PP038025, and PP038027. Additionally, the gp60 sequences of the two subtypes were recognized with accession numbers PP104939 and PP104940.

3. Results

3.1. Identification and source composition of animal species

In this study, all 282 animals were identified as *Suncus murinus*, commonly known as the Asian house shrew. Among them, 26 originated from Yueqing, 68 from Yongjia, and 188 from Rui'an. Meanwhile, 114 (40.4 %) samples were collected in summer, 105 (37.2 %) in autumn and 63 (22.3 %) in spring. No animals were gathered in the winter. The proportions of female and male *S. murinus* were 45.2 % and 54.8 %, respectively (Table 1).

3.2. Occurrence rate of cryptosporidium

The analysis revealed that among the 282 fecal samples tested, 40 were positive for *Cryptosporidium*, yielding an average occurrence rate of 14.2 %, with 23.1 % (6/26) in Yeqing, 17.6 % (12/68) in Yongjia, and 11.7 % (22/188) in Rui'an (Table 1). There were no statistically significant differences in the prevalence of *Cryptosporidium* spp. among the three regions ($\chi^2 = 3.31$, df = 2, $P = 0.19$). In terms of season, the highest frequency of *Cryptosporidium* infection among *S. murinus* was recorded during the summer (20.2 %, 23/114), followed by the spring (14.3 %, 9/63) and the autumn (7.6 %, 8/105) months. For gender, the incidence of *Cryptosporidium* was relatively lower in female rodents (12.9 %, 12/100) than in male rodents (15.4 %, 28/182) (Table 1). The infection rate of *Cryptosporidium* varied significantly across the season groups, with statistical significance ($\chi^2 = 7.08$, df = 2, $P = 0.03$). However, no statistically significant difference was detected between the gender groups ($\chi^2 = 0.61$, df = 1, $P = 0.44$).

3.3. Identification and distribution of cryptosporidium species/genotypes

Analysis of the rRNA sequence products from 40 *Cryptosporidium*-positive samples revealed the presence of three distinct *Cryptosporidium* species, namely *C. ratti*, *C. parvum*, and *C. viatorum*, along with two genotypes: *Cryptosporidium* rat genotype IV and *Cryptosporidium* skunk genotype (Table 1). *C. ratti* was predominant at 40.9 % (19/40), followed by *Cryptosporidium* rat genotype IV ($n = 13$) and the *Cryptosporidium* skunk genotype ($n = 5$), while *C. parvum* and *C. viatorum* exhibited low frequencies, identified in two and one animals, respectively (Table 1).

Table 1
Prevalence and species/genotype of *Cryptosporidium* in *S. murinus* by gender, location and season.

Category	Positive/examined (%)	*Cryptosporidium* spp./genotype (n)
Gender		
Female	12/100 (12.0)	*C. ratti* (8), *Cryptosporidium* rat genotype IV (4)
Male	28/182 (15.4)	*C. ratti* (11), *Cryptosporidium* rat genotype IV (9), *Cryptosporidium* skunk genotype (5), *C. parvum* (2), *C. viatorum* (1)
Location		
Rui'an	22/188 (11.7)	*Cryptosporidium* rat genotype IV (9), *C. ratti* (7), *C. parvum* (2), *Cryptosporidium* skunk genotype (4)
Yongjia	12/68 (17.6)	*C. ratti* (8), *Cryptosporidium* rat genotype IV (4)
Yueqing	6/26 (23.1)	*C. ratti* (4), *C. viatorum* (1), *Cryptosporidium* skunk genotype (1)
Season		
Spring	9/63(14.3)	*C. ratti* (6), *Cryptosporidium* skunk genotype (2), *C. viatorum* (1)
Summer	23/114 (20.2)	*Cryptosporidium* rat genotype IV (13), *C. ratti* (8), *C. parvum* (2)
Autumn	8/105 (7.6)	*C. ratti* (5), *Cryptosporidium* skunk genotype (3)
Total	40/282 (14.2)	*C. ratti* (19), *Cryptosporidium* rat genotype IV (13), *Cryptosporidium* skunk genotype (5), *C. parvum* (2), *C. viatorum* (1)

Variations in the dispersion of *Cryptosporidium* species were observed among the three sampling sites. Specifically, *C. ratti* was detected in all three regions, *Cryptosporidium* rat genotype IV was present in Yongjia and Rui'an, *Cryptosporidium* skunk genotype was found in Yueqing and Rui'an, and *C. parvum* and *C. viatorum* were discovered in Rui'an and Yueqing, respectively (Table 1). Moreover, the distribution of *Cryptosporidium* species in *S. murinus* also varied based on gender and season. Specifically, *Cryptosporidium* rat genotype IV and *C. ratti* were detected in both genders, whereas *C. viatorum*, *C. parvum,* and *Cryptosporidium* skunk genotype were exclusively found in male rodents (Table 1). *Cryptosporidium* rat genotype IV and *C. parvum* were exclusively observed in animals collected during the summer, *C. viatorum* was detected in animals collected in the spring, *Cryptosporidium* skunk genotype was present in both the spring and autumn, and *C. ratti* was detected in all three seasons (Table 1).

3.4. Genetic identification of cryptosporidium species/genotypes

The thirteen SSU rRNA sequences of *Cryptosporidium* rat genotype IV obtained in this study were all identical, exhibiting complete congruency with the previously reported genotype W19 variant sequence (AY737582) that was isolated from storm water samples in the United States. Similarly, 19 SSU rRNA sequences of *C. ratti* identified in our study were identical, but they have not been previously described. Sequence similarity analysis revealed that they share 99.51 % similarity with the JN172971 sequence of *Cryptosporidium* rat genotype I, which was isolated from *Rattus norvegicus* in Sweden. In addition, the two *C. parvum* sequences were identical and shared 100 % similarity with the OM146539 sequence isolated from humans in Sweden. Furthermore, the five sequences of *Cryptosporidium* skunk genotype isolates were identical and novel. These sequences exhibited 99.32 % homology to the MN888751 sequence isolated from *Procyon Lotor* in Germany. Finally, the *C. viatorum* sequence was also novel and exhibited 99.61 % homology to the MK522270 sequence isolated from *Berylmys bowersi* in China.

All three *C. viatorum*- or *C. parvum*-positive samples were successfully subtyped at the gp60 gene. The two *C. parvum* samples shared an identical gp60 sequence and exhibited 100 % homology to the sequence of subtype IIdA15G1 (KJ917586) from *Macaca mulatta* in China. Notably, the *C. viatorum* sequence has not been reported previously and exhibited a nucleotide similarity of 99.51 % with the *C. viatorum* subtype XVdA3 (MK433560) obtained from *Leopoldamys edwardsi* in China.

4. Discussion

In the present study, only *S. murinus* was identified. The positive rate of *Cryptosporidium* among these animals was 14.2 %, similar to that of shrews from Slovakia (14.3 % prevalence) but notably lower than that of shrews from Finland, where a *Cryptosporidium* prevalence of 41.9 % was documented [13,23]. The prevalence of *Cryptosporidium* in shrews from other countries or regions remains unknown. Overall, it is anticipated that the infection rate among shrews is likely to be similar to that among wild rodents given their frequent coexistence or residency in the same habitat. The prevalence of *Cryptosporidium* infection is significantly greater in wild rodents than in humans (17.0 % *vs* 7.6 %) [9,24]. Consequently, it is crucial to enhance surveillance of shrew-like animals to better understand their potential public health significance in the transmission of *Cryptosporidium*.

The present study identified five *Cryptosporidium* species/genotypes, namely, *C. ratti*, *C. parvum*, *C. viatorum, Cryptosporidium* rat genotype IV and *Cryptosporidium* skunk genotype. Among them, *C. ratti* (previously named *Cryptosporidium* rat genotype I) and *Cryptosporidium* rat genotype IV (previously designated as *Cryptosporidium* environmental sequence, genotypes W19 or W19 variant) were previously found in rodents in China, with *C. ratti* in brown rats and red squirrels and *Cryptosporidium* rat genotype IV in Asian house rats, Edward's long-tailed rats, Muridae, and brown rats [25,26]. *Cryptosporidium* rat genotype IV has been identified in rats from Japan, Spain and Sweden [27–29], while *C. ratti* has been detected in rats from Sweden [27], the Philippines [30], the Czech Republic [31] and Nigeria [32]. Furthermore, environmental samples have revealed their presence, comprising untreated water from the UK [33] and wastewater samples from China (Fan et al., 2021) [34], a stream in the US [35], and the South Nation River watershed in Canada [36]. Notably, while *Cryptosporidium* rat genotype IV has been detected in Asiatic black bears and cats from China and one-humped camels from Egypt, limited data exist regarding the potential for infection of humans and other animals by both *C. ratti* and *Cryptosporidium* rat genotype IV [37–39]. This suggests that these two pathogens may have limited zoonotic transmission capabilities. The discovery of *C. ratti* and *Cryptosporidium* rat genotype IV in *S. murinus* in the present study indicates that these genotypes may possess broader host ranges and that *S. murinus* may facilitate their transmission. However, future molecular epidemiological studies are necessary to clarify the exact host distribution of *C. ratti* and *Cryptosporidium* rat genotype IV.

Initially, *Cryptosporidium* skunk genotype was identified in a small number of skunks [40]. Further intensive research revealed that this genotype is prevalent in the raccoon and has been linked to human infections, including in populations in England and Wales and among asymptomatic preschool children in the UK [41]. In addition, 9 % of the water samples from the Wissahickon watershed were identified as *Cryptosporidium* skunk genotypes from May 2005 to April 2008 [33]. This genotype was also found in water samples from Canada [42]. The present study identified the *Cryptosporidium* skunk genotype for the first time in *S. murinus*, which indicates a broader host range for this genotype. Given possible zoonotic transmission, *S. murinus* may serve as a potential reservoir for human cryptosporidiosis caused by *Cryptosporidium* skunk genotype. This finding has major consequences for public health.

C. parvum and *C. viatorum* are two well-known *Cryptosporidium* species that can infect humans and are widely reported worldwide [10]. *C. parvum,* which is responsible for most cryptosporidiosis outbreaks after *C. hominis* [10]. In China, cryptosporidiosis caused by *C. parvum* accounts for 16.7 % (44/263) of the total cases [43]. In addition, *C. parvum* is widely distributed in wildlife and has been documented in more than 40 wild animal species [44]. Similarly, since the initial identification of *C. viatorum* infection in humans in 2012, there have been reported cases of *C. viatorum* infection in humans across 13 countries, encompassing China among them [12,45]. Recent studies have shown that *C. viatorum* also has animal hosts, mainly including some rodents, such as *R. rattus* from France,

R. lutreolus from Australia, and *Leopoldamys edwardsi* and *Berylms bowersi* from China, indicating that rodents are its primary host [9,25]. Although only three animals were identified in this study to infect *C. parvum* and *C. viatorum*, this cannot be overlooked because they can infect humans, with the possibility of transmission from *S. murinus* to humans.

Subtype analysis revealed that *S. murinus* was infected with subtype IIdA15G1 of *C. parvum* and subtype XVdA3 of *C. viatorum*. By analyzing the sequence of the *gp60* gene, researchers have revealed a remarkable genetic diversity among *C. parvum*, encompassing approximately 20 distinct subtype families [44]. The IId subtype family exhibits a widespread global distribution and is primarily responsible for initiating outbreaks of human cryptosporidiosis, stemming from zoonotic infections across New Zealand, along with several regions in the Middle East and Europe [44]. In China, subtypes belonging to the *C. parvum* IId family demonstrate a broad array of host diversity, encompassing humans, ruminants, camelids, equine animals, carnivores, nonhuman primates, as well as numerous rodent species [43,44]. Among these IId subtypes, IIdA15G1 is the most prevalent, with a widespread geographic distribution and occurrence in both humans and animals [43,44]. This widespread occurrence indicates its capacity for cross-species transmission, thereby giving rise to concerns regarding the possibility of zoonotic transmission within China. The current study marks the initial identification of IIdA15G1 in *S. murinus*, emphasizing its extensive host range and sparking concerns regarding the potential risk of transmission of the parasite from *S. murinus* to humans.

Currently, a total of 15 subtypes of *C. viatorum* have been discovered globally [12]. Of the identified subtypes, XVaA3g, XVaA3h, and XVcA2G1 are noteworthy because they are present in both humans and rodents. This shared presence across species suggests a potential link in the evolutionary chain of *C. viatorum*, indicating that the subtype infecting humans may have originated from rodents [9,12]. However, the XVdA3 subtype identified in *S. murinus* in the present study is currently restricted to a single rodent species of *L. edwardsi* and has not yet been found in humans [25]. Therefore, it is crucial to conduct further research to determine the precise host range and transmission dynamics of this subtype, thereby enhancing our comprehensive understanding of its potential impact on human health.

5. Conclusion

For the first time, this study revealed the occurrence of *Cryptosporidium* spp. infecting *S. murinus* in Zhejiang Province, China. Molecular analysis indicated that the infected *S. murinus* primarily harbored nonhuman infectious *C. ratti* and *Cryptosporidium* rat genotype IV, suggesting a significant role for *S. murinus* in maintaining and disseminating *Cryptosporidium* infections among rodent populations. Moreover, the presence of zoonotic *C. parvum*, *C. viatorum*, and the *Cryptosporidium* skunk genotype in some infected *S. murinus*, indicating a potential risk of *Cryptosporidium* transmission from these animals to humans.

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Ethical approval

The Research Ethics Committee of Wenzhou Medical University carried out a review of the protocols pertaining to the present study, ultimately granting its approval with the reference number SCILLSC-2021-01.

Informed consent statement

Not applicable.

Availability of data and materials

All the results of the study are presented within the manuscript. The nucleotide sequences of *Cryptosporidium* obtained in this study have been deposited and registered in GenBank. The SSU rRNA sequences of five species/genotypes were assigned accession numbers PP038021, PP038022, PP038024, PP038025, and PP038027, respectively. Additionally, the gp60 sequences of the two genotypes were recognized with accession numbers PP104939 and PP104940.

CRediT authorship contribution statement

Jiangfeng Li: Writing – original draft, Methodology, Investigation, Formal analysis. **Zhongying Yuan:** Writing – original draft, Investigation, Formal analysis. **Junchen Xu:** Writing – original draft, Investigation, Formal analysis. **Xianming Xin:** Investigation. **Jiani Liu:** Investigation. **Xinrui Zhang:** Investigation. **Shanshan Zhou:** Formal analysis. **Zhen Li:** Investigation. **Shuai Chen:** Methodology. **Huicong Huang:** Supervision. **Wei Zhao:** Writing – review & editing, Writing – original draft, Validation, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yanyan Jiang:** Writing – review & editing, Validation, Software, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Wei Zhao reports financial support was provided by the Basic Scientific Research Project of Wenzhou (Y2023070). Yanyan Jiang reports financial support was provided by the National Key Research and Development Program of China (2021YFC2300902), the National Natural Science Foundation of China (82273693). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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