Kafkas Universitesi Veteriner Fakultesi Dergisi
ISSN: 1300-6045 e-ISSN: 1309-2251
Journal Home-Page: http://vetdergikafkas.org

Kafkas Univ Vet Fak Derg
27 (3): 397-401, 2021
DOI: 10.9775/kvfd.2021.25681

SHORT COMMUNICATION

Identification of a New MLST Subtype of *Cryptosporidium muris* from a Brown Rat *(Rattus norvegicus)* in China

Xuehan LIU [1,a (*)] Lei DENG [2,b] Zhijun ZHONG [2,c] Ziyao ZHOU [2,d] Xuefeng YANG [1,e] Zhixing AN [1,f]

[1] College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, CHINA
[2] College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 625014, CHINA
ORCIDs: [a] 0000-0002-8376-5580; [b] 0000-0003-0391-6180; [c] 0000-0001-8609-448X; [d] 0000-0002-8947-4303; [e] 0000-0002-7037-6607
[f] 0000-0001-7649-5626

Article ID: KVFD-2021-25681 Received: 03.03.2021 Accepted: 22.05.2021 Published Online: 23.05.2021

Abstract

Cryptosporidium muris is a zoonotic protozoan. Characterization of genetic differentiation can be exploited to trace the origins of the parasite. A *Cryptosporidium* isolate from brown rat was obtained by microscopy and genetically characterized. *C. muris* was identified with minor nucleotide differences. Further subtype analysis was performed using a multilocus sequence typing (MLST) tool. At each locus, the *C. muris* isolate was confirmed to be M12, M4, M1 and M2 subtype, representing as a new MLST subtype. Therefore, the results suggest that there was probably a new source of infection for *C. muris* in the study region.

Keywords: *Cryptosporidium muris, 18S rRNA, MLST, Subtype, Brown rat*

Çin'de Kahverengi Sıçanda *(Rattus norvegicus) Cryptosporidium muris*'in Yeni Bir MLST Alt Tipinin Tanımlanması

Öz

Cryptosporidium muris, zoonotik bir protozoondur. Genetik farklılaşmanın karakterizasyonu parazitin kökenini izlemek için kullanılabilir. *Cryptosporidium* izolatı kahverengi bir sıçandan mikroskopi ile elde edildi ve genetik olarak karakterize edildi. Küçük nükleotid farklılıklarla *C. muris* olarak tanımlandı. İleri identifikasyonu, çoklu lokus dizilim analizi (MLST) ile gerçekleştirildi. Her lokusta, *C. muris* izolatının yeni bir MLST alt tipini temsil eden M12, M4, M1 ve M2 alt tipi tanımlandı. Bu nedenle bu sonuçlar, çalışma bölgesinde *C. muris* için muhtemelen yeni bir enfeksiyon kaynağı olduğunu göstermektedir.

Anahtar sözcükler: *Cryptosporidium muris, 18S rRNA, MLST, Alt Tip, Kahverengi sıçan*

INTRODUCTION

Cryptosporidium is a ubiquitous zoonotic protozoan parasite that can infect a variety of vertebrate animals. Depending on the immunological status of hosts, the clinical symptoms of cryptosporidiosis commonly range from acute to chronic diarrhea. Epidemiologic studies indicate that multiple routes of transmission are likely, including direct, zoonotic, foodborne and waterborne transmission may occur in different areas [1,2]. To date, at least 45 valid species and a similar number of genotypes have been described based on molecular characteristics, revealing an extensive genetic diversity [3]. Among those, *C. muris* as gastric protozoa has a wide range of host-adaptations and is generally detected in various rodents [4,5]. Other animals including deer, Tasmanian devil, pig, camel, dog, cat, monkey, and bird can also be infected with *C. muris* at low frequency [6-10]. In addition, a few cases of human cryptosporidiosis have been attributed to *C. muris* in recent years, suggesting the potential for zoonotic transmission [11,12].

Numerous types of molecular diagnostic tools have been used to differentiate *Cryptosporidium* in host and environmental samples at the genotype and subtype levels to trace the likely source of infection [6]. Among those,

How to cite this article?

Liu X, Deng L, Zhong Z, Zhou Z, Yang X, An Z: Identification of a new MLST subtype of cryptosporidium muris from a brown rat *(Rattus norvegicus)* in China. *Kafkas Univ Vet Fak Derg,* 27 (3): 397-401, 2021.
DOI: 10.9775/kvfd.2021.25681

(*) **Corresponding Author**

Tel: +86-0373-3040718
E-mail: liuxuehan1986@126.com (X. Liu)

a multilocus sequence typing (MLST) tool was developed for *C. muris* by employing microsatellite and minisatellite markers with simple tandem repeats, which showed the capacity to distinguish various subtypes [13]. Currently, 14 MLST subtypes have been identified in diverse hosts and geographical regions [13,14]. Additionally, the MLST tool is also helpful for molecular epidemiology and population genetic studies [14].

Reports on *C. muris* infecting brown rats are limited, and subtyping *C. muris* was not conducted in those studies [15]. To our knowledge, there are no reports on brown rats being infected with *C. muris* in China [16]. In the present study, one *C. muris* isolate (BN01) from brown rat was genetically characterized by analysis of three genotyping and four MLST-subtyping genes for the purpose of further tracking of infection sources.

Material and Methods

Ethics Approval

This study was performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the Ministry of Health, China. The research protocol was reviewed and approved by the Research Ethics Committee of the Sichuan Agricultural University.

Cryptosporidium muris Isolate

One brown rat was trapped in the suburbs of Ya'an city and kept in a sterilized mouse cage. Approximately 2-5 g of feces were collected for five consecutive days. The feces were dissolved into 50 mL double-distilled water via stirring. The Sheather's sugar flotation method was used for examining *Cryptosporidium* oocysts by microscopy at x400 magnification as previously described [17]. The specimen was found to be positive. Then, the concentrates containing oocysts were separated to 2.5% potassium dichromate solution and stored at 4°C.

DNA Extraction and PCR Amplification

Oocysts were rinsed twice in double-distilled water and pelleted at 5.000×g centrifugation to remove potassium dichromate. Genetic DNA was extracted using the E.Z.N.A.® Stool DNA Kit (OMEGA Biotek Inc D4015-02, USA) according to the manufacturer's protocol. The extracted DNA was stored at -20°C until the time of PCR analysis.

The nested-PCR was used to amplify the partial 18S rRNA, COWP and CpA135 genes to genotype identification. The primers and amplification conditions were adopted as previously described [17,18]. Premix Ex Taq™ (Takara, Dalian, China) was used for PCR amplification. The secondary PCR products at each genotyping locus were resolved using 1% agarose gel electrophoresis with ethidium bromide staining, and were identified by DNA sequencing (Sangon Biotech, Shanghai, China). Sequence accuracy was confirmed using bidirectional sequencing, and a new PCR product was re-sequenced if necessary.

MLST Subtype

Analysis using the MLST incorporated MS1 (coding for hypothetical protein), MS2 (coding for 90 kDa heat shock protein), MS3 (coding for hypothetical protein), and MS16 (coding for leucine rich repeat family protein) in order to subtype the *C. muris* isolated from a brown rat. Primers and amplification conditions were in accordance with those developed previously *(Table 1)* [13]. PrimeSTAR® DNA Polymerase (TaKaRa, Dalian, China) was used to ensure high sequence fidelity. To neutralize PCR inhibitors, 400 ng/mL of non-acetylated bovine serum albumin (TaKaRa, Dalian,

Table 1. *PCR primers for MLST subtype used in this study*

Genes	Primer	Sequence (5'-3')	Tm	Length	Tandem Repeat Sequence
MS1	F1 R1	ACCATCTAGAGATAACGAGCGA GAATCAGAAGATGAGCGACAA	55°C	~550 bp	GAACGAGATAGG
	F2 R2	CGTGATAGTGGGTATGAATTGGACA CGACTGCGATACTCACGTCCT	55°C		
MS2	F1 R1	TTGCAACTGTACCTAAATTAGTA GTGAGACTTCTGGGGTCCTGA	52°C	~460 bp	CCATATCCC
	F2 R2	TCATGACGCGTCATACCAACA ACTTAGACAGTTCTATGCTGA	55°C		
MS3	F1 R1	AACCAAGTGAATCACGAACTT TCAAGTACAGCAGTCTATTGCTT	55°C	~540 bp	TGTTGG /GCTGCA
	F2 R2	GCAATATCTTCGACGATCCCA ATGGGAATAATTCTTCATCATCAA	55°C		
MS16	F1 R1	GAAGAGGTCGAAGTTAAGCTA GACAATCATCTAAATCGTGTT	50°C	~600 bp	CTTCTTCAT
	F2 R2	AAGTTTCATCTAGGTACACTAAGA CACTACCTAATCTCGTGTACTT	55°C		

F and R: the forward and reverse primers, respectively; 1 and 2: the primary and secondary PCRs, respectively

China) was used in both PCRs at each MLST locus. The detection and DNA sequencing were performed identically to the above manipulations. Likewise, to ensure subtype accuracy, especially in the copy number of minisatellite repeats, PCR products in the subtype loci were sequenced at least twice.

Phylogenetic Analysis

Nucleotide sequences of all 7 genes were aligned with reference sequences in GenBank using ClustalX 1.83 *(ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/)*. Phylogenetic analyses were performed using software PHYLIP v3.69 at each locus *(http://evolution.genetics.washington.edu/phylip.html)*. Neighbor-joining trees were constructed based on evolutionary distances calculated using the Kimura two-parameter model. Phylograms were drawn using MEGA4.0 *(http://www.megasoftware.net/)*. To assess the reliability of these trees, bootstrap analysis using 1,000 pseudo-replicates was carried out with values above 50% reported. Sequence identity was analyzed using MegAlign program in the DNAStar v6.0 software *(https://www.dnastar.com/software/DNASTAR-Cloud/)*.

Through adjustment, the partial nucleotide sequences obtained in this study were deposited in the GenBank under accession numbers: KF419208-KF419214.

Results

Through microscopic examination, it was observed that the oocysts were morphologically bigger than the intestinal *Cryptosporidium*, but similar to the gastric *Cryptosporidium* [4]. Thus, genetic analyses were necessary to distinguish genotypes.

An approximate 830 bp fragment of the 18S rRNA gene was successfully amplified. The resulting sequence was initially identified as *C. muris* with a maximum similarity of 100% when compared to reference *C. muris* isolates from hamster, ostrich, monkey, and human in GenBank. Phylogenetic analysis revealed that the current isolate was clustered with those *C. muris* isolates with high bootstrap values *(Fig. 1)*. In the present study, the observed CpA135 sequence was 99.8% identical to that from house rat-derived *C. muris* (RN66 isolate, HM358026) with a mutation of A to G at nucleotide 6 [18]. For the COWP gene, the isolate shared 100% homology with those *C. muris* reported in rock hyrax (AF161579), whereas 6 nucleotide changes were found compared to the RN66 isolate (DQ060430). Likewise, phylogenetic topology was similar to that in the 18S rRNA gene *(Fig. 1)*. These results demonstrated that this isolate was indeed *C. muris*.

At each MLST locus, the corresponding nucleotide sequences were obtained successfully. Sequence analysis of MS2, MS3 and MS16 genes identified M4, M1 and M2 haplotypes, respectively, which were homologous to those previously deposited in GenBank. However, the sequence at MS1 locus occupied max similarity to M5 haplotype with a single C insertion at nucleotide 471 in the nonrepeat region when compared to all known *C. muris* MS1 sequences. Based on the coding nature of MLST targets, nucleotide insertions and deletions commonly occur in trinucleotide

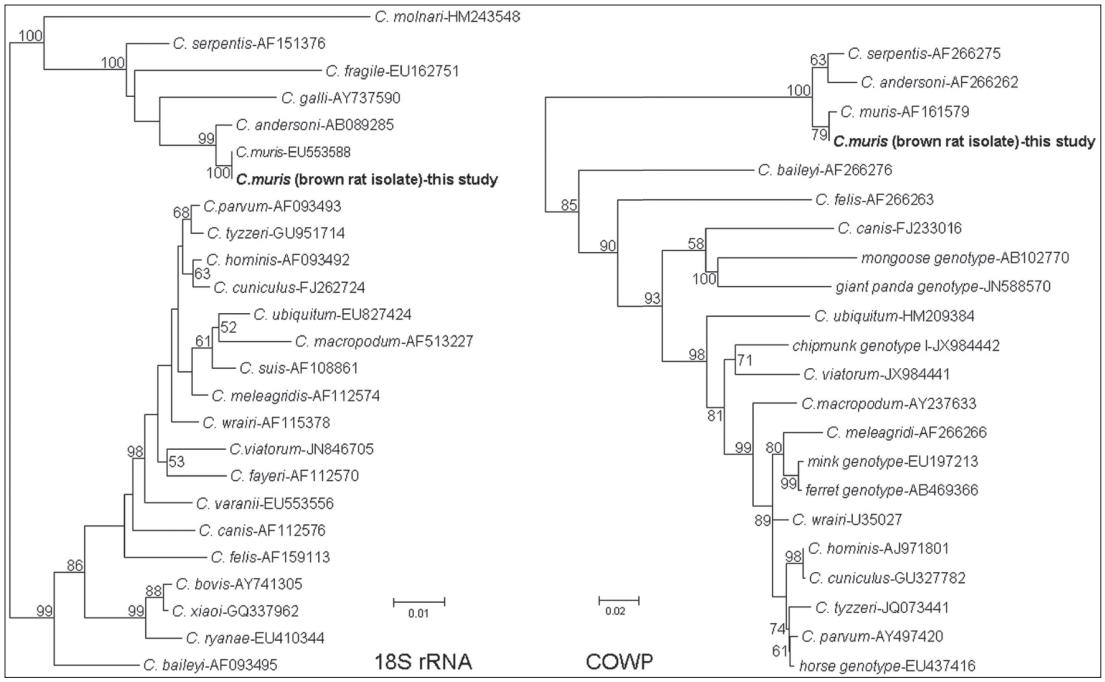

Fig 1. Phylogenetic relationship of *Cryptosporidium* isolated from a brown rat inferred by neighbor-joining analysis of the SSU rRNA and COWP genes based on evolutionary distances calculated using the Kimura two-parameter model. Bootstrap values above 50% are shown using 1.000 pseudoreplicates. Bar = substitutions/site

Table 2. *Subtype of C. muris brown rat isolate in this study and other representative C. muris isolates at four MLST loci*

No.	Specimen	Host	Source	MLST Subtype				Reference
				MS1	MS2	MS3	MS16	
1	RN66	Brown rat	Japan	M5	M4	M1	M5	[13]
2	6853	Human	Lima, Peru	M1	M2	M4	M5	[13]
3	7511	Bactrian camel	Czech Republic	M1	M1	M4	M5	[13]
4	14907	Rat snake	Czech Republic	M2	M2	M3	M5	[13]
5	13469	Camel via mice	Egypt	M5	M4	M2	M3	[13]
6	OH1	Ostrich	Henan, China	M5	M4	M6	M4	[14]
7	14714	Human	Nairobi, Kenya	M6	M4	M1	M2	[13]
8	14906	Lab mouse	Czech Republic	M6	M4	M2	M4	[13]
9	7379	Domestic mouse	Czech Republic	M7	M4	M2	M1	[13]
10	1666	Chipmunk	Czech Republic	M7	M4	M1	M5	[13]
11	7512	Mountain goat	Czech Republic	M8	M4	M2	M4	[13]
12	7380	Mara	Czech Republic	M9	M4	M1	M1	[13]
13	14243	SCID mice	Czech Republic	M10	M5	M5	M6	[13]
14	MC4	Hamster	Henan, China	M11	M4	M6	M1	[14]
15	**BN01**	**Brown rat**	**Sichuan, China**	**M12**	**M4**	**M1**	**M2**	**This study**

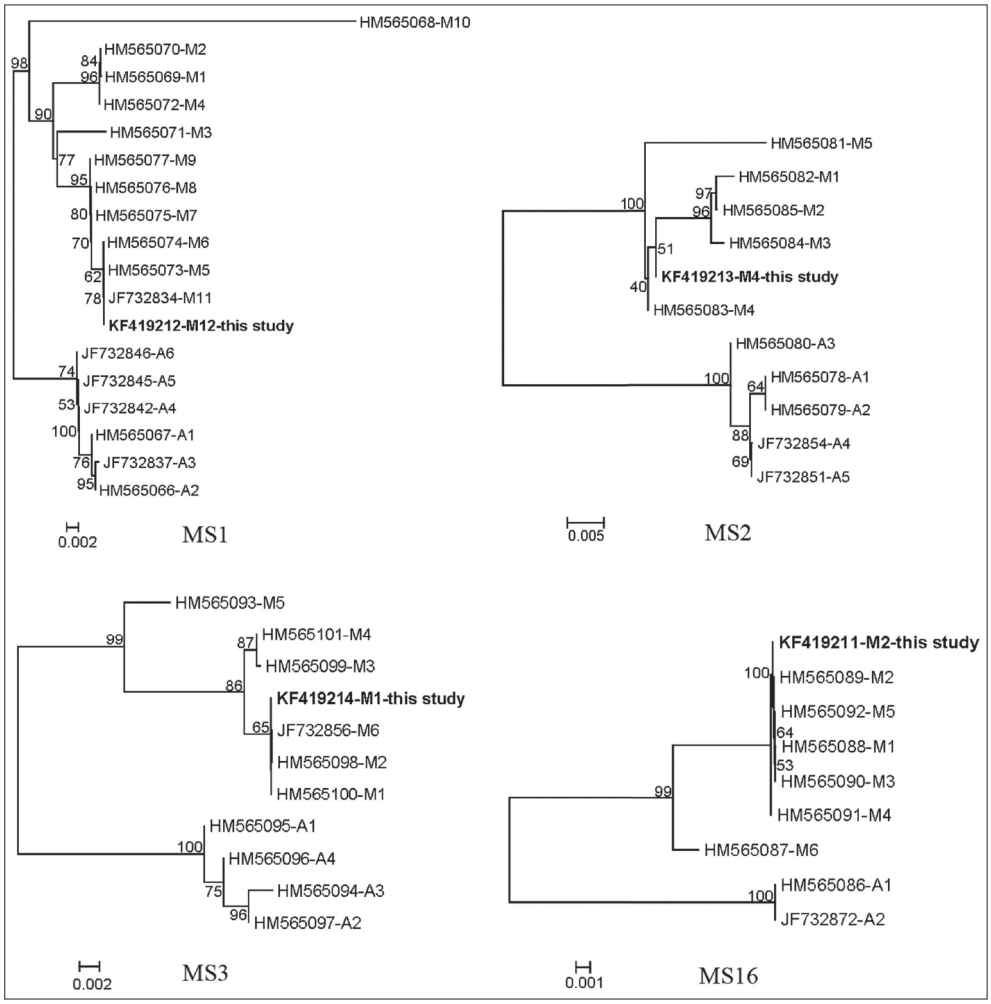

Fig 2. Phylogenetic relationships of *C. muris* and *C. andersoni* subtypes based on MS1, MS2, MS3, and MS16 sequences as assessed by a neighbor-joining analysis of the nucleotide sequences. Distances were calculated by the Kimura two-parameter model. Bootstrap values above 50% are shown using 1.000 pseudoreplicates. Bar = substitutions/site

sets [13]. The *C. muris* analyzed here was considered to be a new haplotype, M12, at MS1 locus and identified as MLST subtype M12, M4, M1 and M2. This characterization was supported by the phylogenetic relationship of each locus *(Table 2, Fig. 2)*.

Discussion

Previously, a total of 11, 5, 6 and 6 haplotypes comprising 14 MLST subtypes were identified for *C. muris* subtype loci *(Table.1; Fig. 2)*. However, the current subtype was distinct

from previous *C. muris*, especially isolate RN66 (subtype M5, M4, M1 and M5). Thus, the *C. muris* isolate in this study was evaluated as a novel MLST subtype.

Subtyping tools have proven useful in the tracing of sources of infection. Of those, the 60 kDa glycoprotein (gp60) gene is a frequently used subtype marker, and is extensively used for distinguishing intestinal *Cryptosporidium* [6,19,20]. In addition, the MLST tool with a high resolution could also accurately describe genetic diversity and perform subtype identification for *Cryptosporidium*, including *C. muris* [6,13,19]. Previous data indicated that genetic differences of *Cryptosporidium* were likely to be related to geographical restriction [6,19]. The presence of a new MLST subtype suggests a new infection source of *C. muris*. Moreover, subtype differences of *C. muris* were observed in different animal hosts. For example, the MLST subtypes from ostriches and other hosts are unique and distinct from each other [13,14]. Thus, the new subtype in this study may be indicative of host-specificity.

Cryptosporidium muris infection in a brown rat was first reported in China, and a new MLST subtype was identified. However, limited information about *C. muris* infection in current areas has been reported. Therefore, there was a need of surveying cryptosporidiosis in extensive areas and hosts to trace the infection sources and characterize the transmission dynamics of *C. muris*.

Acknowledgements

We thank the Xiaoxiao ZHOU and Wei LI for their assistance in feces collection during this study.

Funding

This study was supported by the Chengdu Giant Panda Breeding Research Foundation (No. CPF2012-01) and Program for Changjiang Scholars and Innovative Research Team in University (No. IRT0848).

Author Contributions

Conceived and designed the experiments: X. LIU, Z. ZHONG. Performed the experiments: L. DENG, F. YANG, Z. AN. Analyzed the data: X. LIU, Z. ZHONG, Z. ZHOU. Wrote the paper: X. LIU, L. DENG.

Declaration of Conflict of Interest

The authors declare that they have no competing interests.

References

1. Feng Y, Ryan UM, Xiao L: Genetic diversity and population structure of *Cryptosporidium*. *Trends Parasitol,* 34 (11): 997-1011, 2018. DOI: 10.1016/j.pt.2018.07.009

2. Ungar BL: Cryptosporidiosis in humans *(Homo sapiens)*. **In,** Dubey JP (Ed): Cryptosporidiosis of Man and Animals. 2nd edn., 59-82, CRC Press, Florida, 2018. DOI: 10.1201/9781351071260

3. Jezkova J, Prediger J, Holubova N, Sak B, Konecny R, Feng Y, Xiao L, Rost M, McEvoy J, Kvac M: *Cryptosporidium ratti* n. sp. (Apicomplexa: *Cryptosporidiidae)* and genetic diversity of *Cryptosporidium* spp. in brown rats *(Rattus norvegicus)* in the Czech Republic. *Parasitology,* 148 (1): 84-97, 2021. DOI: 10.1017/S0031182020001833

4. Ryan U, Fayer R, Xiao L: *Cryptosporidium* species in humans and animals: Current understanding and research needs. *Parasitology,* 141 (13): 1667-1685, 2014. DOI: 10.1017/S0031182014001085

5. Garcia-Livia K, Martin-Alonso A, Foronda P: Diversity of *Cryptosporidium* spp. in wild rodents from the Canary Islands, Spain. *Parasit Vectors,* 13:445, 2020. DOI: 10.1186/s13071-020-04330-9

6. Xiao L, Cama VA: *Cryptosporidium* and cryptosporidiosis. **In,** Ortega YR, Sterling CR (Ed): Foodborne Parasites. 2th ed., 73-117, Springer, Berlin, 2018. DOI: 10.1007/978-3-319-67664-7_5

7. Kvac M, Hanzlikova D, Sak B, Kvetonova D: Prevalence and age-related infection of *Cryptosporidium suis*, *C. muris* and *Cryptosporidium* pig genotype II in pigs on a farm complex in the Czech Republic. *Vet Parasitol,* 160 (3-4): 319-322, 2009. DOI: 10.1016/j.vetpar.2008.11.007

8. Pedraza-Diaz S, Ortega-Mora LM, Carrion BA, Navarro V, Gomez-Bautista M: Molecular characterisation of *Cryptosporidium* isolates from pet reptiles. *Vet Parasitol,* 160 (3-4): 204-210, 2009. DOI: 10.1016/j.vetpar.2008.11.003

9. Huang J, Zhang Z, Zhang Y, Yang Y, Zhao J, Wang R, Jian F, Ning C, Zhang W, Zhang L: Prevalence and molecular characterization of *Cryptosporidium* spp. and *Giardia duodenalis* in deer in Henan and Jilin, China. *Parasit Vectors,* 11:239, 2018. DOI: 10.1186/s13071-018-2813-9

10. Wait LF, Fox S, Peck S, Power ML: Molecular characterization of *Cryptosporidium* and *Giardia* from the Tasmanian devil *(Sarcophilus harrisii)*. *PLoS One,* 12 (4): e0174994, 2017. DOI: 10.1371/journal.pone.0174994

11. Guy RA, Yanta CA, Muchaal PK, Rankin MA, Thivierge K, Lau R, Boggild AK: Molecular characterization of *Cryptosporidium* isolates from humans in Ontario, Canada. *Parasit Vectors,* 14:69, 2021. DOI: 10.1186/s13071-020-04546-9

12. Ayinmode AB, Oliveira BCM, Obebe OO, Dada-Adgebola HO, Ayede AI, Widmer G: Genotypic characterization of *Cryptosporidium* species in humans and peri-domestic animals in Ekiti and Oyo States, Nigeria. *J Parasitol,* 104 (6): 639-644, 2018. DOI: 10.1645/17-74

13. Feng Y, Yang W, Ryan U, Zhang L, Kvac M, Koudela B, Modry D, Li N, Fayer R, Xiao L: Development of a multilocus sequence tool for typing *Cryptosporidium muris* and *Cryptosporidium andersoni*. *J Clin Microbiol,* 49 (1): 34-41, 2011. DOI: 10.1128/JCM.01329-10

14. Wang R, Jian F, Zhang L, Ning C, Liu A, Zhao J, Feng Y, Qi M, Wang H, Lv C, Zhao G, Xiao L: Multilocus sequence subtyping and genetic structure of *Cryptosporidium muris* and *Cryptosporidium andersoni*. *PLoS One,* 7 (8): e43782, 2012. DOI: 10.1371/journal.pone.0043782

15. Ng-Hublin JSY, Singleton GR, Ryan U: Molecular characterization of *Cryptosporidium* spp. from wild rats and mice from rural communities in the Philippines. *Infect Genet Evol,* 16, 5-12, 2013. DOI: 10.1016/j.meegid.2013.01.011

16. Lv C, Zhang L, Wang R, Jian F, Zhang S, Ning C, Wang H, Feng C, Wang X, Ren X, Qi M, Xiao L: *Cryptosporidium* spp. in wild, laboratory, and pet rodents in china: Prevalence and molecular characterization. *Appl Environ Microbiol,* 75 (24): 7692-7699, 2009. DOI: 10.1128/AEM.01386-09

17. Wang R, Zhang L, Feng Y, Ning C, Jian F, Xiao L, Zhao J, Wang Y: Molecular characterization of a new genotype of *Cryptosporidium* from American minks *(Mustela vison)* in China. *Vet Parasitol,* 154 (1-2): 162-166, 2008. DOI: 10.1016/j.vetpar.2007.12.038

18. Tosini F, Drumo R, Elwin K, Chalmers R, Pozio E, Cacciò SM: The CpA135 gene as a marker to identify *Cryptosporidium* species infecting humans. *Parasitol Int,* 59 (4): 606-609, 2010. DOI: 10.1016/j.parint.2010.08.011

19. Xiao L: Molecular epidemiology of cryptosporidiosis: An update. *Exp Parasitol,* 124 (1): 80-89, 2010. DOI: 10.1016/j.exppara.2009.03.018

20. Thompson RCA, Ash A: Molecular epidemiology of *Giardia* and *Cryptosporidium* infections - What's new? *Infect Genet Evol,* 75:103951, 2019. DOI: 10.1016/j.meegid.2019.103951