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NOTE

Parasitology

Molecular and histopathological characterization of *Cryptosporidium* and *Eimeria* species in bats in Japan

Fumi MURAKOSHI[1,2], Kenji KOYAMA[3], Takumi AKASAKA[4], Noriyuki HORIUCHI[5] and Kentaro KATO[1]*

[1]National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[2]Department of Infectious Diseases, Kyoto Prefectural University of Medicine, 465, Kawaramachi-hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[3]Laboratory of Veterinary Pathology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[4]Laboratory of Wildlife Ecology, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[5]Laboratory of Veterinary Pathology, Research Center for Global Agro-Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

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ABSTRACT. Bats are potential reservoirs of *Cryptosporidium* and *Eimeria*. The genus *Cryptosporidium* infects various vertebrates and causes a diarrheal disease known as cryptosporidiosis. Many epidemiological studies in wild animals have been performed; however, most of them relied on only PCR-based detection because of the difficulty of performing pathological analyses. Accordingly, the natural host and pathogenicity of *Cryptosporidium* bat genotypes remain unclear. In this study, we captured *Eptesicus nilssonii* (Northern bats) in Hokkaido, Japan. Of the three intestinal samples obtained, two were positive for *Cryptosporidium* spp. and one was positive for *Eimeria* spp. The corresponding microorganisms were also confirmed histopathologically. We detected the novel *Cryptosporidium* bat genotype XII and *Eimeria rioarribaensis* in bat intestine.

KEY WORDS: bat, *Cryptosporidium*, *Cryptosporidium* bat genotype, *Eimeria*, *Eimeria rioarribaensis*

Wild animals, especially bats, are found throughout the world [5], although some species of bat are now endangered. Bats have been implicated as potential reservoirs of many pathogens [1, 12, 18]. Bat *Cryptosporidium* was first reported in the big brown bat (*Eptesicus fuscus*) in the United States [2]. Subsequently, a *Cryptosporidium* sp. closely related to the *Cryptosporidium* mouse genotype was identified in a fecal sample from a large-footed mouse-eared bat (*Myotus adversus*) in Australia [11]. In China, two *Cryptosporidium* genotypes were identified (bat genotypes I and II) from *Rhinolophus sinicus*, *Hipposideros fulvus*, *Rousettus leschenaultia*, and *Aselliscus stoliczkamus* [19]. Bat genotypes III and IV were identified from *Eptesicus fuscus* and *Pipistrellus pipistrellus* in the US and the Czech Republic, respectively [9]. In the Philippines, three *Cryptosporidium* bat genotypes (V–VII) were detected from *Rhinolophus inops*, *Cynopterus brachyotis*, and *Eonycteris spelaea* [13]. Four further *Cryptosporidium* bat genotypes (VIII–XI) and *C. hominis* were described in *Pteropus poliocephalus* in Australia [15]. Despite the potential risk to public health imposed by these pathogens, there have been few pathological or molecular epidemiological studies. Therefore, the natural host and pathogenicity of the *Cryptosporidium* bat genotypes remain unclear.

Eimeria is also a protozoan parasite identified in bats. Traditionally, the identification of *Eimeria* species has relied primarily on oocyst morphology and host specificity. There are no pathological or molecular epidemiological studies of the *Eimeria* infected Japanese bats.

In this study, we detected *Cryptosporidium* and *Eimeria* from the Northern bat (*Eptesicus nilssonii*). We confirmed that a novel *Cryptosporidium* bat genotype (i.e., genotype XII) and *E. rioarribaensis* infected bat intestine.

During 2015, three bats (BT1-3), *Eptesicus nilssonii* (Northern bat), were captured from the same colony located in Tokachi district, Hokkaido, Japan. Species of the bats was confirmed based on morphology. The capture and handling of bats were performed under the license from the Japanese Ministry of Environment (license No. 21-27-0213). The protocol for the

*Correspondence to: Kato, K.: kkato@obihiro.ac.jp

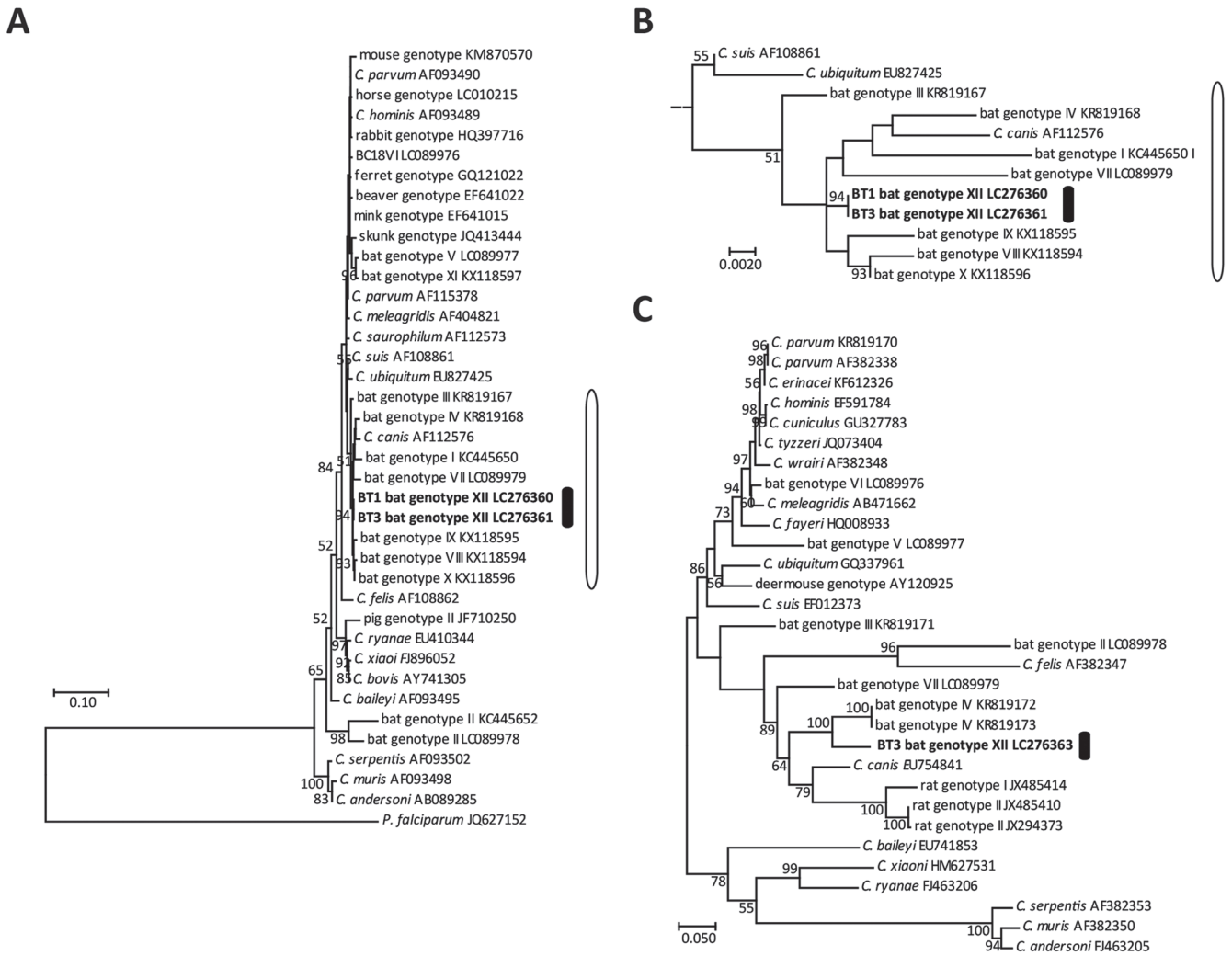

Fig. 1. Phylogenetic trees based on partial sequences of the 18S rRNA and actin genes for *Cryptosporidium* spp. The black marks indicate that sequences detected in this study. The white marks indicate bat genotype clades including sequences detected in this study. Phylogenetic trees based on partial sequences of the 18S rRNA (A, B) and actin genes (C) constructed by ML for *Cryptosporidium* spp. using 712 (18S rRNA gene) and 724 (actin gene) nucleotides without gaps. B is a higher magnification of A. Substitution model and optional parameters=GTR+Γ+I. Only bootstrap values >50% from 500 pseudo-replicates are shown.

experiments was approved by the Committee on the Animal Experiments of the Obihiro University of Agriculture and Veterinary Medicine (Approval number: 260086). The bats did not show any clinical symptom. All animal work has been conducted according to the national guidelines of Japan. The captured bats were euthanized and their intestinal tissues were collected. Tissue samples were immediately immersed in 4% formaldehyde for histopathological analysis or RNAlater (Thermo Fisher Scientific, St. Louis, MO, U.S.A.) and then frozen at −80°C until DNA extraction. The captured bats were identified as *Eptesicus nilssonii* (Northern bat) based on morphology.

DNA was extracted from the intestine by using the Biomasher IV (Funakoshi, Tokyo, Japan) and NucleoSpin® Tissue (Macherey-Nagel, Diiren, Germany). For *Cryptosporidium* detection, ~830-bp and ~850-bp fragments of 18S rRNA and actin genes, respectively, were amplified by using KOD FX Neo (TOYOBO, Osaka, Japan) with the primers described previously [4, 17]. For *Eimeria* detection, 18S rRNA and plastid 23S rRNA gene products of ~1,700 bp and ~1,100 bp were amplified, respectively. 18S rRNA gene was amplified with primers as described previously [7] and the plastid 23S rRNA gene was amplified with forward and reverse primers: Eim23SF (5′-AGGAGCGTTCTATATTTARGAAG-3′) and Eim23SR (5′-GGATCATTAAGACCGACTTCG-3′). All PCR products were cloned (Zero Blunt TOPO PCR Cloning Kit, Thermo Fisher Scientific, St. Louis, MO, U.S.A.) and sequenced in both directions by using an ABI 3130 Genetic Analyzer (Applied Biosystems Japan, Tokyo, Japan). Sequences were aligned by using Clustal X2 [10]. All gaps were eliminated. Maximum likelihood (ML) analyses were performed by using MEGA 7.0.25 [8]. Substitution models and optional parameter sets were also evaluated by using MEGA 7.0.25. We constructed a phylogenetic tree (Figs. 1 and 2), in which the substitution model and optional parameters used were the General Time Reversible model and Hasegawa-Kishino-Yano model [6, 14].

Table 1. Nucleotide identity among 18S rRNA sequences of *Cryptosporidium* genotypes in a bat clade

Genotype	Ref	Bat species in which *Cryptosporidium* was detected	I (Rh)	I (As)	III	IV	VII	VIII	IX	X	XI
Bat genotype I (KC445650)	[19]	*Rhinolophus sinicus*									
Bat genotype I (KC445654)	[19]	*Aselliscus stoliczkanus*	99								
Bat genotype III (KR819167)	[8]	*Eptesicus fuscus*	96	96							
Bat genotype IV (KR819168)	[8]	*Pipistrellus pipistrellus*	97	97	96						
Bat genotypeVII (LC089979)	[13]	*Rhinolophus inops*	95	95	95	96					
Bat genotype VIII (KX118594)	[15]	*Pteropus poliocephalus*	95	95	96	96	95				
Bat genotype IX (KX118595)	[15]	*Pteropus poliocephalus*	96	95	97	97	95	98			
Bat genotype X (KX118596)	[15]	*Pteropus poliocephalus*	96	96	97	97	96	98	98		
Bat genotype XII (BT1) (LC276360)	this study	*Eptesicus fuscus*	96	96	97	98	96	96	97	97	

Rh: *Rhinolophus sinicus*, As: *Aselliscus stoliczkanus*.

The fixed small intestines were divided into three parts (upper, middle, and lower), trimmed, embedded in paraffin, and cut into 3-μm-thick sections. Paraffin sections were stained with haematoxylin and eosin (HE). For *Cryptosporidium* detection, selected sections were subjected to immunofluorescence staining using the Sporo-Glo Cy3 Kit, which contains polyclonal IgG against *Cryptosporidium parvum* (A600Cy3-R-1X, Waterborne, LA, U.S.A.). Microwave antigen retrieval (15 min in 0.01 M citrate buffer, pH 6.0) was also performed. After incubation with Sporo-Glo at 4°C overnight, the sections were washed with phosphate-bufferd saline, mounted with Vectashield (H-1200, Vector Laboratories, Burlingame, CA, U.S.A.), and examined under a BZ-X700 fluorescence microscope (Keyence, Osaka, Japan).

Of the three intestinal samples, two were positive for *Cryptosporidium* spp. and one was positive for *Eimeria* spp. Fragments of the 18S rRNA and actin nucleotide sequences of the *Cryptosporidium,* 18S rRNA and plastid 23S rRNA nucleotide sequences of the *Eimeria* acquired in this study were deposited in GenBank (LC276360–LC276363 and LC371915–LC371916), respectively. Based on the blast search, all of the sequences detected in this study were identifiable as either *Cryptosporidium* or *Eimeria* spp. Of the *Cryptosporidium* 18S rRNA and actin gene sequences obtained, the 18S rRNA sequences from BT1 and BT3 were identical. Actin sequence from BT1 was not amplified by PCR. The sequence similarity between BT1 and the other bat genotypes belonging to the same clade (Fig. 1: white marks) ranged from 95 to 98% (Table 1). For the *Eimeria* 18S rRNA gene sequences, BT2 had 99% (822/823) identity with *E. rioarribaensis* (AF307877) (bat *Eimeria*).

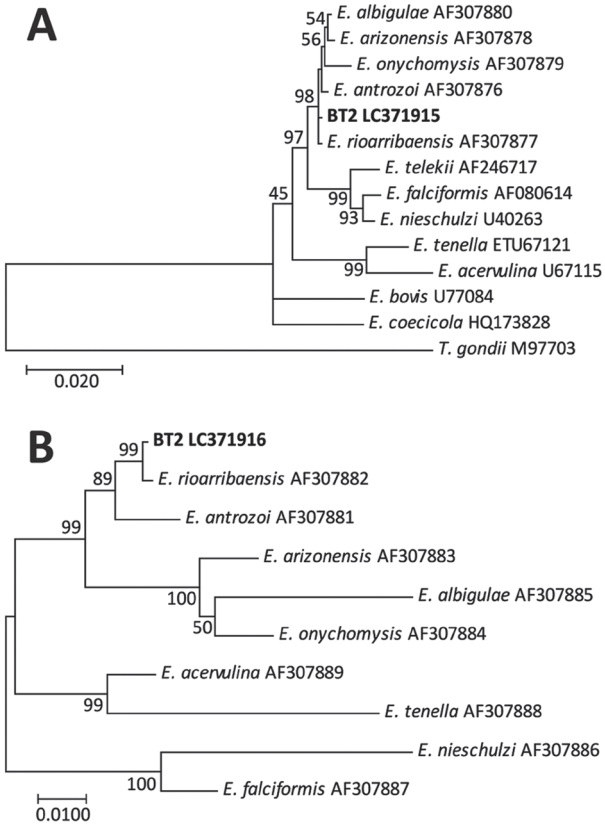

Fig. 2. Phylogenetic trees based on partial sequences of the 18S rRNA and plastid 23S rRNA genes for *Eimeria* spp. Phylogenetic trees based on partial sequences of the 18S rRNA (A) and plastid 23S rRNA genes (B) constructed by ML for *Eimeria* spp. using 1495 (18S rRNA gene) and 1012 (plastid 23S rRNA gene) nucleotides without gaps. Substitution model and optional parameters: (A)=GTR+Γ (B)=HKY+Γ. Only bootstrap values >50% from 1,000 pseudo-replicates are shown.

Figure 1 shows the phylogenetic relationships based on the partial sequences of 18S rRNA and actin among the various *Cryptosporidium* species and genotypes, including the sequences from the Northern bats captured and analyzed in this study. Figure 1B is a higher magnification of Fig. 1A. In Fig. 1B, the *Cryptosporidium* sequences obtained from BT1 and BT3 formed a single clade with the known *Cryptosporidium* bat genotypes (I, III, IV, VII, VIII, IX, and X; white mark). Monophyly among these branches was moderately supported (51%). In Fig. 1C, the actin nucleotide sequence from BT3 made a clade with *Cryptosporidium* bat genotype IV. A phylogeny inferred from the actin sequences was consistent with the phylogenetic relationships based on the partial sequences of the 18S rRNA.

Figure 2 shows the phylogenetic relationships based on partial sequences of 18S rRNA (Fig. 2A) and plastid 23S rRNA (Fig. 2B) genes among various *Eimeria* spp., including the sequences from the Northern bats in this study. In Fig. 2A and 2B, the *Eimeria* sequences detected from BT2 formed a single clade with *E. rioarribaensis*.

In BT1 and BT3, *Cryptosporidium*-like micro-protozoan organisms were observed sparsely as minute basophilic round

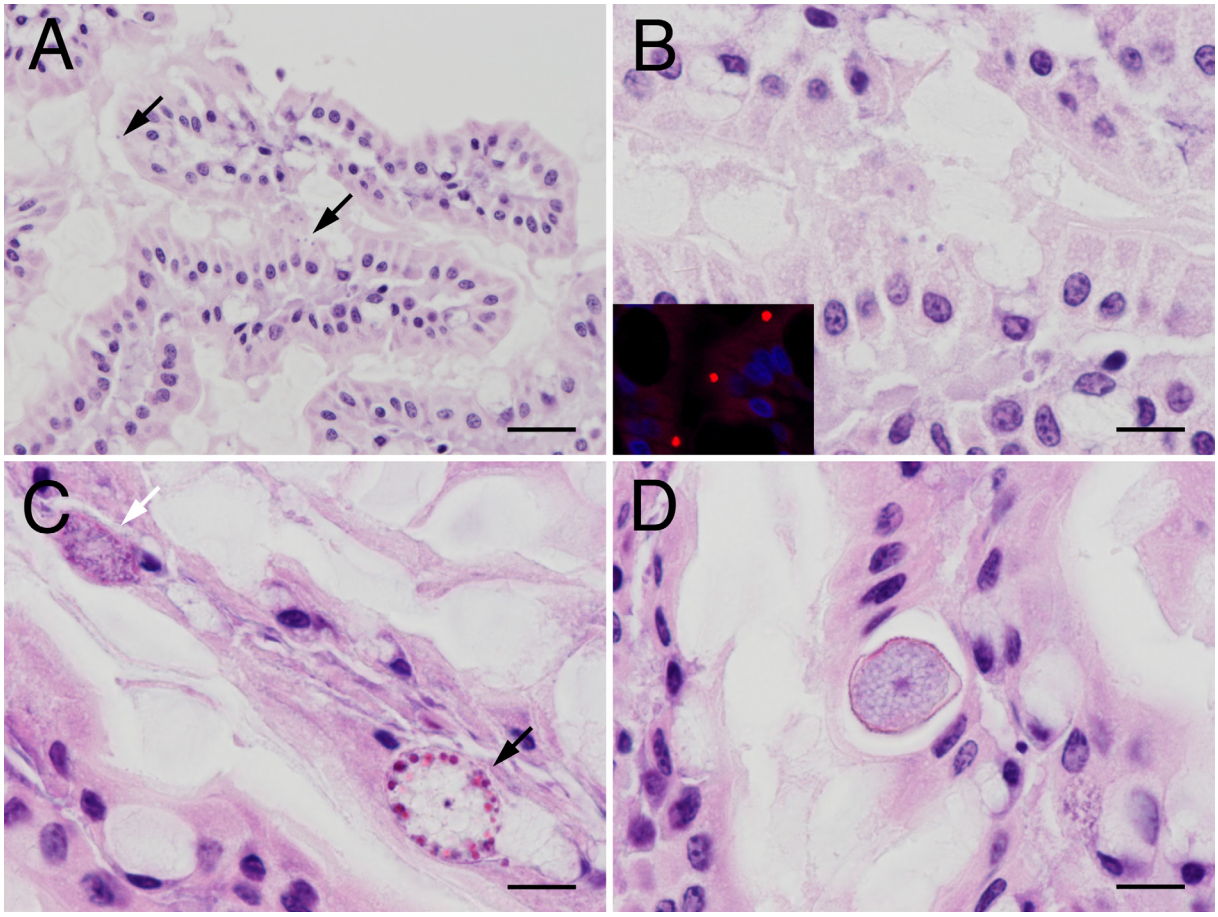

Fig. 3. *Cryptosporidium* and *Eimeria* organisms in the small intestine of bats. (A) Small intestine of BT3. *Cryptosporidium* (arrows) can be seen as minute basophilic round organisms on and within the apical surface of enterocytes. Inflammation was not noted. HE. Bar=25 μm. (B) Higher magnification of (A). HE. Bar=10 μm. Inset. Organisms showing a positive reaction on immunostaining with an anti-*Cryptosporidium parvum* antibody. (C) Small intestine of BT2. A macrogametocyte (black arrow) and a microgametocyte (white arrow) of *Eimeria* are shown. HE. Bar=10 μm. (D) Small intestine of BT2. An oocyst of *Eimeria* is shown. HE. Bar=10 μm.

organisms on and within the apical surface of the enterocytes throughout the small intestine (Fig. 3A and 3B). These organisms were positively stained on immunostaining with an anti-*Cryptosporidium* sporozoite antibody (Fig. 3B, inset). The antibody response was not observed in BT2. There were no other findings, such as villus atrophy or inflammation. In BT2, *Eimeria*-like protozoan organisms at various life cycle stages, including macrogametocytes, microgametocytes, oocysts, and meronts, were scattered in the mucosal epithelium and in the lamina propria mucosae throughout the small intestine (Fig. 3C and 3D). As with BT1 and BT2, no significant lesions indicative of an inflammatory response were observed in the small intestine.

In this study, we analyzed *Cryptosporidium* and *Eimeria* infection in *Eptesicus nilssonii* (Northern bat). Phylogenetic analyses revealed that the BT1 and BT3 sequences were closely related to a bat genotype clade (Fig. 1A and 1B: white marks), but the branches were not well supported (51%) because of the short reference sequence length. Actin sequences were not available for bat genotypes I, III, VII, VIII, IX, and X. The bat genotypes of this clade (Fig. 1A and 1B: white marks) were detected from *Rhinolophus sinicus*, *Aselliscus stoliczkanus*, *Eptesicus fuscus*, *Pipistrellus pipistrellus,* and *Rhinolophus inops* in previous studies in China, the United States and the Czech Republic, the Philippines, and Australia [9, 13, 15, 19]. Minimal intra-clonal variation between bat genotypes I has been reported with nucleotide similarity ranging from 99.4 to 99.8% [15]. In the present study, the sequence similarity between BT1 and the other bat genotypes was between 95% and 98%; therefore, our data suggest that the BT1 and BT3 sequences represent a novel genotype, namely *Cryptosporidium* sp. bat genotype XII. This study represents the first detection of *Cryptosporidium* from bats in Japan. Histopathologically, micro-protozoan organisms like ordinal *Cryptosporidium* species were detected in the absence of indicators of inflammatory response or tissue damage. Taking into account that the captured bats did not show any clinical symptoms, this *Cryptosporidium* species appears to be non-pathogenic for healthy bats. We thus confirmed that bat genotype XII (this study) could infect bat intestine and showed no pathogenicity. The results of the PCR and the histopathological finding stained with haematoxylin and eosin (HE) were corresponded with the immunofluorescence staining results, therefore, we also confirmed that the *Cryptosporidium* antigen cross-reacted with a commercially available anti-

Cryptosporidium parvum sporozoite polyclonal IgG on immunostaining.

The *Eimeria* sequences detected from BT2 was that of *E. rioarribaensis*. *E. rioarribaensis* was first detected from *Myotis ciliolabrum* in North America [3, 16]. We also observed that the macrogametocyte, microgametocyte, oocyst, meront in the intestine. Therefore, it was confirmed that *Eptesicus nilssonii* was one of the natural host of *E. rioarribaensis.*

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REFERENCES

1. Calisher, C. H., Childs, J. E., Field, H. E., Holmes, K. V. and Schountz, T. 2006. Bats: important reservoir hosts of emerging viruses. *Clin. Microbiol. Rev.* **19**: 531–545. [Medline] [CrossRef]
2. Dubey, J. P., Hamir, A. N., Sonn, R. J. and Topper, M. J. 1998. Cryptosporidiosis in a bat (*Eptesicus fuscus*). *J. Parasitol.* **84**: 622–623. [Medline] [CrossRef]
3. Duszynski, D. W., Scott, D. T., Aragon, J., Leach, A. and Perry, T. 1999. Six new *Eimeria* species from vespertilionid bats of North America. *J. Parasitol.* **85**: 496–503. [Medline] [CrossRef]
4. Feng, Y., Ortega, Y., He, G., Das, P., Xu, M., Zhang, X., Fayer, R., Gatei, W., Cama, V. and Xiao, L. 2007. Wide geographic distribution of *Cryptosporidium bovis* and the deer-like genotype in bovines. *Vet. Parasitol.* **144**: 1–9. [Medline] [CrossRef]
5. Gunnell, G. F. and Simmons, N. B. 2012. Evolutionary history of bats: fossils, molecules and morphology. Cambridge University Press, Cambridge.
6. Hasegawa, M., Kishino, H. and Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **22**: 160–174. [Medline] [CrossRef]
7. Honma, H., Yokoyama, T., Inoue, M., Uebayashi, A., Matsumoto, F., Watanabe, Y. and Nakai, Y. 2007. Genetical identification of coccidia in red-crowned crane, Grus japonensis. *Parasitol. Res.* **100**: 637–640. [Medline] [CrossRef]
8. Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **33**: 1870–1874. [Medline] [CrossRef]
9. Kváč, M., Hořická, A., Sak, B., Prediger, J., Salát, J., Širmarová, J., Bartonička, T., Clark, M., Chelladurai, J. R., Gillam, E. and McEvoy, J. 2015. Novel *Cryptosporidium* bat genotypes III and IV in bats from the U.S.A. and Czech Republic. *Parasitol. Res.* **114**: 3917–3921. [Medline] [CrossRef]
10. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947–2948. [Medline] [CrossRef]
11. Morgan, U. M., Sturdee, A. P., Singleton, G., Gomez, M. S., Gracenea, M., Torres, J., Hamilton, S. G., Woodside, D. P. and Thompson, R. C. 1999. The *Cryptosporidium* "mouse" genotype is conserved across geographic areas. *J. Clin. Microbiol.* **37**: 1302–1305. [Medline]
12. Mühldorfer, K. 2013. Bats and bacterial pathogens: a review. *Zoonoses Public Health* **60**: 93–103. [Medline] [CrossRef]
13. Murakoshi, F., Recuenco, F. C., Omatsu, T., Sano, K., Taniguchi, S., Masangkay, J. S., Alviola, P., Eres, E., Cosico, E., Alvarez, J., Une, Y., Kyuwa, S., Sugiura, Y. and Kato, K. 2016. Detection and molecular characterization of *Cryptosporidium* and *Eimeria* species in Philippine bats. *Parasitol. Res.* **115**: 1863–1869. [Medline] [CrossRef]
14. Nei, M. and Kumar, S. 2000. Molecular Evolution and Phylogenetics. Oxford University Press, New York.
15. Schiller, S. E., Webster, K. N. and Power, M. 2016. Detection of *Cryptosporidium hominis* and novel *Cryptosporidium* bat genotypes in wild and captive *Pteropus* hosts in Australia. *Infect. Genet. Evol.* **44**: 254–260. [Medline] [CrossRef]
16. Scott, D. T. and Duszynski, D. W. 1997. *Eimeria* from bats of the world: two new species from *Myotis* spp. (Chiroptera: Vespertilionidae). *J. Parasitol.* **83**: 495–501. [Medline] [CrossRef]
17. Sulaiman, I. M., Hira, P. R., Zhou, L., Al-Ali, F. M., Al-Shelahi, F. A., Shweiki, H. M., Iqbal, J., Khalid, N. and Xiao, L. 2005. Unique endemicity of cryptosporidiosis in children in Kuwait. *J. Clin. Microbiol.* **43**: 2805–2809. [Medline] [CrossRef]
18. Sun, H., Wang, Y., Zhang, Y., Ge, W., Zhang, F., He, B., Li, Z., Fan, Q., Wang, W., Tu, C., Li, J. and Liu, Q. 2013. Prevalence and genetic characterization of *Toxoplasma gondii* in bats in Myanmar. *Appl. Environ. Microbiol.* **79**: 3526–3528. [Medline] [CrossRef]
19. Wang, W., Cao, L., He, B., Li, J., Hu, T., Zhang, F., Fan, Q., Tu, C. and Liu, Q. 2013. Molecular characterization of *Cryptosporidium* in bats from Yunnan province, southwestern China. *J. Parasitol.* **99**: 1148–1150. [Medline] [CrossRef]