Host-adapted Cryptosporidium and Enterocytozoon bieneusi genotypes in straw-colored fruit bats in Nigeria

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\textbf{ABSTRACT}

Few data are available on the distribution and human infective potential of Cryptosporidium and Enterocytozoon bieneusi genotypes in bats. In this preliminary study, we collected 109 fecal specimens during April–July 2011 from a colony of straw-colored fruit bats (Eidolon helvum) in an urban park (Agodi Gardens) of Ibadan, Nigeria, and analyzed for Cryptosporidium spp., Giardia duodenalis and E. bieneusi using PCR targeting the small subunit rRNA gene, triosephosphate isomerase gene, and ribosomal internal transcribed spacer, respectively. Genotypes of these enteric parasites were determined by DNA sequencing of the PCR products. Altogether, 6 (5.5%), 0 and 16 (14.7%) specimens were positive for Cryptosporidium spp., G. duodenalis and E. bieneusi, respectively. DNA sequence analysis of the PCR products indicated the presence of two novel Cryptosporidium genotypes named as bat genotype XIV (in 5 specimens) and bat genotype XV (in 1 specimen) and one known E. bieneusi genotype (Type IV in 1 specimen) and two novel \textit{E. bieneusi} genotypes (Bat1 in 13 specimens and Bat2 in 2 specimens). In phylogenetic analysis of DNA sequences, the two novel Cryptosporidium genotypes were genetically related to Bat genotype II previously identified in fruit bats in China and Philippines, whereas the two novel \textit{E. bieneusi} genotypes were genetically related to Group 5, which contains several known genotypes from primates. With the exception of Type IV, none of the Cryptosporidium and \textit{E. bieneusi} genotypes found in bats in this study are known human pathogens. Thus, straw-colored fruit bats in Nigeria are mainly infected with host-adapted Cryptosporidium and \textit{E. bieneusi} genotypes.

\section{1. Introduction}

Cryptosporidium spp., Giardia duodenalis and Enterocytozoon bieneusi are parasitic protists, causing diarrhea and other gastrointestinal symptoms in humans and animals (Wright, 2012). They are transmitted by direct contact with infected persons (anthropoontic transmission) or animals (zoontic transmission) or through consumption of contaminated food or water (foodborne or waterborne transmission) (Checkley et al., 2015; Matos et al., 2012; Ryan et al., 2018).

Genetic diversity exists in each of the three groups of pathogens. Thus far, there are near 40 named Cryptosporidium species and as many unnamed species known as genotypes, each with some degree of host specificity (Feng et al., 2018). Similarly, there are at least eight genotypes of \textit{G. duodenalis} known as assemblages A to H, which are likely cryptic species with different host ranges (Feng and Xiao, 2011). There are also over 250 \textit{E. bieneusi} genotypes, forming at least 11 genotype groups with different host preferences (Santin et al., 2018; Zhang et al., 2018; Zhong et al., 2017). Only some of the species or genotypes are major human pathogens, such as \textit{C. parvum} and \textit{C. hominis} among \textit{Cryptosporidium} spp., assemblages A and B in \textit{G. duodenalis}, and Group 1 genotypes in \textit{E. bieneusi} (Feng et al., 2018; Matos et al., 2012; Ryan et al., 2018). Molecular diagnostic tools are needed to differentiate the human-infective species and genotypes from animal-specific ones (Ghosh and Weiss, 2009; Xiao and Feng, 2017). As different human-pathogenic species and genotypes have distinct host range, the use of molecular diagnostic tools in epidemiologic investigations has significantly improved our understanding of the transmission of these pathogens in both industrialized and developing countries (Matos et al., 2012; Xiao and Feng, 2017).

Bats are known to play a major role in the transmission of emerging...
pathogens around the world (Han et al., 2015). This is especially the case with viruses such as coronavirus and rabies virus (Brook and Dobson, 2015). This is largely because of their large numbers, mobile nature, and tolerance to many of the pathogens (O’Connor, 2018; Serra-Cobo and Lopez-Roig, 2017). Their role in the transmission of Cryptosporidium spp., G. duodenalis, and E. bieneusi, however, remains unclear. There have been a few recent studies on the identity of Cryptosporidium spp. in bats in Asia, Australia and Europe, which have identified the occurrence of 12 Cryptosporidium genotypes in bats, all of which appear to be bat-specific (Kvac et al., 2015; Murakoshi et al., 2016, 2018; Schiller et al., 2016; Wang et al., 2013). Thus far, there has been no study on G. duodenalis in bats, but six E. bieneusi genotypes were identified in bats in South Korea recently, one of which belongs to Group 1, with the remaining ones belonging to Group 2, which contains E. bieneusi genotypes mostly found in ruminants (Lee et al., 2018).

In this preliminary study, we have examined the occurrence and identity of Cryptosporidium spp., G. duodenalis, and E. bieneusi in straw-colored fruit bats in a popular public park in Ibadan, Nigeria.

2. Materials and methods

2.1. Specimens

The study was conducted with fecal specimens collected from straw-colored fruit bats (Eidolon helvum) living in the Agodi Gardens (N 07.40614; E 003.90073), a popular public park in central Ibadan, Nigeria (Fig. 1). It is located between a University College Hospital and a residential area and is 60.7 hectares in size. It had one single colony of bats with no interspecies co-roosting, with thousands of mixed ages of bats on trees with thick canopy. It was estimated that there were about 30,000 straw-colored fruit bats at the time of sampling, with no other species of bats in presence. Fecal droppings from bats hanging on tall forest trees were collected during April–July 2011 at 109 individual points. Only fresh droppings were collected from various locations on two separate occasions. They were stored at −20 °C prior to DNA extraction. There were no direct interactions between sampling personnel and animals at the time of sampling.

2.2. Detection of Cryptosporidium spp., G. duodenalis and E. bieneusi

DNA was extracted from 200 μl of stored fecal specimens using the FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA). This technique was shown to be better in removing PCR inhibitors in environmental samples than other common commercial DNA extraction kits (Jiang et al., 2005). The extracted DNA was stored at −80 °C before analysis by PCR. To detect Cryptosporidium spp., a ∼830-bp fragment of the small subunit (SSU) rRNA gene was amplified by nested PCR, and Cryptosporidium genotypes were initially identified by restriction fragment length polymorphism (RFLP) analysis of the secondary PCR products using restriction enzymes SspI and VspI (New England BioLabs, Massachusetts, USA) (Xiao et al., 1999). To detect G. duodenalis, a ∼530-bp fragment of the triosephosphate isomerase (tpi) gene was amplified by nested PCR (Sulaiman et al., 2003a). To detect E. bieneusi, a 392-bp fragment of the rRNA unit containing the entire internal transcribed spacer (ITS) was amplified by nested PCR (Sulaiman et al., 2003b). Each specimen was analyzed by PCR twice using 1 μl of

![Fig. 1. Location of the straw-colored fruit bats examined in the study.](image-url)
extracted DNA per PCR, with DNA from *C. canis* as the positive control for the *SSU rRNA* PCR, DNA from *G. duodenalis* assemblage C as the positive control for the *tpi* PCR, and DNA from *E. bieneusi* genotype PtEb IX as the positive control for the *ITS* PCR. Two negative controls (reagent-grade water) for primary PCR and secondary PCR were further used in each PCR run. Non-acetylated bovine serum albumin (Sigma-Aldrich, St, Louis, MO, USA) was used at the concentration of 400 ng/μl in the primary PCR to neutralize residual PCR inhibitors in DNA, as previously described (Jiang et al., 2005).

3. Results

3.1. Occurrence of *Cryptosporidium* spp., *G. duodenalis* and *E. bieneusi* in straw-colored fruit bats in Ibadan, Nigeria

| Pathogen          | No. of specimens | No. positive (%) | Genotype                        |
|-------------------|------------------|------------------|---------------------------------|
| *Cryptosporidium* | 109              | 6 (5.5%)         | Bat genotype XIV (5), Bat genotype XV (1) |
| *G. duodenalis*   | 109              | 0                | –                               |
| *E. bieneusi*     | 109              | 16 (14.7%)       | Type IV (1), Bat1 (13), Bat2 (2) |

Table 1

Fig. 2. Genotyping of *Cryptosporidium* spp. in straw-colored fruit bats by small subunit rRNA-based PCR-RFLP. Upper panel: SspI RFLP patterns; lower panel: VspI RFLP patterns; M: 100-bp molecular markers; H: *C. hominis* positive control; P: *C. parvum* positive control; B1: *Cryptosporidium* bat genotype XIV; B2: *Cryptosporidium* bat genotype XV.

3.2. *Cryptosporidium* genotypes in bats

RFLP analysis of the *SSU rRNA* PCR products revealed a unique banding pattern for the *Cryptosporidium* spp. detected. All *SSU rRNA* products produced the same banding pattern, with the SspI restriction enzyme failing to digest the PCR product, while the VspI restriction enzyme producing a banding pattern that was similar to *C. parvum* (Fig. 2). DNA sequence analysis revealed the presence of two novel *Cryptosporidium* genotypes that were closely related to each other and to *Cryptosporidium* bat genotype II (Fig. 3). They were named as bat genotypes XIV and XV. The former was found in five specimens while the latter was found in one specimen. They differed from each other by about 28 single nucleotide polymorphisms (SNPs). Within *Cryptosporidium* bat genotype XIV, one specimen (No. 32604) produced a sequence that had one two-nucleotide insertion, one four-nucleotide deletion and one two-nucleotide deletion in two polymorphic areas of the *SSU rRNA* gene compared with other bat genotype XIV specimens.

3.3. *E. bieneusi* genotypes in bats

DNA sequence analysis showed the presence of three genotypes among the 16 *E. bieneusi*-positive specimens, including one known one
and two novel ones. The former included Type IV, which was found in one specimen, while the latter were represented by two closely related genotypes named as Bat1 and Bat2, which differed from each other by 6 SNPs and were found in 13 and 2 specimens, respectively. Phylogenetically, the two new E. bieneusi genotypes belonged to Group 5, which contains several known genotypes from primates, such as CAF4, PtEb XII and KB-6 (Fig. 4).

4. Discussion

Results of this preliminary study have shown the occurrence of Cryptosporidium spp. and E. bieneusi, but not G. duodenalis, in fruit bats living in an urban public park in Nigeria. The 5.5% infection rate for Cryptosporidium spp. is in line with the 2.1–8.9% infection rates of Cryptosporidium spp. reported in previous studies of several species of bats in Australia, USA, Czech Republic, China and Philippines (Kvac et al., 2015; Murakoshi et al., 2016, 2018; Schiller et al., 2016; Wang et al., 2013). The 14.7% detection rate of E. bieneusi in fruit bats examined in this study was significantly higher than the 1.9% detection rate of E. bieneusi in eight species of bats analyzed recently in South Korea (Lee et al., 2018). Giardia duodenalis was not examined in any of the previous studies of enteric protozoa in bats. Despite the use of a PCR assay that is designed to detect divergent Giardia species (Sulaiman et al., 2003a), we failed in obtaining any expected PCR products, indicating that G. duodenalis is not common in the bat species examined in Nigeria.

The two Cryptosporidium genotypes found in bats in this study are not known human pathogens. This agrees with previous characterizations of Cryptosporidium spp. in bats in several countries, where mainly novel Cryptosporidium genotypes were detected in both fruit bats and insectivorous bats (Kvac et al., 2015; Schiller et al., 2016). Similarly, the two common E. bieneusi genotypes found in fruit bats in this study are not known human pathogens, only with one fruit bat positive for Type IV, a common zoonotic E. bieneusi subtype in humans (Matos et al., 2012). Thus, in contrast to their role in the transmission of emerging pathogens, bats can be only minor reservoirs of human-pathogenic Cryptosporidium spp. and E. bieneusi.

The two novel Cryptosporidium genotypes identified in straw-colored fruit bats in Nigeria appear to be host-adapted Cryptosporidium spp. Phylogenetically, the two Cryptosporidium genotypes in this study formed the basal branch of the SSU rRNA-based Bayesian inference tree, together with Cryptosporidium bat genotypes II and XIII previously identified in fruit bats in China and Philippines (Murakoshi et al., 2016; Wang et al., 2013). Similarly, bat genotypes V and XI from fruit bats in
Australia and Philippines formed another cluster (Murakoshi et al., 2016; Schiller et al., 2016), while bat genotypes VIII, IX, and X from fruit bats in Australia formed a third cluster (Schiller et al., 2016). In contrast, other six Cryptosporidium genotypes identified in insectivorous bats are mostly dispersed over the SSU rRNA-based tree, probably as a result of the more diverse nature of their hosts (Fig. 3). As only a small number of bat species have been examined in a few countries, more novel Cryptosporidium genotypes are likely to be identified in future.

The two novel E. bieneusi genotypes identified are very divergent from common human-pathogenic E. bieneusi genotypes, which are mostly belong to Group 1. In phylogenetic analysis of the ITS sequences, they formed a cluster with several E. bieneusi genotypes from primates. They, however, have 10–13 SNPs compared to other genotypes in the group, thus could be bat-adapted E. bieneusi genotypes rather than related genotypes from primates. The host specificity of the various E. bieneusi genotype groups described thus far appears to be less strict than previously believed (Guo et al., 2014).

In conclusion, this preliminary study has shown the occurrence of Cryptosporidium spp. and E. bieneusi, but not G. duodenalis, in straw-colored fruit bats in the present study. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Fig. 4. Phylogeny of Enterocytozoon bieneusi genotypes in bats based on Bayesian inference analysis of sequences of the internal transcribed spacer of the rRNA gene. The posterior probability values are indicated on the branches. Red ones are E. bieneusi genotypes identified in straw-colored fruit bats in the present study. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Declaration of interest

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

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31425025).

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