NOTE *Bacteriology*

Detection of *Campylobacter jejuni* in rectal swab samples from *Rousettus amplexicaudatus* in the Philippines

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ABSTRACT. Bats are the second diversity species of mammals and widely distributed in the world. They are thought to be reservoir and vectors of zoonotic pathogens. However, there is scarce report of the evidence of pathogenic bacteria kept in bats. The precise knowledge of the pathogenic bacteria in bat microbiota is important for zoonosis control. Thus, metagenomic analysis targeting the V3-V4 region of the 16S rRNA of the rectal microbiota in *Rousettus amplexicaudatus* was performed using high throughput sequencing. The results revealed that 103 genera of bacteria including *Camplyobacter* were detected. *Campylobacter* was second predominant genus, and *Campylobacter coli* and *Campylobacter jejuni* were identified in microbiome of *R. amplexicaudatus*. Campylobacteriosis is one of the serious bacterial diarrhea in human, and the most often implicated species as the causative agent of campylobacteriosis is *C. jejuni*. Therefore, we investigated the prevalence of *C. jejuni* in 91 wild bats with PCR. As a result of PCR assay targeted on 16S-23S intergenic spacer, partial genome of *C. jejuni* was detected only in five *R. amplexicaudatus*. This is the first report that *C. jejuni* was detected in bat rectal swab samples. *C. jejuni* is the most common cause of campylobacteriosis in humans, transmitted through water and contact with livestock animals. This result indicated that *R. amplexicaudatus* may be a carrier of *C. jejuni*.
KEY WORDS: 16S rRNA, bat, *Campylobacter*, metagenome, microbiota

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Bats are the second diversity species of mammals in the world, consisted of approximately 1,100 species [25]. Bats have a variety of food choices, including fruits, insects, small vertebrate and blood. Furthermore, bats are known as reservoir hosts and vectors of zoonotic pathogens. Previous and ongoing researches in bats mainly focus on viruses, including Rabies virus [9], Nipah virus [5, 6] and Hendra virus [18] as well as European and Australian bat lyssaviruses [3]. In contrast, the knowledge of pathogenic bacteria in bats is scarce. Only a few bacteria pathogenic to humans, such as *Salmonella* spp. [23] and *Clostridium* spp. [14], were isolated from bats. Therefore, it is important to characterize rectal microbiota and identify pathogenic agents in bats from the viewpoint of public health.

Gut microbiota has been investigated and characterized by a variety of methods. These methods include culture-based analysis, DNA sequencing using the Sanger method and fluorescence *in situ* hybridization targeting the 16S rRNA gene [10]. Most of the previous approaches are limited in scope. Traditional DNA-based approaches have targeted phylogenetic genes, which provide limited information, especially when a large amount of microbial groups must be classified.

The number of metagenomic studies has increased in recent years, because of the availability of high throughput sequencing technologies. High throughput sequencing al-

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lows the production of millions of short sequence reads in a single run and do not require additional cloning steps like the traditional Sanger sequencing [17]. The enormous amount of data collected by high throughput sequencing indicates that a variety of microbiota has not been analyzed previously [16]. High throughput sequencing technologies have proven usefulness for studying the diversity and dynamics of bacterial species, even in complex systems like the gut [12].

Despite of the importance from the viewpoint of public health, little information on bat microbiota is available. In this study, we performed metagenomic analysis of the 16S rRNA of rectal microbiota in wild bats using high throughput sequencing to detect pathogenic bacteria.

A total of 88 megachiropteras, comprised of 67 *Rousettus amplexicaudatus*, 14 *Eonycteris spelaea*, 5 *Cynopterus brachyotis* and 2 *Macroglossus minimus*, and 3 microchiropteras comprised of 3 *Hipposideros diadema* were collected from five sites located in the Davao region of the Philippines: Dadatan (6°56′N; 125°40′E), Guilon (7°03′N; 125°44′E), Lavigan (6°16′N; 126°11′E), the forest of Davao (7°11′N; 125°25′E) and Sion (6°56′N; 125°45′E). Bats were captured with the permission of the regional executive director of the Department of Environment and Natural Resources (DENR).

Rectal swab samples were collected under anesthesia and suspended in 500 μl of PBS. The samples were stored at −80°C until DNA extraction. To extract DNA, 250 μl of the swab suspension were centrifuged at 10,000 × *g* for 5 min, and supernatant was removed. DNA samples were extracted from the pellet using prepGEM Bacteria (ZyGEM, Hamilton, New Zealand) according to the manufacturer's protocol.

To identify the microbiome, DNA sample from *R. amplexicaudatus* captured at Lavigan was used for high throughput sequencing analysis. For high throughput sequencing of the 16S rRNA gene, PCR fragments of the V3-V4 region were amplified using the universal primers F341 5′-CCTACGGGNGGCWGCAG-3′) and R805 (5′-GACTACHVGGGTATCTAATCC-3′) [11]. These primers were modified according to the high throughput sequencing protocol (Illumina, San Diego, CA, U.S.A.). For PCR, the reaction mixture (50 μl) contained 25 μl Premix EX Taq (TaKaRa-Bio, Otsu, Japan), 1 μM each primer and 5 *n*g isolated DNA. The PCR conditions were as follows: 98°C for 3 min; 25 cycles of 98°C for 10 sec, 55°C for 30 sec, 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR products were purified using the MonoFas DNA purification kit (GL Science, Tokyo, Japan) and used as template in the second PCR round to attach dual indices and Illumina sequencing adapters. For the second round PCR, the reaction mixture (50 μl) contained 25 μl Premix EX Taq, 1 μM each primer and 10 *n*g purified amplicons. The PCR conditions were as follows: 98°C for 3 min; 12 cycles of 98°C for 10 sec, 55°C for 30 sec, 72°C for 30 sec; and a final extension at 72°C for 10 min. The second round PCR products were purified as above and then were adjusted to 10–20 *n*g of DNA. Metagenomic sequencing was carried out on a MiSeq sequencer (Illumina) using the MiSeq Reagent kit v3 (600 cycles) with 300 paired-end reads, and data analysis was performed using the MiSeq Reporter software with the Greengenes database.

Table 1. Family level classification of microbiome in *R. amplexikaudatus*

Family[a)]	Reads (%)
Clostridiaceae	162,921 (69.3)
Campylobacteraceae	56,852 (24.2)
Enterobacteriaceae	11,898 (5.1)
Bacillaceae	589 (0.3)
Streptococcaceae	467 (0.2)
Enterococcaceae	350 (0.1)
Others	602 (0.3)
Unclassified	850 (0.4)
Total	234,529

a) Family obtained over 0.1% of total reads was shown.

The metagenomic reads are stored at the National Center for Biotechnology Information Sequence Read Archive under BioProject PRJDB4733 (Accession nos. experiment: DRX053603, run: DRR059024).

A total of 469,058 reads were obtained. Of those, 619 reads contained an ambiguous base (N). In the diversity of microbiota, the predominant phyla were Firmicutes. Family level classification resulted in the identification of 66 families. The predominant families were *Clostridiaceae* (69.5%), *Campylobacteraceae* (24.2%) and *Enterobacteriaceae* (5.1%) (Table 1). In Genus level classification, 103 genera were identified. The predominant genera were *Clostridium* (65.7%) and *Campylobacter* (24.2%) (Table 2). Species level classification resulted in the identification of 170 species. The predominant species were *Clostridium butyricum* (21.0%) and *Campylobacter coli* (19.3%) (Table 3). In a previous study, 71 genera were identified on metagenomics analysis of the fecal bacterial flora of *Myotis daubentonii*, and genera *Leuconostoc*, *Betaproteobacteria* and *Enterobacter* dominated [27]. Common genera between *R. amplexicaudatus* and *M. daubentonii* were 21, and dominated genera were different between them. These differences might be due to the difference of food habit. *R. amplexicaudatus* is frugivorous, and *M. daubentonii* is insectivorous.

Campylobacter was second predominant genus, and *C. coli* and *C. jejuni* were identified in microbiome of *R. amplexicaudatus*. Campylobacteriosis in one of the most important infectious disease in children and has increased over the world in the last decade [2, 13]. Campylobacteriosis is attributed to contamination of poultry product [1, 20], or drinking and environmental water [19, 24]. In *Campylobacter* spp., *C. jejuni* is the most common cause of acute bacterial diarrhea in humans [8]. Genus *Campylobacter* dominated secondly in the flora of *R. amplexicaudatus*, and to further evaluate the risk of spreading *Campylobacter* spp. by wild bats, we investigated the prevalence of *C. jejuni* in these 91 bats. To determine the prevalence of *C. jejuni* in bats, conventional PCR assay was conducted on all the fecal DNAs. The intergenic spacer 16S–23S (ITS) of *C. jejuni* was amplified by PCR using the primers CampyForw (5′-CTGATAAGGGTGAGGTCACAAGT-3′) and Campy-Rev (5′-CTTGCTTGTGACTCTTAACAATG-3′) [7]. The

Table 2. Genus level classification of microbiome in *R. amplexikaudatus*

Genus[a)]	Reads (%)
Clostridium	154,182 (65.7)
Campylobacter	56,846 (24.2)
Escherichia	9,720 (4.1)
Sarcina	7,495 (3.2)
Serratia	1,526 (0.7)
Bacillus	588 (0.3)
Streptococcus	379 (0.2)
Enterococcus	328 (0.1)
Alkaliphilus	273 (0.1)
Gemella	149 (0.1)
Chlamydophila	128 (0.1)
Others	612 (0.3)
Unclassified	2,303 (1.0)
Total	235,429

a) Genus obtained over 0.1% of total reads was shown.

Table 3. Species level classification of microbiome in *R. amplexikaudatus*

Species[a)]	Reads (%)
Clostridium butyricum	49,319 (21.0)
Campylobacter coli	45,167 (19.3)
Clostridium saccharobutylicum	11,320 (4.8)
Clostridium saccharoperbutylacetonicum	10,219 (4.4)
Escherichia albertii	9,396 (4.0)
Clostridium cavendishii	9,249 (3.9)
Sarcina maxima	7,411 (3.2)
Campylobacter helveticus	3,970 (1.7)
Clostridium chartatabidum	3,011 (1.3)
Campylobacter peloridis	2,886 (1.2)
Clostridium cadaveris	2,353 (1.0)
Clostridium histolyticum	1,746 (0.7)
Serratia entomophila	1,524 (0.6)
Clostridium vincentii	999 (0.4)
Clostridium sardiniense	759 (0.3)
Clostridium baratii	754 (0.3)
Bacillus butanolivorans	478 (0.2)
Campylobacter insulaenigrae	278 (0.1)
Campylobacter subantarcticus	272 (0.1)
Campylobacter volucris	262 (0.1)
Clostridium taeniosporum	261 (0.1)
Clostridium bovipellis	242 (0.1)
Campylobacter jejuni	237 (0.1)
Clostridium nitrophenolicum	234 (0.1)
Clostridium sulfidigenes	219 (0.1)
Enterococcus lactis	214 (0.1)
Clostridium chromoreductans	211 (0.1)
Clostridium fallax	141 (0.1)
Others	1,222 (0.5)
Unclassified	70,175 (29.9)
Total	234,529

a) Species obtained over 0.1% of total reads was shown.

reaction mixture (50 μl) contained 25 μl Premix EX Taq (TaKaRa-Bio), 1 μM each primer and 5 *n*g isolated DNA. The PCR conditions were as follows: 98°C for 3 min; 35 cycles of 98°C for 10 sec, 60°C for 30 sec, 72°C for 30 sec; and a final extension at 72°C for 10 min. PCR products were purified as described above, and then, the products were sequenced using the BigDye Terminator v3.1 Cycle Sequence kit (Applied Biosystems, Foster City, CA, U.S.A.) on a 3130 Genetic Analyzer (Applied Biosystems) and analyzed by Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for validation. These results confirm the accurate identification of *C. jejuni*. From 91 samples, *C. jejuni* was detected only in *R. amplexicaudatus* with a prevalence of 7.5% (5/67), and these five bats were captured at two sites, three at Lavigan and two at Sion. Another 43 bats (17 *R. amplexicaudatus* and 3 *H. diadema* at Lavigan, 22 *R. amplexicaudatus* and 1 *E.spelaea* at Sion) were negative at both sites.

The genus *Campylobacter* currently consists of 25 species, some of which are important human pathogens. *Campylobacter enteritis* causes acute bacterial diarrhea in humans, mainly in the developed world. The most often implicated species as the causative agent of campylobacteriosis is *C. jejuni*, followed by *C. coli* [26], *C. upsaliensis* [15] and *C. lari* [22]. Palmer and colleagues speculated that human campylobacteriosis may be the result of water contaminated by bird or bat feces [21], and enteric bacteria were isolated or detected with high throughput sequencing from bats [1, 27]. In these studies, only microchiroptera was surveyed, and *Campylobacter* spp. were not isolated or detected from their fecal or enteric swab samples. In this study, however, we detected *C. jejuni* in rectal swab samples from megachiroptera, *R. amplexicaudatus*, for the first time. *C. jejuni*, especially, could enter a viable, but non-culturable (VBNC) state under stressed condition, such as nutrient starvation, osmotic shock and fluctuations in temperature and pH and is thought to be a possible cause of water borne infections. *C. jejuni* in VBNC form was able to remain in this form for several months and then recover their culturability in the mouse intestine [4]. Therefore, *C. jejuni* might also be transmitted from wild bats to humans via water contaminated by bat feces.

In conclusion, the microbiota of bat was characterized using high throughput sequencing. Furthermore, we showed *R. amplexicaudatus* is potential carrier of *C. jejuni* in the Philippines. Bats can acquire infectious agents from their diet and other environmental sources and subsequently may transfer those agents to humans and livestock. Therefore, it is necessary to detect other bacterial pathogen to human from bats continuously.

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