NOTE *Wildlife Sicence*

Isolation of multiple drug-resistant enteric bacteria from feces of wild Western Lowland Gorilla (*Gorilla gorilla gorilla*) in Gabon

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ABSTRACT. Prevalence of drug-resistant bacteria in wildlife can reveal the actual level of anthropological burden on the wildlife. In this study, we isolated two multiple drug-resistant strains, GG6-2 and GG6-1-1, from 27 fresh feces of wild western lowland gorillas in Moukalaba-Doudou National Park, Gabon. Isolates were identified as *Achromobacter xylosoxidans* and *Providencia* sp., respectively. Minimum inhibitory concentrations of the following 12 drugs—ampicillin (ABPC), cefazolin (CEZ), cefotaxime (CTX), streptomycin (SM), gentamicin (GM), kanamycin (KM), tetracycline (TC), nalidixic acid (NA), ciprofloxacin (CPFX), colistin (CL), chloramphenicol (CP) and trimethoprim (TMP)—were determined. Isolate GG6-2 was resistant to all antimicrobials tested and highly resistant to CTX, SM, TC, NA and TMP. Isolate GG6-1-1 was resistant to ABPC, CEZ, TC, CL, CP and TMP.
KEY WORDS: intestinal bacteria, multiple drug-resistance, wild gorilla

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A multidisciplinary study has been conducted on the ecosystems of the tropical forest in Moukalaba-Doudou National Park (MDNP), Gabon, since 2009 [21]. Our studies focused on the conservation of biodiversity in the tropical forest through sustainable coexistence between humans and wild animals. In tourism that targets endangered wild animals, such as the gorilla, it is important to reduce the human impact on the wildlife [13]. Transmission of human-borne microbes, especially pathogens, to wild animals must be controlled in this context.

In addition to viral, bacterial and protozoal pathogens [19], we studied the distribution of drug-resistant bacteria in wild gorillas. Since the emergence and spread of antibiotic-resistant bacteria was caused by humans [11], we thought that the level of drug-resistant bacteria in wildlife might reveal the actual level of the anthropological burden on the wildlife in MDNP.

In a preliminary survey, we have detected a range of antibiotic-resistant genes from the fecal Enterobacteriaceae of wild gorillas in MDNP [22]. The most prevalent genes were responsible for resistance against β-lactam antibiotics. This is not surprising, because some species of Enterobacteriaceae naturally harbor such resistance [14]. The second most prevalent gene was aminoglycoside-resistance genes, followed by genes responsible for tetracycline resistance and chloramphenicol resistance. It was also suggested that some of the isolates hosted multiple resistances. We presumed that it would not be plausible for wild animals in MDNP to be free from antibiotic-resistant enterobacteria of anthropological origin. MDNP was once exploited by a logging company with more than 1,000 employees living in bunkhouses surrounding the forest. After the logging operations were withdrawn in 1989, a group of the bunkhouses became villages, and a crop plantation surrounds the forest; therefore, the wild animals in MDNP may have contact with humans in close proximity. In this study, we tried to isolate multidrug-resistant enterobacteria from wild gorillas in MDNP to measure the level of anthropological impact on wild animals in MDNP before the full-scale introduction of tourism in this forest.

Feces of wild western lowland gorillas were collected during research trips organized in August and December 2013 and in February 2014. In total, 20 fresh feces were sampled randomly from 4 groups of gorillas and one solitary individual in the summer of 2013 in the forest of Boutsiana and Mt. Doudou in MDNP. Four more feces were sampled in December 2013, and 3 fresh feces were sampled in February 2014 in the forest of Moukalaba in MDNP. The location of the sampling site was shown elsewhere [33]. The fecal samples were collected and inoculated on the plate media as described previously [36]. Briefly, fresh gorilla feces were sampled by localization and subsequent following the groups of gorillas or solitaires with a distance of ca. 15–20 m which enable to collect fecal samples just after

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Table 1. Summary of sampling results

Period	Sampling location in MDNP	Number of feces collected	Number of colonies developed	Number of feces carrying resistant bacteria
Aug-13	Boutsiana	10	3	3[a)]
Aug-13	Mt. Doudou	10	0	0
Dec-13	Moukalaba	4	3	3[b)]
Feb-14	Boutsiana	3	2	1

a): GG6-2 was purified; b): GG6-1-1 was purified. The locations are shown elsewhere [33].

Table 2. Identification, antimicrobial susceptibility and resistance genes of 2 isolates

Strain	Identification[a)]	MIC (μg/m*l*)[b)]												Resistance Gene[c)]
		ABPC	CEZ	CTX	SM	GM	KM	TC	NA	CPFX	CL	CP	TMP	
GG6-2	*Achromobacter xylosoxidans*	32	128	>64	>128	32	64	>64	128	2	2	32	>16	N.D.
GG6-1-1	*Providencia* sp.	32	>128	≤0.5	8	4	2	64	4	≤0.03	>16	32	16	*tetB*

a): Identified by 16S rRNA gene sequences. Accession numbers are LC009440 and LC009441. b): Abbreviations and the range of antimicrobial concentration (μg/m*l*) in MIC testing are as follows: ABPC, ampicillin (1–128); CEZ, cefazolin (1–128); CTX, cefotaxime (0.5–64); SM, streptomycin (1–128); GM, gentamicin (0.5–64); KM, kanamycin (1–128); TC, tetracycline (0.5–64); NA, nalidixic acid (1–128); CPFX, ciprofloxacin (0.03–4); CL, colistin (0.12–16); CP, chloramphenicol (1–128); and TMP, trimethoprim (0.25–16). c): Results from PCR amplification with specific primers for bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, plasmid-mediated AmpC, *tetA*, *tetB*, *tetC*, *tetD*, *tetE* and *tetG*. N.D., not detected.

defecation. A loopful of feces was placed on tryptic-soy agar (TSA) (Tryptic Soy Broth (Becton, Dickinson and Co., Sparks, MD, U.S.A.) supplemented with 1.5% agar) plates supplemented with defibrinated horse blood (5% v/v) and a mixture of antibiotics (ampicillin, tetracycline, gentamycin and chloramphenicol with final concentrations at 4 μg/m*l*) that corresponded to the major resistance genes detected in the previous survey and the major class of antibiotics used in Gabon thus far. After returning to the camp site, the fecal specimen on the plate was streaked and placed in a styrene foam box with hand warmers to keep the temperature in the box as close as possible to 37°C. After visible colonies were grown (up to 48 hr incubation), the plates were placed in a dark, cool place or in a water stream beside the camp site for safer preservation. After returning to the laboratory in Libreville, colonies were picked and transferred to the fresh TSA plates supplemented with antibiotics at the same concentration. For the preliminary identification, DNA was extracted from single colony of each isolate by DNA Stool Mini Kit (QIAGEN, Tokyo, Japan), and resultant DNA solutions were transported to Kyoto for partial sequencing (ca. 500 bps) of 16S rRNA gene as indicated elsewhere [35]. After species level identification, two isolates were transported to Japan by a commercial parcel service for further analyses.

After receipt of these bacteria in Japan, DNA was again isolated from these bacteria with the DNA Stool Mini Kit, and 16S rRNA genes were amplified with primers 63f and 1387r [20]. Amplicons were subjected to direct sequencing at FASMAC Co., Ltd. (Atsugi, Japan) by the dye-terminator method. A range of resistance genes was examined by PCR amplification. The PCR protocol was the same as indicated elsewhere [16, 17, 24, 37]. The drug susceptibility of the isolates was determined according to the Clinical and Laboratory Standards Institute (CLSI) with the Performance Standards for Antimicrobial Susceptibility Testing (M31-A3) [9]. Briefly, Dry Plate® (Eiken Chemical, Tokyo, Japan) was applied to determine the minimum inhibitory concentrations (MIC) of the following 12 drugs: ampicillin (ABPC), cefazolin (CEZ), cefotaxime (CTX), streptomycin (SM), gentamycin (GM), kanamycin (KM), tetracycline (TC), nalidixic acid (NA), ciprofloxacin (CPFX), colistin (CL), chloramphenicol (CP) and trimethoprim (TMP). Bacteria were suspended in McFarland No. 1 with Tryptosoy Bouillon Medium (Eiken Chemical) and further mixed with Mueller-Hinton Bouillon Medium (Eiken Chemical) and inoculated in plate wells to obtain 2.5×10^4 CFU/well. The plates were incubated at 37°C for 18 hr in ambient air. Growth was checked visually according to the manufacturer's instructions. MIC breakpoints used for GG6-1-1 and GG6-2 were those established by CLSI for Enterobacteriaceae [10] and other non-Enterobacteriaceae, such as *Achromobacter*, respectively [2, 10].

In the summer of 2013 survey, we detected three colonies on the plates from three individual fecal samples. Among these three colonies, two of them were contaminated by yeast and mobile Gram staining negative bacteria. These two colonies could not be purified through repeated transfers to fresh TSA plates with antibiotics. The remaining colony, designated as GG6-2, was, therefore, subjected to a further study (Table 1). In the 2013–2014 survey, we have detected 5 colonies from 7 fecal samples; however, as in the 2013 research trip, one colony was only obtained as a purified enterobacterial isolate. This isolate was designated as GG6-1-1 (Table 1).

Two gorilla isolates, both Gram staining negative rods, GG6-2 and GG6-1-1, were, respectively, identified as *Achromobacter xylosoxidans* (1,261 bp/1,261 bp, identity=100%) and *Providencia* sp. (1,274 bp/1,278 bp, identity=99%).

The latter is highly similar to *P. rettgeri* (1,273/1,278 bp, identity=99%) in its 16S rRNA gene sequences. Their 16S rRNA gene sequences were registered at the DNA Database of Japan (DDBJ) with accession numbers LC009440 and LC009441, respectively.

Achromobacter spp. are considered to be environmental bacteria [3, 23]. However, this group of bacteria was detected in a wide range of clinical specimens, including feces, and was recognized as human nosocomial bacterium [1, 3, 5]. As shown in Table 2, isolate GG6-2 was resistant to all antimicrobials tested and highly resistant to CTX, SM, TC, NA and TMP. *Achromobacter* naturally harbors various β-lactamase including AmpC type [15]. It is obvious that strain GG6-2 showed innate resistance to ABPC. This isolate also showed resistance to third-generation cephalosporin as well as to aminoglycosides and tetracycline. *A. xylosoxidans* has been shown to be innately resistant to cephalothin, cefoxitin, cefotaxime, aztreonam and aminoglycosides [5, 6]. Acquired resistance to carbapenems, ceftazidime and ciprofloxacin is also frequent [3, 4, 18]. In addition to these resistances, our isolate harbors tetracycline resistance and chloramphenicol resistance.

Isolate GG6-1-1 was resistant to ABPC, CEZ, TC, CL, CP and TMP, but susceptible to aminoglycosides and quinolones. *Providencia* also naturally harbors AmpC-type β-lactamase [14]. Because of its susceptibility to CTX, it is expected that GG6-1-1 is naturally resistant to ABPC and CEZ. In addition to β-lactam, clinical isolates of *P. stuartii* are innately resistant to tetracycline with varying resistance to aminoglycosides [31]. GG6-1-1 is close to *P. rettgeri*, which has multi-drug resistance to a lesser extent as compared with *P. stuartii* [31].

Isolates GG6-2 and GG6-1-1 showed resistance to tetracycline and aminoglycoside as well as to β-lactam. The resistance of GG6-1-1 shown in Table 2 is regarded as innate, according to the literature [31], and its susceptibility to CTX and CPFX suggests that this strain has characteristics in common with *P. rettgeri*. However, in the case of GG6-2, high resistance to the third-generation cephalosporin, CTX, was shown. At present, we cannot conclude the origin of such resistance in GG6-2. It is possible that wild gorillas represent a reservoir of multi-drug resistance, as discussed elsewhere [12, 25, 26, 28, 32]. As shown in a study in a remote area of Africa, the glacier snow samples from Mt. Stanley of Ruwenzori, Uganda, contained high levels of resistance genes to carbapenem (*blaIMP*), chloramphenicol (*cmrA*) and tetracycline (*tetG*), followed by resistance genes to aminoglycoside (*aac*(3), *aac*C and *strA*); the bacteria harboring the resistance genes may have spread out all over the African continent through global atmospheric circulation [30]. Moreover, *A. xylosoxidans* is commonly detected in various environments [23]. In this circumstance, wild gorillas could acquire several resistant bacteria to exhibit multidrug resistance in their digestive tracts. In fact, Benavides *et al*. [7], who surveyed the prevalence of antibiotic-resistant *Escherichia coli* in wild gorillas and villagers living adjacent to the Lopé National Park in Gabon, concluded that there was no or very low direct transmission of resistant *E. coli* from humans to wild gorillas in Lopé National Park. However, the anthropological burden of gorillas in MDNP is not the same for those in Lopé National Park; the human population density around Lopé was 4 times lower (0.2 person/km^2) than that around MDNP (0.8 person/km^2) [8, 34]; the latter had intensive logging activity along with agricultural activity. It is, therefore, not safe to exclude the transmission of human-borne resistant bacteria in MDNP as suggested by Cohen [11], who concluded that the emergence and spread of multidrug-resistant enterobacteria is, in principle, considered human-borne. Acquisition of resistance to 3rd-generation cephalosporin supports this hypothesis. In fact, there were several surveys regarding the antibiotic resistance of infectious bacteria in 1997 and 2009 in a hospital located adjacent to MDNP that clearly indicated a significant progress in the resistance of diarrheagenic *E. coli* isolates to second-generation cephalosporin, third-generation cephalosporin, carbapenem, aminoglycosides and the new quinolones [27, 29]. The multidrug-resistant *Achromobacter* presently found in gorillas together with previously suggested resistant *Enterobacter* and *Klebsiella* [22] in wild western lowland gorillas may suggest that gorillas in Gabon have been exposed to human-borne resistant bacteria. Although the prevalence of multi-drug resistant bacteria was not high (Table 1), wild animals like gorilla in MDNP may have received the stronger anthropological influences comparing to those in Lopé National Park in terms of transmission of bacteria.

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