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Bacteria culturing and isolation under field conditions of Moukalaba-Doudou National Park, Gabon, and preliminary survey on bacteria carrying antibiotic resistance genes

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ABSTRACT Culturing the intestinal bacteria of wild animals is a difficult task under field conditions, although isolation and characterization of bacteria are absolutely necessary to evaluate the transmission of bacteria from human to animal or *vice versa*. We have developed a protocol for intestinal bacteria culturing from feces of wild animals such as gorillas in Moukalaba-Doudou National Park in Gabon, where no laboratory settings were available. The prevalence of resistance genes in Enterobacteriaceae isolates estimated by real time PCR array was higher for aminoglycosides resistance, followed by tetracycline resistance, except for possible naturally occurring β -lactam resistance. The detection level of resistance genes was higher for isolates from humans than those from gorillas. Occasional monitoring of this prevalence may help to measure the intensity of introduction of human-borne bacteria to wildlife.

Key words: Bacteriology, Field conditions, Gabon, Wild animal

INTRODUCTION

Tourism focusing on great apes in their natural habitat has grown in recent days. Observations at a short distance often allow contact between animals and tourists (Homsy, 1999). This type of eco-touristic activity may increase the incidence of epidemics for the animals, which could decrease the populations of primates (Kalema-Zikusoka et al. 2000, Leendertz et al. 2006, Köndgen et al. 2008, Pusey et al. 2008).

In the forest of Moukalaba-Doudou National Park (MDNP), Gabon, eco-tourism will be introduced in the near future as a result of human habituation of groups of west lowland gorillas (*Gorilla gorilla gorilla*) (Yamagiwa 2006, Matsuura et al. 2013). It would be important to protect both humans and wild animals, great apes in particular, from mutual transmission of epidemics (Dörnath Aguirre Alvarez and Völlm, 2004). In our previous studies, we have detected both human- and non-human-type intestinal lactic acid bacteria in wild chimpanzees (Uenishi et al. 2007, Ushida 2008). Little is known about how the exchanges of bacteria between humans and wildlife took place. To elucidate this, the bacteria in question should be isolated from wildlife by classic bacteriological techniques to evaluate the mutual transmission of bacteria, although DNA-based meta-genomic

analyses have been quite successful in the study of intestinal bacteria of great apes (Uenishi et al. 2007, Moeller et al. 2012). Indeed, the transmission of bacteria can only be traced by studying the isolates at strain level identification. The first step of our study, therefore, was to develop an appropriate methodology to isolate and cultivate bacteria from the feces of wildlife on site where none of the laboratory facilities for bacteriology were available. A previous study shows some possibilities for anaerobic cultivation in field conditions (Ushida et al. 2010a). However, these techniques were developed in Bossou, Guinea, where a manipulation room was available within the field. Recent study in Lopé National Park, Gabon, also showed successful cultivation of *Escherichia coli* from wild animals such as gorillas (Benavides et al. 2012). Such study was based on the laboratory facility on site. Since a similar level of facilities was not available in our current study field, MDNP, the original methodology for the bacteria culturing had to be adapted to the situation: a camping site with only tents and an absence of electricity (therefore, no incubator and no refrigerator) and clean water supply, which creates major limitations for the culture and isolation of bacteria in the field. Difficulties in contamination control in addition to temperature control for media preservation before use and after colony development were the major problems we experienced. In this report, we show

the techniques for so-called “field-bacteriology” developed in the past 4 years in a SATREPS project (Yamagiwa 2006, Matsuura et al. 2013) to study the intestinal bacteria of wild gorillas and local workers, particularly antibiotic resistant bacteria. We theorize antibiotic-resistant bacteria may be good indicators for transmission from humans to wild animals if we isolate the same resistant bacteria at strain level from both sources.

In this work, human fecal samples were requested by written informed consent. The study was approved (CENA REST research permission No. 277) by the Centre National de la Recherche Scientifique et Technologique (CENAREST), the Ministry of Education and the Higher education of Gabon.

MATERIALS AND METHODS

Media: The following culture media were used in this study: deoxycholate-hydrogen sulfide-lactose (DHL) agar (Nissui Pharmaceutical, Tokyo), Salmonella-Shigella (SS) agar (Nissui Pharmaceutical, Tokyo), MacConkey (MC) agar (Oxoid, Hampshire, United Kingdom), Gifu Anaerobic Medium (GAM) agar (Nissui Pharmaceutical, Tokyo), and Trypticase soy (TS) agar, with three ways of culturing on site as described below. Except for MC and TS, anaerobiosis (GAM plates) or micro-aerobiosis (DHL plates, SS plates) were achieved by using Anaeropouch® or Anaeropouch-MicroAero® (Mitsubishi Gas Chemicals, Tokyo).

Source of bacteria and on-site cultivation: Fecal samples were collected from individuals belonging to a habituated group of gorillas (*Group Gentil* (GG)) and non-habituated groups of gorillas (*Group Martial* or *Group Méchand* (GM)) in the forest of Boutsiana, a gorilla group in habituation (group 8 (G8)) in the Dougets forest, and other gorillas, mostly solitary individuals, that live in these research fields. During the studies, feces of chimpanzees were occasionally collected. Feces of humans were also collected in this study from local villagers and researchers in accordance with informed consent.

During the dry season (August 6–14) in 2010, 50 fecal samples were collected and subjected to on-site culturing as follows. Fresh feces were collected in plastic bags with a pair of sterile tweezers. Precautions were taken to avoid soil contamination. After the prompt return to the camping site in the forest, a loopfull fecal specimen, collected from the inside of the feces, was streaked on DHL and SS plates (90 mm ϕ) using sterile plastic loops. Incubation was done according to a protocol previously established (Ushida et al. 2010a), except that Anaeropouch-MicroAero® (Mitsubishi

Gas Chemicals, Tokyo, Japan) was used instead of Anaeropouch® (Mitsubishi Gas Chemicals). Plates were placed in an airtight nylon bag and kept in a styrene foam box. The temperature of the box was kept as close as possible to 37°C by hand warmers with periodical (every 3 to 4 h) verification of the temperature. After 24-h incubation, 1–3 colonies were selected from a plate according to the colony morphology and transferred at the camping site to a 2-ml plastic tube containing the same medium. Tubes were incubated for another 24 h as indicated above. After transportation to the laboratory of IRET in Libreville, the isolates were repeatedly purified on the same medium in nearly the same manner as on-site culturing because some critical equipment such as incubators and clean benches, were not available.

During 2011 (from March to December), we went 6 times to the field to collect 120 fecal samples from gorillas (GG, G8, and solitaires) and 26 from humans as indicated above. In this period, we attempted to culture the fecal bacteria on site using 2-ml plastic screw cap tubes containing slant or stub media. We had chosen the 2-ml screw cap tube for the cultivation on site due to its compact size and easy incubation in 37°C water in a thermos. After inoculation by streaking or stabbing using sterile plastic loops, incubation in water at 37°C in a thermos was done for 24 h. For the anaerobic medium, GAM, inoculation was achieved by stabbing, while streaking was used for other media. Incubation was conducted by periodically checking tap water at 37°C. Once visible colonies were obtained, tubes were placed in a dark place or in fresh water until transportation to the laboratory in Libreville. Tubes were packed in a plastic liquid-tight container and submerged in a stream. In the laboratory, the colonies were transferred to the plates with the same medium. Purification was repeated on the same medium as conducted in the 2010 study.

During the studies in 2012 and 2013, we collected fecal samples in June, July, and November 2012 and in January and February 2013. In these periods, we collected 107 samples of feces from gorillas (GG and G8) and 5 from humans as indicated above. Unlike the 2010 or 2011 seasons, we used dishes of 60 mm in diameter because 2-ml screw cap tube and dishes of 90 mm in diameter were inconvenient for the on-site culturing. Indeed, head space gas of 2-ml screw cap tubes could not be controlled during inoculation of fecal specimens and the dishes required too much space for the field research particularly for the transportation in our condition. One loop-full fecal specimen was collected on site from the inside of the feces, quickly placed on a plate medium and returned to an airtight nylon bag with or without Anaeropouch-MicroAero®. After returning to the

camp site, Petri dishes were carefully re-opened to streak the fecal specimens. This manipulation was carried out due to a risk of contamination with airborne microorganisms, yeast in particular, if we streaked fecal specimen in the forest or savannah, where significant soil dust in the air was observed. Finally, the plastic bags with plates were kept in a styrene foam box as in the 2010 study. When bacterial colonies had grown, the plates were kept in a liquid-tight plastic container under cooler conditions, e.g., in a dark place or submerged in a stream until they were transported to the laboratory. The isolation and purification of colonies were conducted in a laboratory of the Research Institute of Tropical Ecology in Libreville.

Biochemical identification and phylogenetic analyses: The strains successfully purified were subjected to biochemical identification by API 20 E (bioMérieux, Marcy l'Etoile, France) (Swanson and Collins, 1980). For some strains, a 16S rRNA gene was sequenced after PCR amplification with universal primers (Tsukahara and Ushida 2002). PCR reaction mixture was prepared with 1.0 µl each of primer solution (10 pmol), 25 µl of GoTaq polymerase solution (Toyobo, Osaka), and 20 µl of distilled water. A colony developed on DHL or SS was picked with sterile plastic loops, and three loopfuls of bacteria were suspended in boiling water (100 µl). This bacterial suspension (3 µl) was mixed with the above-mentioned PCR mixture (the final volume was 50 µl). The thermal program consisted of an initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 58°C for 30 sec, and 72°C for 30 sec and terminated with the final elongation step of 72°C for 3 min. The sequence was conducted by a dye-terminator method at Hokkaido System Science (Sapporo, Japan). The obtained sequences were analyzed by BLAST at DDBJ (Mishima, Japan) to identify the strains. Phylogenetic trees were constructed by the Neighbor-joining method using MEGA ver. 5.05 (Tamura et al. 2011) (Fig. 1).

Analyses on antibiotic resistance genes: The profile of the antibiotic resistance genes was obtained by a real-time PCR-array approach (Segawa et al. 2013), which had a set of 95 resistance genes to be analyzed by a Microfluidic PCR (BioMark 48 X 48 Dynamic Array; Fluidigm, South San Francisco, CA, USA) for microaerophilic isolates in the 2010 and 2011 studies.

RESULTS

Thirty five isolates were obtained from 50 gorilla fecal samples by DHL and SS plates at first in the 2010 study. However, 22 isolates from 12 fecal samples were viable

after storage on site, transportation, and re-purifications. These 22 isolates were all gram-negative enterobacteria according to API 20E.

In the 2011 study, all tubes carried colonies after 24-h incubation in water at 37°C. After isolation and purification were carried out in the laboratory of Libreville, 110 bacteria were isolated (43 were from DHL and 67 were from SS) from gorilla and human subjects. Among 110 isolates, 80 were sequenced for 16S rRNA. The data, based on the successful identification, of the prevalence frequency of enterobacteria are shown in Table 1. The most prevalent enterobacteria for the gorilla was *Enterobacter. cloacae* (44.0 %), and it was followed by *Klebsiella variicola* (20.0 %), *K. pneumoniae* (16.0 %), and *Cedecea davisae* (12.0 %). The other minor bacteria belonged to *Ent. ludwigii* (4.0 %) and *Ent. cancerogenus* (4.0 %). In human fecal samples, the most prevalent bacterium was *E. coli* (61.3 %), followed by *K. pneumoniae* (19.4 %) and *Ent. hormaechei* (12.9 %); *Ent. cloacae* was a minor bacterium (6.5 %).

In the 2012 study, we collected 112 fecal samples, which allowed us to obtain 146 isolates, of which 139 were from gorillas (25 from DHL, 25 from SS, and 89 from MC) and 7 from humans (1 from DHL, 2 from SS, and 4 from MC). The prevalence of each bacterium identified is shown in Table 2. The most prevalent bacterium in the gorilla was *Ent. cloacae* (24.8 %), followed by *E. coli* (20.4 %), *Serratia odorifera* (19.0 %), *Erwinia sp.* (7.3 %), *Providencia rettgeri* (6.6 %), and *Salmonella sp.* (4.4 %). Other minors were *Ser. liquefaciens* (3.6 %), *Raoultella planticola* (2.9 %), *R. ornithinolytica* (2.2 %), *Kluyvera sp.* (1.4 %), *Proteus mirabilis* (1.5 %), *Citrobacter freundii* (0.7 %), *Ent. cancerogenus* (0.7 %), *Hafnia alvei* (0.7 %), *K. pneumoniae* (0.7 %), *Pseudomonas fluorescens* (0.7 %), *Ser. ficaria* (0.7 %), *Ser. marcescens* (0.7 %), and *Ser. rubidaea* (0.7 %). However, in the human, the most prevalent bacterium was *Ent. cloacae* (66.7 %) followed by *E. coli* (16.7 %) and *Pr. vulgaris* (16.7 %). (Table 2).

In the 2010 and the 2011 study, antibiotic-resistance genes against β -lactam (*ampC/blaACT*, *blaSHV*, *blaCTX*), aminoglycoside (*aadA*, *aadB*, *aac(3)*, *strA*, *strU*), tetracycline (*tetA*, *tetD*, *tetG*, *tetU*), and/or chloramphenicol (*catI*, *cmrA*) were detected (Table 3 and Fig. 1) from gorillas and humans. The prevalence of resistance genes (number of genes/number of isolate tested) was higher for strains isolated from humans (31/31) than those from gorillas (44/47). In the 2010 and 2011 study, the most prevalent resistance gene in enterobacteria obtained from gorillas was the gene against β -lactam such as *blaACT* (34 % prevalence) carried by a variety of enterobacteria. In the case of humans, the most prevalent resistance gene in enterobacte-

Fig. 1. Phylogenetic tree of Enterobacteriaceae isolated from feces of a group of wild gorilla (*Group Gentil*) and human subjects in Moukalaba-Doudou National Park. Fecal samples were collected in one sampling trip in the 2011 study. The tree was constructed by the neighbor-joining method with MEGA ver. 5.0.5. The bar represents the distance. Numbers at nodes show the bootstrap values obtained from 1,000 replicates. The triangles preceding the strain number indicate the human isolates. Others are all gorilla isolates.

Table 1. Prevalence of enterobacteria in the feces of gorilla and human subjects in the 2011 study

Source	Enterobacteria	Number of isolate	Frequency (%)
Gorilla	<i>Cedecea davisae</i>	3	12.0
	<i>Enterobacter cancerogenus</i>	1	4.0
	<i>Enterobacter cloacae</i>	11	44.0
	<i>Enterobacter ludwigii</i>	1	4.0
	<i>Klebsiella pneumoniae</i>	4	16.0
	<i>Klebsiella variicola</i>	5	20.0
Human	<i>Enterobacter cloacae</i>	2	6.5
	<i>Enterobacter hormaechei</i>	4	12.9
	<i>Escherichia coli</i>	19	61.3
	<i>Klebsiella pneumoniae</i>	6	19.4

Table 2. Prevalence of enterobacteria in wild gorilla in Moukalla-ba-Doudou National Park and human subjects adjacent to National Park in 2012 study

Source	Identification	Number of isolates	Frequency (%)
Gorilla	<i>Citrobacter freundii</i>	1	0.7
	<i>Enterobacter cancerogenus</i>	1	0.7
	<i>Enterobacter cloacae</i>	34	24.8
	<i>Erwinia sp.</i>	10	7.3
	<i>Escherichia coli</i>	28	20.4
	<i>Hafnia alvei</i>	1	0.7
	<i>Klebsiella pneumoniae</i>	1	0.7
	<i>Kluyvera sp.</i>	2	1.5
	<i>Proteus mirabilis</i>	2	1.5
	<i>Providencia rettgeri</i>	9	6.6
	<i>Pseudomonas fluorescens</i>	1	0.7
	<i>Raoultella ornithinolytica</i>	3	2.2
	<i>Raoultella planticola</i>	4	2.9
	<i>Salmonella sp.</i>	6	4.4
	<i>Serratia ficaria</i>	1	0.7
	<i>Serratia liquefaciens</i>	5	3.6
	<i>Serratia marcescens</i>	1	0.7
	<i>Serratia odorifera</i>	26	19.0
	<i>Serratia rubidaea</i>	1	0.7
Human	<i>Enterobacter cloacae</i>	4	66.7
	<i>Escherichia coli</i>	1	16.7
	<i>Proteus vulgaris</i>	1	16.7

Isolates were identified by API 20E. Details, see text.

ria was the gene against β -lactam such as *blaACT* (19% prevalence) and *blaCTX* (16%) carried also by a variety of enterobacteria. The second most prevalent resistance gene was aminoglycoside resistance in gorillas, such as *strA*, *strU*, and *aadA*. In human, the second most prevalent resis-

tance gene was *catI* followed by resistance genes against aminoglycosides. In the case of gorillas, *blaACT*-positive *Cedecea* showed high frequency (67% in the *Cedecea* isolates), followed by *blaACT*-positive *Enterobacter* (38% in the *Enterobacter* isolates). In the case of humans, *blaACT*-positive *Enterobacter* showed high frequency (83% in the *Enterobacter* isolates), followed by *blaCTX*-positive *Escherichia* (26% in *Escherichia* isolates). We did not detect the same enterobacterial species carrying the same resistance genes both in gorillas and humans. Gorillas carried resistant bacteria belonging to genus *Enterobacter* rather than other Enterobacteriaceae, while human subjects carried resistant bacteria belonging to genus *Escherichia* rather than other Enterobacteriaceae. *Klebsiella* sp. strain PG32 isolated from humans might have a relation to *Klebsiella* sp. strain G2 from gorillas, since these two strains are phylogenetically nearly identical (>99.5% identical for partial 16S rRNA gene sequence). Particular points are raised for the absence of a common bacterium carrying the same resistance gene for humans and gorillas in Fig. 1.

DISCUSSION

Development of field bacteriology

Cultivation and isolation of fecal bacteria in field conditions is still a difficult task in comparison with DNA-based analysis. For the latter, we are only required to collect a small amount of fecal samples into tubes containing preservatives of bacterial DNA such as ethanol (Uenishi et al. 2007) or a detergent solution with EDTA (Hayaishi and Kawamoto 2006). However, phylogenetic information does not provide functional information (Riesenfeld et al. 2004, Tringe et al. 2005). Recently developed metagenomic analyses may help such limitations of phylogenetic analyses. However, the contigs constructed in metagenomics only suggest the presence of a particular type of functional protein, such as enzymes (*i.e.*, a fibrobacterial type of glucanase or Prevotellal type of glycosidase), according to the sequence similarity to known sequences in databases. The analysis of contigs does not necessarily confirm the functionality of the "sequence" obtained. We believe that isolation of live bacteria still has tremendous value for understanding the functionality of fecal microbiota. Moreover, transmission of bacteria from humans to animals or *vice versa* can be followed only by tracing particular isolates. These show the limitation of DNA-based meta-analyses. Accordingly, we think that bacterial isolates should be obtained for further analyses from wild animals as well as hu-

Table 3. Prevalence of enterobacteria carrying antibiotic resistance gene in wild gorilla in Moukalaba-Doudou National Park and human subjects adjacent to this area (2010 and 2011 studies) Genes were detected by Real-time PCR-Array, Phylogenetic identification was done by 16S rRNA gene sequencing. Details are given in the text

Source	Detected genes	Frequency (%) of resistance gene in total resistance gene detected	Number of strains harboring the resistance gene	Carried by	Frequency (%) resistance gene within the same species
Gorilla	<i>aadA</i>	2.3	1	<i>Cedecea davisae</i>	33.3
	<i>aadB</i>	2.3	1	<i>Klebsiella pneumoniae</i>	9.0
	<i>aph(3')</i>	2.3	1	<i>Enterobacter asburiae</i>	16.6
	<i>ampC</i> *	18.2	2	<i>Enterobacter cloacae</i>	15.3
			1	<i>Enterobacter asburiae</i>	16.6
			1	<i>Escherichia coli</i>	25.0
			2	<i>Klebsiella pneumoniae</i>	18.1
			2	<i>Klebsiella variicola</i>	50.0
	<i>blaACT</i> *	34.1	2	<i>Cedecea davisae</i>	66.7
			1	<i>Enterobacter asburiae</i>	16.6
			6	<i>Enterobacter cloacae</i>	46.1
			1	<i>Enterobacter ludwigii</i>	33.3
			1	<i>Enterobacter cancerogenus</i>	50.0
			1	<i>Escherichia coli</i>	25.0
			3	<i>Klebsiella pneumoniae</i>	23.0
	<i>blaSHV</i>	9.1	1	<i>Enterobacter asburiae</i>	16.6
			1	<i>Escherichia coli</i>	25.0
			2	<i>Klebsiella pneumoniae</i>	18.1
	<i>cmrA</i>	2.3	1	<i>Enterobacter cloacae</i>	7.7
	<i>strA</i>	2.3	1	<i>Enterobacter cloacae</i>	7.7
	<i>strU</i>	13.6	1	<i>Enterobacter asburiae</i>	16.6
			3	<i>Klebsiella pneumoniae</i>	27.2
			2	<i>Klebsiella variicola</i>	40.0
	<i>tetA</i>	2.3	1	<i>Klebsiella pneumoniae</i>	9.1
	<i>tetD</i>	2.3	1	<i>Enterobacter cloacae</i>	7.7
	<i>tetG</i>	2.3	1	<i>Klebsiella variicola</i>	20.0
	<i>tetS</i>	2.3	1	<i>Enterobacter asburiae</i>	16.6
	<i>tetW</i>	4.5	2	<i>Klebsiella pneumoniae</i>	18,1
Human	<i>aac(3)</i>	9.7	3	<i>Escherichia coli</i>	15.8
	<i>aadA</i>	9.7	2	<i>Escherichia coli</i>	10.5
			1	<i>Klebsiella pneumoniae</i>	16.7
	<i>blaACT</i> *	19.4	2	<i>Enterobacter cloacae</i>	100.0
			3	<i>Enterobacter hormaechei</i>	75.0
			1	<i>Escherichia coli</i>	5.3
	<i>blaCTX</i>	16.2	5	<i>Escherichia coli</i>	26.3
	<i>blaSHV</i>	3.2	1	<i>Klebsiella pneumoniae</i>	16.7
	<i>catI</i>	19.4	4	<i>Escherichia coli</i>	21.1
			2	<i>Klebsiella pneumoniae</i>	33.3
	<i>strA</i>	9.7	2	<i>Enterobacter hormaechei</i>	10.5
			1	<i>Escherichia coli</i>	25.0
	<i>strU</i>	3.2	1	<i>Klebsiella pneumoniae</i>	16.7
	<i>tetA</i>	6.5	2	<i>Enterobacter cloacae</i>	10.5
	<i>tetD</i>	3.2	1	<i>Klebsiella pneumoniae</i>	16.7

* *ampC* and *blaACT* were amplified by different primer set (Ushida et al. 2010b)

mans living adjacent to wildlife area.

In usual cases, cultivation, isolation, and purification of bacteria should be manipulated under equipped-laboratory conditions. Such conditions are needed to minimize the risk of contamination. Basic techniques of bacteriology have been developed on the premise of such conditions. For example, selective media are conditioned for specific microorganisms with particular temperature and gas-phase composition needs. In our study, SS and DHL were not necessarily selective for *Shigella*-*Salmonella* or *Enterobacteriaceae*. Yeast contaminants often heavily colonized on these media, which inhibited the growth of target bacteria. Such contamination may have been caused by poor control of the incubation temperature, which is crucial for the selectiveness of these media. Storage of media before use is another problem for selective agents, as they often lost their ability to select the target organisms when storage conditions are not optimum. Antibiotics in this study seemed to have lost their functionality when used in field conditions over a week after preparation. The temperature in the dark place of camp has varied between 22.7°C and 31.5°C, which was not good for storage of media. As mentioned above, the conditions for inoculation, incubation, and storage after incubation could not be fully controlled in the MDNP, in contrast to the conditions in Bossou or Lopé, where one of the authors worked on bacteriology on wild chimpanzees in the former, wild gorillas in the latter. In Bossou, we had a clean laboratory adjacent to the field with virtually no electricity supply, although it was equipped with a kerosene refrigerator, which enabled us to preserve spent media after colony formation in addition to keeping unused media intact. These tools were not completely unavailable in the present study field. As shown in the results, we have developed an original technique for inoculation, incubation, and isolation on site. To minimize the time for Petri dish to be open, which increases tremendously the risk of contamination by fungi or yeasts, it was not recommended to streak the inoculum in open forest area. To that end, we also selected a small size of Petri dish (60 mm ϕ) rather than screw capped tubes. The maintenance of anaerobiosis was the reason for this selection, because gas-phase of screw cap tubes was uncontrollable once opened in the field. Storage in a river (<20°C) was the only possible way to avoid storage at an undesirably high temperature (>33°C). As a result, we have now established a protocol for the bacteria isolation from the feces of wild animals. With these techniques, isolation seemingly improved in the 2012 study (Table 2). Indeed, the number of bacterial isolates relative to the number of fecal specimens increased from 22 to 146 when comparing the results in 2010 with those in 2012.

Some of the bacteria found in gorillas and humans in this study can be considered environmental and not intestinal. *Bacillus altitudinis*, *Burkholderia cepacia*, *Bur. multivorans*, *Ralstonia solanacearum*, and *R. thomasi* for the 2010 study and *B. altitudinis*, *B. pumilus*, and *Chromobacterium violaceum* for the 2012 study (data not shown) were typical soil bacteria that may have contaminated the plate when it was opened in the forest or at least can be considered transient bacteria. A protocol established in the 2012 study improved the level of contamination due to the minimized risk for contamination by airborne environmental bacteria and yeasts.

Antibiotic resistance

Transmission of bacteria from animals to humans (zoonosis) and in the opposite direction is traced only by strain-level identification. As the preliminary study, we tried to estimate the prevalence of antibiotic resistance in bacteria isolated from the feces of wild gorillas by comparing them with those isolated from human subjects. We detected a wide variety of resistance genes from a range of bacteria isolated from both gorillas and humans. Among the genes detected, genes against β -lactum such as *blaACT* were the most frequently detected in enterobacteria of both gorillas and humans (Table 3). However, at least some of these resistances can be regarded as naturally occurring resistance in enterobacteria (Bradford 2001). Resistance against β -lactum was followed by resistance to aminoglycosides such as *strA* and *strU* and then by resistance to tetracyclines such as *tetA* and *tetD*. A similar tendency was observed in Lopé National Park, where the prevalence of antibiotic-resistant *E. coli* was studied in wild gorillas and villagers living adjacent to the national park (Benavides et al. 2012). These authors reported that the most prevalent resistance in *E. coli* from both gorillas and humans was ampicillin resistance, followed by streptomycin resistance and tetracycline resistance. Although a low level of frequency (2.3 %) for chloramphenicol resistance was recorded in enterobacteria isolated from gorillas in our study (Table 3), these authors did not detect chloramphenicol resistance in *E. coli* isolated from gorillas (Benavides et al. 2012). However, they detected some level of chloramphenicol resistance in human isolates of *E. coli* (4 % of isolates). In our study, the level of *catI*-positive *E. coli* in human isolates was as high as 21 %, which may explain the detection of a chloramphenicol resistance gene in enterobacteria at a low level (2.3 %) in gorillas. These genes are associated with therapeutic antibiotics commonly used in Gabon, including the

villages adjacent to MDNP. An overlapping pattern of resistance in *E. coli* between humans and wild animals was also shown in a similar study conducted in Michigan, United States, where resistance to streptomycin, tetracycline, and cephalothin was detected as the most prevalent resistance in *E. coli* isolated both from wild animals and humans (Sayah et al. 2005). In Chobe National Park, Botswana, where resistance to ampicillin was the most prevalent both in banded mongooses and humans, followed by resistance to tetracycline, streptomycin, doxycycline, and sulfamethoxazole-trimethoprim (Pesapane et al. 2013). In all cases, resistance to ampicillin is the most prevalent in both wild animals and human subjects, but the values for wild animals are significantly smaller than those for humans. This tendency suggests the transmission of human-borne resistant bacteria to wildlife. It is noteworthy to mention that significant overlapping was also shown between wild animals and farm animals in the resistance to neomycin, chloramphenicol, ampicillin, sulfamethoxazole-trimethoprim, and sulfisoxazole in addition to streptomycin, tetracycline, and cephalothin (Sayah et al. 2005). Since very high prevalence of antibiotic resistance was demonstrated in farm animals such as pigs (Sayah et al. 2005, Kozak et al. 2008, Mariano et al. 2009, Allen et al. 2011), intensive farming should not be ruled out as the major source of transmission of resistant bacteria to wild animals (Kaneene et al. 2007). However, at our study site, there were few farm animals (goats and chicken) in the village, which were apparently raised without antimicrobials. Due to this, we eliminated the possibility of transmission from livestock in the village to wildlife in MDNP. Transmission of bacteria from humans to wild gorillas may have occurred in an unconscious way as shown in the studies on tourist resorts (Hamelin et al. 2006, Wheeler et al. 2012). In addition to such local transmission, a worldwide transmission of resistant bacteria can occur. Research on the distribution of antibiotic-resistance genes in remote areas such as glaciers suggests that antibiotic-resistant bacteria can be transmitted by global atmospheric circulation (Ushida et al. 2010b, Segawa et al. 2013). However, resistant bacteria may not be preserved in the intestines of animals for a long period if antibiotics are not applied. The wild gorillas, unlike captive gorillas in zoos or the livestock under intensive farming, have no history of antibiotic dosing. Therefore, we theorized that the repeated detection of resistant bacteria in gorilla feces may reflect the continuous transmission of human-borne bacteria. At present, it is difficult to analyze the frequency of such transmission; the prevalence of resistant bacteria (*i.e.*, not resistance genes) seems to be low enough. Nonetheless, the prevalence estimated by PCR-array approach in the 2010

and 2011 studies (Table 3) showed a relatively high values for gorillas in MDNP (25 %) compared with those reported in *E. coli* isolated from wild gorillas in Lopé National Park (ca. 7 %) and baboons in Tanzania (up to 6 %) (Routeman et al. 1985). However, the value is lower than or similar to those in *E. coli* isolated from raccoons (*Procyon lotor*) in Canada (from 17 % to 54 %) (Jardine et al. 2012), in *E. coli* and *Salmonella* isolated from Russian rooks (*Corvus frugilegus*) in the Czech Republic (13 %) (Literák et al. 2007), in *E. coli* isolated from sympatric wild rodents in the United Kingdom (20 %) (Williams et al. 2011), in *E. coli* of wild rats (*Rattus* spp.) in Trinidad (36.3 %) (Nkogwe et al. 2011), in *E. coli* of rats (*Rattus* spp.) in Kenya (20 %) (Gakuya et al. 2001), and in *E. coli* isolated from the banded mongoose (*Mungos mungo*) in northern Botswana (40.9 %) (Pesapane et al. 2013). Since the real-time PCR approach targets the relatively short sequence of the gene in question, MICs or cultures with antibiotics are needed to confirm the presence of the resistance. In this context, our study about antibiotic resistance is of a preliminary nature. For the 2011 study, we could not apply MICs to the strains harboring the resistance genes for practical reasons. Therefore, the prevalence of resistance genes by PCR array in the 2011 study possibly led to overestimation of the presence of resistant bacteria. In fact, in our recent study in 2013, only a few (3 strains) highly resistant bacteria were isolated from 20 fecal samples of gorillas in the same area using a TS agar plate containing ampicillin, gentamicin, chloramphenicol, and tetracycline at 4 µg/ml (data not shown). Although with some methodological limitations, the 2011 study showed that the level of contamination, particularly in *E. coli*, was lower in wild gorillas than in human subjects. Moreover, little overlap was seen between human isolates and those from gorillas; only one strain (PG32) of *Klebsiella* sp. may have a relation with that of gorillas (strain G2). Accordingly, the level of human bacterial contamination of wildlife in MDNP seemed not to be higher than expected taking into account the history of the active use of the forest by a logging company until 1989.

We are now preparing for eco-tourism in this area in the near future. Occasional monitoring of antibiotic resistant bacteria in the feces of gorillas subjected to this sightseeing may help to measure the effects of tourism in this area. If the forthcoming prevalence of antibiotic-resistant bacteria is significantly increased from the current level, it may suggest that touristic activity would be too heavy (Wheeler et al. 2012). This may be true if we consult the previous study on wild baboons in Kenya (Rolland et al. 1985), which demonstrated the contamination of wild baboons by antibiotic-resistant bacteria due to access to the tourist lodge.

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