Research Article

Faecal Antibiotic Resistome of Nigerian Chimpanzees from a Wildlife Sanctuary in Cross-River State, Nigeria

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Abstract | Out of prescription, indiscriminate use, misuse and abuse of antibiotic administration and/or usage in both humans and farm animals have led to a consistent interference and contamination of biomes and ecobiomes. These resultantly give platforms for positive selection of resistant pathogens and high levels of Antibiotic Resistance Genes (ARGs). We examined Nigerian Chimpanzees in Boki Afi Mountain Wildlife Sanctuary, Cross-River State, Nigeria, to detect ARGs. Faecal samples from 15 Chimpanzees in pristine enclosures of Non-Human Primates in the Wildlife Sanctuary were analyzed. All faecal samples were pooled, then resuspended in phosphate-buffered saline. Subsequently, nucleic acid was extracted from the suspension and Illumina sequencing performed. ARGs in the raw reads were determined and assembled using the KmerResistance tool v2.2. From the 2,763,954 reads generated, 14 ARGs with statistically significant reads were identified. Precisely, 90.5% (12/14) of the ARGs detected target drugs that inhibit translation, of which 66.6% (8/12) were tetracycline resistance (TC-r) genes, while remaining 9.5% (2/14) inhibit cell wall synthesis (*cfxA3_1* and *cfxA6_1*). Eight (*aph(3')-III_1*, *cfxA3_1*, *cfxA6_1*, *erm(B)_10*, *tet(Q)_1*, *tet(Q)_2*, *tet(Q)_4*, *tet(W)_5*) of the ARGs detected were predicted to be plasmid-borne. We report using a cultivation-independent approach the presence of ARGs in Nigerian Chimpanzees. Findings suggest Nigerian Chimpanzees may constitute a hitherto overlooked source of antibiotic resistance in the environment. These ARGs may have been exchanged with handlers and rural dwellers around the Sanctuary. Surveillance of sympatric human faecal and environmental microbiota and their resistomes at the Wildlife Sanctuary are merited to inform public health interventions and decrease ARGs dissemination.

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Introduction

The environment, humans, and animals play a significant role in the epidemiology of antibiotic resistance genes (ARGs). Out of prescription, indiscriminate use, misuse and abuse of antibiotic administration and or usage in both humans and farm animals have led to a consistent interference and contamination of biomes and ecobiomes. These resultantly give platforms for positive selection of resistant pathogens and high levels of ARGs. Various studies have supported the view that antibiotics used in animal husbandry and the long-term use of antibiotics are the key element and determinants of the antibiotic resistance genes repertoire in the human gut microbiome (Forslund et al., 2013; Yongfei et al., 2014). We recently found 21 ARGs that target broad-spectrum antibiotics that inhibit translation, cell wall synthesis and nucleic acid synthesis in Pigs farmed on a small-scale piggery in Ibadan, south-west, Nigeria, suggesting that Swine industry in Nigeria might play a crucial role as reservoir of ARGs (unpublished). Exposure of wildlife living close to the contaminated environment to resistant bacteria and ARGs may in turn rapidly spread the ARGs.

Niger Delta; a region in South-south, Nigeria is home to over 150 Ecoregion and part of the Guinean Forest hotspot harbouring many locally and globally endangered Non-human primate (NHP) species including the Nigerian chimpanzee, Pan troglodytes *ellioti*. Morgan et al. (2011) reported that Nigerian chimpanzees are one of the most endangered subspecies with population estimated at 3,500-9,000. Subsequently, the subspecies has been included on the red list as one of the "threatened species" by the International Union for Conservation of Nature and Natural Resources (Humle et al., 2016). The largest population of Pan *troglodytes ellioti* are mostly found within National Parks such as Cross River National Park and Gashaka-Gumti National Park, and Wildlife Sanctuary such as Boki Afi Mountain (Ogunjemite et al., 2010).

Frequency of interaction between humans and primates has dramatically increased due to increased ecotourism, subsistence/slash and burn agriculture and industrial agriculture which could facilitate bacteria exchange. Meat from wild animals, including NHPs, serve as a source of protein and income (bush-meat trading) for most rural community dwellers in Africa (Wilkie, 2001; Fa et al., 2003). Glover (2014), while evaluating the enteric bacteria of monkeys with three levels of human contacts, observed that the closer monkeys were to humans, the more resistant were their enteric bacteria to antibiotics. Furthermore, Rolland et al. (1985) found a high proportion of antibiotic-resistant enteric bacteria in Yellow baboon (Papio *Cynocephalus*) population in the Amboseli National Park of Kenya that fed on human debris than those without human contact. Tsukayama et al. (2018) recently observed significant changes in the microbiota composition of baboons on exposure to human antibiotics. However, Costa et al. (2008) reported a high prevalence of tetracycline, streptomycin, and ampicillin resistance in *E. coli* isolated from wild animals that had not been previously treated with antibiotics in numerous Natural Parks of Portugal. In this study, we present data on the diversity of antibiotic resistance gene of a captive Non-human primate population, namely, Nigerian Chimpanzee, in the Boki Afi Mountain Wildlife Sanctuary. Our results demonstrate the preponderance of ARGs that target broad-spectrum antibiotics and that are likely plasmid-borne.

Materials and Methods

Sample collection

Faecal samples from 15 Nigerian Chimpanzees were analyzed in this study. The faecal samples were collected between June and July 2017 from the enclosures of NHPs in Boki Afi Mountain Wildlife Sanctuary in Cross-River State, Nigeria. Fresh faecal samples were collected before 10:00am in the morning. This is about the first sets night soil produced to give a good metabolic resource for the study. The samples

were collected in 15mL sterile centrifuge tubes and transported to the laboratory in an ice-chest. At the laboratory, it was stored at -20°C till the end of the sample collection period. Subsequently, samples were transported in cold chain to the Department of Virology, College of Medicine, University of Ibadan, Nigeria.

Sample processing for illumina sequencing

Precisely, one gram of each of the 15 samples was first resuspended in 3ml phosphate-buffered saline (PBS), and equal volumes (300µL) of the dilutions were pooled and briefly vortexed. Subsequently, nucleic acid was extracted from 100µL of the suspension using the Virus DNA/RNA extraction kit (Jena Biosceince, Jena, Germany) according to the manufacturer's instructions. This was then shipped to a commercial facility (MR DNA, Texas, USA) where library preparation and sequencing was done. The library was prepared using the Nextera DNA Sample Preparation Kit (Illumina) as recommended by the manufacturer. Subsequently, sequencing was done paired-end for 300 cycles using the HiSeq system (Illumina).

Bioinformatic analysis

Assessment of the quality of the raw reads was done using the FASTQC tool v1.0.4. Subsequently, the ARGs in the raw reads were determined and assembled using the Kmer Resistance tool v2.2 (https://cge.cbs.dtu.dk/services/KmerResistance/) which uses global alignment to assign the reads to redundant databases (Clausen et al., 2018). The identity threshold was set at 70% while the Depth threshold was set at 10%. The detected and assembled ARGs were then downloaded, visually screened, and those for which single contigs exceeded 200bp were further analyzed and submitted to GenBank under the accession numbers MT050494 to MT050500.

Results and Discussion

From the 2,763,954 reads generated, fourteen (14) ARGs with statistically significant reads were identified (Table 1). All the ARGs detected targeted broad-spectrum antibiotics, fell into three modes of action and target four classes of antimicrobials (Table 2).

Precisely, 90.5% (12/14) of the ARGs detected target drugs that inhibit translation. The remaining 9.5% (2/14) inhibit cell wall synthesis (cfxA3_1 and cfxA6_1). Estimates of how good the depth of the current template is compared to that found in host (Table 1; Depth corr >0.8) suggest that some (aph(3')-III_1, cfxA3_1, cfxA6_1,erm(B)_10, tet(Q)_1, tet(Q)_2, tet(Q)_4, tet(W)_5) of the ARGs detected are likely to be plasmid-borne (Clausen et al., 2018). Though the data supports the ermB gene being plasmid-borne, it is not clear whether the ermF gene (Table 1; Depth corr >0.7259) is too.

The complete coding sequence (CDS) of seven (cfxA3_1_AF472622, cfxA6_1_GQ342996, erm (B)_10_U86375, erm (F)_3_M17808, tet (Q)_1_ L33696, tet (Q)_4_Z21523 and tet (W)_5_ AJ427422) of the genes were recovered.

The apparent misuse and abuse of antimicrobial drugs in humans, veterinary and agricultural practices have resulted in the emergence and rapid spread of antimicrobial-resistant bacteria and genes, posing a significant public health threat. In this study using faecal samples collected from captive chimpanzee from South-South Nigeria, we were able to detect 14 ARGs targeting broad-spectrum antibiotics (Table 1). The existence of a shared pool of antibiotic resistance genes in bacteria from wildlife with no prior exposure to antimicrobials have been reported (Rolland et al., 1985; Costa et al., 2008), raising the question about the mechanism of emergence and rapid spread of antimicrobial-resistant bacteria and genes. Previous surveys of environmental, farm animal and human resistome have also observed the widespread antibiotic resistance gene in even the most remote location (D'Costa et al., 2011; Clemente et al., 2015; Pawlowski et al., 2016). Recently, Tsukayama et al. (2018) reported a shift in wild and captive Zambian baboon gut microbiota composition and resistome upon exposure to humans and their activities. In our case, other factors such as diet, habitat, social interaction and host species may be responsible for the observed ARGs (Tung et al., 2015; Ren et al., 2016).

Strikingly, 12 (90.5%) of the ARGs detected target drugs that inhibit translation. The remaining 2 (9.5%) inhibit cell wall synthesis (*cfxA3_1* and *cfxA6_1*). We also observed a relative abundance, 8 (57.1%) of tetracycline resistance (TC-r) genes in faeces of Nigerian Chimpanzees (Table 2). We identified the genes *tet*(32), *tet*(40), *tet*(Q), *tet*(W) and *tet*(O), with *tet*(Q) being the most prevalent. The predominant

Table 1: *Antibiotic Resistance Genes recovered from NHP faeces.*

S. No	Antibiotic resistance gene	Score	Expected	Template length	q_value	p_value	Template id	Template coverage	Query id	Query coverage	Depth	Depth corr
1	ant(6)-Ia_1_AF330699	1088	58	909	924.58	1.00E-26	77.12	77.45	99.57	129.12	1.29	0.5318
2	aph(3')-III_1_M26832	3195	50	795	3046.6	1.00E-26	98.24	98.99	99.24	101.02	4.18	0.9145
3	cfxA3_1_AF472622	13656	57	966	13483.68	1.00E-26	100	100	100	100	14.43	0.9998
4	cfxA6_1_GQ342996	22667	56	996	22498.52	1.00E-26	99.7	100	99.7	100	23.06	1
5	erm(B)_10_U86375	4712	46	738	4574.34	1.00E-26	100	100	100	100	6.51	0.9783
6	erm(F)_3_M17808	1760	51	801	1611.89	1.00E-26	85.02	85.02	100	117.62	2.2	0.7259
7	tet(32)_2_EF626943	3341	121	1920	2992.33	1.00E-26	51.15	51.56	99.19	193.94	1.81	0.6552
8	tet(40)_2_AM419751	2662	77	1221	2437.36	1.00E-26	81.08	82.56	98.21	121.13	2.26	0.7354
9	tet(O)_3_Y07780	1916	122	1920	1576.86	1.00E-26	45.68	45.83	99.66	218.18	1.01	0.448
10	tet(Q)_1_L33696	58118	84	1926	57864.91	1.00E-26	99.53	99.9	99.64	100.1	31.31	1
11	tet(Q)_2_X58717	9429	118	1926	9080.46	1.00E-26	75.13	76.43	98.3	130.84	5.12	0.9508
12	tet(Q)_4_Z21523	20133	110	1926	19803.2	1.00E-26	92.26	92.68	99.55	107.9	10.8	0.9983
13	tet(W)_5_AJ427422	18814	111	1920	18482.71	1.00E-26	100	100	100	100	9.95	0.9971
14	tet(O/32/O) 5_FP929050	4986	120	1920	4634.98	1.00E-26	76.61	78.02	98.2	128.17	2.71	0.7969

Antibiotic Resistance Gene: shows the name of the template sequences; Score: is the global alignment score of the template; Expected: is the expected alignment score if all mapping reads where smeared over all templates in the database; Template length: is the template length in nucleotides; q_value: is the quantile in a standard Pearson Chi-square test, to test whether the current template is a significant hit; p_value: is p-value corresponding to the obtained q-value; Template_id is the percent identity of the found template, over the full template length; Template_coverage is percent of the template that is covered by the query; Query_id is the percent identity between the query and template sequence, over the length of the matching query sequence; Query_coverage is the length of the matching query sequence divided by the template length; Depth: is the number of times the template has been covered by the query; Depth_Corr: is an Estimate of how good the depth of the current template is compared to the found host, a low value would point towards contamination, a value around 0.5 would indicate that the gene is located on the host genome and a value close to 1.0 would indicate that this template is plasmid borne.

Table 2: *Classification and Mechanism of action of the Antibiotic Resistance Genes detected during this study.*

S. No	Mechanism of action	Antibiotic resistance gene	Class of target drug	Mode of action
1	Inhibition of Translation	ant(6)-Ia_1_AF330699	Aminoglycoside	Aminoglycoside adenyltransferases
2	Inhibition of Translation	aph(3')-III_1_M26832	aminoglycoside	Aminoglycoside phosphotransferases
3	Inhibition of Translation	erm(B)_10_U86375	macrolide	Erm 23S rRNA methyltransferases
4	Inhibition of Translation	erm(F)_3_M17808	macrolide	Erm 23S rRNA methyltransferases
5	Inhibition of Translation	tet(32)_2_EF626943	Tetracycline	Tetracycline resistance ribosomal protection proteins
6	Inhibition of Translation	tet(40)_2_AM419751	Tetracycline	Tetracycline resistance ribosomal protection proteins
7	Inhibition of Translation	tet(O)_3_Y07780	Tetracycline	Tetracycline resistance ribosomal protection proteins
8	Inhibition of Translation	tet(Q)_1_L33696	Tetracycline	Tetracycline resistance ribosomal protection proteins
9	Inhibition of Translation	tet(Q)_2_X58717	Tetracycline	Tetracycline resistance ribosomal protection proteins
10	Inhibition of Translation	tet(Q)_4_Z21523	Tetracycline	Tetracycline resistance ribosomal protection proteins
11	Inhibition of Translation	tet(W)_5_AJ427422	Tetracycline	Tetracycline resistance ribosomal protection proteins
12	Inhibition of Translation	tet(O/32/O)_5_FP929050	Tetracycline	Tetracycline resistance ribosomal protection proteins
13	Inhibition of cell wall synthesis or disruption of membrane	cfxA3_1_AF472622	beta-Lactamase	Inhibition of cell wall synthesis or disruption of membrane
14	Inhibition of cell wall synthesis or disruption of membrane	cfxA6_1_GQ342996	beta-Lactamase	Inhibition of cell wall synthesis or disruption of membrane

mechanism of TC-r involves the use of active efflux pumps and ribosomal protection proteins (Thaker et al., 2010). This distribution of TC-r genes is consistent with the report from Jeters et al. (2009) who also found in a high proportion TC-r in samples from captive baboons, but less frequently in the wild baboons with tet(Q) genes equally found only in the captive baboons, not in the wild baboon. The TC-r genes described in this study may be a stable part of the genomes of normal Chimpanzee microflora (Mitchell et al., 2015). Erythromycin ribosome methylase (erm) genes were less frequently detected. The ermB and ermF genes were found in the captive chimpanzee samples. However, ermG gene was not detected in the sample. The erm genes encode ribosomal methylase that methylates specific adenine residues A2058/2059 in the peptidyl transferase region of 23S rRNA domain V and are responsible for macrolide antibiotic resistance (Weisblum, 1995; Tait-Kamradt et al., 2000).

There was a moderately high proportion of about 57% Plasmid borne ARGs detected, suggesting high genetic mobility within the faecal microbiome. The captive chimpanzees' faecal samples also contained class A broad-spectrum beta-lactamase commonly associated with plasmids found in human pathogens (Jolivet-Gougeon et al., 2004; Pehrsson et al., 2016). It is also possible that contact with handlers, tourist, and rural dwellers around the NHP sanctuary over time may have provided the opportunity for transfer of the ARGs between humans and Chimpanzees (Clayton et al., 2016)

Conclusions and Recommendations

Our resistome survey showed that chimpanzee contains genes encoding Aminoglycoside adenyltransferases, Aminoglycoside phosphotransferases, Erm 23S rRNA methyltransferases, Tetracycline resistance ribosomal protection proteins and class A broad-spectrum beta-lactamase commonly associated with plasmids found in human pathogens. Our results suggest that Nigerian Chimpanzees may constitute a hitherto overlooked source of antibiotic resistance in the environment. It is plausible that these ARGs might be exchanged with handlers and rural dwellers around the NHP sanctuary in recent times. Future studies to characterize sympatric human faecal and environmental microbiota and their resistomes at the NHP sanctuary are merited to inform public health interventions and decrease ARG dissemination. It is also desirable to explore other possible sources of ARGs other than drug in the food of Chimpanzees and wildlife generally.

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Novelty Statement

The study confirms prevalence of ARGs that target broad-spectrum antibiotics and are possibly plasmid-borne. This adds to information on nonhuman primate resistome research.

Author's Contribution

UEG, OAA, TOCF, OMA. and JAA conceptualized the research. UEG and AOO coordinated sample collection. OGO, OAA, UEG and TOCF were responsible for laboratory preparation of samples. TOCF and UEG analyzed the data and interpreted the results. OMA and JAA supervised the work. All authors were responsible for writing and reviewing the final manuscript.

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Ethics approval

Approval for the study was obtained from appropriate authority. However, it is pertinent to note that sampling was not invasive and was precisely done without contact with the NHPs.

Consent to participate

Not applicable

Consent for publication

All authors read the manuscript and consent to submission for publication.

Availability of data and material

Sequence data generated from the study have been submitted to GenBank under the accession numbers MT050494 to MT050500.

Code availability

Not applicable.

Conflict of interest

The authors have declared no conflict of interests.

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