Captivity and geography influence the antibiotic resistome of non-human primates

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Introduction: Antibiotic resistance poses a serious threat for animals and humans health worldwide. Yet a comprehensive exploration of the influence of captivity and geography on non-human primate (NPH) gut antibiotic resistance remains incomplete.

Methods: In this study, 131 metagenomic sequencing datasets of five species of NHPs included different regions and lifestyles were selected to perform the antibiotic resistance analysis.

Results: Nineteen related resistance antibiotics and 325 antibiotic resistance genes (ARGs) were obtained. A significantly higher abundance and diversity index of ARGs in the captive NHPs than in the wild was found but not for all of the samples. The biomarker-tracking of ARGs analysis identified key ARGs related to aminoglycoside resistance genes and tetracycline resistance genes.

Discussion: These results suggest that captivity and geography changes associated with human activities can lead to marked changes in the ecology of the NHP gut flora ARGs.

KEYWORDS antibiotic resistance genes, captivity, geography, metagenome, non-human primate fecal

Introduction

Antibiotic resistance genes (ARGs) existed before antibiotics were reported and did not pose a threat to humans at that time. However, the widely used antibiotics in clinical, aquaculture, animal husbandry, and planting accelerated the propagation and dissemination of ARGs (1). Nowadays, antibiotic resistance has been recognized as a vital global threat to human and public health by World Health Organization (2). The World Bank warned that antibiotic resistance would cause 10 million deaths and 100 trillion dollars losses in global economic by 2050 if unchecked (3, 4). European Antimicrobial Resistance Surveillance Network (http://www.ecdc.europa.eu/en/activities/surveillance/EARS-Net) and the US National Antimicrobial Resistance Monitoring System for Enteric Bacteria (http://www.cdc.gov/narms/) focus on the clinical and public dosage of antibiotics and the isolation of drug-resistant bacteria in public health laboratories, also illustrating that antibiotic resistance has been a wide concern (5). In almost all environments, sediment (6), surface water (7, 8), sewage (9–11), sludge (12), medical waste (13), and animal feces (14–16) serve as important reservoirs of ARGs that provide for reproduction and propagation conditions.
At the same time, ARGs can transmit by horizontal gene transfer (17–20) between pathogen or host (21) via mobile genetic elements (4, 22, 23), which will increase the difficulty of environmental governance and disease treatment. Thus, under the One-Health concept, effective measures should be researched to understand and control ARGs transmission.

Non-human primates, as close living relatives of human beings in the world, have a high degree of genetic and physiological similarity to humans (24). They are considered as the best model animal to explore the occurrence and development of human disease in medicine. However, due to the study of animals referred to ethical issues and part of the NHPs being endangered or protected animals, samples of them are hard to get. The high-throughput sequencing-based metagenomic analysis is a powerful tool that provided conveniences for rare animal samples microbiota analysis. Thus, systematic research on the resistome of NHPs by metagenomic analysis will provide us with the prevention of human diseases. Gut microbial communities, affected by several factors, including host lifestyle, host species, and geography (25), can generally shape the resistome (26, 27). Therefore, hypothesis that NHPs in wild might harbor more diverse ARGs and lower abundance than that in captivity were put forward since their surroundings were varied and they almost free from antibiotic selection pressure. Comparison of the gut resistome of NHPs in wild and in captivity can help us have a deep understanding of how the lifestyles influence the gut resistome. Besides, geography may also be an important factor that influences the ARGs abundance and diversity. To uncover these mysteries, five species of NHPs from different regions and lifestyles were included in our study.

Studies that looked at ARGs in gut microbiota of human or other animals have usually been performed at a local scale, which reduced the statistical power of the analyses. To break through this limitation, ARGs abundance and diversity of NHPs from a large scale were obtained. In this study, Macaca mulatta (M. mulatta) datasets sampled and sequenced in previous studies were included. Metagenomic datasets of NHPs worldwide including white-faced capuchins (Cebus capucinus), Macaca sp, Macaca fuscicularis (M. fuscicularis), and Rhinopithecus roxellana (R. roxellana) that held in the Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) were downloaded. Metagenomic data of white-faced capuchins, Macaca sp., M. mulatta and M. fuscicularis could be classified by the host, lifestyle, and geography and datasets for R. roxellana were classified into captive, semi-wild, and wild groups. Hence, this research aimed to (1) investigate the distribution of gut resistome from large-scale NHPs; (2) analyze the biomarker ARGs of captive NHPs by Linear discriminant analysis (LDA) effect size (LEfSe) algorithm; (3) speculate the influence of captivity and geography on the abundance and diversity of NHPs gut resistome.

Materials and methods

Dataset collection

A total of 131 metagenome datasets were included in this study. Among them, 34 M. mulatta (Guangxi) were from the datasets of samples collected in the early stage sequenced by Illumina X-ten strategies, and 19 white-faced capuchins (PRJNA485217, Costa Rica), 23 Macaca sp. (PRJNA478556, USA, Minneapolis), 17 M. fuscicularis (PRJEB22765, China, Beijing), and 38 R. roxellana (PRJNA456633, China, Hubei) were downloaded from the SRA database of National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/sra?term=) (1, 28). The sample ID, sequencing platform, reads length, reads number, data size, and accession numbers of the downloaded datasets were listed in Supplementary Table S1. The datasets of SRA format were converted to the fastq format by the fastq-dump module integrated in the NCBI SRA Toolkit (http://www.ncbi.nlm.nih.gov/Traces/sra/view5software) for subsequent analysis.

Bioinformatics analysis

The sequencing reads were aligned to a hierarchical structured Structured Antibiotic Resistance Genes (SARG) database (containing 24 ARG types and 1209 ARG subtypes) to characterize the resistance structure by the ARGs-OAP pipeline (version 2.0) (29). Specifically, 131 metagenomic datasets were searched against the integrated structured ARG database by the Usearch sequence analysis tool with the default parameter (25 aa, E-value of 1 × 10⁻⁷, identity of 80%). Potential ARG-like reads obtained from the Usarch results were searched against the integrated structured ARG database [ARDB (Antibiotic Resistance Genes Database), CARD (The Comprehensive Antibiotic Resistance Database), and NCBI-NR] by the Basic Local Alignment Search Tool to ensure accurate annotation. A sequence was annotated as an ARG-like read by meeting the following standards: an E-value cutoff of 1e-7, amino acid similarity of 80%, and minimum alignment length of 25 amino acids. The ARGs-OAP pipeline provided three standardized information including total read number, 16S rRNA gene copy number, and cell number. The abundance of ARG profiles of different sample groups was compared at the type level, the subtype level, and the gene level (the reference sequence level). Results of 16S rRNA gene copy number were selected for the following analysis.

Antibiotic resistance mechanisms analysis

The CARD database (30) (http://arpcard.mcmaster.ca) was downloaded to classified ARGs detected in the 131 NHPs gut
microbiota into different mechanism categories. We mapped the detected ARGs were mapped to CARD database to obtain the resistance mechanism of each ARG. Remarkably, the 15 unassigned subtypes were named ‘others’ in this study.

**Statistical analysis**

Principal Components Analysis (PCA) and heatmap analysis were performed by STAMP v2.0. Histogram and unpaired t-test analysis were performed by GraphPad v7.0. Alpha-diversity indexes were calculated to estimate microbial diversity between Macaca sp., M. mulatta, M. fascicularis, and white-faced capuchins groups. Differences in the relative abundance of the gut microbial ARG features were determined by LEfSe (http://huttenhower.sph.harvard.edu/lefse/).

**Results**

**The ARG diversity of Macaca sp., white-faced capuchins, M. mulatta, and M. fascicularis**

Except for goods_coverage index, all the other 7 indexes showed that white-faced capuchins had the highest index, followed by the wild M. mulatta (Figure 1). Macaca sp. in captive and M. fascicularis had the lowest index. This indicated that the diversity of ARGs in white-faced capuchins was the highest (Figures 1B,C). However, the goods_coverage indexes showed that M. fascicularis and Macaca sp. in captive had the highest index, and white-faced capuchins and M. mulatta in wild had a relatively low index (Figures 1D,E). The Shannon index, Simpson index, Pielou index, Chao index and ACE index of the M. mulatta were higher than the captive Macaca sp. (Figures 1B–D,F,G).

The **ARG profiles of white-faced capuchins, Macaca sp., M. fascicularis, and M. mulatta**

In the study, 19 ARG types were annotated from 131 samples by using the ARGs-OAP pipeline. Seventeen ARG types were identified in white-faced capuchins and R. roxellana (Supplementary Figure S1, Figures 2A,B). Sixteen ARG types were found in the wild M. mulatta and 12 ARG types were annotated in the captive Macaca sp. (Figure 3B). M. fascicularis was resistant to 15 ARG types (Figure 3B). The top 10 antibiotic resistance types of white-faced capuchins, Macaca sp., M. fascicularis, and M. mulatta were presented in Figures 4A–D, respectively. Tetracycline resistance genes had the highest relative abundance in 131 samples, followed by macrolides-lincosamids-streptogramins (MLS), multidrug, and beta-lactam. At the subtype level, 325 ARGs were annotated in 131 samples. Among them, 310 subtypes were classified into four mechanism categories based on the CARD database, of which 145 subtypes belonging to antibiotic inactivation, 110 subtypes to antibiotic efflux, 48 subtypes to antibiotic target alteration, and 7 to antibiotic target replacement (Supplementary Table S2). The top 10 ARGs were presented in Figures 4E–H. TetQ had the highest relative abundance in 131 samples, followed by tetW, multidrug_transporter, and CfxA2. The major ARGs of the 3 NHPs differed from one another.

**Influence of captivity on R. roxellana gut ARGs**

Thirty-eight datasets of R. roxellana were downloaded from the NCBI SRA database, including captive group, semi-wild group, and wild group. Seventeen ARG types were found in 38 samples, and the relative abundance of the top 10 ARG types were shown by a heat map (Figures 2A,B). The relative abundance of ARGs in the captive group was significantly higher than that in the semi-wild and wild groups (2.063 ± 0.025 vs. 0.002 ± 0.004, P < 0.0001, and 2.063 ± 0.025 vs. 0.0007 ± 0.001, P < 0.0001), consistent with the result of our datasets (Figure 2C). The relative abundance of ARGs in the captive group was up to 14-fold higher than that in the wild group. In the captive group, tetracycline was the primary ARG, with relative abundance ranging from 0 to 0.145, followed by 0–0.030 in semi-wild and 0–0.007 in wild. In semi-wild and wild R. roxellana, vancomycin, bacitracin, and multidrug had high relative abundance (Figures 2A,B). The PCA analysis confirmed that the three lifestyles clustered separately (Figure 2D). The similar result was seen in the heatmap (Supplementary Figure S3). Diversity analysis found that apart from Pielou index, the diversity index of the captive R. roxellana and semi-wild were higher than the wild R. roxellana (Supplementary Figure S2). Besides, the marker-ARG of captive R. roxellana were tetracycline resistance genes, tetW, tetQ, tetracycline resistance protein, and tetO (Supplementary Figures S4, S5).

**Influence of geographic location on Macaca sp., M. fascicularis, and M. mulatta gut ARGs**

Macaca spp. from three places were included to explore whether ARGs were associated with geographic factors (the wild M. mulatta from Hezhou, Guangxi, the captive M.
From Beijing, China, and the captive Macaca sp. from Minneapolis, USA. At last, 17 ARG types were annotated. The dominant antibiotic resistance of M. mulatta and M. fascicularis in China were tetracycline, which accounted for 65.71% and 81.27%, respectively. The primary ARG abundance in captive Macaca sp. from USA was beta-lactam, accounting for 56.99% (Figures 3A, B). The total relative abundance of ARGs in captive Macaca spp. was significantly higher than the wild (unpaired t-test, P < 0.0001) (Figure 3C), consistent with the result above. PCA analysis was used to evaluate the similarities of the compositions of ARG types in the three groups of Macaca spp. (Figure 3D). The samples from the same place clustered more closely, and M. fascicularis from China and Macaca sp. from USA formed different clusters. Additionally, the captive Macaca sp. from the USA was different from those in China, reflecting that the diversity of ARG type was also related to host geography.

Identification of discriminative ARG relative to captivity and geography

LEfSe is an algorithm for high-dimensional biomarker discovery and explanation that identifies genomic features
Comparison of *R. roxellana* gut microbiota ARGs between captive, semi-wild, wild.

(A) Clustering heat map of antibiotics with high relative abundance in samples.

(B) Relative abundance of ARG types of *R. roxellana*.

(C) Barplot of total ARG relative abundance of *R. roxellana*.

(D) PCA plot of ARG relative abundance of *R. roxellana*. 

**FIGURE 2**

Comparison of *R. roxellana* gut microbiota ARGs between captive, semi-wild, wild. (A) Clustering heat map of antibiotics with high relative abundance in samples. (B) Relative abundance of ARG types of *R. roxellana*. (C) Barplot of total ARG relative abundance of *R. roxellana* (t-test, **** *P* < 0.0001). (D) PCA plot of ARG relative abundance of *R. roxellana*. 

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Comparison of Macaca spp. gut microbiota ARGs from three regions. (A) Heat map clustering 17 antibiotics of 74 samples. (B) Relative abundance of 17 antibiotics of 74 samples. (C) Barplot of total ARG relative abundance of 74 samples (t-test, ****P < 0.0001). (D) PCA plot of ARG relative abundance of 74 samples.
Non-human primates are closely genetically related to humans and share many cognitive, physiological and morphological similarities with humans (31). In this study, a total of 131 NHPs were selected, including *Macaca* sp., *M. mulatta*, *M. fascicularis*, white-faced capuchins, and *R. roxellana*, to study the impact of captivity and geographic location on the distribution of the NHPs gut resistome. Researches noted that there were 19 related resistance antibiotics and 325 ARGs in the fecal microbiota of 131 NHPs. Study reveals that both the *M. mulatta* in captive and wild were resistant to tetracycline and with higher abundance compared to tetracycline resistance genes *tetQ*, *tetW*, and *tet37* were enriched in wild *M. mulatta* (Figures 5, 6).

**Discussion**

Non-human primates are closely genetically related to humans and share many cognitive, physiological and morphological similarities with humans (31). In this study, a total of 131 NHPs were selected, including *Macaca* sp., *M. mulatta*, *M. fascicularis*, white-faced capuchins, and *R. roxellana*, to study the impact of captivity and geographic location on the distribution of the NHPs gut resistome. Researches noted that there were 19 related resistance antibiotics and 325 ARGs in the fecal microbiota of 131 NHPs. Study reveals that both the *M. mulatta* in captive and wild were resistant to tetracycline and with higher abundance compared
Cladogram of the significantly different ARGs between white-faced capuchins, Macaca sp. (captive), M. mulatta (wild) and M. fascicularis samples. The inner circles are at the ARG type level, while the outer circles are at the subtype level.

with other groups. The major resistance of M. fascicularis was tetracycline, too. Tremendous tetracycline resistance genes were found in the above three kinds of NHPs, liking tetQ, tetW, tetO, and tet40. Yang et al. (32) uncovered that the most abundant ARGs of the gut microbiota of the M. mulatta were tetracycline resistance genes and verified by isolation and culture experiments (32). This was also consistent with this study. Aminoglycosides, tetracyclines, and macrolides are the most widely used antibiotics in veterinary medicine worldwide for animal growth promotion and disease control (33, 34). Especially, tetracyclines are broad-spectrum antibiotics and popular first-line antibiotics in human infectious disease treatment (25). The habitat of M. mulatta Gupo Mountain in Hezhou is a tourist attraction that produces a lot of human activities. Although tetracycline antibiotics were not used in the wild M. mulatta, long-term human activities and widespread use of tetracycline antibiotics in other species have caused an extensive global tetracycline resistance (31, 35, 36). It may be explained the prevalence of tetracycline resistance genes in NPHs. Besides, this study found that 145 subtypes belong to
FIGURE 6
LDA score distribution of the discriminative ARGs between white-faced capuchins, *Macaca* sp. (captive), *M. mulatta* (wild) and *M. fascicularis* fecal microbiomes determined by LEfSe analysis. The red rectangle, green rectangle, blue rectangle, purple rectangle represent white-faced capuchins, *Macaca* sp. (captive), *M. fascicularis* samples, and *M. mulatta* (wild), respectively, of which ARGs’ LDA score are more than 2.
antibiotic inactivation. Similar results have been reported that antibiotic inactivation was the dominant resistance mechanism in swine and humans (37).

The effect of captivity on intestinal microbial resistance in animals is still unclear. Previous researches show that captivity influences the gut resistome structure by altering the gut microbiota composition. Thus, comparison with wild animals, captive breeding may affect the diversity/abundance of resistant genes by affecting their own intestinal microbial structure in a variety of ways, such as dietary changes, drug treatment, and reducing contact with other individuals, species, and variable environmental substrates as a source of bacterial diversity (16, 38, 39). Previous research also reveals that semi-captive wildlife might harbor a higher diversity of antimicrobial-resistant genes (40). Research on captive and wild baboon gut microbiota and their resistome found that exposure to humans is associated with changes in gut microbiota composition and resistome expansion compared (15). Rolland et al. (41) found that compared with baboons that do not contact humans, baboons that directly contact humans can detect a higher level of antibiotic resistance (41). In these results, both white-faced capuchins and M. mulatta in wild had high diversity index. Further analysis of R. roxellana found that captive R. roxellana had significantly higher diversity and abundance than wild and semi-wild. Therefore, diversity index analysis can not tell the direct relationship between captive and wild NHPs. It is certain that lifestyle is an important factor that influences the ARGs diversity of NHPs. Besides, a comparison of M. mulatta in China and the USA revealed that ARG was also influenced by geography but to a lesser extent. Consequently, a concluded that gut resistome of NHPs were more substantially affected by the captive environment than geography or host.

Compared with the gut microbiota ARGs of M. mulatta and M. fascicularis, the diversity of gut microbiota ARGs of white-faced capuchins was the highest. Various ARGs of white-faced capuchins that were significantly different from M. mulatta and M. fascicularis in this study, indicating that the ARGs may have spread to the habitats of white-faced capuchins. Strangely, tetracycline resistance genes tetQ, tetW and tet37 were found in the wild gut microbiota of M. mulatta. The spread of ARGs in wild NHPs is more worrisome because it may lead to a large-scale environment polluted that increasingly animal antibiotic-resistant infections cannot be treated including endangered animals. Research showed that the frequent use of antibiotics has led to antibiotic selection pressure causing the prevalence of antibiotic-resistant bacteria (42). Therefore, antibiotics must be used reasonably to reduce the selection pressure. Measures should be taken to reduce resistance development and spreading of resistant bacteria. Under the one-health approach, a holistic perspective on antibiotic resistance, including humans, animals, and the external environment needs to take on Bengtsson-Palme et al. (43). Furthermore, models should be built to demonstrate and predict how resistance emerges and disseminates by increasing knowledge of influence factors and the mechanism of transmission of drive resistance. All in all, these results provide references for NHPs’ gut resistome studies.

Conclusion

The study described a comprehensive R. roxellana, M. mulatta, Macaca sp. M. fascicularis, and white-faced capuchins gut resistance gene catalog and revealed the abundance and diversity of ARGs of them. The total ARGs relative abundance in the captive animal was higher than in the wild and tetracycline was the predominant resistance in both captive and wild NHPs. Captivity was considered to be the primary influence factor of host gut microbiota ARGs abundance and diversity. This study helps to improve the knowledge and understanding of NHPs’ gut flora ARGs and strengthen the management of antibiotics and the prevention of human diseases.

Data availability statement

Publicly available datasets were analyzed in this study. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Author contributions

HH designed and constructed the manuscript, downloaded and analyzed of the datasets, generated the major figures and tables, and completed the writing.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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