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SHORT COMMUNICATION

Bacterial isolation of oral, rectum and anus swabs from *Macaca fascicularis* and *Macaca namestrina* in Kemasul, Pahang, Malaysia

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ABSTRACT

This study was conducted with the aim of isolating and identifying pathogenic bacterial communities from actively shedding anatomical sites of *Macaca fascicularis* and *M. namestrina* in Jambu Rias (JR) and Chemomoi (CM) in Kemasul Forest Reserve, Pahang and to determine the antibiotic susceptibility of these isolates. The findings show that *M. fascicularis* had higher bacterial density and ten different isolates were identified from these samples. The antibiotic susceptibility tests determined that ciproflaxin and vancomycin as most effective antibiotic towards these isolates.

Keywords: Macaques, bacteria isolation, antibiotic, susceptibility, zoonotic diseases

INTRODUCTION

Human and non-human primates (NHP) are close evolutionary relatives that are economically and ecologically interconnected in many parts of the world (Fuentes and Hockings, 2010). The long-tailed or crab-eating macaque, *Macaca fascicularis*, is widely spread throughout the mainland of Southeast Asia. This species has been listed as a "Least Concern Species" by International Union for Conservation of Nature (IUCN) and thus, considered to be one of the most abundant NHP species within its native range (Umapathy *et al.*, 2000). *Macaca namestrina*, also known as the pig-tailed macaque, is also one of the essential and abundant NHP species, especially in the biomedical field, providing a valuable model for human diseases, as well as a model for primate evolutionary process. Contrary to *M. fascicularis*, *M. namestrina* is listed as vulnerable under the Red List of Threatened Species.

The populations of *M. namestrina* and *M. fascicularis* are believed to be rapidly declining in many areas due to habitat loss, forest degradation, conflict with humans and trapping for commercial trade. The continuous and extensive conversion of tropical rainforests is widely believed to be a fundamental threat to the survival of wild populations of terrestrial and arboreal animals, including arboreal NHPs such as these two-macaque species. In addition, the management authorities in the areas with human and macaque conflict often regard them as an expendable pests species with little ecological or conservation value (Foden *et al.*, 2008). Populations of *M. namestrina* and *M. fascicularis* living in proximity to humans are more prevalent due to the natural habitat shrinkage, thus increasing the potential for interface with humans and often resulting in conflict situations. As the macaques live near humans, the inevitable consumption of human food waste by the macaques would lead to infections by a variety of bacteria from the wastes (Albert *et al.*, 2013). These infected macaques would harbor infectious agents and would eventually pose a risk of transmitting zoonotic diseases to human populations where human-macaque conflicts occur.

There have been no reported studies conducted on the identification of bacteria that are found in actively shedding anatomical sites such as oral, rectum and anus of *M. fascicularis* and *M. namestrina* especially in Peninsular Malaysia, thus this study is essential to enlighten the potential bacterial agents harboured by these species. These actively shedding anatomical sites were chosen as they were in direct contact with the environment and can harbour pathogenic bacteria and may lead to the pathogenesis of zoonotic disease. It is important to understand the microbial agents carried by these species due to the close-relationship between

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humans and macaques, geographical ranges and habits of human feeding the wild macaques. This research is fundamental to sites of *M. fascicularis* and *M. namestrina*, as well as the bacterial determine the type of bacteria found in different anatomical resistance towards common antibiotics. Further understanding regarding the identified bacteria can then be used to determine whether they can be transmitted to humans and cause any significant harm due to the possibility of zoonosis.

MATERIALS AND METHODS

Study areas

This study was conducted within the Kemasul Forest Reserve, located in the state of Pahang. This forest has undergone forest plantation program apart from intense agricultural activities, leaving several patches of forest fragments scattered in the forest. Two different sites within a long stretch of the forest fragment were chosen, namely Jambu Rias (03°27'18.3" N, 102° 08' 17.5" E) and Chemomoi area (03°19'34.2" N, 102° 15'01.6" E). Jambu Rias were surrounded with oil palm plantation, whereby Chemomoi were surrounded with acacia plantation.

Sampling of macaques

The field sampling was conducted for 20 days in both study sites, within four months between August and November 2015 where six trapping lines of 50 m long were situated at different locations in each forest. The macaques were captured using 30 units of large wire-mesh trap (38 cm × 38 cm × 106 cm), baited with jackfruits. The captured macaques were euthanised using 0.5-1 mL of Zoletil 100, prior to obtaining the samples. Triplicate swab samples from different anatomic areas (rectum, oral and anus) of each macaque were collected by using sterile swabs and then stored in vials. These vials were then kept in liquid nitrogen for preservation and further analysis was conducted at the Environmental Microbiology Laboratory of Universiti Kebangsaan Malaysia (UKM).

Ethical note

All scientific procedures conducted were approved by Universiti Kebangsaan Animal Ethical Committee under the reference number (FST/2016/SHUKOR/18-MAY/750-MAY-2016-SEPT.-2018-AR-CATS).

Colony Forming Unit (CFU/mL)

The swab samples were proceeded with serial dilution (10^{-2}, 10^{-4}, 10^{-6} and 10^{-8}) and 100 µL of the diluted mixtures were then spread unto nutrient agar and incubated at 37 °C for 24 h. The CFU/mL was determined using the standard CFU/mL formula. Each experiment was conducted in triplicate.

Identification of bacteria

Pure cultures of isolated bacteria were characterised using Gram stain. Further phenotypic identification was performed based on morphology and biochemical tests on the isolated bacteria based on Bergey's Manual of Determinative Bacteriology. Biochemical testing that were used for bacterial characterisation were Indole, Voges Proskauer's, Methyl Red, Catalase, Citrate Utilisation, Oxidase Triple Sugar Iron tests. 16S rRNA identification was also carried out to identify isolates to genus level. DNA was isolated with the PrestoTM Mini gDNA Bacteria Kit and the PCR reaction was conducted with specific primers for bacteria, E8F (5'-AGAGTTGATCCTGGCTCAG-3') and U1510R (5'-GGTTACCTTGTTACGACTT-3'). The PCR mix was made up to a total of 10 µL and this consisted of 5 µL GoTaq Green (Promega), 0.5 µL each upstream and downstream primers, 1 µL DNA template and 3 µL ddH_2O. The polymerase reaction was performed over 30 cycles using the following protocol; pre-denaturation (95 °C, 2 min), denaturation (92 °C, 30 sec), annealing (55 °C, 30 sec), elongation (72 °C, 1 min), and post elongation (72 °C, 5 min). The amplified DNA then was electrophoresed at 80 V for 30 min. The sequenced samples were Blasted using NCBI and phylogenetic trees were generated using MEGA6 software.

Antibiotic susceptibility test

The test was performed via the disc-diffusion method, where the antibiotics that were used were Ampicillin, Penicillin G, Streptomycin, Kanamycin, Polymyxin B, Ciprofloxacin, Neomycin N-5, and Vancomycin. The pure isolates were enriched in nutrient broth and were incubated at 37 °C for 24 h. The culture from each pure isolate was swabbed evenly onto the entire surface of Mueller-Hinton agar plate by using a sterile cotton bud for antibiotic susceptibility test. Eight small filter paper disks of different types of antimicrobial agents were placed by using sterile forceps on the agar surface before incubation at 37 °C for 24 h. The diameter of inhibition zones was measured to determine the resistance and susceptibility of the bacteria towards these antibiotics. DMSO was used as a negative control in this experiment.

RESULTS AND DISCUSSION

The growth of bacteria colony was generally higher in *M. fascicularis* at both Jambu Rias and Chemomoi. The highest number of the bacterial colony for *M. fascicularis* was from rectum swabs of macaques located in Jambu Rias with 5.3×10^6 CFU/mL. On the other hand, bacterial density was higher in oral samples of *M. namestrina* (2.9×10^6 CFU/mL) from Jambu Rias, compared to rectal samples in Chemomoi (1.9×10^6 CFU/mL). Bacterial growth from anus was generally low in both macaques species at both sites (Table 1). Jambu Rias generally exhibited more disturbed habitat due to the dominance of oil palm plantation compared with Chemomoi which was

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surrounded by acacia plantation. *M. fascicularis* showed higher density of microbes in all sites tested compared to *M namestrina*. This may be due to this species living a closer proximity to human settlements and live most successfully in disturbed habitats (Karimullah and Anuar, 2012). The interaction of animal-microbiome, specifically their role in the biological cycle is out of our expectation. Organisms such as bacteria can be found to coexist with other organisms in large neighbourhoods in a more complexed and diverse ecosystems.

Table 1: Total CFU /mL of bacteria isolated in different anatomical sites from 3 individuals of *Macaque* sp.

Sites	Macaque spesies	Location of anatomical sites		
		Oral	Rectum	Anus (PV)
		No. of CFU count (CFU/mL ±sd)	No. of CFU Count (CFU/mL±sd)	No. of CFU Count (CFU/mL±sd)
Jambu Rias	*Macaca fascicularis*	$4.8\times10^6\pm$ 0.45	5.3×10^6 ±0.29	4.6×10^6 ±0.34
	Macaca namestrina	$2.9\times10^6\pm$ 0.36	2.4×10^6 ±0.47	2.4×10^6 ±0.28
Chemomoi	*Macaca fascicularis*	$3.3\times10^6\pm$ 0.41	2.8×10^6 ±0.38	3.3×10^6 ±0.44
	Macaca namestrina	$1.5\times10^6\pm$ 0.38	1.9×10^6 ±0.31	1.1×10^6 ±0.28

Higher diversity of bacteria was isolated from various anatomical sites of *M. fascicularis* with 4 species from both oral and rectum sites and 3 species from anus site at both Jambu Rias and Chemomoi areas. *Macaca nemestrina* on the other hand only recorded 1 species from oral site, 2 species from anus, but no bacteria was successfully isolated from rectum site (Table 2). Bacteria isolated from the oral sample of *M. fascicularis* and *M. namestrina* identified with biochemical test, namely *Staphylococcus aureus*, *Staphylococcus* sp. and *Clostridium* sp. *Staphylococcus aureus* is known as a component of the normal flora and is generally found in respiratory tracts, on the skin and nose. However, it is the most important pathogen of human diseases causing infections such as gastrointestinal diseases, meningitis, skin infections, endocarditis and pneumonia. The transmission between human and primates has been documented and the infection of non-human primates has been detected in the natural environment (Schaumburg *et al.*, 2012). In Africa, *S. aureus* has been found in wild monkeys showing a newly high divergent isolation with a new species. Other studies in Zambia, Uganda and Gabon, found greater transmission between humans and non-primates (Schaumburg *et al.*, 2012). The possibilities of direct contact through the skin, human secretion or faeces in soil or water could enhance the efficacy of the transmission. *Clostridium* sp. is the most versatile bacteria as it was found in different anatomical sites from oral, rectum and anus in both macaque species in both study sites. It also exists as a normal flora located in the intestinal tract of human and animals. In terms of zoonosis, there was no evidence showing that *Clostridium* sp. can be transmitted directly from animals to humans. *Clostridium* sp. can be transmitted by contamination of wound sites and they breach the gastrointestinal tract whereby resulting in spontaneous infections (Chipp *et al.*, 2009).

Neisseria sp. were isolated from *M. fasicularis* of Jambu Rias whilst *Corynebacterium kutscheri* were isolated from *M. fasicularis* of Chemomoi. *Corynebacterium sp.* is a part of the normal flora, with low pathogenicity*. Corynebacterium* and *Neisseria* which usually inhabit the skin has been known to cause animal diseases (Vela *et al.*, 2006). Rayan *et al*. (1987) have isolated about 19.5% of *Neisseria* sp. from tongues of healthy rhesus monkeys. The infection by *Neisseria* sp. can also be transferred to other organisms over time. Disease transmission between humans and monkeys were rarely reported. However, recently, Vecten *et al.* (2017) reported that a 65-year-old patient was infected with *N. macacae* endocarditis with complicated aortic valve and peri-aortic abscess. *Salmonella* sp. on the other hand are pathogenic bacteria and also a zoonotic bacterium of public health concern, particularly in the food-borne disease transmission (Botti *et al.*, 2013). In this study, *Salmonella* sp. was isolated from *M. fascicularis* species of Jambu Rias. Therefore, this study suggests the potential transmission of this bacterium from contaminated foods or the environment due to anthropogenic factors. The behaviour and feeding habits of wild animals influence the likelihood of *Salmonella* infection. Macaques could acquire this bacterium by scavenging on contaminated human leftover or through surface water runoff.

Selected isolates have been identified using PCR-based method of 16S rDNA profiling. Isolate 09-T-1 (Mf-JR-oral) was identified as *Bacillus aryabhattai* with similarity level 100%, isolate 03-T-3 (Mn-CM-oral) was *Acinetobacter schindleri* with similarity 100% and isolate 02-PV-1 (Mn-JR-Anus) was *Bacillus cereus* with similarity level 99%. *B. aryabhattai* is a rhizobacterium promoting plant growth and naturally occurring bacteria associated with plant roots (Park *et al.*, 2017). *Bacillus cereus* is broadening in nature and often isolated from soil and growing plants, but it is also well adapted for growth in the intestinal tract with their ability to grow in foods and cause diseases in mammals and insects. Wijnands (2008) reported that macaques can directly be infected by *B. cereus* through soil and plants since those bacteria are commonly found in soil and the environment. *Acinetobacter* spp. can be part of the human flora. However *A. schindleri* is an opportunistic bacterium which is responsible for nosocomial infection and outbreaks (Montaña *et al*., 2018).

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Table 2: Biochemical tests for oral, rectum and anus swabs of *Macaca fascicularis* and *Macaca namestrina* at different study sites.

Anatomical sites	Sample Code	Sample source	Site	Gram staining	Microscopic	Morphology	Spore	Anaerobic	MCA	EMB	Oxidase	Catalase	MSA	Glucose	Starch	SIM	Citrate	Bacteria species
Oral	03-O-3	M*f*	JR	-	C	W			+	+/DB	+	+						-
	13-O-1	M*f*	JR	+	C	Y						+	+/Y	+				*S.aureus*
	18-O-2	M*f*	CH	+	C	Y						+	+/Y	+				*S.aureus*
	30-O-3	M*n*	CH	+	C	W						+	+/Y	+				*Staphylococcus* sp.
	08-O-1	M*f*	JR	+	R	W	+	+			-	+		-	-			*Clostridium* sp.
	09-O-1	M*f*	JR	+	R	W	-	+			-	+		-	+			-
	29-O-1	M*f*	CH	+	R	W	+	+			-	+		-	+			*Clostridium* sp.
Rectum	03-R-2	M*f*	JR	-	R	Y			+/Y/C	+/P	-	+						*Neiserria* sp.
	13-R-1	M*f*	JR	+	C	W						+	+/Y	+				*S.aureus*
	09-R-1	M*f*	JR	+	C	Y						+	+/Y	+				*S.aureus*
	09-R-2	M*f*	JR	+	R	W	+	+			-	+	+/Y	+				*Staphylococcus* sp.
	16-R-2	M*f*	CH	+	R	W	+	+			-	+		-	-			*Clostridium* sp.
	18-R-1	M*f*	CH	+	R	W	+	+			-	+		-	+			*Corynebaterium kutscheri*
Anus	29-A-2	M*f*	JR	-	R	W			+/Y/C	+/P	-	+				M:+ I:- S:R B:R	+	*Salmonella* sp.
	09-A-2	M*f*	JR	-	R	W			+/Y/C	+/P	+	+				M:+ I:- S:R B:Y	-	*Pseudomonas* sp.
	13-A-2	M*f*	JR	+	C	W						+	+/Y	+				*S. aureus*
	30-A-2	M*n*	CH	+	C	Y						+	+/Y	+				*S. aureus*
	02-A-1	M*n*	CH	+	R	W	+	+			-	+		-	+			-
	29-A-1	M*f*	CH	+	R	W	-	+			-	+		-	+			*Corynebacterium kutsceri*
	42-A-1	M*n*	CH	+	R	W	+	+			-	+		-	-			*Clostridium* sp.

R=Rod
C= coccus
+= Possitive
-= Negative

DB= Dark Blue Green
P= Pink Colony
Y=Yellowish colony
W=White colony

M=Motality Test
I= Indole Test
S=Slant
B=Butt

M*f* = *M. fascicularis*
M*n* = *M.Namestrina*
JR= Jambu rias
CH=Chemomoi

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In terms of biosafety, the high resistance level of penicillin G, ampicillin and polymyxin B were found not to be the ideal drug of choice to treat bacterial infections for most of the bacteria isolated from this study (Table 3). It may be an outcome of abusive usage of antimicrobial agents in an agricultural area as stated by Okwori *et al*. (2011). In contrast, various bacteria in this study are susceptible to ciproflavin and vancomycin indicating their suitability in treating these infections. Antimicrobial-resistant bacterial isolates originating from wildlife species were described for the first time from Japanese wild birds (Sato *et al*., 1978). In different continents, the occurrence of antimicrobial-resistance in bacterial species from wildlife has been widely reported mainly for *Salmonella* sp. and *Staphylococcus aureus*. According to Molina-Lopez *et al*. (2011), resistance towards antimicrobial agents in *Salmonella* sp. is increasing due to the widespread use of antimicrobial agents. Recently, *Cornybacterium* sp. resistance to penicillins, erythromycins, and clindamycin has been reported (Reddy *et al*., 2012). The uncontrolled usage of antibiotics also enhances the mechanism of resistance in new drugs within a short period. The occurrence of drug resistance by *S. aureus* becomes more crucial with the ability of this bacterium to change their host species, resulting in new adaptations in a fresh environment whereby resulting in a wider spread in host (Shepheard *et al*., 2013).

CONCLUSION

Macaques intrusion in human settlements could increase pathogen transmission in zoonotic diseases. This study provides information on multiple bacteria located in different actively shedding anatomical sites of *M. fascicularis* and *M. nemestrina* in Kemasul Forest Reserve, Pahang, with a high influence of anthropogenic activities. No resistance was reported on ciproflaxin and vancomycin antibiotics, whilst a high resistance in penicilin G and ampicilin was observed in most of the isolated bacteria. Furthermore, all the bacteria reported may lead to zoonosis due to the close phylogenetic traits in human and non-human primates, thus, measures must be undertaken to prevent a global outbreak.

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