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Identification and Antibiotic Resistance Profile of Bacteria From Fruit Bat (*Chironax melanocephalus*)

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ABSTRACT
Antibiotic resistance is one of the most serious health issues in the world which is commonly attributed to the overuse of antibiotics whether in human or animal treatment. The dispersion of antibiotic resistance genes not only found in bacteria from the clinical sample but also found in wildlife animals. Therefore, the aim of this research is to isolate, identify and perform antibiotic resistance assay in bacterial isolates from fruit bat *Chironax melanocephalus*. Animal samples were taken by using a mist net installed in one of the spots in Gunung Halimun Salak National Park (TNGHS). Bacteria sample was taken using an oral and rectal swab. The bacteria were then isolated in the laboratory by using xylose lactose deoxycholate (XLD) agar medium. There were two bacteria successfully isolated and identified using 16s rDNA amplification. The identification was performed by comparing the 16s rDNA genes to the two online databases *i.e* BLAST-N in Genebank and 16s-Based ID in the EzBioCloud database. According to the identification results, isolate 4A.1 was identified as *Proteus mirabilis* while isolate 5.B.2 is identified as *Serratia marcescens*. Then antibiotic susceptibility test was performed using the disk diffusion method using four different antibiotics disk i.e oxacillin, cefoxitin, amikacin and amoxicillin-clavulanic acid. The resistance profile of each bacteria is different between *P. mirabilis* and *S. marcescens* in each antibiotic. However, all isolates were found susceptible against amikacin 30 µg.

Keywords: *Antibiotic resistance, identification, bacteria, wildlife*

1. INTRODUCTION

Antibiotic resistance is one of the major health problems attributed to the overuse and misuse of antibiotics, whether in human or animal treatment. These antimicrobial-resistant bacteria spread not only around the clinical isolates but also found in the natural environment. The mechanisms of the dissemination of antibiotic resistance among bacteria in the natural environment are still not clearly understood. Several studies reported that bacteria isolated from areas which anthropogenically affected and less touched areas have different antibiotic-resistant patterns [1], [2].

Fruit bat (*Chironax melanocephalus*) is one of the wild animals which considered as reservoir of zoonotic pathogen and multi-drug resistant microorganisms. Previous studies reported that bat as reservoir for common pathogenic bacteria such as enteric bacteria (e.g. *Shigella, Salmonella, Campylobacter and Yersinia* spp.), arthropod-borne bacterial pathogen (*Borrelia* spp., *Bartonella* and several Rickettsiales), and pathogenic *Leptospira* species [3]. Several studies also reported bats harboring antibiotic-resistant bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella*, and *Escherichia coli* that resistant to erythromycin and streptomycin [4], [5].

The researches on antimicrobial resistance in Indonesia especially related to wildlife are very limited. Previous research was performed in 1988 and found several enteric pathogens such as *E. coli*, *Klebsiella*, and *Enterobacter* has antibiotic resistance to sulphamethoxazole, cephalothin, ampicillin, gentamycin, and chloramphenicol [6]. The further researches were more focused on the livestock-related animal such as cattle and broiler chicken [7], [8], [9]. Therefore, the aim of this study is to isolate the bacteria from fruit bat *Chironax melanocephalus*

in Gunung Halimun Salak National Park (TNGHS) and analyze the antibiotic resistance profile of the bacterial isolates. This study is a preliminary study about antibiotic resistance surveillance among wild animals.

2. MATERIALS AND METHODS

2.1. Samples Collection

Two animal samples were taken from Cikaniki area, in Gunung Halimun Salak National Park (TNGHS) using a mist net. The net was installed 48 hours and regularly checked. The bacterial isolates were obtained by using the swab method (oral and rectal swab) and stored in Amies transport medium agar (Citotest-CN) for further processing in the laboratory. Bacterial isolation was performed by streaking the cotton swab in the semi selective medium xylose lactose deoxycholate (XLD) agar medium (Himedia Lab-IN) and incubated at 28°C for 48 hours. The different and dominant colonies of bacteria were taken and purified in a similar medium then identified by the molecular method through PCR amplification of 16S rDNA.

2.2. Identification of Bacteria

The total genomic DNA of the bacterial isolates was extracted using the boiling method at 90°C for 15 minutes and spun down for 5 minutes. The crude DNA then used as a template for PCR amplification using 16S universal primer 27F/ 1492R [10]. PCR product then sequenced and analyzed using ChromasPro (Technelysium- AU) for the sequence assembly then compared in two online databases for the type strain *i.e* BLAST-N in Genebank [11] and 16S-Based ID in EzBioCloud [12]. The phylogenetic tree was generated using the neighbor-joining method with 1000x bootstrap in MEGA 7.0 [13].

2.3. Antibiotic Resistance Assay

The disk infusion method was used to perform antibiotic resistance assay which according to the protocol from European Committee on Antimicrobial Susceptibility Testing (EUCAST). Prior to the assay, the bacterial isolates were grown overnight in the LB medium, then streaked onto Mueller Hinton agar medium for antibiotic resistance assay. Several antibiotic disks used in this study such as Amoxicillin-clavulanic acid (AMC; 30 µg), Oxacillin (OX; 5 µg), Cefoxitin (FOX; 30 µg), and Amikacin (AK; 30 µg) were purchased from Oxoid (Oxoid Ltd-USA). For the analysis of minimum inhibitory concentrations (MICs) and zone diameters, EUCAST breakpoint tables were referred to determine the susceptibility [14].

3. RESULT AND DISCUSSION

There are four isolates collected from two fruit bat (*Chironax melanocephalus*) caught in a mist net. These four isolates are the dominant bacteria isolated in XLD medium. Isolate 4.A.1 and 4A.2.1 are isolated from oral swab while 5.B.2 and 5.B.1.2 isolated from the rectal swab. The morphology characteristic of isolate 4.A.1 and 4.A.2.1 are blackish gray and pale red for isolates 5.B.2 and 5.B.1.2 in XLD medium (Fig 1). The XLD medium is the selective and differential medium that is generally used to perform isolation of salmonellae and shigellae. The medium uses sodium deoxycholate as selective inhibitory and three indicator systems such as xylose, lactose, sucrose combined with phenol red; lysine hydrochloride with phenol red; sodium thiosulphate and iron (ferric ammonium citrate). Iron salts in the medium reacted with hydrogen sulphide produced from sodium thiosulphate resulting the black stain of the colony [15].

Identification of the bacteria was performed using molecular method through 16S rDNA sequencing which currently becomes a common standard for bacterial identification. According to the identification results, isolate 4.A.1 identified as *Proteus mirabilis* and 5.B.2 as *Serratia marcescens* whether in Genebank or EzBioCloud (Table 1). The usage of two online databases is recommended in order to confirm that the identification is valid. Generally, the Genebank database is often preferable due to the huge number of 16S rDNA data, however, sometimes the status of the strain is not clearly known [16]. Instead of identification through two online databases, phylogenetic analysis also performed using neighbor-joining method, with 1000x bootstrap replicates (Fig. 2) and the result is in line with the previous identification.

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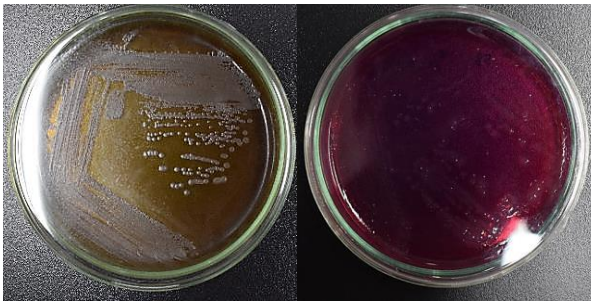


(a) (b)

Figure 1. Pure bacterial isolates (a) *Proteus mirabilis* 4A.1 and (b) *Serratia marcescens* 5B on XLD agar

Table 1. Identification of bacterial isolates based on two online databases

Isolate code	GeneBank (NCBI)			EzBiolab (ChunLab)		
	Identification	Similarity (%)	Accession	Identification	Similarity (%)	Accession
4.A.1	*Proteus mirabilis* JCM 1669	99.93	LC060912.1	*Proteus mirabilis* ATCC 29906	99.78	ACLE01000013
5.B.2	*Serratia marcescens subsp. marcescens* ATCC 13880	98	CP041233.1	*Serratia marcescens* ATCC 13880	95	JMPQ01000005

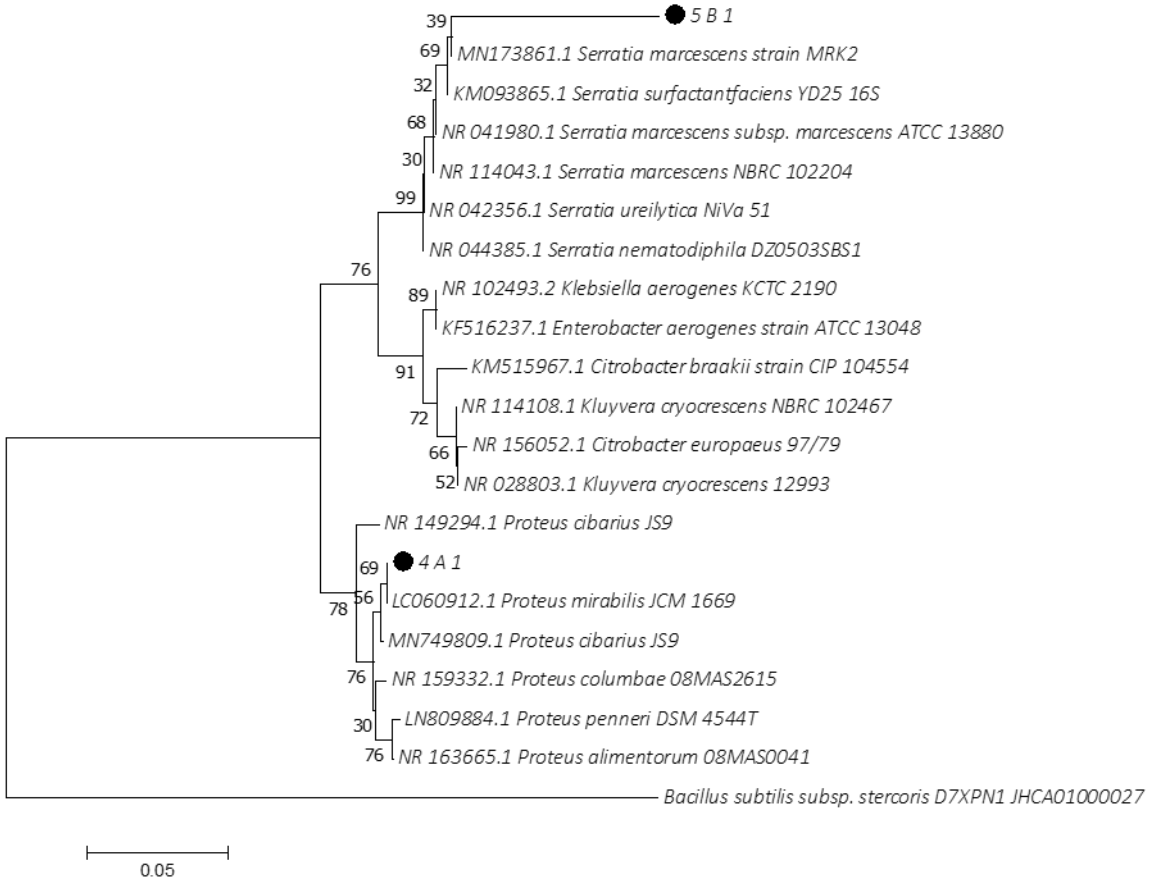


Figure 2. Neighbor-joining tree of isolate 4.A.1 and 5.B.1 with 1000x bootstrap replicates.

In animals, *P. mirabilis* had been reported as a cause of urinary tract infections in dogs [17], suppurative nephritis [18] and endometritis in cattle [19], to salpingitis in laying hens [20]. Meanwhile, *S.*

marcescens is known to be associated with abortion [21] and mastitis outbreaks in dairy cows [22], inflammation of the digestive tract in hummingbirds [23], and necrotizing fasciitis in dogs [24]. Both *P. mirabilis* and *S. marcescens* are opportunistic pathogens in humans [25] and can be potentially transmitted through contaminated food [26]. Thus, fruit bat consumption should be avoided [27].

According to antibiotic resistance assay, both isolates *P. mirabilis* 4.A.1, 4.A.2.1 and *S. marcescens* 5.B.2, 5.B.1.2 resistant to oxacillin (5 µg) and susceptible to amikacin (30 µg). Antibiotic-resistant profiles also show *P. mirabilis* susceptible against cefoxitin (30 µg) and amoxicillin-clavulanic acid (30 µg), while *S. marcescens* resistant against these two antibiotics (Table 2). Oxacillin is a β-lactam class antibiotic that has a limited spectrum of activity. The primary target of this antibiotic is gram-positive bacteria. The report of resistance to oxacillin is quite common and most of the resistant bacteria are gram-negative enteric bacteria [28]. Mechanism of resistance to a β-lactam antibiotic is conducted by two general mechanisms i.*e* hyperproduction of β-lactamase and the production of supplemental penicillin-binding protein (PBP 2 or PBP2a) which encoded by the *mecA* gene [29]

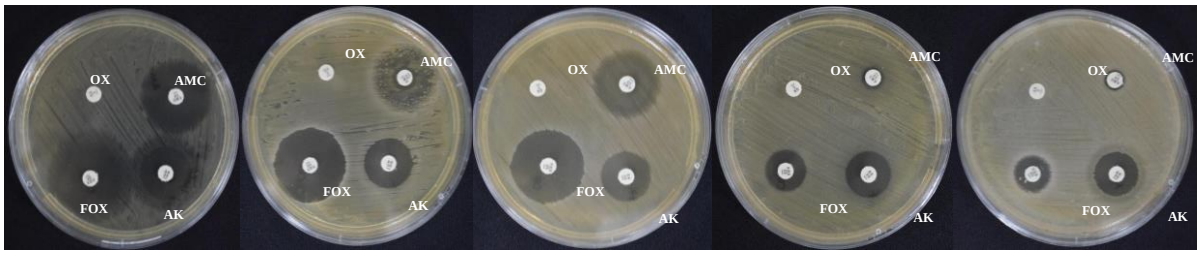
In contrast to oxacillin, all isolates found in this study are susceptible to amikacin (30 µg) (Table 2). Amikacin is the most commonly used semisynthetic aminoglycoside antibiotic. Among other aminoglycosides currently available, amikacin is considered to be the most resistant to the aminoglycoside modifying enzyme [30].

Naturally, *P. mirabilis* is sensitive to aminoglycoside antibiotics and several groups of β-lactam such as the penicillin group (amoxicillin, amoxicillin-clavulanic acid), cephalosporin (cefoxitin, cefazoline, cefotiam), carbapenems, and monobactams [31]. Another study reported that *P. mirabilis* from clinical samples shows 54.9% resistance to cefoxitin and 37.3% resistance to amikacin, while, *P. mirabilis* from animal samples have no resistance against cefoxitin and only 2.7% resistant to amikacin [32].

Table 2. Antibiotic resistance profile of bacterial isolates

Isolate code	Identification	Clear Zone Diameter (mm)							
		OX	**R/I/S**	**FOX**	**R/I/S**	**AK**	**R/I/S**	**AMC**	**R/I/S**
4.A.1	*Proteus mirabillis*	-	R	31	S	21	S	28	S
4.A.2.1		-	R	30	S	20,5	S	29,5	S
5.B.2	*Serratia marcescens*	-	R	16,5	R	18,5	S	9	R
5.B.1.2		-	R	16	R	19	S	8,5	R
InaCC B5	*Escherichia coli*	-	R	28,5	S	22	S	30,5	S

OX: Oxacillin (5 µg); **FOX**: Cefoxitin (30 µg); **AK**: Amikacin (30 µg); **AMC**: Amoxicillin-clavulanic acid (30 µg), **R**: Resistant; **I**: Intermediate; **S**: Susceptible



(a) (b) (c) (d) (e)

Figure 3. Antibiotic susceptibility test for each bacterial isolate using 4 different antibiotics (a) *E. coli* InaCC B5, (b) *P. mirabilis* 5.B.2 (c) *P. mirabilis* 5.B.1.2 (d) *S. marcescens* 5.B.2 and (e) *S. marcescens* 5.B.1.2

In this study, *Serratia marcescens* has higher resistance against antibiotics tested compared to *P. mirabilis*. These two bacteria are enteric pathogenic bacteria from enterobacterales order. *S. marcescens* reported has a plasmid-mediated adenyl-transferase which also present in *E. coli, Klebsiella pneumoniae*, and *Proteus vulgaris.* It caused the bacteria able to use amikacin as the substrate, which leads to amikacin resistance [30], [33]. *S. marcescens* also reported naturally resistant to several antibiotics from the penicillin group of β-lactam such as oxacillin, amoxicillin, amoxicillin/clavulanate, and ampicillin. It also considered intermediate to resistant against and cephalosporins groups such as cefoxitin and cefotiam [34].

4. CONCLUSION

The bacteria isolated from *C. melanocephalus* were successfully identified using the molecular method through two online databases. Isolate 4.A.1 is identified as *P. mirabilis* and 5.B.2 is identified as *S. marcescens*. The identification is also supported in phylogenetic analysis. Antibiotic resistance test shows that all isolates are resistant to oxacillin (5 µg) and susceptible to amikacin (30 µg). *P. mirabilis* also susceptible to cefoxitin (30 µg) and amoxicillin-clavulanic acid (30 µg) while *S. marcescens* are found resistant to these antibiotics.

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