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Morphological and molecular identification of multi-antibiotic resistant bacteria in the wound site of diabetic ulcers

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Abstract. *Risqiyah W, Narulita E, Rofiqoh A, Ludfi AS, Iqbal M. 2022. Morphological and molecular identification of multi-antibiotic resistant bacteria in the wound site of diabetic ulcers. Biodiversitas 23: 663-670.* The study aimed to determine the characteristics of diverse species of bacteria found in diabetic ulcers and the inhibition of antibiotics against these bacteria. The method used is Gram staining for morphological characterization, identification using 16S rRNA gene, and pour plate for antibiotic resistance test. The morphological characterization result showed that the colonies had white color, circular shape, flat elevation, entire edge, small size, and basil shape with Gram negative type of bacteria. Another result showed yellow, greenish, pellucid, and green color, irregular shape, raised elevation, undulate and lobate edge, medium size, coccus and Gram positive type of bacteria. Query cover of molecular identification showed 73-100% and 77.61-96.77% for the result of similarity identification with *Alcaligenes faecalis*, *Shigella flexneri*, *Enterococcus faecalis*, *Proteus mirabilis*, and *Acinetobacter seohaensis*. The identification and antibiotic resistance testing showed that the bacterial species found in diabetic ulcers was *A. faecalis* strain NRBC 13111, which was resistant to all tested antibiotics, except *A. faecalis* strain NRBC 13111 from the UB 2.3K and *A. faecalis* strain NRBC 13111 from the sample UB 3.4M. *Enterococcus faecalis* strain ATCC 19433 was resistant to ceftriaxone, ceftazidime, clindamycin and metronidazole, intermediates to cefoperazone, and sensitive to ciprofloxacin. *Proteus mirabilis* strain JCM 1669 was resistant to clindamycin and metronidazole, sensitive to ceftriaxone and ciprofloxacin, intermediates to ceftazidime and cefoperazone. *Proteus mirabilis* strain ATCC 29906, *A. seohaensis* strain SW-100 and *S. flexneri* strain ATCC 29903 were resistant to all tested antibiotics.

Keywords: *Alcaligenes faecalis*, antibiotics, diabetic ulcers, resistance

Abbreviations: CAX: Ceftriaxone; CAZ: Ceftazidime; CDM: Clindamycin; CFP: Cefoperazone; CLSI: Clinical and Laboratory Standards Institute; CP: Ciprofloxacin; E: EMBA/Eosin Methylene Blue Agar; GN: Gram-negative bacteria; GP: Gram-positive bacteria, I: Intermediate, R: Resistant; K (-): negative control; K: King's B media; M: MSA/Maltose Salt Agar; MTZ: Metronidazole; S: Sensitive; UB x.yZ (UB: Bacterial Ulcers; x: sample number; y: colony number; Z: growth medium)

INTRODUCTION

Diabetes is characterized by a state of hyperglycemia that can occur due to decreased insulin secretion or impaired insulin activity. Diabetes mellitus is classified into type 1 diabetes mellitus, known as insulin dependent, in which the pancreas fails to produce insulin characterized by a lack of insulin production and type 2 diabetes mellitus, known as non insulin dependent, due to the body's inability to effectively use the insulin produced by the pancreas. Diabetes mellitus which accounts for 90% of cases worldwide is type 2 diabetes which is known as non-insulin dependent (Sudoyo et al. 2007). The number of people with diabetes in the world was 463 million in 2019, Indonesia ranks seventh in the world with around 10.7 million people with diabetes and is predicted to increase to 16.2 million in 2045 (International Diabetes Federation 2019). East Java Province ranks the ninth highest diabetes prevalence in Indonesia (Kominfo 2015). One of the districts with quite high cases of diabetes 2 is Jember district as evidenced by data on the number of patient visits in 2015 (Dinkes Jember 2015) and increased from January to December 2016, reaching 10,941 (Sasmita et al. 2019). The prevalence of diabetes continues to increase in several districts in the district of Besuki (Riskesdas 2018).The highest number of diabetes mellitus cases was at the Patrang Public Health Center which had 371 cases (Sasmita et al. 2019). It is known that the prevalence of diabetes mellitus in Banyuwangi Regency from 2013-2018 has increased, from 1.5% to 2%. Bondowoso Regency also increase from 1% in 2013 to 1 .5% in 2018. Meanwhile, Situbondo Regency increased from 2% in 2013 to 3% in 2018 (Riskesdas 2018). This triggers an increasing number of patients makes an ulcer complications of peripheral neuropathy complications and it causes a damage to nerve cells and blood vessels (Ghotasiou et al. 2018). One of the contributing factors is its control against bacterial infections (Rahmadiliyani and Muhlisin 2008). Bacterial infections occur due to high glucose levels which are strategic places for various bacteria to breed (Sri and Setyawati 2016), such

as *Staphylococcus aureus* and non-haemolytic *Staphylococcus* sp. (Donastin et al. 2019). Control of bacterial growth in diabetic ulcer patients is generally given empirically (Donastin et al. 2019). One thing that needs to be considered in choosing empiric therapy is the type of bacteria (Katarnida 2014). The type of antibiotic used to treat infection must be appropriate and wise because the microorganisms that infect patients with diabetic ulcers are very diverse. The precise use of antibiotics in treatment will provide better therapeutic results, reduce the number of antibiotic resistance, reduce the incidence of amputations, and reduce mortality rates (Sari et al. 2018). Therefore, there is a need for further studies on bacterial resistance found in diabetic ulcer patients to antibiotics commonly prescribed by doctors such as ceftriaxone (Agistia et al. 2017), ceftazidime, cefoperazone, metronidazole, ciprofloxacin (Sari et al. 2018) and clindamycin (Sugiyono and Padmasari 2019) to determine their inhibition against bacteria. Hence, identifying macro and micro is very necessary to know the characteristics and species of bacteria (Rinanda 2011). The present study deals with morphological and molecular identification of multi-antibiotic resistant bacteria in the wound site of diabetic ulcers.

MATERIALS AND METHODS

Isolation of bacteria

Bacteria were isolated from sample of patients with diabetic ulcers at Dr. Soebandi Hospital and Diabetics Clinic at Jember, East Java, Indonesia and performed wound care with inclusion criteria, having ulcer grade I or II. Isolated bacteria were grown onto nutrient agar (NA) plate, then continued grown onto maltose salt agar (MSA), eosin methylene blue agar (EMBA) and King's B medium (Table 1). Observations on macro and micro characteristics was determined based on color, colony shape, elevation, edge, size, and shape/type of Gram bacteria (Table 2). ests for antibiotic resistance of bacteria isolated from diabetic ulcers were carried out using disk paper, six antibiotics with 3 replications, and ddH2O as a negative control.

Based on the result of bacterial growth on three kinds of medium, sample UB 1.1- UB 5.8 grow well on maltose salt agar, eosin methylene blue agar and King's B agar, and the other samples grow on two or one kind of medium with different colors each on medium.

Isolation and extraction of Bacterial DNA

The bacteria were grown in nutrient broth (NB) medium for 24 hours at 37ºC which then 1000 µL aliquot was retrieved and centrifuged at 4°C, 1500 rpm for 10 minutes. The obtained supernatant contained DNA transferred to other eppendorf tubes, added with 180µL digestion buffer, 20µL proteinase K and mixed by vortex. Two hundred microliters lysis/binding buffer was added then. Five hundred microliters volumes of cold absolute ethanol and washing buffer were added, then centrifuged for 1 minute in 1000 g. The pellet was dried and resuspended in 25-200 µL elution buffer (Zimbro et al. 2009).

DNA amplification and quantification

The 16S rRNA genes were amplified by using the universal primer of 27F 5'- GAG AGT TTG ATC CTG GCT CAG -3' and 1495R 5'- CTA CGG CTA CCT TGT TAC GA -3'.The first PCR amplification process was conducted using gradient temperature to obtain optimum temperature for each primer pairs. The gradient temperature setting was based on melting temperature (Tm) of each primer at 5°C below of Tm with 35 cycles. The PCR condition was pre-denatured at 95°C for 1 minute, denaturation at 94°C for 45 seconds, followed by 35 cycles annealing temperature at 53°C for 30 seconds, and extension at 72°C for 2 minutes, and final extension at 72°C for 10 seconds (Sunar et al. 2014). The amplification products were separated on 1.2% agarose gel stained with 3 µg/mL ethidium bromide using 1kb DNA ladders as molecular-weight size marker (Tilahun et al. 2018).

Table 1. Bacterial growth on maltose salt agar, eosin methylene blue agar, and King's B agar medium

Sample	Maltose salt agar	Eosin methylene blue agar	Kings' B agar
UB 1.1	White	Pink Purplish	White
UB 1.2	White	Pink Purplish	Pellucid
UB 2.3	White	Pink purplish	Pellucid
UB 3.4	White	Pink purplish	White
UB 4.5	White	Pink purplish	White
UB 4.6	White	Pink Purplish	Pellucid
UB 5.7	White	Pink Purplish	Pellucid
UB 5.8	White	Pink Purplish	Pellucid
UB 6.15	White	Pellucid	-
UB 6.16	-	-	Pellucid
UB 7.17	-	-	Pellucid
UB 7.18	-	-	Pellucid
UB 8.9	Yellow	-	-
UB 8.10	Yellow	-	-
UB 9.11	-	Purplish pink	-
UB 10.12	-	Purplish pink	-
UB 11.13	Putih	Pellucid	-
UB 12.14	White	Pellucid	-
UB 13.19	-	-	Pellucid
UB 13.20	-	-	Pellucid
UB 14.21	-	-	Pellucid
UB 14.22	-	-	Pellucid

Note: - (not growing).

Table 2. Macroscopic and microscopic bacterial morphological

Sample	Macro characteristics					Cell shape/type of gram bacteria
	Color	Colony shape	Elevation	Edge	Size	
UB 1.1M	Yellow	Circular	Flat	Entire	Small	Coccus (GN)
UB 1.1E	White	Circular	Raised	Entire	Small	Coccus (GN)
UB 1.1K	White	Circular	Raised	Entire	Small	Basile (GN)
UB 1.2M	White	Circular	Raised	Entire	Medium	Basile (GN)
UB 1.2E	White	Circular	Flat	Entire	Medium	Coccus (GN)
UB 1.2K	White	Irregular	Flat	Serrate	Point	Coccus (GN)
UB 2.3M	White	Circular	Flat	Entire	Medium	Coccus (GN)
UB 2.3E	White	Circular	Raised	Entire	Point	Basile (GN)
UB 2.3K	White	Circular	Flat	Entire	Small	Basile (GN)
UB 3.4M	White	Circular	Flat	Entire	Medium	Basile (GN)
UB 3.4E	White	Circular	Raised	Entire	Small	Coccus (GN)
UB 3.4K	White	Circular	Flat	Entire	Small	Basile (GN)
UB 4.5M	White	Circular	Flat	Entire	Medium	Basile (GN)
UB 4.5E	Greenish	Circular	Raised	Entire	Small	Basile (GN)
UB 4.5K	White	Circular	Raised	Entire	Medium	Basile (GN)
UB 4.6M	Yellow	Circular	Flat	Entire	Small	Coccus (GP)
UB 4.6E	Yellow	Circular	Raised	Entire	Point	Basile (GN)
UB 4.6K	White	Irregular	Flat	Undulate	Point	Basile (GN)
UB 5.7M	White	Circular	Flat	Entire	Point	Basile (GN)
UB 5.7E	White	Circular	Flat	Entire	Medium	Basile (GN)
UB 5.7K	White	Circular	Flat	Entire	Small	Basile (GP)
UB 5.8M	White	Circular	Flat	Entire	Small	Coccus (GN)
UB 5.8E	White	Circular	Raised	Entire	Medium	Coccus (GP)
UB 5.8K	White	Circular	Flat	Entire	Small	Basile (GN)
UB 6.15M	White	Circular	Raised	Entire	Small	Basile (GN)
UB 6.15E	Pellucid	Circular	Raised	Entire	Medium	Basile (GN)
UB 6.16K	White	Circular	Raised	Entire	Small	Basile (GN)
UB 7.17K	White	Circular	Raised	Entire	Point	Coccus (GP)
UB 7.18K	White	Circular	Raised	Entire	Medium	Basile (GN)
UB 8.9M	Golden	Circular	Flat	Entire	Small	Basile (GN)
UB 8.10M	White	Circular	Flat	Entire	Small	Basile (GN)
UB 9.11E	White	Irregular	Flat	Serrate	Small	Coccus (GN)
UB 10.12E	White	Circular	Flat	Entire	Small	Coccus (GP)
UB 11.13M	Green	Circular	Raised	Entire	Small	Basile (GN)
UB 12.14M	Green	Circular	Flat	Entire	Small	Basile (GN)
UB 12.14E	Yellow	Circular	Raised	Entire	Point	Basile (GN)
UB 13.19K	White	Circular	Flat	Entire	Small	Basile (GN)
UB 13.20K	White	Circular	Flat	Entire	Point	Basile (GN)
UB 14.21K	Pellucid	Circular	Flat	Entire	Small	Basile (GN)
UB 14.22K	White	Irregular	Raised	Lobate	Small	Basile (GN)

Note: GN- Gram negative; GP- Gram Positive.

RESULTS AND DISCUSSION

The selected samples were UB 1.2E, UB 2.3K, UB 3.4, and UB 4.5M showed the similarities with *Alcaligenes faecalis* strain NRBC 13111 observed by morphological characteristics in the form of *Bacillus*, including Gram-negative thype of bacteria (Table 2). Thats four bacterial samples had different characters from other samples, which were both grown in white on MSA medium, purplish-pink on EMBA, and on white King's B (Tabel 1). *Alcaligenes faecalis* strain NRBC has a tolerance to high salt content of almost 10%. However, the growth was not as much as when the salt concentration was below 7% (Suhartati et al. 2018). *Alcaligenes faecalis* can grow in EMBA differential selective medium. EMBA contains eosin Y as a pH indicator and inhibits the growth of Gram-positive bacteria. The inability of *A. faecalis* to ferment sucrose and glucose makes the colony color purplish pink (Omer et al. 2017). This medium is used to confirm the presence or absence of *Pseudomonas aeruginosa* based on its fluorescence. A positive result indicates the presence of luminescence green-yellow fluorescent pigment on *Pseudomonas aeruginosa* when placed under a UV lamp with a wavelength of 366 nm (Quinn et al. 2004). Case infection *A. faecalis* in diabetic ulcers has rarely been reported in the literature. There were only 4 cases worldwide in 1952, 1997, 2019, and 2020 (Sommeng et al. 2019). Based on the molecular identification showed the similarities until 96.77% with *A. faecalis* strain NRBC 13111 (Table 3).

Sample UB 6.15E is known to have similarities with *Shigella flexneri* strain ATCC 29903 observed by molecular identification (Table 3). *Shigella flexneri is* a Gram-negative Bacilli. Based on the results of macroscopic morphological observations, *S. flexneri* also grew on white

MSA medium and clear colored EMBA medium. The growth of *S. flexneri* on MSA medium is due to *S. flexneri* having a tolerance of salt levels up to 8% to still survive on MSA medium (Huang 2020). *Shigella flexneri* is a bacterium that cannot ferment lactose, so when grown in EMBA medium, it will be clear or colorless. *Shigella flexneri* grown dimedium time EMBA, large colony size and colorless (Zaika et al. 2002). In general, *S. flexneri* is a bacterium that does not have flagella, is aerobic, does not form spores, causes diarrhea and dysentery. Its habitat is in the digestive tract with infection through the mouth. The morphological characters were clear colony color, raised elevation, entire edge, medium colony size, and smooth surface. This is in accordance with the observations that have been made (Table 2). *Shigella flexneri* has also been found in the bloodstream of patients with uncontrolled diabetes mellitus. If these bacteria survive long enough in large numbers, it can cause serious infections (Power and Johnson 2009).

Sample UB 7.17K *(Enterococcus faecalis)* was a facultative anaerobe, had a white colony color, around colony shape, a smooth surface (Khamid and Mulasari 2012). This was also suitable for observing morphological results (Table 4). Samples UB 11.13M and UB 12.14M based on molecular identification were similar to *Proteus mirabilis* bacteria but different strains. UB 11.13M was similar to *P. mirabilis* strain JCM 1669 while UB 12.14M was similar to *P. mirabilis* strain ATCC 29906. *Proteus mirabilis* was a Gram-negative Bacilli bacteria (Table 2). Sample UB 13.20K was known to have similarities with *Acinetobacter seohaensis* strain SW-100 (Table 3), with morphological characteristic was a Gram negative-Bacilli bacteria (Table 2).

The antibiotics resistance test results showed that all *A. faecalis* strain NRBC 13111 were resistant to all tested antibiotics. *Enterococcus faecalis* strain ATCC 19433 and *P. mirabilis* strain JCM 1669 are resistant, intermediate and sensitive to several antibiotics. *Proteus mirabilis* strain ATCC 29906, *A. seohaensis* strain SW-100 and *S. flexneri* strain ATCC 29903 were resistant to all the antibiotics tested (Table 4) and (Figure 1).

Sample UB 7.17K is similar to bacteria *E. faecalis* strain ATCC 19433. *Enterococcus faecalis* is coccus-shaped and includes Gram positive bacteria. The growth of *E. faecalis* on King's B medium other microbes could be grown other then *Pseudomonas aeruginosa*. *Enterococcus faecalis* also did not grow in MSA medium because the tolerance limit for NaCl was 6.5% only (Ninan et al. 2016). *Enterococcus faecalis* is a facultative anaerobe, has white colony color, rounded colony shape, smooth surface (Ahmad et al. 2002). This is also in accordance with the observations (Table 2). *Enterococcus faecalis* can grow at a high pH of 4.8-9.6, with no spores, and non-motile. These bacteria can cause urinary tract infections and are found in diabetic ulcers, but their abundance is about 8% (Khamid et al. 2012).

Table 3. Molecular identification of bacteria causing diabetic ulcers

Sample	Species	Max score	Query cover (%)	E-value	Identity (%)	Accession number	Length (bp)
UB 1.2E	*Alcaligenes faecalis* strain NRBC 13111	1956	100	0.0	96.77	NR_113606.1	1462
UB 2.3K	*Alcaligenes faecalis* strain NRBC 13111	2132	99	0,0	95.15	NR_113606.1	1462
UB 3.4M	*Alcaligenes faecalis* strain NRBC 13111	422	73	3e-117	77.61	NR_113606.1	1462
UB 4.5M	*Alcaligenes faecalis* strain NRBC 13111	1884	97	0.0	90.86	NR_113606.1	1462
UB 6.15E	*Shigella flexneri* strain ATCC 29903	1803	100	0.0	91.80	NR_026331.1	1530
UB 7.17K	*Enterococcus faecalis* strain ATCC 19433	1229	90	0.0	83.97	NR_115765.1	1483
UB 11.13M	*Proteus mirabilis* strain JCM 1669	2100	96	0.0	95.46	NR_113344.1	1465
UB 12.14M	*Proteus mirabilis* strain ATCC 29906	915	90	0.0	85.24	NR_114419.1	1497
UB 13.20K	*Acinetobacter seohaensis* strain SW-100	1960	99	0.0	92.20	NR_115299.1	1493

Table 4. Measurement results of antibiotic clear zone against bacteria that cause diabetic ulcer

Sample	Antibiotics treatment	Paper disk conc. * (µg/mL)	Inhibition zone criteria			Average (mm)	Result
			S	I	R		
Alcaligenes faecalis strain NRBC 13111 (UB 1.2E)	CAX	30	≥ 23	20-22	≤19	0	R
	CAZ	30	≥ 21	18-20	≤17	0	R
	CFP	75	≥ 21	16-20	≤15	0	R
	CDM	2	≥ 21	15-20	≤14	0	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	0	R
	K(-)/ddH_2O	-	-	-	-	0	-

Alcaligenes	CAX	30	≥ 23	20-22	≤19	14.83	R
faecalis strain	CAZ	30	≥ 21	18-20	≤17	11.66	R
NRBC 13111	CFP	75	≥ 21	16-20	≤15	12.06	R
(UB 2.3K)	CDM	2	≥ 21	15-20	≤14	0	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	25.43	S
	K(-)/ddH_2O	-	-	-	-	0	-
Alcaligenes	CAX	30	≥ 23	20-22	≤19	21.86	I
faecalis strain	CAZ	30	≥ 21	18-20	≤17	0.17	R
NRBC 13111	CFP	75	≥ 21	16-20	≤15	10.4	R
(UB 3.4M)	CDM	2	≥ 21	15-20	≤14	0	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	0	R
	K(-)/ddH_2O	-	-	-	-	0	-
Alcaligenes	CAX	30	≥ 23	20-22	≤19	18.06	R
faecalis strain	CAZ	30	≥ 21	18-20	≤17	4.88	R
NRBC 13111	CFP	75	≥ 21	16-20	≤15	8.33	R
(UB 4.5M)	CDM	2	≥ 21	15-20	≤14	0	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	0	R
	K(-)/ddH_2O	-	-	-	-	0	-
Shigella flexneri	CAX	30	≥ 23	20-22	≤19	7.56	R
strain ATCC	CAZ	30	≥ 21	18-20	≤17	0	R
29903	CFP	75	≥ 21	16-20	≤15	2.83	R
(UB 6.15E)	CDM	2	≥ 21	15-20	≤14	0	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	0	R
	K(-)/ddH_2O	-	-	-	-	0	-
Enterococcus	CAX	30	≥ 23	20-22	≤19	14.33	R
faecalis strain	CAZ	30	≥ 21	18-20	≤17	14.43	R
ATCC 19433	CFP	75	≥ 21	16-20	≤15	16.3	I
(UB 7.17K)	CDM	2	≥ 21	15-20	≤14	5,9	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	23.5	S
	K(-)/ddH_2O	-	-	-	-	0	-
Proteus	CAX	30	≥ 23	20-22	≤19	26.23	S
mirabilis	CAZ	30	≥ 21	18-20	≤17	19.23	I
strain JCM 1669	CFP	75	≥ 21	16-20	≤15	16.1	I
(UB 11.13M)	CDM	2	≥ 21	15-20	≤14	0	R
	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	25.9	S
	K(-)/ddH_2O	-	-	-	-	0	-
Proteus	CAX	30	≥ 23	20-22	≤19	17.97	R
mirabilis	CAZ	30	≥ 21	18-20	≤17	17	R
strain ATCC	CFP	75	≥ 21	16-20	≤15	12.93	R
29906	CDM	2	≥ 21	15-20	≤14	0.38	R
(UB 12.14M)	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	0.96	R
	K(-)/ddH_2O	-	-	-	-	0	-
Acinetobacter	CAX	30	≥ 23	20-22	≤19	0	R
seohaensis	CAZ	30	≥ 21	18-20	≤17	0	R
strain	CFP	75	≥ 21	16-20	≤15	0	R
SW-100	CDM	2	≥ 21	15-20	≤14	0	R
(UB 13.20K)	MTZ	**5	≥ 21	16-21	≤16	0	R
	CP	5	≥ 21	16-20	≤15	0	R
	K(-)/ddH_2O	-	-	-	-	0	-

Note: *Standard of antibiotic concentration according to (CLSI 2017); ** Concentration according to (Fabanyo et al. 2017).

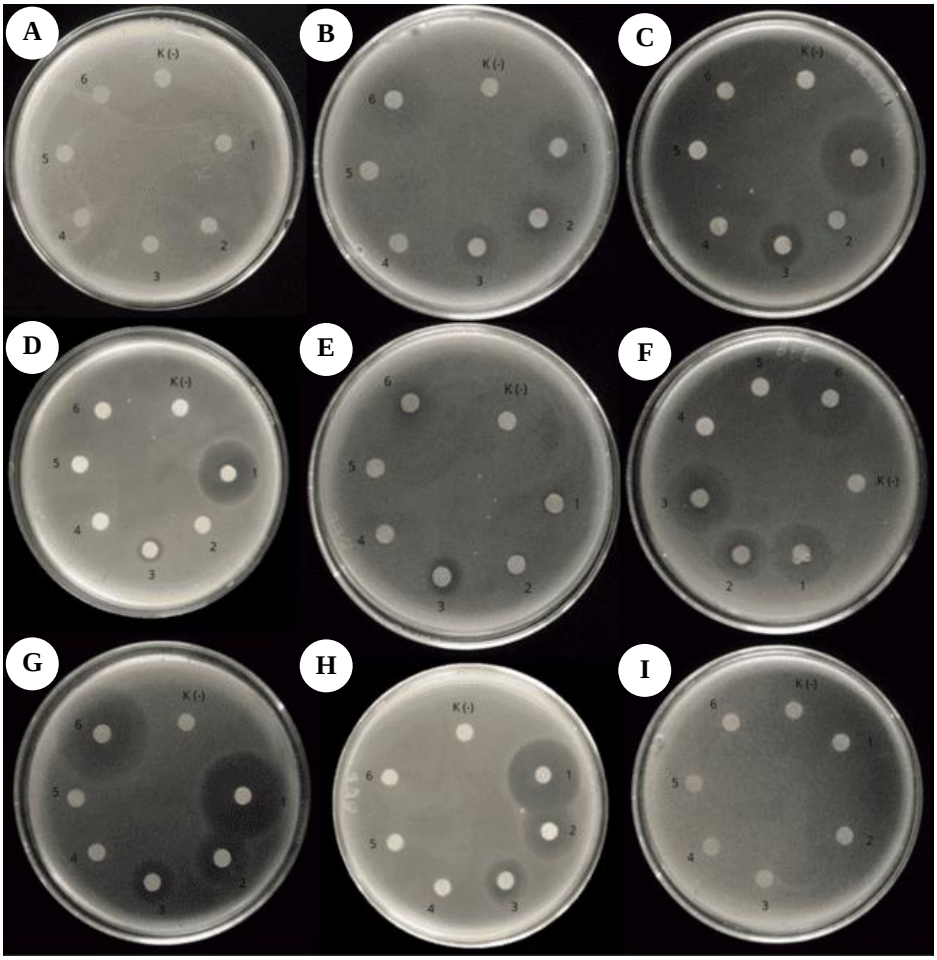

Figure 1. Antibiotics resistance: A. *Alcaligenes faecalis* strain NRBC 13111 (UB 1.2E); B. *Alcaligenes faecalis* strain NRBC 13111 (UB 2.3K); C. *Alcaligenes faecalis* strain NRBC 13111 (UB 3.4M); D. *Alcaligenes faecalis* strain NRBC 13111 (UB 4.5M); E. *Shigella flexneri* strain ATCC 29903 (UB 6.15E); F. *Enterococcus faecalis* strain ATCC 19433 (UB 7.17K); G. *Proteus mirabilis* strain JCM 1669 (UB 11.13M); H. *Proteus mirabilis* strain ATCC 29906 (UB 12.14M); I. *Acinetobacter seohaensis* strain SW-100 (UB 13.20K). (1) CAX; (2) CAZ; (3) CFP; (4) CDM; (5) MTZ; (6) CP

UB 11.13M and UB 12.14M samples have similarities with bacteria *P. mirabilis* but different strains. UB 11.13M looks like *P. mirabilis* strain JCM 1669 while UB 12.14M has similarities with *P. mirabilis* strain ATCC 29906. *Proteus mirabilis* is a Gram-negative bacterium in the form of a bacilli, does not form spores, is facultatively anaerobic, moves with flagella and is a pathogenic bacterium in the intestines of both humans and animals. This bacterium is often found in wound infections and is a urogenital pathogen. This is because *P. mirabilis* has a virulent gene that causes it to become pathogenic. The presence of *P. mirabilis* in diabetic ulcers is quite high at 17.5% (Putri et al. 2018).

Sample UB 13.20K is known to have similarities with *A. seohaensis* strain SW-100. This bacterium is a gram negative bacterium in the form of a bacillus. This *A. seohaensis* can be isolated from seawater (Nur et al. 2016) and there are no studies previously found *A. seohaensis* in diabetic ulcers or wound infections. This can happen because a diabetic ulcer is a strategic place for the proliferation of various kinds of bacteria so that when the ulcer is still in the treatment process, it can be contaminated with bacteria from the air, surrounding objects and from the closest people, especially if the ulcer is not bandaged for a long time.

The types of bacteria found in diabetic ulcers may vary and differ in each region because bacteria are microorganisms that are easy to mutate so that they can form new strains with different characteristics such as resistance to antibiotics. This is evidenced by the results of antibiotic resistance tests (Table 4) and (Figure 1) showing that *A. faecalis* strain NRBC 13111 is known to be resistant to all the antibiotics tested, except *A. faecalis* strain NRBC 13111 from UB sample 2.3K which is sensitive to ciprofloxacin and *A. faecalis* strain NRBC 13111 derived from UB sample 3.4M intermediate to ceftriaxone. This is reinforced by the case of diabetic ulcers with the infection *A. faecalis* in 2019 and known that *A. faecalis* was resistant to antibiotics ciprofloxacin, ceftriaxone, and ceftazidime (Ahmad et al. 2002).

Shigella flexneri strain ATCC 29903 is known to be resistant to all antibiotics which was tested and

strengthened by research conducted by Ninan et al. 2016 *S. flexneri* was resistant to ceftazidime and ceftriaxone. *Enterococcus faecalis* strain ATCC 19433 is resistant to ceftriaxone, ceftazidime, clindamycin and metronidazole, intermediate to cefoperazone, and sensitive to ciprofloxacin. *Proteus mirabilis* strain JCM 1669 and *P. mirabilis* strain ATCC 29906 had different results even though the bacteria were the same. *Proteus mirabilis* strain JCM 1669 is resistant to clindamycin and metronidazole, sensitive to ceftriaxone and ciprofloxacin, intermediate to ceftazidime and cefoperazone. Meanwhile, *P. mirabilis* strain ATCC 29906 was resistant to all the antibiotics tested. This matter can occur because of the presence or absence of resistance genes in these bacteria and the differences in strains that occur due to mutations can change the nature of these bacteria to antibiotics (Yoon 2007). *Acinetobacter seohaensis* strain SW-100 is also known to be resistant to all tested antibiotics.

Ceftriaxone and ceftazidime are broad-spectrum antibiotics and widely used for curing diabetic ulcers. Ceftriaxone and ceftazidime are used as antibiotics that can control gram bacterial infections negatively and have a low effect on Gram-positive bacteria. Metronidazole and clindamycin are often added in combination antibiotics because clindamycin has an optimal spectrum against Gram-positive cocci and Gram-positive bacteria. Anaerobic bacteria, while the administration of metronidazole depends on the patient's ulcer condition, metronidazole is directly given to patients with chronic ulcers, ulcers which has a deep odor (Huang 2016) because it is effective against anaerobic protozoan parasites, anaerobic Gram-negative bacilli, and spore-forming anaerobic Gram-positive bacteria.

Metronidazole is ineffective against aerobic bacteria, because aerobic bacteria do not have electron transport components like anaerobic bacteria. In this study, all bacteria isolated were aerobic and facultative anaerobes, therefore clindamycin and metronidazole in bacteria were found to have no effect. The occurrence of bacterial sensitivity to ciprofloxacin is caused because ciprofloxacin is a quinolone class of antibiotics with strong antibacterial activity against Gram-negative bacteria. The presence of the bacteria is still sensitive to ciprofloxacin can get into the cell by passive diffusion through the protein channel filled with water on the outer membrane of bacteria intracellularly. Ciprofloxacin inhibits bacterial DNA replication by interfering with the work of DNA during bacterial growth and reproduction (Huang 2020).

In conclusion, identification of molecular and morphological bacterial species found a various of bacteria with the multi-antibiotic resistant in patients with diabetic ulcers at the former Besuki Residency, namely *A. faecalis* strain NRBC 13111 which was resistant to all tested antibiotics, except *A. faecalis* strain NRBC 13111 from UB sample 2.3K sensitive to ciprofloxacin and *A. faecalis* strain NRBC 13111 from UB sample 3.4M intermediate to ceftriaxone. *Enterococcus faecalis* strain ATCC 19433 is resistant to ceftriaxone, ceftazidime, clindamycin and metronidazole, intermediate to cefoperazone, and sensitive to ciprofloxacin. *Proteus mirabilis* strain JCM 1669 was resistant to clindamycin and metronidazole, sensitive to ceftriaxone and ciprofloxacin, intermediate to ceftazidime and cefoperazone. *Proteus mirabilis* strain ATCC, 29906 *A. seohaensis* strain SW-100 and *S. flexneri* strain ATCC 29903 were resistant to all tested antibiotics.

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