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Genetic Identification of Superbugs from River Streams in State University of Malang

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ABSTRACT

Superbugs revert to bacterial strains, which exhibit resistance to antibiotics. These bacteria could cause some problems in disease treatment and the environment. This research aimed to identify the presence of superbugs in the river stream that flows through State University of Malang campus area. Amoxicillin, tetracycline, and chloramphenicol were selected to test the possibly found resistant bacteria. Water samples taken from a decided spot was spread over Luria Bertani's agar on both antibiotic supplemented and antibiotics-free LB agar media with no delay. Molecular identification was carried out using 16S rRNA gene in DNA barcoding approach, completed with morphological and Gram staining analyses. A total of 16 isolates of gram-negative colonies were found in the form of bacilli, diplobacilli, cocci, and diplococci. The genetic identification of eight resistant colonies led us to suggest that the isolates may belong to *Aeromonas*, *Shigella*, and *Bacillus*. Further studies are still required to get a clearer view of the correct taxonomical position of those resistant isolates.

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INTRODUCTION

Superbug is a term used to describe strains of microorganisms, which are resistant to one or more antibiotics (Rajendran, 2018). Antibiotic resistance is a phenomenon that occurs when bacteria cannot be killed or their growth is not inhibited by the administration of antibiotics (World Health Organization, 2014). There are more than 1.5 million deaths worldwide yearly caused by superbug infection (Hall *et al.*, 2018).

Various pathogenic bacteria, including *Escherichia coli*, *Mycobacterium tuberculosis*, *Clostridium perfringens*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Shigella flexneri*, *Vibrio cholera*, and *Salmonella enterica* have been detected in wastewater (Cai & Zhang, 2013). Among those pathogenic bacteria, several strains of *P. aeruginosa*, *E. coli*, *Acinetobacter* sp., and *Enterobacteriaceae* has been known to be resistant to antibiotics (Jia & Zhang, 2019). Reports on resistant bacteria are still very limited in Indonesia. Verawaty *et al.* (2020) reported the presence of *E. coli* that is resistant to ampicillin, tobramycin, and tetracycline in the waters of Palembang, meanwhile Suhartono *et al.* (2021) reported the presence of *Salmonella* spp. that are resistant to azithromycin, tetracycline, and streptomycin in the Lamnyong and Krueng waters, Aceh. Research on resistant bacteria in Indonesian waters is still limited, providing the basis for carrying out this research.

The main cause of antibiotic resistance is the misuse of antibiotics, especially in humans and livestock (Parathon *et al.*, 2017), due to the easy access of the public to antibiotics without a prescription either in drugstores or food stalls (Yarza *et al.*, 2015). Amoxicillin was the most widely

consumed antibiotic in Indonesia during 2008-2010, while tetracycline and chloramphenicol were ranked fifth and tenth, respectively (Pradipta *et al.*, 2015). Amoxicillin is commonly used to treat respiratory infections (Akhavan *et al.*, 2021) as well as tetracycline (Bidell *et al.*, 2020), while chloramphenicol is used to treat typhoid fever (Hanekamp & Bast, 2015). Amoxicillin is a ß-lactam antibiotic of the penicillin class (amino-penicillin), which acts as a bactericide (Bernatová *et al.*, 2013). Tetracycline is a tetracycline-class antibiotic that works as a bacteriostatic to inhibit bacteria replication (Shutter, 2022). Meanwhile, chloramphenicol works through both bacteriostatic and bactericidal mechanisms at high concentrations (Oong & Tadi, 2021).

Superbug have also reported to be found in the air environment of hospitals, livestock sector, and sewage treatment plants (Zhou *et al.*, 2020). In the aquatic environment, superbugs can also be found due to the fact that in many areas, water bodies become the estuaries of domestic waste (Puspita *et al.*, 2016), hospital waste (Sarkar *et al.*, 2019), agricultural waste (Habibi *et al.*, 2014), mining waste (Mulyadi *et al.*, 2020), and industrial wastewater (Rahardjo & Prasetyaningsih, 2021). This causes adverse ecotoxicological effects (Smith *et al.*, 2020). In many cases, the disposal of wastewater treatment plants located upstream is a major source of antibiotic-resistant bacteria in waters (Ren & Hongqiang, 2019). Surface and groundwater have been reported in several studies to have a role in causing the emergence of antibiotic-resistant organisms (Li *et al.*, 2014; Hughes *et al.*, 2013; Bradford *et al.*, 2017).

The river stream within State University of Malang Campus is an estuary of domestic waste disposal originating from densely populated residential areas upstream. Wastewater has been reported to be one of the reservoirs for superbugs and an important source for the spread of antibiotic resistance (Jia & Zhang, 2019). Taking into consideration the negative impact of resistant bacteria on the treatment of diseases and environment, we studied the possible existence of superbugs in the river flow within the campus. Molecular identification of superbugs was done using 16S rRNA gene sequence based on the report that 16S rRNA gene is highly conserved which only undergoes slight nucleotide changes in the evolutionary process (Lan *et al.*, 2016). This research aimed to identify the presence of superbugs in the river stream that flows through Malang State University campus area.

MATERIALS AND METHODS

Location and time of observation

This explorative descriptive research was carried out from September 2021 through August 2022 at Biology Laboratory of the State University of Malang. Sampling was taken at river flow of the State University of Malang, Jl. Semarang No.5, Malang, Indonesia. The location of sampling coordinates is located at 7°57'41.0"S, 112°37'06.9"E.

Culture Medium

Luria Bertani (LB) agar and Luria Bertani broth were used as a bacterial culture medium. All media and tools were sterilized in an autoclave at 121^{0}C, 15 lbs. for 15 minutes. As much as 32 mg/L of amoxicillin, 16 mg/L of tetracycline, or 32 mg/L of chloramphenicol were added to LB agar media (Huang et al., 2012; Tahrani et al., 2015) to detect the possible existence of antibiotic resistant bacteria. The media were then stored in the refrigerator until being used.

Bacteria Sample

Bacterial samples were isolated from water taken at 7 a.m. from the river behind the Green House area of State University of Malang. A total of 15 mL of river water in a sterile centrifuge tube was brought to the laboratory in a cooler box. Water quality parameters were measured, including water pH using the Hanna Instrument pH 211 and water turbidity using a digital turbidity meter, SGZ-200BS. Water sample was diluted gradually (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}) and homogenized in 0.1% peptone media. A total of 0.1 mL of water from each sample was inoculated on both LB agar and antibiotic added LB agar media in three repetitions. Incubation was carried out for 1 x 24 hours at 37^{0}C (Bergeron *et al.*, 2015).

Colony Morphological Analysis and Bacterial Gram Staining

Each colony was purified using the quadrant streak plate method to obtain a single colony. Each isolated colony was observed for colony morphology, which included: colony shape, colony edge, colony elevation, and colony color. The isolated colonies were also inoculated on slanted agar media for short-term stock and preserved in glycerin solution for extended time storage. Bacterial Gram and cell shape were microscopically observed. Gram staining was performed using ammonium oxalate crystal violet reagent, iodine, 96% alcohol, and safranin.

Genetic Identification (DNA Barcoding)

For genetic identification purposes, each of antibiotic resistant isolates was cultured in 2 ml of liquid LB medium with respective antibiotic added in duplicate. Incubation was carried out for 2x24 hours. Total bacterial DNA isolation was carried out following the protocol provided by the manufacturer (Geneaid Presto™ Mini gDNA Bacteria Kit). The quality and concentration of total DNA obtained were measured using a Spectrophotometer NanoDrop ND-2000. Target gene amplification was carried out by PCR technique using universal primers for 16S rRNA gene. The forward primer used was 27F, 5'-AGAGTTTGATCMTGGCTCAG-3', and reverse primer used was R1492, 5'-TACGGYTACCTTGTTACGACT-3' (Pertiwi *et al.*, 2018). Pre-denaturation was carried out at 95°C for 3 minutes, followed by 40 amplification cycles consisting of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 1 minute, and the final extension was carried out at 72°C for 10 minutes. Electrophoresis was performed to visualize the PCR product on 1% agarose gel running in 50 volts electrical current for 60 minutes (Pertiwi *et al.*, 2018). The results of electrophoresis are visualized using a UV trans illuminator. The sequence data was taken for eight isolates demonstrating antibiotic resistance. Sequencing process was carried out by First BASE Laboratories, Malaysia.

Data Analysis

Colony morphology, bacterial Gram, and sequence data were analyzed descriptively. The target gene sequences were read using Finch TV. Consensus sequences from forward and backward sequencing of each sample were constructed using ClustalX, then compared to the respective sequence databases available in the Gene Bank NCBI using online Basic Local Alignment Search Tool (BLAST). Phylogenetic tree reconstruction in order to analyze the taxonomic position of each bacterial isolate was carried out using MEGA-11 software for Neighbor Joining (NJ) model with the Kimura-2-Parameter in a 1000 bootstrap value (Prasetio *et al.*, 2021).

RESULTS AND DISCUSSION

Water Parameter

Water sample taken from a decided spot within State University of Malang was neutral (pH 7,33) and clear (0,1 NTU turbidity).

Colony Morphology and Gram Staining

Bacterial colonies were growing on both LB and antibiotic added LB media (Figure1 & 2). From the original inoculation plates, we subcultured in order to purify the distinct colonies. In LB media we found eight different colonies. Meanwhile, three different colonies were growing on both Amoxicillin and Chloramphenicol media, and only two different colonies were growing on Tetracycline media. The observed colonies were dominated by circular opaques with entire margin, and only a few showing other shapes of their edges and being translucent in color (Table 1). The observation through Gram staining showed that all colonies growing on LB media were bacilli, while those growing on antibiotic added media were bacilli, diplobacilli, cocci, or diplococci (Table 1, Figure 2).

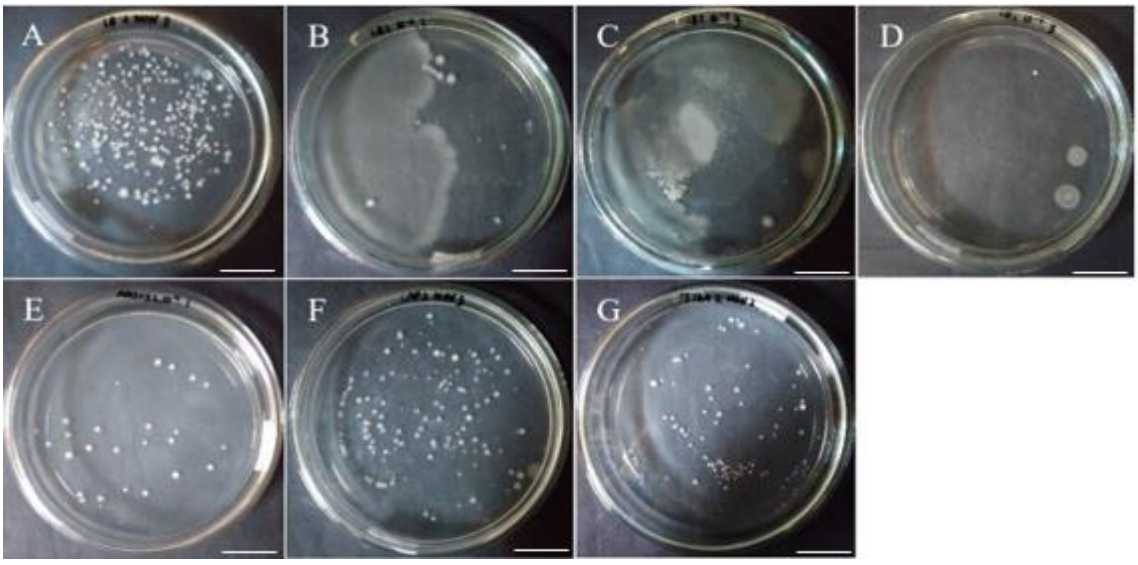

Figure 1. Initial inoculation on LB Agar media and antibiotic added LB Agar media. Scale bar = 2 cm

Table 1. Morphologies and Gram-staining of 16 distinct purified colonies

No	Control (LB)	Gram	LB + Antibiotic	Gram
1	2.1 circular; entire; raised; opaque; milky white	Negative bacilli	Amoxicillin 2.1 circular; entire; raised; opaque; milky white	Negative bacilli
2	2.2 circular; entire; flat translucent; clear white	Negative bacilli	Amoxicillin 2.2 circular; entire; flat; translucent; clear white	Negative bacilli
3	2.3 circular; wooly; flat; opaque; murky white	Negative bacilli	Amoxicillin 2.3 circular; irregular; flat; opaque; murky white	Negative diplo bacilli
4	2.4 circular; entire; flat; opaque edge; milky white	Negative bacilli	Chloramphenicol 2.1 circular; entire; raised; opaque; milky white	Negative cocci
5	2.5 circular; irregular; flat; opaque; milky white	Negative bacilli	Chloramphenicol 2.2 circular; entire; flat; translucent; clear white	Negative bacilli
6	2.6 circular; undulate; flat; opaque; milky white	Negative bacilli	Chloramphenicol 2.3 circular; entire; raised; opaque; murky white	Negative bacilli
7	2.7 circular; wooly; flat; opaque; milky white	Negative bacilli	Tetracycline 2.1 circular; entire; raised; opaque; milky white	Negative bacilli
8	2.8 circular; ciliate; flat; opaque; murky white	Negative bacilli	Tetracycline 2.2 circular; entire; flat; translucent; clear white	Negative diplococci

Among all subcultured colonies, we identified that there were colonies growing on two different antibiotic media, i.e: those which were growing on both Amoxicillin (AMX 2.1; Figure 2I) and Tetracycline (TET 2.1; Figure 2O) and those which were growing on both Amoxicillin (AMX 2.2; Fig. 2J) and Chloramphenicol (CAP 2.2; Figure 2M). A total of 16 bacterial isolates were found. On control media grew, eight isolates, on amoxicillin and chloramphenicol media, three isolates were grown, and on tetracycline media grew only two isolates. It can be understood that those number are less than the number of bacterial isolates that grew in LB media. The presence of antibiotic eliminates the non-resistant ones that grow naturally on the control media due to the absence of any agents that inhibit bacterial growth. The inhibition of bacterial growth in antibiotic supplemented media may be caused by the bacteriostatic effect, which caused disruption of bacterial replication (Bernatová *et al.*, 2013; Nemeth *et al.*, 2015) and/or bactericidal effect of given antibiotics that cause bacterial death (Baquero & Levin, 2021).

The number of isolates grown on amoxicillin and chloramphenicol media was greater than that grown-on tetracycline media (Table 1; Figure 2). We suggest that this finding resulted from the high usage of amoxicillin and chloramphenicol in society in the area upstream to the river. Amoxicillin is widely used to treat various bacterial infections, such as pneumonia, tonsillitis, ear infections (Akhavan *et al.*, 2021), sinusitis dermatitis (Sartelli *et al.*, 2018), and urinary tract infections (Tan & Chlebicki, 2016). Chloramphenicol is used to be applied to treat typhoid fever (Hanekamp & Bast, 2015), bacterial conjunctivitis (eye infection) and otitis externa (Oong, 2021). Tetracyclines are common to be used to treat respiratory tract infections (Bidell *et al.*, 2020) and certain skin inflammations (Orylska *et al.*, 2022).

As it has been well understood that the excessive metabolites will be removed from body through excretory system, either through feces, urine, or sweat. (Garza *et al.*, 2020). Then, in turn, the disposal of house or hospital sewage, which may contain excess antibiotics, directly into any type of water body will not only pollute the water, but also induce the living organism including bacteria, to develop their resistance.

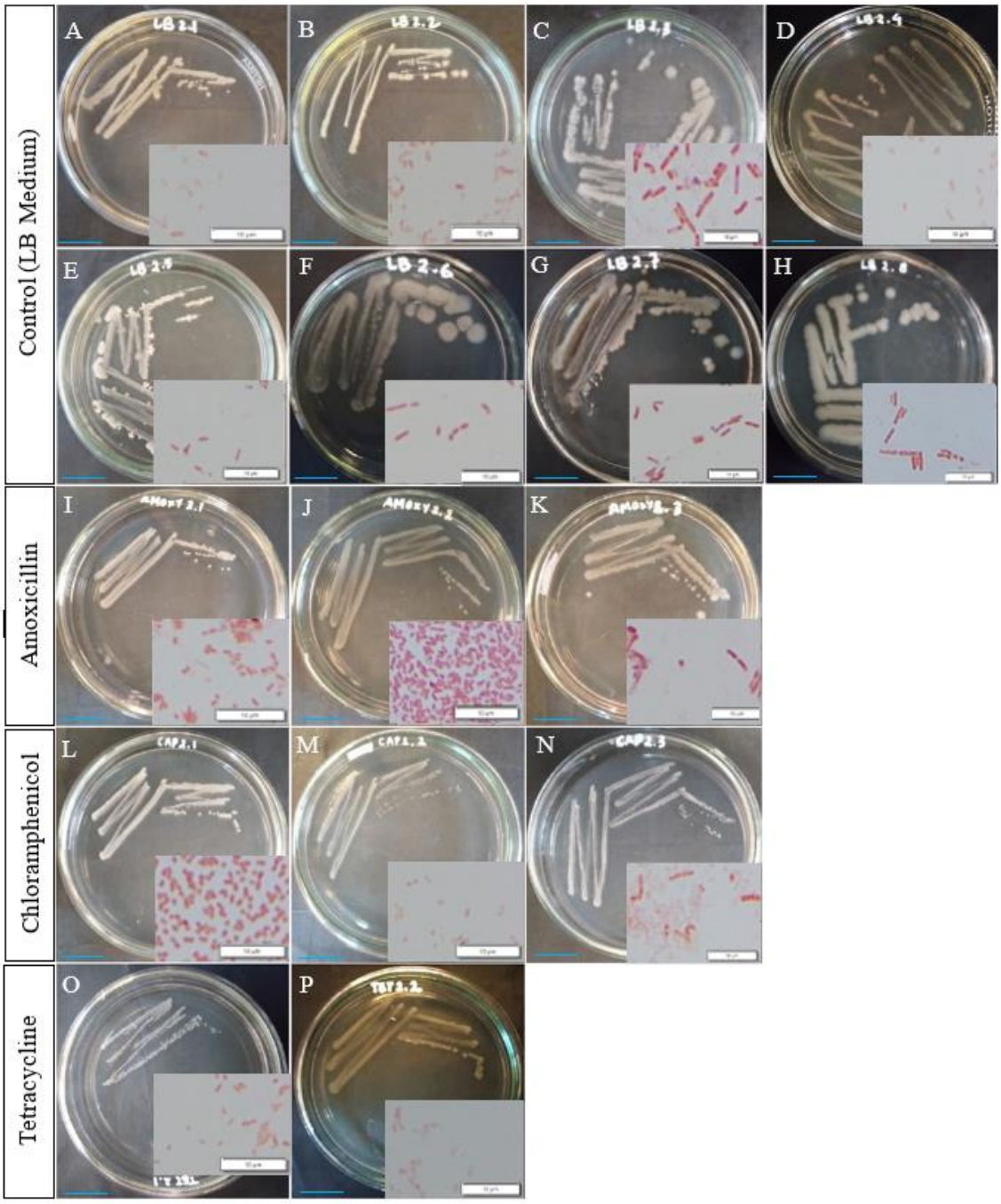

Figure 2. Colony morphology and Gram type. Blue Scale bar = 10 µm

Amoxicillin works by binding to penicillin-binding protein, activating autolytic enzymes, and thereby causing cell wall lysis (Akhavan *et al.*, 2021). Certain antibiotic resistant bacteria, such as *E. coli*, produce ß-lactamase enzymes. This enzyme degrades ß-lactam ring of the antibiotic which resulting in the deactivation of its function (Wu *et al.*, 2021). Chloramphenicol works by interfering the protein synthesis on the 50S ribosomal subunit and inhibiting aminoacyl-tRNA binding at the ribosomal site (Blanco & Antonio, 2017). Thus, the mechanism of bacterial resistance against chloramphenicol is different from the mechanism of resistance to amoxicillin. It can be through drug inactivation, inhibiting protein synthesis (Kapoor *et al.*, 2017), or the existence of an efflux system mechanism (Reygaert, 2018). Tetracyclines work by binding to 16S rRNA and inhibiting tRNA from binding to the ribosome site (Chukwudi, 2016). It had been reported that tetracycline resistance in Gram-negative bacteria can occur through the efflux system mechanism. The efflux pump gene codes for a membrane protein that pumps tetracycline out of cells, rendering the drug ineffective (Amaral *et al.*, 2014). The presence of superbugs in amoxicillin, chloramphenicol, and tetracycline media suggested

that the isolated bacteria in this study may develop resistance mechanisms. Further studies are required to fully understand this finding.

Identification of bacteria based on the 16S rRNA gene

From all antibiotic resistant isolates, we got 955 to 1460 bp length fragments. Reconstructed phylogenetic tree analysis revealed that two isolates of Amoxicillin resistant bacteria (AMX 2.1 and AMX 2.2) were sitting among *Aeromonas* species with low bootstrap value. A similar situation was shown by one Chloramphenicol-resistant isolate (CAP 2.2), which was sitting among *Shigella* species with relatively low bootstrap value. It means that position is not confidently accepted and needs more genetic analysis to clarify this result. On the other hand, TET 2.1, CAP 2.3, AMX 2.3, AND CAP 2.1 were forming a distinct clade splited from *Bacillus* spesies with high bootstrap value (Figure 3).

The results of the phylogenetic tree reconstruction showed that AMX 2.1 and AMX 2.2 isolates were suspected to be *Aeromonas caviae* or *Aeromonas dhakensis*. A. *dhakensis* causes soft tissue infections, bloodstream infections in immunocompromised individuals with malignancy and cirrhosis. A higher mortality rate was observed in cases of bacteremia (Puah *et al*., 2022). It has been reported that *A. veronii* isolated from Lake Erie showed resistance to tetracycline (Skwor *et al*., 2014). *Aeromonas* spp. isolates were susceptible to chloramphenicol found in freshwater fish (Fauzi *et al*., 2021). Resistance of *A. dhakensis* to erythromycin, amoxicillin, and ampicillin has also been reported (Soto-Rodriguez *et al*., 2018).

This study also revealed that AMX 2.3 isolate, CAP 2.1 isolate, CAP 2.3 isolate, and TET 2.1 isolate were suspected to be species of the genus *Bacillus*. Some species of *Bacillus* are involved in food poisoning, except for *B. anthracis* which causes anthrax (Tarek *et al*., 2021). Furthermore, some *Bacillus* sp. was found to have high resistance to β-lactams, fluoroquinolones, and tetracyclines (Mbhele *et al*., 2021).

Analyzing the position of CAP 2.2 isolate in the phylogenetic tree, we suggest that the isolate belongs to *Shigella* sp. Contamination of *Shigella* spp. to the water can be occurred through human and pet waste (Garcia *et al*., 2017). *Shigella* spp. is the most common bacterium that causes bacillary dysentery (shigellosis), a disease characterized by damage to the colonic mucosa caused by bacterial invasion (Garcia *et al*., 2017). This member of *Shigella* has also been reported to be resistant to several antibiotics, such as ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulfamethoxazole (Meiyanti *et al*., 2016). Again, a thorough investigation is required to prove it.

The TET 2.2 isolate was suspected to be *Aeromonas veronii* since this isolate stayed in the same clade with a convincing bootstrap value. *A. veronii* is a facultative anaerobic, gram-negative, and bacillus-shaped bacterium (Li *et al*., 2020). This species had been known to cause diarrhea and sepsis in humans (Fernández-Bravo *et al*., 2020). In particular, *A. veronii* is a common pathogen in aquaculture, which infects freshwater fish. Drug sensitivity tests showed that the *A. veronii* isolate strain 18BJ181 was resistant ~~to~~ against four antibacterial drugs, including amoxicillin, madinomycin, penicillin, and sulfamethoxazole, while quite sensitive to erythromycin and rifampin (Wang *et al*., 2021). Resistance to tetracyclines has also been reported in isolates of *A. veronii* which have been confirmed to have tetA and tetE genes (Kim *et al*., 2020).

The AMX 2.3, CAP 2.1, CAP 2.3, and TET 2.1 isolates, which were found to be Gram negative, phylogenetically formed an exclusive clade inside *Bacillus* genus big cluster which well known as Gram positive bacteria. Considering carefully the finding that these isolates were multi-antibiotic resistant, thus we suggest that both morphological and physiological changes have been occurred. Those changes might be caused by certain significant mutations. On the other hand, changes in bacterial colony morphology and cell shape found in this study, might be possible as a result of antibiotic treatment and the concentration given in this experiment as of reported by Peach *et al*. (2013) and the length of exposure (Cushnie *et al*., 2016). The morphology of *E. coli* bacterial cells exposed to the antibiotics after three hours showed a change in cell shape to resemble to the diplobacilli-like, diplococci, or forming a typical intracellular inclusion body (Dufour *et al*., 2017).

Overall, our findings in this study are still far from clear. More genetic studies including DNA Barcoding approaches using stronger markers, genetic analysis on responsible genes that may induce antibiotic resistance, and enzymatic bioassays are urgently required. Enzymatic analysis would confirm whether the isolates produce β-lactamase that makes it resistant. Meanwhile, genetic analysis which focusing on certain genes mutation may reveal how those isolates developed their resistance, especially those that became multi-antibiotic resistant while phylogenetically coexisting Gram-

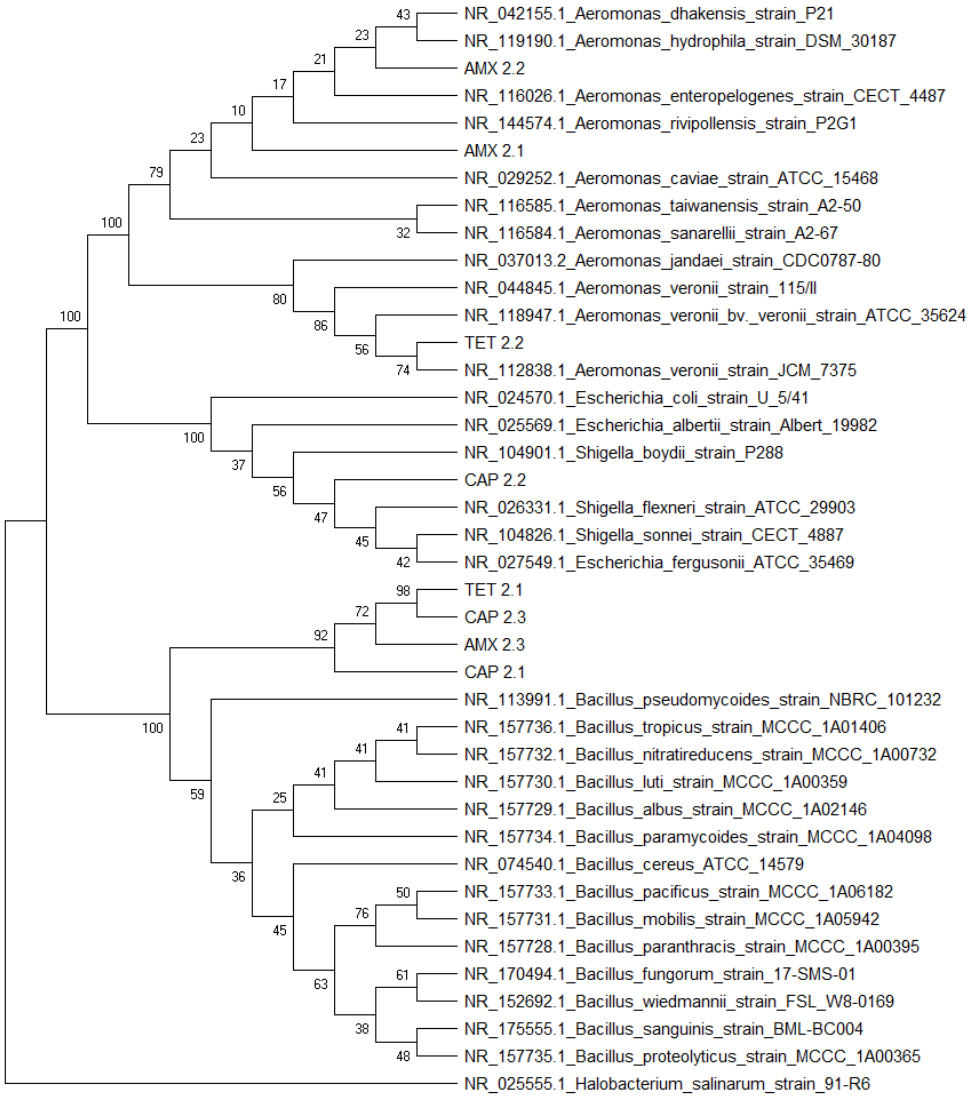

Figure 3. Isolates taxonomical position based on 16S rRNA in Neighbor Joining method

positive bacteria, may help to determine the possible mutations which lead to the resistance to the given antibiotic.

CONCLUSION

This study found eight isolates grew on LB media, three isolates grew on amoxicillin media, three isolates grew on chloramphenicol media, and two isolates grew on tetracycline media. Based on colony morphology and gram staining, we found that one isolate was multi-resistant to amoxicillin and tetracycline, and the other was multi-resistant against amoxicillin and chloramphenicol. Genetically, the isolates found were suggested to be members of *Aeromonas*, *Shigella*, and *Bacillus*. Further research is needed to ensure the accuracy of isolated species found.

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