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Synergisitc Effect of Antibiotics Against *Aeromonas* Isolates

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Abstract
Aeromonas species is a pathogenic bacteria and life threatening toward humans and animals. It has been implicated with several diseases including necrosis fasciitis, gastrointestinal and wound infections which can lead to death. This bacterial species is resistant to a wide range of antibiotics. Antibiotic-resistant bacteria can spread both directions between humans and animals. The use of antibiotics in agriculture and medicine has contributed to the development of several antibiotic-resistant pathogenic bacteria. Higher antibiotic concentration is required due to antibiotic resistance. Using several combinations of two antibiotics such as tetracycline with ciprofloxacin, ampicillin with chloramphenicol and ampicillin with tetracycline may help to reduce the emergence of resistant bacteria. The disc diffusion method was used for the antibiotic sensitivity test, and the zone of inhibition was used to determine if the bacteria were resistant, susceptible, or intermediate. After that, the next procedure was carried out using microdilution broth method, MBC analysis, and checkerboard assay method to investigate the combination of two antibiotics. To validate if the combination of antibiotics has a synergistic effect on bacteria, no interaction, or antagonistic effects, the fractional inhibitory index (FICI) value was calculated. According to the combination's data, all of the chosen combinations have an antagonistic effect on bacteria.
Keywords: *Aeromonas sp.*; *Aeromonas Sobria*; Minimum Inhibitory Concentration; Minimum Bactericidal Concentration; Checkerboard assay

Introduction

Antimicrobial resistance (AMR) is a worldwide pandemic that threat modern medicine (Krochmal & Wicher, 2021). Overuse and misuse of antibiotics endangered the efficacy of antibiotics have changed the medicinal history and saved millions of lives by the emergence of antibiotics resistance bacteria (Ventola, 2015). It was first noticed as a common medical condition in patients with hospital-acquired infections, especially those who were critically ill or extremely immunocompromised (Zain et al., 2021). There is a lack of effective medication treatment due to the slow pace, high cost, and low realistic sales prices of new antibiotic monotherapies.

Aeromonas species is from Aeromonads family and a gram-negative rod that are found in soil, foods, and the aquatic environment. They are hallophillic, nonacid fast, and nonspore forming (Spadaro et al., 2014). It can cause necrotizing fasciitis, acute gastroenteritis, hepatobiliary tract infection, pneumonia, empyema, meningitis, septic arthritis, osteomyelitis, endocarditis, bacteremia, 2 burn and wound infection in human. *A. sobria* that was infecting to the patients with predisposing factors such as B-cell acute lymphoblastic leukemia, diabetes mellitus and local wound trauma have cause death. These patients were treated with several combination of antibiotics before which is ceftazidime and teicoplanin, cefuroxime and gentamicin but it was not successful and they were died several days after

the antibiotic treatment (Spadaro et al., 2014).

Antibiotic combination therapy, which is employed by nature but is under-explored and could give a solution to AMR, is a new source of optimism. In order to cure patients with bacterial infections, it is important to not only actively find out new therapeutic strategies, but also to carefully choose antibiotics based on a range of criteria, including microbiological ones. In vitro levels of sensitivity or resistance of various bacterial strains to administered antibiotics are defined by the minimal inhibitory concentration (MIC). The ability to accurately measure MIC has a substantial impact on the therapeutic method chosen, which impacts the efficacy of infection treatment (Krochmal & Wicher, 2021).

Materials and methods

Two isolates of *Aeromonas. sp* that have been previously characterized to be resistant to several antibiotics through disk diffusion method from wastewater treatment plant at Taman Medini and Taman Selesa source were cultured from the glycerol stock. The glycerol stock of the isolates that stored at -80°C was removed with a sterile loop to obtain frozen bacteria off the top. Later on, it will be streaked on MHA and incubated overnight at 37°C (Yung, 2021).The antibiotic susceptibility profile of the strain *Aeromonas sp.* was determined using microdilution broth method on a 96-well plate, following Clinical and Laboratory Standards Institute (CLSI) criteria (Clinical and Laboratory Standards Institute, 2012). Ampicillin, chloramphenicol, ciprofloxacin, gentamicin, sulfafurazole and tetracycline were among the antibiotics used in this study. These antibiotics were chosen because of their broad spectrum of activity, which targets various elements of bacterial growth and resistance is likely to be displayed *Aeromonas sp.* from previous research.

The workflow was begin by making an antibiotic stock solution with a 50X concentration for ampicillin and a 30X concentration for the remaining antibiotics. Before using the microdilution broth method to determine the MIC, the antibiotic powder were dissolved in the suitable diluent and diluted to the working concentration required. Mueller–Hinton broth (MHB) were poured in each well of a 96-well plate. To obtain the appropriate working concentration, 2X higher concentration of each antibiotic were generated from the stock antibiotic solution and applied to the first well of each column, for example, well A1. Antibiotics were serially diluted two times towards column 10 (A1 to A10), with 100 ul of the combination discarded at the last well. To make the bacterium solution, inoculating colonies in sterile MHB was used to generate McFarland 0.5, which is equivalent to 1-2 x 108 cfu/ml. A spectrophotometer was used to verify the concentration of bacteria suspension at 600nm wavelength, where the absorbance should be between 0.08 and 0.15. (Wiegand, Hilpert & Hancock, 2008). The bacteria suspension then diluted 30 times, resulting 5 × 106 cfu/ml in each well. Columns 11 and 12 were the last two to be served. Row A to G contained each antibiotic tested as the sequence from ampicillin, chloramphenicol, ciprofloxacin, gentamicin, sulfafurazole, tetracycline, ampicillin to rifampicin. The range of each antibiotic tested were as follow: ampicillin (1024 - 2 µg/ml); chloramphenicol (128 - 1 4 µg/ml); ciprofloxacin (16 - 1 32 µg/ml); gentamicin (16 - 1 32 21 µg/ml); sulfafurazole (1024 - 2 µg/ml) and tetracycline (512 - 1 µg/ml). In the case where MIC could not be determined from the range, the concentration was further lowered down.

Classification of antibiotics' activity whether bactericidal or bacteriostatic was carried out by MBC after determination of MIC which the lowest concentration of antibiotic required to kill bacteria or reduces viability of initial bacterial inoculum 99.9% over an extended time (El-azizi, 2016). 4X MIC was required to record MBC where colony is observed on the highest MIC plate. The antibiotic then categorized as bacteriostatic antibiotic. By accessing FICI, MIC of antibiotics was carried out to determine the synergistic effect of double antibiotic combination. 96-well plates were used to perform this assay by 2 plates method with some modification (Xu et. al, 2018). To interpret the checkerboard assay, two different antibiotics were used such as tetracycline and ciprofloxacin . Dilution of tetracycline is from 8X MIC in horizontal direction from right to left which is column 8 to 2 by two-fold serial dilution method with 100 uL of mixture discarded at column 2. Preparation of ciprofloxacin dilutions from 4X MIC to 1/16 MIC is in microcentrifuge tubes by 2X serial dilution method and correspondingly added into designed wells on 96-well plates to obtain different proportions with

tetracycline. Determination of tetracycline MIC in row A meanwhile for ciprofloxacin MIC in column 1. Quality control segment with growth control and sterility control was performed in column 11 and 12. For column 9 and 10 will leave as blank. Suspension of bacteria is prepared in the same method as the previous and will be added into each well to achieve the final desired inoculum at 5 x 10^5 cfu/mL. To evaluate the combination effect of the antibiotics, FICIs values was calculated based on Equation 3.2. The interpretation of FICI is as followed where synergy, FICI ≤ 0.5; indifference, 0.5 < FICI ≤ 4; and antagonism, FICI ≥ 4.0 (Xu et al., 2018). The procedure of checkerboard assay was repeated for the combination of ampicillin with tetracycline and ampicillin with chloramphenicol.

Results and discussion

The Aeromonas isolates, MED(I)2 and TSE(E)2 has been previously characterized to antibiotics resistant. Both isolates were isolated from waste waster treatment plant where MED(I)2 was isolated from Taman Medini and TSE(E)2 was isolated from Taman Sejahtera (Yung, 2021). Broth microdilution method was used to accessed the MIC of the strains as described in CLSI M07. Figure 1 and figure 2, determination of MIC on 96-well plate are illustrated. Higher concentration of antibiotic in the first trial leads to suppresses growth of bacteria. The concentration needs to be adjusted to lower concentration for both Aeromonas isolates. Growth control in all wells column 11 shows pink colour formation while sterility control in all wells column 12 stay blue colour of resazurin. The susceptibility of the strains toward each antibiotic were determined by comparing MIC with breakpoints in CLSI M100 under section for other non-Enterobacteriaceae. The antibiotics susceptibility profile of both strains is tabulated in Table 1. MED(I)2 is sensitive to tetracycline, gentamicin and rifampicin. This isolate show lower activity toward ciprofloxacin, chloramphenicol, sulfafurazole and ampicillin. Thus, it indicates its resistant ability. For TSE(E)2 isolate, it is susceptible towards all antibiotics.tested except for ampicillin and sulfafurazole. Both strains show different susceptibility profile from the MIC determined. Therefore, this finding similar with previous study which stated Aeromonas isolates are chromosomally mediated resistant or intrinsic against ampicillin (Med. L. J, 2013). Different sources of bacteria were isolated is related to the different in antibiotic susceptibility profile. The concentration for individual antibiotics determined in the influent sample was lower than in the effluent sample, Kortesmäki. E. et al (2020). This may link to the sludge that contain antibiotic-resistant bacteria that was used as fertilizer on agriculture difference antibiotic susceptible profile is commonly observed even in the same to have a lower MIC values. However, the resistant strains have markedly higher MIC values (Rodloff, 2008).

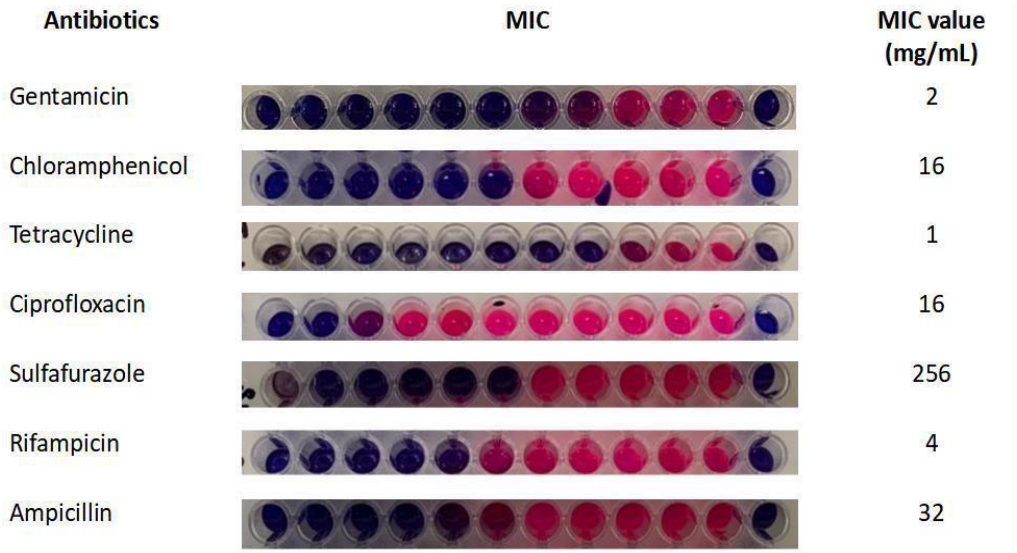

Figure 1: Minimum Inhibitory Concentration (MIC) for MED(I)2

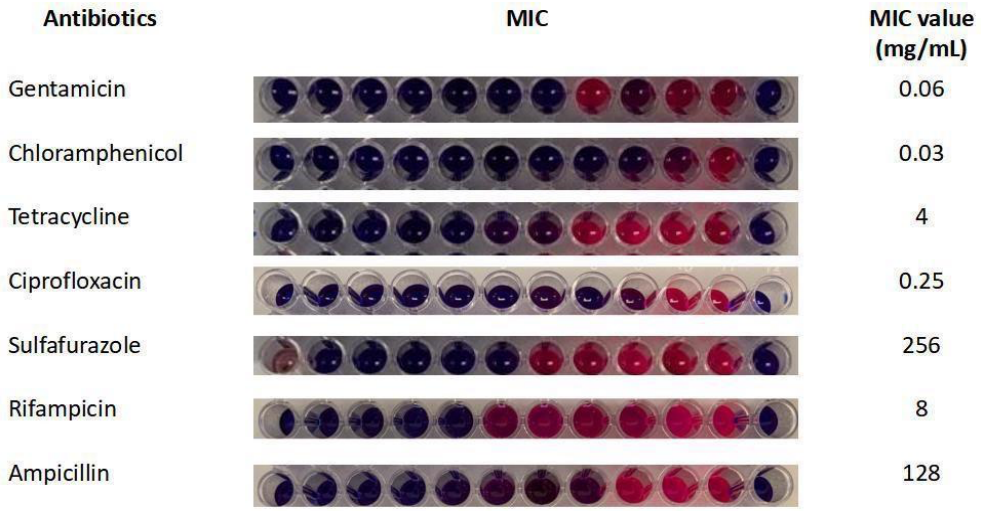

Figure 2: Minimum Inhibitory Concentration (MIC) for TSE(E)2 (A.sobria)

Table 1: MICs of *Aeromonas sp.* of MED(I)2 and TSE(E)2 isolate

Antibiotic	Minimum inhibitory concentration (ug/mL)			
	MED(I) 2	Susceptibility	TSE(E)2	Susceptibility
Gentamicin	2	N.A.	$\frac{3}{50}$	N.A.
Chloramphenicol	16	I	$\frac{3}{100}$	N.A.
Tetracycline	1	N.A.	4	R
Ciprofloxacin	16	S	$\frac{1}{4}$	N.A.
Sulfafurazole	256	R	256	R
Rifampicin	4	I	8	N.A.
Ampicillin	32	R	128	N.A.

*N.A. indicates for that particular data is not applicable. S: susceptible; I: intermediate; R: resistant

Table 2 shows the summary of antibiotics tested on *Aeromonas sp.* of MED(I)2 and TSE(E)2 isolates that classified into bactericidal or bacteriostatic by determining the MBC. MBC is the lowest concentration required for an antibiotic to kill a bacterium or reduces viability of the initial bacterial inoculum 99.9% over an extended period (El-Azizi, 2016). The antibiotic gives bactericidal effect toward bacteria if the MBC is less than 4X MIC meanwhile the antibiotic gives bacteriostatic effect toward bacteria if the MBC is more than 4X MIC (Panthong et al., 2020). The definition of bactericidal is when the antibiotic able to kill the bacteria. For bacteriostatic, the antibiotic keeps the bacteria in the stationary phase. It means that the antibiotic able to suppress or prevent the growth of bacteria (Bernatova. S. et al., 2013). From table, all antibiotics show bacteriostatic effect toward MED(I)2 isolate meanwhile only tetracycline and gentamicin act as bactericidal toward TSE(E)2 isolate. Ampicillin, rifampicin, chloramphenicol, ciprofloxacin and sulfafurazole show bacteriostatic effect toward this isolate.

Table 2: Summary table of MIC, MBC and antibiotics

Antibiotics	MED(I)2			TSE(E)2		
	MIC (ug/ml)	MBC (ug/ml)	Antibiotic activity	MIC (ug/ml)	MBC (ug/ml)	Antibiotic activity
Gentamicin	2	>8	Bacteriostatic	$\frac{3}{50}$	$\frac{1}{4}$	Bactericidal
Chloramphenicol	16	>64	Bacteriostatic	$\frac{3}{100}$	$>\frac{1}{8}$	Bacteriostatic
Tetracycline	1	>4	Bacteriostatic	4	8	Bactericidal
Ciprofloxacin	16	>64	Bacteriostatic	$\frac{1}{4}$	>1	Bacteriostatic
Sulfafurazole	256	>1024	Bacteriostatic	256	>1024	Bacteriostatic
Rifampicin	4	>16	Bacteriostatic	8	>32	Bacteriostatic
Ampicillin	32	>128	Bacteriostatic	128	>512	Bacteriostatic

Checkerboard assay method applied to combine two different antibiotics in order to increase the concentration to provide a final classification of the combined antibiotics based on FICI. The FICI value of synergy is ≤ 0.5, indifferent range is between 0.5 – 4 and antagonism ≥ 4 (Costa et al., 2019). Discovering combinations of antibiotics can be beneficial as they may act synergistically hence improve the bactericidal activity of one antibiotic alone. Three double antibiotic combinations each was carried out to determine the synergism of antibiotics against *Aeromonas sp*., MED(I)2 and TSE(E)2 isolate. The combination of both isolates is based on previous study that proved tetracycline has improved its activity significantly with ampicillin and chloramphenicol. The FICI and interpretation on double antibiotic combination effects are shown in Table 3. From the result of checkerboard assay obtained, all antibiotics show antagonistic effect toward both MED(I)2 and TSE(E)2 isolate. Antagonistic effect can occur because the first drug may defeat or suppress the activity of the second drug which means the susceptible bacteria can grow in the presence of the drug if high concentration of the second drug is present. Once bacteria develop resistance to the first drug, the suppressive effects of the second drug are removed, resulting in a concentration regime where susceptible bacteria will outgrow resistant bacteria while both drugs are present. In addition, if the bacteria develop specific resistance mutations, antagonistic drugs that fail to kill susceptible bacteria can become synergistic (Baym et al., 2016).

Table 3: Double antibiotic combinations with FICI of each combinations

Aeromonas sp. strain	Combination			FICI	Interpretation
MED(I)2	No.	Antibiotic A	Antibiotic B		
	1	Ampicillin	Chloramphenicol	10	Antagonism
	2	Ampicillin	Tetracycline	66	Antagonism
	3	Tetracycline	Ciprofloxacin	4	Antagonism
TSE(E)2	1	Ampicillin	Chloramphenicol	12802	Antagonism
	2	Ampicillin	Tetracycline	66	Antagonism
	3	Tetracycline	Ciprofloxacin	2007	Antagonism

Conclusion

The objectives of this paper are to identify the antibiotic susceptibility profile of Aeromonas sp. of MED(I)2 and TSE(E)2 isolates previously isolated from WWTPs samples respectively towards different classes of antibiotics include gentamicin, chloramphenicol, tetracycline, sulfafurazole, ciprofloxacin, rifampicin and ampicillin. Checkerboard assay was proceeded after obtaining the **r** value

of MIC to identify the double antibiotic combinations that could result synergistic effect to inhibit the growth of both bacterial strains. The test revealed that Aeromonas sp. of MED(I)2 isolate shows resistant to sulfafurazole, intermediate to chloramphenicol and susceptible to ciprofloxacin. Otherwise, TSE(E)2 isolate was resistant to tetracycline and sulfafurazole. This isolate susceptible to all antibiotics with known breakpoints including gentamicin, chloramphenicol and ciprofloxacin. However, there are no available antibiotic susceptibility breakpoints for ampicillin and rifampicin in CLSI M100. Thus, these two antibiotics have antibiotic susceptibility based on previous study. Moreover, same species of bacteria not necessarily to have same antibiotic susceptibility profile because every source of isolation has different antibiotic resistant bacteria. For example, MED(I)2 isolate was susceptible toward tetracycline but not for TSE(E)2 isolate. Determination of antibiotics activities by MBC are necessary to ensure either it is bactericidal or bacteriostatic. All antibiotics were bacteriostatic toward MED(I)2 isolate meanwhile for TSE(E)2 isolate, the antibiotic showed bacteriostatic activity to all antibiotics except for tetracycline and gentamicin. The overgrowth of bacteria of MED(I)2 proved that it has high functional immune system as a host. The synergistic effect of double antibiotics was determined by checkerboard assay method. All antibiotics demonstrate antagonism effect toward both MED(I)2 isolate and TSE(E)2 isolate with an extremely high FICI which values more than 4. To sum up, this study gives an idea the antibiotic combination could solve the problem of multiple antibiotic resistance bacteria as it is rising as a health concern that requires immediate action to be taken in order to prevent an increase in mortality rate resulting from bacterial infection. Identification of antibiotic combination that works on them are highest priority in the present time. Therefore, it is crucial to figure the antibiotic combinations by in-vitro analysis before trying in-vivo.

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