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Synergistic Effect of Antibiotic Against *Aeromonas media* and *Aeromonas caviae*

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Abstract
The focus of this research is to provide knowledge on previously undiscovered synergy effects against *Aeromonas media* and *Aeromonas caviae*. Nowadays, there are lot of diseases cause by the bacteria necessitating antibiotics as a treatment to treat bacterial infections. However, bacteria may develop resistance toward antibiotics if it were misuse or overuse. Because of this event, antibiotics are becoming increasingly ineffective as antibiotic resistance grows over the world. Not only restricted to one antibiotic but more than two antibiotics. Thus, making infections more difficult to be treated, hence, lead to increasing mortality rate. Therefore, combination of antibiotics treatment has been proposed over monotherapy to treat bacterial infections. Furthermore, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were done in advance before determination of synergistic effect by checkerboard assay on *Aeromonas* isolates such as *A.media* isolate and *A.caviae* isolate. The strains that used for this research were isolated from wastewater treatment plant in Johor. The susceptibility profile of both isolates showed that they were resistant toward ampicillin and rifampicin. Meanwhile, all antibiotics tested were bacteriostatic toward *A. media* isolate and *A. caviae* isolate. Above all, synergistic result obtained from this research was antagonism. Overall, this research shows how combining antibiotics can fight multidrug-resistant microorganisms. Antibiotic resistance threatens global health, food security, and development. Finding effective antibiotic combinations is a top priority.
Keywords: *Aeromonas media*; *Aeromonas caviae*; Minimum Inhibitory Concentration; Minimum Bactericidal Concentration; Checkerboard assay

Introduction

Antibiotics are drugs that are used to treat infections caused by bacteria in many medical conditions such as urinary tract infection, gastroenteritis, and pneumonia (Haque et al., 2019). Unfortunately, every time antibiotics are misused or overused; they can contribute to antibiotics resistance (AMR). The reasons behind the increasing number in antibiotic resistance are driven by poor antibiotic degradations (Zain et al., 2021). As a consequence of this event, antibiotics are becoming increasingly ineffective as antibiotic resistance grows over the world, making infections and death more difficult to treat (WHO, 2021). At least 2.8 million people in the United States are infected with antibiotic-resistant bacteria or fungi each year, with more than 35,000 people died as a result (CDC, 2020).

Aeromonas is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria that resemble members of the Enterobacteriaceae family morphologically. *Aeromonas* species can be found in a variety of aquatic settings around the world, including rivers, lakes, and drinking water treatment plants and distribution systems. In addition, meat and dairy products include the majority

of pathogenic *Aeromonas* species (Drancourt, 2017). There are now 36 species in this genus, two of them are *Aeromonas caviae* and *Aeromonas media* (Lamy et al., 2022). Besides, both of these species lead to several diseases such as infections in fish, human diarrheal and wound infections (Baron et al., 2017). Moreover, there are many virulence factors, including hemolysins, enterotoxins, cytotoxins, and adhesins, are produced by various species of *Aeromonas*, and these factors have been linked to the pathogenicity of this microorganism (Martins et al., 2002).

This study focused on both *Aeromonas caviae* and *Aeromonas media* which they had been resistant to several antibiotics (Azzam-Sayuti et al., 2021). Furthermore, the objectives of this study were to provide understanding on the previously synergy effect of antibiotics agents against *A. media* and *A. caviae* isolates. The severe bacterial infections caused by *A. caviae* and *A. media* strains are typically difficult to treat with antibiotics, and the process by which the genus *Aeromonas* develops resistance while infecting human hosts is a topic that must be addressed as soon as possible.

Materials and methods

Two isolates of *A. caviae* and *A. media* that have been previously characterized to be resistant to several antibiotics through disk diffusion method from wastewater treatment. Source were cultured from the glycerol stock. The glycerol stock of the isolates that stored at -80°C was scraped with a sterile loop to obtain frozen bacteria off the top. Later, it was streaked on Mueller Hinton agar (MHA) and incubated overnight at 37°C (Yung, 2021).

It is necessary to apply "Clinical breakpoints" to investigate the antimicrobial susceptibility of *Aeromonas spp*. To interpret an in vitro measurement or estimate of the Minimal Inhibitory Concentration (MIC), clinical breakpoints must be used (Baron et al., 2017). The antibiotics used in the study were chloramphenicol, ciprofloxacin, tetracycline, gentamicin, ampicillin, rifampicin and sulphafurazole. The choices of antibiotics were due to their broad spectrum of activity that targets different aspects of bacterial growth, and the resistance was expected to be demonstrated by *Aeromonas spp*. as according to previous study. Firstly, the antibiotics stocks solution of 50X concentration for chloramphenicol and 30X concentration for the rest of antibiotics were prepared. The antibiotics powder was dissolved in respective diluent and was diluted to working concentration needed before determination of MIC by broth microdilution method.

Mueller–Hinton broth (MHB) were placed in every well in a 96-well plate from column 1 to 12. 2X higher concentration of each antibiotic was prepared from the stock antibiotic solution and was added to the first well of each column, for example well A1, to yield the working concentration desired. Two- fold serial dilution of antibiotics towards column 10 (A1 to A10) was carried out with 100 µl mixture discarded at the last well. The bacteria suspension was prepared by inoculating colonies in sterile MHB to obtain McFarland 0.5 that is equivalent to 1-2 x 108 cfu/ml. The concentration of bacteria suspension was checked with a spectrophotometer at 600 nm wavelength where the absorbance shall fall between the range 0.08 – 0.15 (Wiegand, Hilpert & Hancock, 2008).

Next, the bacteria suspension was diluted 30X to yield 5 x 106 cfu/ml. The final concentration of bacteria 5 x 105 cfu/ml was achieved when 10 µl of bacteria suspension was inoculated into each well containing 50 µl of antibiotics and 50 µl of MHB. A replicate 96-well plate was carried out with the same concentration and content of each well. The last two columns, columns 11 and 12 served as a quality control segment of growth control and sterility control, respectively. Growth control column contained 10 µl of bacteria suspension with 100 µl of MHB without addition of antibiotic agents while the sterility control column contained MHB.

Finally, the 96-well plate was wrapped with aluminum foil to prevent drying and placed in an air incubator at 35±2°C for 16 to 20 hours within 15 minutes of adding the inoculum. Following the incubation period, 5 µl resazurin at a concentration of 6.75 mg/ml will be added to each well and incubated for an additional 4 hours to complete the colour development. As a dye, resazurin can be reduced by the bacteria cell to a pink fluorescence known as resofurin, which is produced by the oxidoreductase found in live cells (Teh et al., 2017). After the incubation period, pink colour formation indicated the presence of bacteria cells while blue to purple colour formation indicated absence of bacterial cells. MIC of the antibiotic agents against the bacteria will be able to be identified which is the

lowest concentration of antibiotic agents that were effective in inhibiting bacterial growth that was the first well before a pink well. The antibiotics susceptibility of the bacteria was identified when MIC was compared with the breakpoint value in CLSI standard of M100-Performance Standards for antimicrobial susceptibility testing on whether the bacteria is resistant, intermediate, or susceptible to the antibiotics (Yung, 2021).

After MIC was determined, to be bactericidal, an antimicrobial medication must have a minimum bactericidal concentration (MBC). Broth diluents that restrict growth of bacteria can be detected by subculturing them (re-culturing) (Sykes & Rankin, 2014). The method was as described by (El-Azizi, 2016). The schematic diagram of subculturing from the 96-well. Generally, 10 µl portions of MIC, 2X MIC and 4X MIC from 96- well plates incubated with resazurin were taken and spread onto MHA and further incubated overnight. MBC was recorded at the concentration where no colony growth was observed on the plate and the antibiotic was said to be a bactericidal antibiotic; MBC was recorded higher than 4X MIC where colony growth was observed on the highest MIC plated and the antibiotic was said to be a bacteriostatic antibiotic.

Checkerboard assay as referred to the MICs of antibiotics was carried out to determine the synergistic effect of double antibiotic combination by accessing FICI. The assay was performed on 96- well plates by 2 plates method as according to the method described by Xu et al. (2018) with some modification. Combination of tetracycline/ciprofloxacin was used as an example for interpreting the checkerboard assay where tetracycline represents antibiotic A and ciprofloxacin represents antibiotic B. Briefly, tetracycline dilutions from 8X MIC to 1/8 MIC in the horizontal direction from right to left (column 8 to 2) was carried out by two-fold serial dilution method with 100 µl of mixture discarded at column 2. Ciprofloxacin dilutions from 4X MIC to 1/16 MIC were prepared in microcentrifuge tubes by two-fold serial dilution method and were correspondingly added into the designed wells on the 96-well plates to obtain different proportions with tetracycline. Row A served as MIC determination of tetracycline while column 1 served as MIC determination of ciprofloxacin. Columns 11 and 12 served as quality control segment with growth control and sterility control, respectively. Columns 9 and 10 were leaved blank. Bacteria suspension was prepared in the same method as previously and was added into each well to achieve the final desired inoculum at 5 x 105 cfu/ml. The incubation period of the tray will be set at 35±2°C for 16 to 20 hours. 5 µl resazurin was added into each well to observe colour formation and was interpreted in the same manner as previously. To evaluate the combination effect of the antibiotics, FICIs values will be 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).

Results and discussion

Both *A. media* isolate and *A. caviae* isolate are sensitive towards all seven antibiotics tested. Both isolates have a similar antibiotic susceptibility profile, as indicated by their MIC values, which are susceptible to chloramphenicol, ciprofloxacin, gentamicin and sulphafurazole. The antibiotic susceptibility profile for both of *A. media* and *A. caviae* isolates dissimilar for tetracycline. *A. caviae* showed intermediate response towards tetracycline while *A. media* illustrated susceptible toward tetracycline. Hence, the purple colour indicated intermediate response occur. The difference in antibiotic susceptibility profile can be related back to the source from which the bacteria was isolated. Both *A. media* and *A. caviae* isolates were isolated from wastewater treatment plant in Skudai, Johor Bahru. In this context, some antibiotic agents particularly ciprofloxacin, ampicillin, and tetracycline would use in wastewater treatment plant (Rodriguez-Mozaz et al., 2020). The susceptibility MIC of the strains were observed by broth microdilution method as described in CLSI M100 and shown in Table 1 and Table 2.

Table 1: Susceptibility MIC of *A. media* isolate

ANTIBIOTIC	MIC RESULT	MIC VALUE (µg/ml)	SUSCEPTIBILITY
GENTAMICIN		0.0156	S
CHLORAMPHENICOL		0.0156	S
CIPROFLOXACIN		0.0156	S
SULPHAFURAZOLE		8	S
TETRACYCLINE		0.5	S
AMPICILLIN		1024	R
RIFAMPICIN		64	R

*S: susceptible; I: intermediate; R: resistant

Table 2: Susceptibility MIC of *A. caviae* isolate

ANTIBIOTIC	MIC RESULT	MIC VALUE (µg/ml)	SUSCEPTIBILITY
GENTAMICIN		0.0156	S
CHLORAMPHENICOL		0.0156	S
CIPROFLOXACIN		0.0156	S
SULPHAFURAZOLE		8	S
TETRACYCLINE		0.5	S
AMPICILLIN		1024	R
RIFAMPICIN		64	R

*S: susceptible; I: intermediate; R: resistant

Antibiotic tested on *A. media* and *A. caviae* isolates are classified into bactericidal or bacteriostatic by determining the Minimum Bactericidal Concentration (MBC) as tabulated in Table 3 and Table 4. MBC can be used to determine the activity of antibiotics, where MBC less than 4X MIC indicates a bactericidal antibiotic and MBC greater than 4X MIC indicates a bacteriostatic antibiotic. MBC was done on MHA plates for both isolates with MIC, 2X MIC and 4X MIC. Besides, in Table 3 and Table 4 demonstrate all seven antibiotics (gentamicin, chloramphenicol, ciprofloxacin, sulphafurazole, tetracycline, ampicillin and rifampicin) were bacteriostatic antibiotics. Hence, there were positive growth observed at all dilution plated up to 4X MIC.

Table 3: MBC and antibiotic activity of *A. media* isolate

Antibiotics	Dilution			MBC (µg/ml)	Activity
	MIC	2X MIC	4X MIC		
Gentamicin	+	+	+	>0.0625	Bacteriostatic
Chloramphenicol	+	+	+	>0.0625	Bacteriostatic
Ciprofloxacin	+	+	+	>0.0625	Bacteriostatic
Sulphafurazole	+	+	+	>32	Bacteriostatic
Tetracycline	+	+	+	>2	Bacteriostatic
Ampicillin	+	+	+	>4096	Bacteriostatic
Rifampicin	+	+	+	>512	Bacteriostatic

* + indicates growth; - indicates no growth.

Table 4: MBC and antibiotic activity of *A. caviae* isolate

Antibiotics	Dilution			MBC (µg/ml)	Activity
	MIC	2X MIC	4X MIC		
Gentamicin	+	+	+	>0.25	Bacteriostatic
Chloramphenicol	+	+	+	>2	Bacteriostatic
Ciprofloxacin	+	+	+	>8	Bacteriostatic
Sulphafurazole	+	+	+	>512	Bacteriostatic
Tetracycline	+	+	+	>32	Bacteriostatic
Ampicillin	+	+	+	>2048	Bacteriostatic
Rifampicin	+	+	+	>512	Bacteriostatic

* + indicates growth; - indicates no growth.

The checkerboard assay uses increasing concentrations of two antibiotics to classify the combined antibiotics based on their FICI. Moreover, checkerboard synergy assay results were interpreted using the fractional inhibitory concentration index as the determining factor (FICI) (Bonapace et al., 2002). FICI of ≤ 0.5 indicates synergy, FICI > 0.5 or ≤ 4 means indifferent and FICI ≥ 4.0 implies antagonism (Costa et al., 2019). Therefore, three double antibiotic combinations each was carried out to determine the synergism of antibiotic towards A. media isolate and A. caviae isolate. The combination of antibiotics for both isolates were based on the antibiotic susceptibility profile where the combinations were done between resistant and susceptible, resistant and intermediate, and intermediate and susceptible response antibiotics for A. media isolate and A.caviae isolate. From the result checkerboard assay, all three combinations of the antibiotics for both isolates showed antagonism affects with FICI value which was greater than 4. Table 5 tabulate double antibiotic combinations with FICI of each combination.

Table 5: Double antibiotic combinations with FICI of each combination

Isolate	Combination			FICI	Interpretation
	No.	Antibiotic A	Antibiotic B		
A. media	1	Ampicillin	Chloramphenicol	>4	Antagonism
	2	Ampicillin	Tetracycline	>4	Antagonism
	3	Tetracycline	Ciprofloxacin	>4	Antagonism
A. caviae	1	Ampicillin	Chloramphenicol	>4	Antagonism
	2	Ampicillin	Tetracycline	>4	Antagonism
	3	Tetracycline	Ciprofloxacin	>4	Antagonism

Conclusion

The MICs obtained was proceeded to checkerboard assay to identify the double antibiotic combinations that could result in synergistic effect to inhibit the growth. Furthermore, from the determination of antibiotic susceptible profile, the test revealed that *A. media* and *A. caviae* isolates were susceptible to all antibiotics with known breakpoints including chloramphenicol, ciprofloxacin, gentamicin, and sulphafurazole. However, antibiotic susceptibility for tetracycline demonstrates intermediate in *A. caviae*. On the contrary, both isolates show resistant to ampicillin and rifampicin. Besides, the classification of antibiotics into their activities of either bactericidal or bacteriostatic was done by determining MBC. All the antibiotics tested showed bacteriostatic activity towards both isolates. Double antibiotic combination was tested in a checkerboard assay to see if there was a synergistic response. Unfortunately, it turned out all three combinations on both isolates were antagonism effect with FICI greater than 4. Further validation by time kill assay cannot be done as synergism was not achieved.

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