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Comparison of synergistic effects of multiple combinations of anti-*pseudomonas* antibiotics against *Pseudomonas aeruginosa* pan drug resistance in *in vitro* test with AZDAST Method

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ABSTRACT

Background: Antimicrobial resistance is a global threat to public health. *Pseudomonas aeruginosa Pan Drug-Resistant* requires the latest antimicrobials. This limitation of therapy requires a breakthrough in the treatment of this infection. Combining antimicrobials with a synergistic effect is thought to increase the cure rate in clinical use. This study aimed to determine the synergistic effect of several combinations of *anti-pseudomonas* antibiotics against *Pan Drug-Resistant Pseudomonas aeruginosa* in an *in vitro* test using the *AZDAST* method.

Method: An Antibiotic combination test was carried out using the *AZDAST* method to assess *in vitro* synergistic activity. The antibiotic single disk used was *Amikacin 30 µg, Ceftazidime 30 µg, Meropenem 10 µg, Ciprofloxacin 5 µg*; double disk antibiotic is *Amikacin 30 µg, Ceftazidime 30 µg, Meropenem 10 µg, Ciprofloxacin 5 µg* and combination antibiotic disks *Amikacin 30 µg-Ceftazidime 30 µg, Amikacin 30 µg-Meropenem 10 µg, Amikacin 30 µg-Ciprofloxacin 5 µg* in which two antibiotic paper disks are combined stacked together, with a 24 mm gap between the other antibiotic combinations.

Result: The results showed that combining the four antibiotics had a synergistic effect. The zone of inhibition resulting from testing the combination of several antibiotics against *Pan Drug Resistant Pseudomonas aeruginosa* showed no statistical significance ($p > 0.05$) compared to all antibiotics and comparisons in the combination group of antibiotics only.

Conclusion: The combination of *anti-pseudomonas* antibiotics synergizes with *Pan Drug-Resistant Pseudomonas aeruginosa* in the *AZDAST* method *in vitro* test.

Keywords: *Pseudomonas aeruginosa*, *Pan Drug-Resistant*, Combination antibiotic, the AZDAST method.
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BACKGROUND

Antimicrobial resistance is a global threat to public health. 1.2 Data for 2019, there were 2.8 million people infected with antibiotic-resistant bacteria, and 35,000 deaths caused by these bacteria.[1] In February 2017, WHO published a list of pathogens with a high priority to resistance. The list of referenced pathogens is ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) which are designated as "priority status".[1,2] National data for 2018 of 4873 *Pseudomonas aeruginosa* isolates found 1625 (33%) were resistant to antibiotic drugs.[3] Regional General Hospital Data Dr. Saiful Anwar Malang in 2021 from the ICU, 15.5% of 349 samples were positive for *Pseudomonas aeruginosa* infection.[4]

Pseudomonas aeruginosa is an organism that is very difficult to control with antibiotics or disinfectants, this bacterium very easily forms resistance due to several things such as the formation of resistance to antimicrobial agents due to low cell wall permeability, has the genetic capacity to express a wide repertoire of resistance mechanisms, and can acquire additional resistance genes from other organisms via plasmids, transposons, and bacteriophages or phages.[5] The management of resistant *Pseudomonas aeruginosa* bacteria requires up-to-date antimicrobials. The last recommended line of antimicrobials are colistin and polymyxin B, however, these antibiotics should not be given monotherapy because they have the potential to cause resistance to these antibiotics.[6] This limitation of therapy requires a breakthrough in treating this infection. The combination of antimicrobials with a synergistic effect is thought to increase the cure rate in clinical use.[7]

The antimicrobials tested in this study included the aminoglycoside group, namely Amikacin, the third generation cefalosporin group here, namely

Ceftazidime, the fluoroquinolone group, namely Ciprofloxacin, and the carbapenem group, namely Meropenem. The four antimicrobials were chosen because they were active *in vitro* against the bacterium *Pseudomonas aeruginosa* and came from four different classes of antibiotics.[8]

The study used the *Ameri-Ziaei Double Antibiotic Synergism Test (AZDAST)* method. This method is a new method and the development of clinical microbiology in evaluating antimicrobial synergism. This method is carried out using routinely available laboratory materials and using daily test procedures carried out, as well as easy-to-understand interpretations.[9] The size of the growth inhibition zone is affected by the depth of the agar, because antimicrobials diffuse in three dimensions, so the depth of the disc on the agar media will produce a zone.[9,10] So this study aims to see whether several combinations of *anti-pseudomonas* antibiotics synergistically affect *Pseudomonas aeruginosa Pan Drug Resistant* in the *AZDAST* method *in vitro* test.

MATERIALS AND METHODS

Design, Time and Place of Research

This research was conducted in an experimental laboratory randomization *in vitro*. This research was conducted at the Clinical Microbiology Laboratory of RSUD. Dr. Saiful Anwar Malang and Laboratory of Microbiology, Faculty of Medicine, University of Brawijaya Malang, in August - September 2022.

Sampling Technique

The sampling technique in this study used a random sampling method for each treatment on the *Pan Drug-Resistant Pseudomonas aeruginosa* bacteria. Enter the inclusion and exclusion criteria.

Diffusion Agar Disk with AZDAST Method

The *Ameri-Ziaei Double Antibiotic Synergism Test (AZDAST)* is the latest antimicrobial synergism or interaction evaluation method developed based on the important role of the laboratory in testing antibiotic combinations. This method can be categorized as a double disk diffusion antibiotic synergism test. In the AZDAST method, the diameter of the bacterial

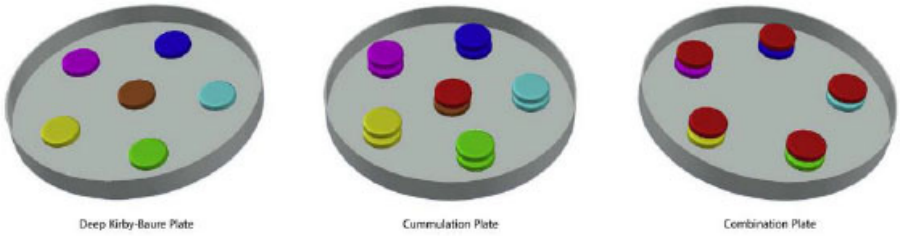

Figure 1. *Arrangement of antibiotic disks in the AZDAST petri dish.*

Table 1. Test results for the *Amikacin-Meropenem* antibiotic combination

Antibiotic Testing	Mean ± SD	Effect
Amikacin 30 µg	10,56 ± 9,65*	
Meropenem 10 µg	0*	
Amikacin 30 µg –*Amikacin* 30 µg	15 ± 2,31*	**Synergistic**
Meropenem 10 µg –*Meropenem* 10 µg	0*	
***Amikacin* 30 µg –*Meropenem* 10 µg**	**26,31 ± 3,41***	

Description: * significant at p <0,05

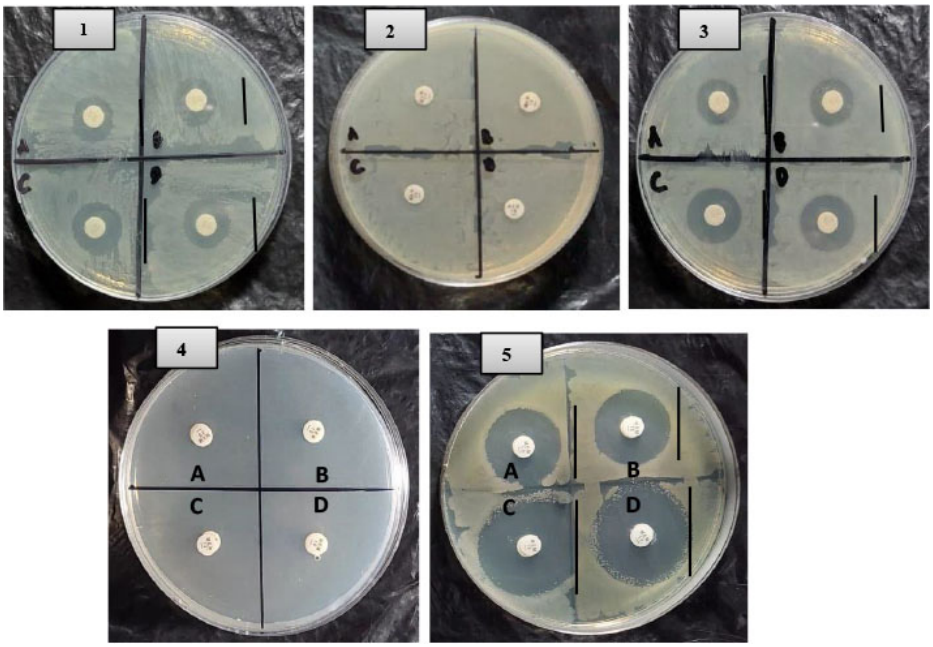

Figure 2. An Inhibition zone formed in the antibiotic test on Pan Drug-Resistant Pseudomonas aeruginosa. (1) A zone of inhibition was formed in the Amikacin 30 µg test. (2) No zone of inhibition was formed in the 10 µg Meropenem test. (3) An inhibition zone was formed in the Amikacin 30 µg–Amikacin 30 µg test. (4) No zone of inhibition was formed in the Meropenem 10 µg –Meropenem 10 µg test. (5) A zone of inhibition was formed in the Amikacin 30 µg –Meropenem 10 µg test.

Table 2. Test results for the *Amikacin-Ceftazidime* antibiotic combination

Antibiotic Testing	Mean ± SD	Effect
Amikacin 30 µg	10,56 ± 9,65*	
Ceftazidime 30 µg	1,5 ± 3*	
Amikacin 30 µg –*Amikacin* 30 µg	15 ± 2,31*	**Synergistic**
Ceftazidime 30 µg – *Ceftazidime* 30 µg	2,75 ± 5,5*	
***Amikacin* 30 µg - *Ceftazidime* 30 µg**	**25,12 ± 2,22***	

Description: * significant at p <0,05

growth inhibition zone is the same as in other disk diffusion methods.[11]

This method is carried out by preparing sterile loops and transferring several bacterial isolates from solid media to a test tube containing sterile 0.9% NaCl. Vortexing was carried out for bacterial homogenization with a micropipette, samples were taken and read using a spectrophotometer or a nephelometer (0.5 McFarland).

Mueller-Hinton media was made according to the instructions from the media manufacturer. Separate 10 ml in a separate tube before autoclaving, then sterilize using an autoclave at 121˚C for 15 minutes. Arrange on the bottom of the petri disk plate for the antibiotic disk to be tested according to the picture.

The petri dish for a single antibiotic disk consists of 4 discs, including Ceftazidime 30 µg, Meropenem 10 µg, Ciprofloxacin 5 µg, and Amikacin 30 µg, with a distance of 24 mm between the other antibiotics. Petri dishes for double antibiotic disks consist of 4 types of double antibiotic disks, including Ceftazidime 30 µg + Ceftazidime 30 µg, Meropenem 10 µg + Meropenem 10 µg, Ciprofloxacin 5 µg + Ciprofloxacin 5 µg, Amikacin 30 µg + Amikacin 30 µg, in 2 ways Antibiotic paper disks are stacked together and spaced 24 mm between double antibiotic disks. Petri dishes for combination antibiotic disks consist of 3 types of combination antibiotic disks Amikacin 30 µg + Ceftazidime 30 µg, Amikacin 30 µg + Meropenem 10 µg, Amikacin 30 µg + Ciprofloxacin 5 µg, using 2 combined antibiotic paper disks stacked together and spaced 24 mm between antibiotic combinations. Paste the antibiotic on the floor of the inner petri dish using 10 ml of Mueller-Hinton so that it is still liquid which has been separated and sterilized so that it does not move or float during Mueller-Hinton so that the other is poured into the petri dish until it fills half the height of the cup (approximately 20 - 25 ml in a 90 mm cup).

Then incubated at 37˚C for 18-24 hours with the petri dish upside down. The interpretation was carried out after incubation by observing and measuring the zone of inhibition. If a clear zone is found around the disc, it indicates that the antibiotic being tested can inhibit bacterial growth. The zone is measured in diameter with a ruler and reported in mm. According to the Clinical and Laboratory Standards Institute edition M100-S25 (CLSI), the level of inhibition of bacteria can be categorized into susceptibility, intermediate, and resistance.

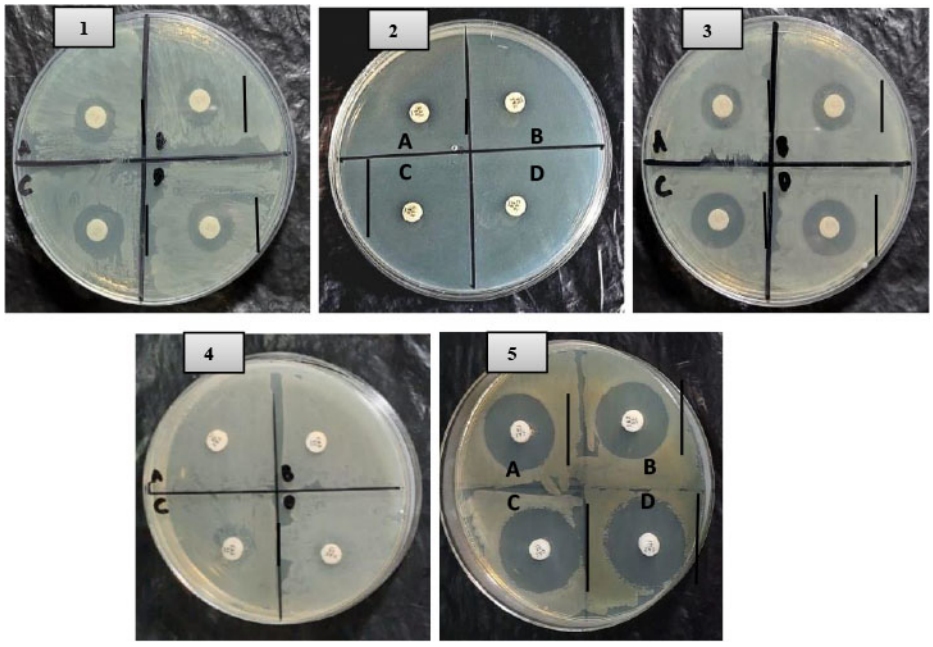

Figure 3. An inhibition zone formed in the test on *Pan Drug-Resistant Pseudomonas aeruginosa* bacteria. (1) A zone of inhibition was formed in the Amikacin 30 µg test. (2) A zone of inhibition was formed in the Ceftazidime 30 µg test. (3) A zone of inhibition was formed in the Amikacin 30 µg–Amikacin 30 µg test. (4) An inhibition zone was formed in the Ceftazidime 30 µg – Ceftazidime 30 µg test. (5) A zone of inhibition was formed in the Amikacin 30 µg - Ceftazidime 30 µg test.

Table 3. Test results for the *Amikacin-Ciprofloxacin* combination of antibiotics

Antibiotic Testing	Mean ± SD	Effect
Amikacin 30 µg	10,56 ± 9,65*	
Ciprofloxacin 5 µg	0*	
Amikacin 30 µg –*Amikacin* 30 µg	15 ± 2,31*	**Synergistic**
Ciprofloxacin 5 µg –*Ciprofloxacin* 5 µg	3 ± 6*	
***Amikacin* 30 µg -*Ciprofloxacin* 5 µg**	**23,12 ± 2,25***	

Description: * significant at p <0,05

Statistical Data Analysis

The results were evaluated using a ruler with an accuracy of 1 mm and processed using the Statistical Package for the Social Sciences (SPSS) to obtain the mean and standard deviation. The combination antibiotic sensitivity test was tested using One-Way ANOVA and Post Hoc Tukey on normally distributed data or by Kruskal Walis and Mann Whitney on non-normally distributed data to determine comparisons. Results were evaluated using the AZDAST method, results were said to be synergistic if AB > A&B or AA and or BB, potentiation if A/B = 0 and AB > A & B or AA and or BB, antagonistic if AB < A & B or AA and or BB , additive if AB = AA and or BB or A & B, not distinguishable if AB = A or B A+B is greater than A and B and less or greater than A+A or B+.[9]

RESULTS

Zone of Inhibition Results for *Amikacin - Meropenem* Antibiotic Combination

The results of the inhibition zone test of Amikacin 30 µg –Meropenem 10 µg combination against *Pan Drug-Resistant Pseudomonas aeruginosa* produced a synergistic effect. Significant differences were obtained in comparisons made using the AZDAST method (p <0.05).

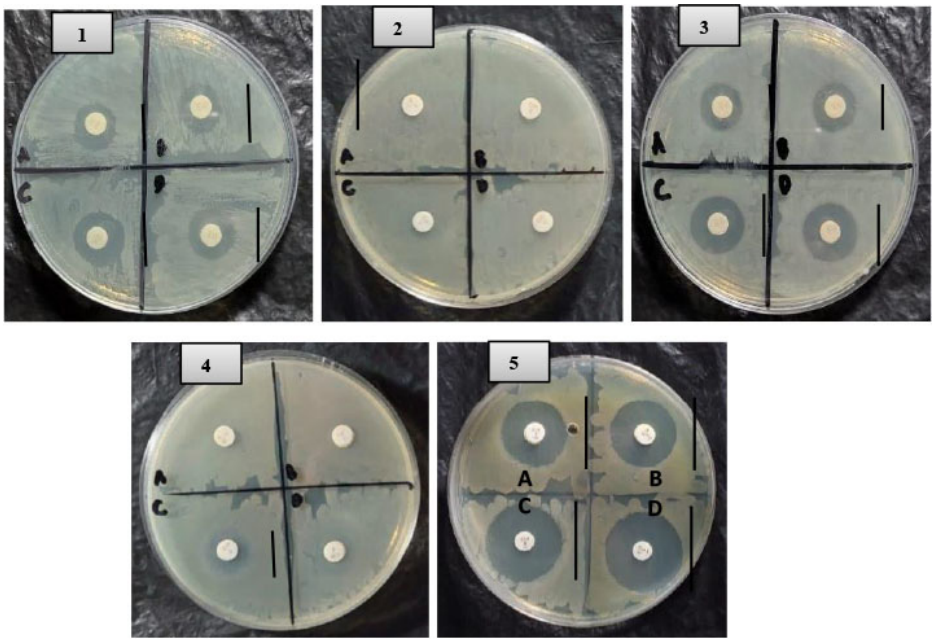

Figure 4. An inhibition zone formed in the test on Pan Drug-Resistant Pseudomonas aeruginosa bacteria. (1) A zone of inhibition was formed in the Amikacin 30 µg test. (2) No zone of inhibition was formed in the 5 µg Ciprofloxacin test. (3) A zone of inhibition was formed in the Amikacin 30 µg –Amikacin 30 µg test. (4) An inhibition zone was formed in the Ciprofloxacin 5 µg – Ciprofloxacin 5 µg test. (5) A zone of inhibition was formed in the Amikacin 30 µg - Ciprofloxacin 5 µg test.

Table 4. Comparison Results of Testing Three Combinations of Antibiotics

Antibiotic Testing	Mean ± SD
Amikacin 30 µg –*Meropenem* 10 µg	26,31 ± 3,41
Amikacin 30 µg - *Ceftazidime* 30 µg	25,12 ± 2,22
Amikacin 30 µg -*Ciprofloxacin* 5 µg	23,12 ± 2,25

Zone of Inhibition Results on *Amikacin - Ceftazidime* Antibiotic Combination

The results of the inhibition zone test of Amikacin 30 µg - Ceftazidime 30 µg combination against Pan Drug-Resistant Pseudomonas aeruginosa produced a synergistic effect. Significant differences were obtained in comparisons made using the AZDAST method ($p < 0.05$).

Zone of Inhibition Results on Amikacin - Ciprofloxacin Antibiotic Combination

The results of the zone of inhibition of the Amikacin 30 µg – Ciprofloxacin 5 µg combination test against Pan Drug-Resistant Pseudomonas aeruginosa produced a synergistic effect. Significant differences were obtained in comparisons made using the AZDAST method ($p < 0.05$).

Comparison of Several *Anti-Pseudomonas* Antibiotic Combinations

The results of the inhibition zone resulting from testing the combination of several antibiotics against Pan Drug-Resistant Pseudomonas aeruginosa showed that the zone of inhibition of the antibiotic combination from largest to smallest was produced by Amikacin 30 µg–Meropenem 10 µg (26.31 ± 3.41), followed by Amikacin 30 µg Ceftazidime 30 µg (25.12 ± 2.22) and Amikacin 30 µg-Ciprofloxacin 5 µg (23.12 ± 2.25). The resulting zone of inhibition was not statistically significant ($p > 0.05$) either in comparison to the antibiotics as a whole or in the antibiotic combination group alone.

DISCUSSION

Antibiotic therapy for gram-negative bacterial infections is often given a combination of two drugs that are susceptible to organisms in *in-vitro* tests, the drugs that are usually combined are β-lactam groups together with aminoglycoside class antibiotics. In more serious infections, combination antibiotic therapy has its appeal in the face of the doctor. The results of the inhibition zone from the disc diffusion test have different diameters for each antibiotic. The standard for each inhibition zone diameter is also a special consideration.[11]

The use of beta-lactam antibiotics and aminoglycosides together has been widely published, in which the combination was reported to have synergistic effects for both Gram-positive and Gram-negative organisms. The current mechanism of action addresses the synergistic effect of the combination of the two antibiotics via beta-lactam antibiotics increasing the porosity of the bacterial cell wall, resulting in greater penetration of the aminoglycosides and access to the target ribosome.[12]

The synergistic mechanism of the combination of β-lactam and cephalosporin groups may be achieved because β-lactam when hydrolyzed acts as a competitive β-lactamase inhibitor.[13] So that the combination of the two antibiotics works together in sticking or efflux to the bacterial cell wall, this theory is widely accepted in explaining the mechanism of an antibiotic combination which until now has not been known with certainty until now.[14] The combination between Amikacin and Ceftazidime has been reported to have a good synergistic effect which can be seen in clinical improvement without changing the predetermined dose.[12]

The mechanism of the combination of aminoglycoside and quinolone class antibiotics cannot be explained clearly. Changes in efflux pumps mediated by the outer membrane porin Omp (outer membrane protein) are the key that can explain this mechanism. These changes can contribute to the entry of antibiotic combination agents into Pseudomonas aeruginosa bacteria.[15] The mechanism of action of the two combinations inside the cell makes this combination unable to work quickly in inhibiting the growth of resistant bacteria.[16,17]

CONCLUSION

The combination of anti-pseudomonas antibiotics synergistically affects Pan Drug-Resistant Pseudomonas aeruginosa in the AZDAST method in vitro test.

AUTHOR CONTRIBUTION

All author contributed equally to prepare this manuscript.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest associated with this study.

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ETHIC APPROVAL

This study had been ethically approved by ethical commission of Faculty of Medicine, University of Brawijaya, Malang, Indonesia

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