ANTIBIOTIC SUSCEPTIBILITY TEST OF *PSEUDOMONAS AERUGINOSA* AND *STAPHYLOCOCCUS AUREUS* WITH DISK DIFFUSION AND DILUTION METHOD

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ABSTRACT

The susceptibility test is a test used to measure bacteria's sensitivity and vulnerability towards antibiotics. This study was to determine sensitivity of *Pseudomonas aeruginosa* and *Staphylococcus aureus* towards amoxicillin, neomycin, and sulfanilamide. In this study, the methods used in susceptibility tests are disk diffusion method and serial dilution. The disk diffusion method is a method with paper disks that already saturated the antibiotics, with one paper disk for each antibiotic, put the disk on the agar media that had been inoculated by the bacteria, then incubated and measured the inhibition zone. The dilution method was done by making a series of antibiotic dilutions in liquid media which added microbes inside. This test is to estimate the smallest concentration or Minimum Inhibitory Concentration (MIC) in the dilution series with no bacterial growth. The diffusion test shows no clear visible zone at all, so *Pseudomonas aeruginosa* and *Staphylococcus aureus* are resistant to amoxicillin, neomycin and sulfanilamide. The dilution test shows the clearest sample was on 0,25% concentrate, so amoxicillin's Minimum Inhibitory Concentration towards *Staphylococcus aureus* is 0,25%.

Keywords: Antibacterial; Dilution; Disk method; *P. aeruginosa*; *S. aureus*

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INTRODUCTION

Antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome. In this study, we focused on the use of antimicrobial testing methods for the *in vitro* investigation of extracts and pure drugs as potential antimicrobial agents. The growth of bacteria that cause infection and disease needs to be inhibited with antibacterials. Antibacterials are substances that can inhibit the growth of bacteria and can kill pathogenic bacteria.

The diffusion method is a method used to determine the sensitivity of microbes to antimicrobial agents. The principle of this method is the diffusion of antibacterial compounds in a solid medium containing inoculated microbes. The well method, one of the diffusion techniques employed to assess the sensitivity of microbes to antimicrobial agents, operates on the principle of the diffusion of antibacterial compounds in a solid medium containing previously inoculated microorganisms. In this method, solid media with microbial cultures are prepared with wells of 6-8 mm using an aseptic tip.

Subsequently, antimicrobial agents are introduced into these wells in volumes ranging from 20 to 100 ml. The culture is then incubated for 18-24 hours at a temperature of 35°C. The evaluation of antimicrobial activity is based on the observed effects within the created wells. This method offers a practical approach to studying the impact of antimicrobial agents on microbial growth and provides insights

into the effectiveness of these agents against a variety of microorganisms (Lilih, 2020).

The cylinder method, a diffusion technique employed in assessing microbial sensitivity to antimicrobial agents, involves using sterile glass cylinders placed on agar containing a frozen microbial suspension. These cylinders are then filled with the test substance and incubated at 35°C for 18-24 hours. Post-incubation, the diameter of the inhibition zone is measured. This method offers a notable advantage due to the clarity it provides when introducing substances into the agar, enabling precise measurements. However, a significant drawback is the heightened risk of cylinder displacement during the process, potentially introducing inaccuracies and compromising result reliability. Understanding these pros and cons is crucial for researchers employing the cylinder method to ensure the accuracy and validity of their antimicrobial susceptibility testing (Lilih, 2020).

Disk diffusion is a method to determine microbial susceptibility to antibiotics in which filter paper disks containing known concentrations of antibiotics are placed on the agar surface that has been previously inoculated with the bacteria of interest. The disk diffusion method is performed using Mueller-Hinton Agar (MHA), which is the best medium for routine susceptibility tests because it has good reproducibility, low in sulfonamide, trimethoprim, and tetracycline inhibitors, and gives satisfactory growth of most bacterial pathogens.

Dilution methods are the most appropriate ones for the determination of MIC values, since they offer the possibility to estimate the concentration of the tested antimicrobial agent in the agar (agar dilution) or broth medium (macrodilution or microdilution). Either broth or agar dilution method may be used to quantitatively measure the *in vitro* antimicrobial activity against bacteria and fungi. The dilution method is a method used to determine the potential of a compound for microbial activity by measuring the Minimum Inhibitory Concentration and Minimum Kill Concentration.

a. Liquid dilution

Liquid seed dilution consists of two methods, namely macrodilution and microdilution. In principle, microdilution and macrodilution are the same, the only difference is in volume. For macrodilution, a volume of more than 1 ml is used, while for microdilution, a volume of 0.05 ml to 0.1 ml is used. The antimicrobials used are provided in various types of dilutions, usually in units of µg/ml, the concentration varies depending on the type and nature of the antibiotic, for example cefotaxime for sensitivity testing against *Streptococcus pneumonia*, the dilution should not exceed 2 µg/ml, while for Escherichia coli the dilution is carried out up to 16 µg/ml or more. In general, for MIC determination, dilution of antimicrobials is carried out based on decreasing the concentration by half, for example starting from 16, 8, 4, 2, 1, 0.5, 0.25 µg/ml, the lowest concentration that shows the most significant growth inhibition, is called the power concentration. minimum inhibitory concentration/MIC (minimum inhibitory concentration).

b. Agar dilution

In the agar dilution method, antibiotics appropriate to the dilution will be added to the agar, thus requiring an agar seed that corresponds to the amount of dilution. Then, one more agar seed was added as a control without adding antibiotics. One of the advantages of the agar dilution method is to determine the MIC of Neisseria gonorrhoeae which cannot grow using the liquid seed dilution technique. Determination of the minimum concentration of antibiotics that can concentration (MBC) is carried out by planting bacteria in liquid seeds used for MIC into agar and then incubating overnight at 37^0C. MBC occurs when there is no more bacterial growth on the agar.

The basis for determining antimicrobials in vitro is based on MIC (Minimum Inhibition Concentration) and MBC (Minimum Bactericidal Concentration). MIC is the lowest

concentration of bacteria that can inhibit bacterial growth through results seen from colony growth on agar or turbidity in liquid culture. Meanwhile, MBC is the lowest concentration of antimicrobial which can kill 99.9% of microbial colonies during the specified time.

c. Dilution Method

The dilution method is a method used to determine the potential of a compound for microbial activity by measuring the Minimum Inhibitory Concentration (MIC) and Minimum Kill Concentration (KBM) (Fitriana, 2020). The principle of the dilution method is to make a series of dilutions of certain antibacterials in liquid/solid media to which the test microbes have been added. The liquid dilution method is used to measure the MIC (minimum inhibitory content) while the solid dilution method is used to determine the KBM (minimum bactericidal content). The solid dilution method is carried out by inoculating test microbes on agar media containing antimicrobial agents. The advantage of this dilution method is that one concentration of the antimicrobial agent being tested can be used to test several test microbes (Pelczar, 2019).

MATERIALS AND METHODS

a. Materials

Antibiotics amoxicillin, neomycin, sulfanilamide, paper disks containing antibiotics, blank disk paper, *Pseudomonas aeruginosa* and ATCC *Staphylococcus aureus* (6538) bacterial culture, Ethanol 70%, NaCl 0,9%, aquadest, Nutrient Agar (Himedia), Nutrient Broth (Himedia), and Muller Hinton Agar (Himedia) medium.

b. Method

The method section is explaining the research activities. This test used the Kirby-Bauer Method (disk) Diffusion Test and Liquid Dilution Test. Disk diffusion tests performed by paper disks contained antimicrobial agents. Subsequently, the paper disk placed on the surface of an agar medium inoculated with the microbial test culture and incubated at 100°C 35±2°C for 18 to 24 hours. At the same time, a liquid dilution test is performed by performing a series of antimicrobial dilutions in liquid culture medium supplemented with the test microorganism. The smallest concentration of an antimicrobial test solution that appears clear without growing the test bacteria is called the MIC. Both test methods were performed to obtain qualitative and quantitative experimental results. The diffusion test is used to obtain qualitative results and the dilution test is used to obtain quantitative results.

RESULT

Table 1. Observations bacterial growth inhibition zone

Sample	Bacterial Growth Inhibition Zone (mm)			
	Staphylococcus aureus			Mean ± SD
	R1	R2	R3	
Control (*blank disc)*	0 mm	-	-	
Amoxicillin	 0 mm	 0 mm	 0 mm	0 mm
Neomycin	 0 mm	 0 mm	 0 mm	0 mm
Sulfanilamide	 0 mm	 0 mm	 0 mm	0 mm
Pseudomonas aeruginosa				
Control (*blank disc*	0 mm	-	-	

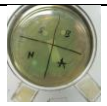
Sample	Bacterial Growth Inhibition Zone (mm) *Pseudomonas aeruginosa*			Mean ± SD
	R1	R2	R3	
Amoxicillin	 0 mm	 0 mm	 0 mm	0 mm
Neomycin	 0 mm	 0 mm	 0 mm	0 mm
Sulfanilamide	 0 mm	 0 mm	 0 mm	0 mm

Table 2. Observation results of liquid diluted tests on the growth of *Staphylococcus aureus*

Turbidity of Samples			
Staphylococcus aureus			
Sample	 Bacteria Control	 Solvent Control	 Media Control
Amoxicillin Concentration			
 0,25%	 0,5%	 1%	 2%

DISCUSSION

In the discussion include theories that support or convey previous research (if any) for comparison and are written in Times New Roman font, font 12, space 1 and the size of the paper used is A4, with margins Top 1 ", Bottom 1", Left 0 , 75 "and Right 0.75" (in inches), or Top margin 2.54 cm, Bottom 2.54 cm, Left 1.90 cm and Right 1.90 cm (in centimeters). Images and photos must be clear and must include a proportional image or photo description. use page layout with columns Two

Diffusion Method Susceptibility Test

Sensitivity testing using the agar plate diffusion method can be carried out using the Kirby Bauer method using the disc diffusion technique or the welling technique. Apart from that, this disc or paper disc method has other advantages, such as being easy to do, does not require special equipment and is relatively cheap. Meanwhile, the weakness is that the size of the clear zone formed depends on the incubation conditions, inoculum, prediffusion and preincubation as well as the thickness of the medium. If these four factors do not match then the results of the disk method are usually difficult to interpret. In addition, this disc method cannot be applied to microorganisms that grow slowly and microorganisms that are obligate anaerobes (Prayoga, E. 2013).

In the tests that have been carried out, the preparation of *Staphylococcus aureus* and Pseudomonas aeruginosa bacterial suspensions was standardized to the 0.5 McFarland standard. The McFarland standard is an equalization of microbial concentrations that aims to replace individual bacterial counts and to estimate the number of cell densities that will be used in antimicrobial testing procedures. The McFarland standard consists of a wide range of numbers ranging from 0.5-4. The McFarland standard of 0.5 or the equivalent of $1.5x10^8$ CFU/ml is the turbidity recommended by the Clinical Laboratory Standards Institute (CLSI) for sensitivity tests and the basis for antimicrobial susceptibility experiments and bacterial culture results experiments (Murtafi'ah, 2023). McFarland 0.5 solution is also commonly used as a comparison for the turbidity of bacterial cultures in liquid medium with a density between $1x10^7$ cells/mL - $1x10^8$ cells/mL (Aviani and Pujiyanto, 2020).

Table 3. Antibiotic Sensitivity determination table (diameter of inhibition zone in mm) CLSI (2020)

	Disk Content	Inhibition zone diameter (mm)		
		Susceptible	Intermediate	Resistant
Staphylococcus Aureus				
Trimethoprim Sulfamethoxazole	1.25/23.75μ g	≥ 16	11–15	≤ 10
Sulfonamides	250 or 300μg	≥ 17	11–16	≤ 12
Trimethoprim	5 μg	≥ 16	11–15	≤ 10

Mcfarland 0.5 standard solution is made by pipetting 0.05 mL of 1% Barium Chloride (BaCl2) solution to which 9.95 mL of 1% Sulfuric Acid (H2SO4) solution is completely mixed. The 0.5 McFarland standard is used compared to other McFarland standards because there will be too many bacteria growing on the agar medium, making it difficult to observe. It is hoped that the presence of a smaller quantity or number of bacteria will make it easier to observe the inhibition zone.

Diffusion tests were carried out on *Staphylococcus aureus* and *Pseudomonas aeruginosa* bacteria. Each bacteria was inoculated on agar media and a paper disc containing antibiotics in the form of amoxicillin, sulfanilamide and neomycin was added. Based on observation data, the results showed that no inhibition zone was formed for the three antibacterials tested. This shows that the antibiotics amoxicillin, sulfanilamide, and neomycin are resistant to the bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Determination of antibiotic sensitivity (inhibition zone diameter in mm) can be determined based on the antibacterial susceptibility table standardised by CLSI (Clinical and Laboratory Standards Institute).

In accordance with the CLSI table (2020), the inhibition zone for sulfonamide antibiotics against *Staphylococcus aureus* is sensitive if ≥17mm, intermediate if 13-16 mm, and resistant if ≤12mm. Meanwhile, the antibiotics amoxicillin and neomycin are not in the CLSI table (2020), this could be because these antibiotics are no longer used in the treatment and inhibition of the growth of *Staphylococcus aureus* because they are resistant. This also applies to Pseudomonas aeruginosa bacteria, by administering the antibiotics amoxicillin, sulfanilamide and neomycin (Rahman, 2021). *Staphylococcus aureus* and *Pseudomonas aeruginosa*, when given the antibiotics amoxicillin, neomycin and sulfanilamide did not produce inhibition zone as results of the test. This test results correlated with the latest CLSI data in 2020. In the CLSI most recent antibacterial susceptibility standard stated that the inhibition zone for sulfonamide antibiotics against *Staphylococcus aureus* is sensitive if

≥17mm, intermediate if 13-16 mm, and resistant if ≤12mm. Meanwhile, the antibiotics amoxicillin and neomycin are not in the CLSI table (2020), this could be because these antibiotics are no longer used in the treatment and inhibition of the growth of *Staphylococcus aureus* because they are resistant. This also applies to the bacteria Pseudomonas aeruginosa.

Staphylococcus aureus resistance to amoxicillin can be mediated by a decrease in the amount of Penicillin Binding Protein (PBP-1A) produced by the bacteria or a decrease in the affinity of amoxicillin for PBP-1A (Setiawati, 2015). *Staphylococcus aureus* resistance to neomycin is caused by cytoplasmic aminoglycoside modification of the enzyme encoded by genetic transfer (Jensen & Liyon, 2009). Bacteria that are resistant to sulfanilamide do not need PABA from outside the cell, but can use folic acid so that sulfanilamide, which competes with PABA, has no effect on cell metabolism (Marhamah, 2016).

In Pseudomonas aeruginosa, resistance can occur to the antibiotic amoxicillin because

this bacteria can produce the beta-lactamase enzyme which can cause hydrolysis of the drug. The beta-lactamase enzyme will break the beta lactam ring so that the antibiotic amoxicillin cannot inhibit the work of the transpeptidase enzyme which forms the peptidoglycan needed by bacteria (Kong K., 2010). Administration of neomycin can cause the Pseudomonas aeruginosa bacteria to undergo enzymatic modifications which can inactivate aminoglycoside antibiotics such as neomycin. These enzymes will phosphorylate antibiotics, allowing interference with the antibiotics. In addition, modification of membrane permeability and target sites causes the binding of antibiotics to their targets to be reduced. Bacterial resistance to sulfonamide antibiotics also occurs because resistant bacteria do not use PABA from outside the cell, but instead use folic acid so that sulfanilamide which competes with PABA has no effect on cell metabolism (Krause K.M, 2016).

a. Amoxicillin

Amoxicillin is a penicillin whose substituent at position 6 of the penam ring is the 2-amino-2-(4-hydroxy phenyl) acetamido group which acts as an antibacterial drug. Amoxicillin is a long-standing FDA-approved prescription antibacterial drug for the treatment of certain bacterial infections, such as community-acquired pneumonia; infections of the ears, nose, and throat; infections of the genitourinary tract, and infections of the skin and respiratory tract. Amoxicillin competitively inhibits penicillin-binding proteins, causing upregulation of autolytic enzymes and inhibition of cell wall synthesis. Amoxicillin has a long duration of action because it is usually given twice a day. Amoxicillin has a wide therapeutic range as mild overdose does not cause significant toxicity. However, patients should be counseled regarding the risk of anaphylaxis, Clostridium difficile infection, and bacterial resistance (NCBI, 2023).

Amoxicillin is included in the beta-lactam antimicrobial group. Beta-lactams work by binding to penicillin-binding proteins that inhibit a process called transpeptidase (a cross-linking process in cell wall synthesis), leading to the activation of autolytic enzymes in the bacterial cell wall. This process causes lysis of the cell wall, thereby destroying the bacterial cell. This type of activity is called bactericidal killing. Administration of amoxicillin can also be combined with beta-lactamase inhibitors. Some examples are clavulanic acid and sulbactam. These beta-lactamase inhibitors work by irreversibly binding to the catalytic site of the organism's beta-lactamase enzyme, causing resistance to amoxicillin's native beta-lactam ring. These drugs do not have bactericidal activity but they can expand the spectrum of amoxicillin to organisms that produce beta-lactamase enzymes when combined with amoxicillin (Bernatova et al, 2013).

Amoxicillin is one of the antibiotics most commonly used in primary health care. Amoxicillin is an amino-penicillin made by adding an extra amino group to penicillin to fight antimicrobial resistance. Amoxicillin covers a wide range of gram-positive bacteria, with some additional gram-negative coverage compared to penicillin. Like penicillin, it covers most species of Streptococcus and is also effective against Listeria monocytogenes and Enterococcus species. It also includes Haemophilus influenzae, some Escherichia coli, Actinomyces species, Clostridium species, Salmonella species, Shigella species, and Corynebacteria species (NCBI, 2023).

b. Neomycin

Neomycin is an aminoglycoside antibiotic that works by inhibiting bacterial protein synthesis, thereby causing a bactericidal effect on gram-

negative bacteria. This drug is used to treat bacterial infections in the outer ear (otitis externa), skin, or eyes, and is available in various forms such as eye drops, ear drops, ointment, cream, or gel. Neomycin works by binding to the 30s ribosomal unit in susceptible bacteria, causing inhibition of protein synthesis and resulting in errors in the transcription of the genetic code. Neomycin is known to be especially active against almost all Gram-negative bacteria except Pseudomonas aeruginosa, as well as anaerobic organisms such as Bacteroides (Tortora et al., 2010).

Neomycin also has some activity against Gram-positive bacteria such as Staphylococcus bacteria. However, the use of neomycin may increase the risk of side effects on the kidneys or nervous system, especially in patients with impaired kidney function, high dose therapy, or long-term therapy. Neomycin is included in the classification of protein synthesis inhibitors. This antimicrobial is selectively toxic to the human body because bacterial ribosomes (sites of protein synthesis) consist of 50s and 30s subunits, while human ribosomes have 60s and 40s subunits. Examples of antibiotics in this class are tetracyclines and aminoglycosides whose actions are bound to the transferase of the 50s ribosomal subunit. Macrolides bind to the 50s subunit, Linezolid binds to the 50s subunit (Tortora et al., 2010).

c. Sulfanilamide

Sulfanilamide acts as a competitive antagonist and structural analogue of p-aminobenzoic acid (PABA) in the synthesis of folic acid which is important for DNA production in bacteria. The structural similarity to PABA allows sulfanilamide to inhibit and replace PABA to bind to the dihydropteroate synthetase (DHPS) enzyme whose activity is important for folate production. This causes inhibition of the formation of dihydrofolate, tetrahydrofolate, and subsequently the formation of bacterial DNA. Inhibited DNA formation causes disruption of bacterial cell replication because folate compounds for the formation of purines and pyrimidines in the de novo nucleotide synthesis pathway are inhibited (Zessel et al., 2014).

Dilution Method Susceptibility Test

The dilution test was carried out using three test controls as a comparison. The function of solvent control, bacterial control, and media control is to ensure that the results of this lab are free from external contamination factors. The function of bacterial control is to prove that the bacteria used in the practicum can remain alive after treatment, thus proving that the work procedures are correct. For making solvent control and media control, the function is almost the same, namely to prove that the solvent used is free from contamination and ensure that the media used can become a place for microbial culture, as well as avoiding external contamination factors (Astutiningsih, 2014).

Based on the test results obtained, the liquid dilution test shows that the clearest, or most effective, sample is at a concentration of 0.25%. This means that at this concentration amoxicillin is effective in inhibiting the growth of Staphylococcus aureus bacteria, in accordance with the MIC theory, which is the lowest concentration that is still able to inhibit bacterial growth. So, in this context, we can know that the MIC of amoxicillin against the Staphylococcus aureus bacteria is at a concentration of 0.25%. The results of the amoxicillin MIC experiment on Staphylococcus aureus bacteria showed that the minimum concentration needed to inhibit bacterial growth was 0.25%. Media with this concentration appears clear and there is no presence of bacterial growth. The ability to inhibit bacteria that arises comes from the mechanism of amoxicillin, where amoxicillin can increase the regulation of autolytic enzymes and the mechanism of beta lactam

antibiotics, namely inhibiting cell wall synthesis (Tjay & Rahardja, 2015). From the observations, it can be said that the MIC value shows qualitatively that the antibiotic mechanism of amoxicillin does have antibacterial activity at certain concentrations. So the results of the dilution test are in accordance with the mechanism of the antibiotic amoxicillin.

CONCLUSION

Antibacterial sensitivity tests of diffusion and dilution methods were carried out against *Staphylococcus aureus* and Pseudomonas aeruginosa bacteria using samples of amoxicillin, neomycin, and sulfanilamide. Based on the results of the diffusion method, there is no clear zone in all observations (0 mm). So, it can be concluded that the bacteria *Staphylococcus aureus* and Pseudomonas aeruginosa are resistant to the antibacterial tested when viewed in the CLSI table. While the results of the amoxicillin antibacterial dilution method in *Staphylococcus aureus* bacteria obtained a KHM value of 0.25%.

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