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Research

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Effect of solvent variation on results of antibiotic susceptibility test using the disk diffusion method against *Staphylococcus aureus*

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Abstract

Background Mueller-Hinton agar (MHA) is widely used for disk diffusion tests to assess antibiotic susceptibility in non-fastidious bacteria. The type of water used to prepare the MHA may have affected the test outcomes.

Objective This study evaluated the effect of different water types as solvents on the antibiotic susceptibility test results of tetracycline and gentamicin against Staphylococcus aureus.

Methods MHA was prepared using four types of water: distilled (control), bottled, reverse osmosis (RO), and municipal tap water (PDAM). Antibiotic susceptibility testing was performed using the Kirby-Bauer disk diffusion method, and data were analyzed using One-Way ANOVA and Tukey's Post Hoc test.

Results The mean inhibition zones for tetracycline were 23.8 ± 0.99 mm (distilled), 23.4 ± 0.37 mm (bottled), 23.0 ± 0.43 mm (RO), and 17.8 ± 1.41 mm (tap), categorized as sensitive except for tap water (intermediate). For gentamicin, the zones were 11.7 ± 0.37 mm (distilled), 12.7 ± 0.77 mm (bottled), 9.8 ± 0.18 mm (RO), and 17.6 ± 1.19 mm (tap), with the first three classified as resistant and tap water as sensitive. The tap water results were significantly different ($p<0.05$) from those of the both antibiotics.

Conclusion The use of non-standard solvents in MHA preparation, particularly tap water, may lead to inconsistent antibiotic susceptibility results. Standardized use of distilled water is recommended to ensure test accuracy and reliability.

Keywords antibiotic susceptibility test | Kirby-Bauer disk diffusion method | Mueller Hinton agar | solvent variation | *Staphylococcus aureus*

Introduction

Culture media are essential components in microbiology that support the growth of microorganisms and facilitate the analysis of bacterial and fungal characteristics. These media play a critical role in laboratory research, particularly in antibiotic susceptibility testing, which aims to determine the efficacy of antimicrobial agents against specific pathogens (Atmanto *et al.*, 2022). Among the various available media, Mueller-Hinton agar (MHA) has been recognized as the international standard for antibiotic susceptibility testing (CLSI, 2023). It is recommended by the Food and Drug Administration (FDA) and the World Health Organization (WHO) because of its ability to support the growth of non-fastidious bacteria and maintain antibiotic stability (Jorgensen & Ferraro, 2009).

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The main components of the culture medium included peptone, meat extract, and water. Water, as a solvent, plays a crucial role in ensuring the homogeneity and stability of the media. The type of water used can significantly affect the susceptibility testing outcomes. Water with extreme pH levels (either too acidic or too alkaline) can alter the chemical composition of the medium, inhibit bacterial growth, or interfere with antibiotic diffusion in the agar matrix (Fajar *et al*., 2022). Moreover, the mineral content of water may also influence microbial growth. Some studies have suggested that mineral-rich water can enhance microbial proliferation compared to mineral-free water (Dong *et al*., 2022).

Distilled water is mineral-free water, which is most commonly used as a solvent, favoring its neutrality. Bottled water and reverse osmosis (RO) water are increasingly being evaluated as alternative and more accessible solvents. Darwin *et al*. (2021) reported that bottled water could be used to prepare nutrient agar (NA) without causing significant differences in microbial growth compared with distilled water. However, the use of PDAM water (mineral-rich water that often contains residual disinfectants such as chlorine) and RO water (mineral-free water) as solvents for agar media remains undocumented. Despite this, RO water is permitted for healthcare use in Indonesia, as it complies with Ministry of Health Regulation No. 492/MENKES/PER/IV/2010, which stipulates zero presence of E. coli and coliform bacteria per 100 mL sample. Nonetheless, specific evaluations of RO water as an agar medium solvent for bacterial growth are lacking.

Staphylococcus aureus is a common pathogenic bacterium found on body surfaces and is responsible for various infections including dermatitis, endocarditis, and pneumonia (González *et al*., 1999). Resistance of *S. aureus* to antibiotics, particularly penicillin and methicillin, has led to increased use of alternative agents such as tetracycline and gentamicin. These antibiotics have distinct mechanisms of action; tetracycline inhibits bacterial protein synthesis, whereas gentamicin disrupts ribosomal function (Li *et al*., 2024). The selection of these antibiotics in the present study was based on resource availability, specific research objectives, and clinical and environmental relevance, representing two different classes that are frequently used in medical practice.

Comparative studies of the types of water used in agar preparation for antibiotic susceptibility testing are limited. Differences in solvent types may result in variability in the test outcomes. Therefore, this study aimed to evaluate the effectiveness of different types of water as solvents in the agar media used for the disk diffusion method employing tetracycline and gentamicin against *S. aureus*. These findings are expected to provide insights into the feasibility of using alternative solvents beyond distilled water for antibiotic susceptibility testing.

Methods

Ethical approval, study period, and location

This study was approved by the Health Research Ethics Committee of Politeknik Kesehatan Kementrian Kesehatan (Poltekkes), Bandung, Indonesia (approval number No. 14/KEPK/EC/XII/2024). This research was conducted from September to October 2024 at the Bacteriology and Basic Chemistry Laboratories, Department of Medical Laboratory Technology, Poltekkes Bandung.

Experimental design

This study employed a quasi-experimental, *post-test only control group* design. The method used was static group comparison, in which measurements were taken only after the treatment groups received the intervention. Each treatment was replicated five times, following Federer's formula for experimental design (Hanafiah, 2014). The independent variable was the type of solvent used in the agar medium and the dependent variable was the diameter of the inhibition zone. The antibiotics tested were tetracycline and gentamicin, and the solvents consisted of distilled water (control), bottled water, reverse osmosis (RO) water, and municipal tap water (PDAM). The bacterial isolate used was Staphylococcus aureus strain SAU01, obtained from normal human skin and provided by the Medical Laboratory of Poltekkes Bandung.

Water and media preparation

Distilled water, commercial bottled water, RO water (Saba RO, Indonesia), and tap water from PDAM Tirta Raharja, Bandung were used as solvents. The pH of each water type (1 L) was tested using pH indicator strips (Merck, Germany).

Tryptic Soy Broth (TSB, Merck, Germany) was prepared by dissolving 3 g in 10 mL distilled water, heated on a hotplate until fully dissolved, and autoclaved at 121°C for 15 min under 1 atm pressure. Sterilized medium was poured into test tubes (10–15 mL) and incubated for 24 h.

Blood agar (Merck, Germany) was prepared by dissolving 4.024 g in 100 mL of distilled water, heated, autoclaved (121°C, 15 min, 1 atm), and supplemented with 3 mL of fresh human blood type O while still warm. The mixture was homogenized, poured into sterile petri dishes, and allowed to solidify.

Mannitol Salt Agar (MSA, Merck, Germany) was prepared by dissolving 11.002 g in 100 mL of distilled water, heated, autoclaved (121°C, 15 min), poured into sterile petri dishes, and allowed to solidify.

Mueller Hinton agar (MHA, Merck, Germany) was prepared by dissolving 3.8 g in 100 mL of each tested solvent in a sterilized 250 mL Erlenmeyer flask. The

mixture was boiled, covered with gauze containing cotton, and autoclaved at 121°C for 15 min. After cooling to approximately 50°C, the medium was poured into Petri dishes (±20 mL each) and allowed to solidify prior to use.

Propagation of S. aureus on TSB, blood agar, and MSA

The isolate was propagated in TSB and incubated at 37°C for 24 h. Turbidity in TSB indicated bacterial growth, whereas contamination was assessed via colony morphology on solid media or confirmed using staining and identification methods (Missiakas & Schneewind, 2013). Confirmation of *S. aureus* was performed using blood agar to observe colony morphology and β-hemolytic activity, as indicated by the clear zones around colonies (Turista & Puspitasari, 2019). MSA has been further identified used MSA, a selective and differential medium (Sharp & Searcy, 2006). *S. aureus* colonies that ferment mannitol undergo a color change from red to yellow.

Gram staining

Gram staining has been used to classify bacteria by gram reaction and morphology (Sarudji *et al.*, 2017). Smears were prepared from blood agar cultures, heat-fixed, stained with crystal violet (Merck, Germany) for 1–2 min, rinsed with tap water, treated with Lugol's iodine (Merck) for 30 s, and rinsed again. Decolorization was performed briefly using 96% ethanol, followed by rinsing and counterstaining with safranin (Merck, Germany) for 2 min. After drying, the slides were observed under a microscope using a 100× oil-immersion lens. *S. aureus* appears as Gram-positive cocci in clusters (Ibrahim, 2017).

Biochemical tests

Catalase and agglutination tests were performed. The catalase test confirmed the *Staphylococcus* genus by placing an inoculum from blood agar and one drop of H_2O_2 side by side on a slide, followed by mixing. A positive reaction was indicated by effervescence (O_2 formation), distinguishing *Staphylococcus* from catalase-negative *Streptococcus* (Reiner, 2010).

The agglutination test involved three glass slides: a positive control, negative control, and test sample. Each colony was mixed with a drop of citrated human plasma on a slide. Positive agglutination, indicated by clumping or granule formation, confirmed S. aureus, whereas no clumping suggested the presence of a different *Staphylococcus* species.

Antibiotic susceptibility testing

Antibiotic susceptibility was tested using the Kirby-Bauer disk diffusion method. A 0.5 McFarland turbidity standard was prepared by mixing 9 mL of 1% H_2SO_4 and 1 mL of 1% $BaCl_2$. The standard, equivalent to 1.5×10^8 CFU/mL, was used to adjust the turbidity of S. aureus suspensions (Suryantarini et al., 2024). The accuracy of the bacterial concentration was verified using UV-Vis spectrophotometry (5.0; Agilent, USA) at 625 nm.

Sterile swabs were dipped into the adjusted bacterial suspension, pressed against the inner wall of the tube to remove excess fluid, and evenly spread across the MHA plate surface. The plates were left for 5–15 min to allow absorption. Antibiotic disks (tetracycline and gentamicin) were then placed on the agar surface spaced approximately 15 mm apart. The disks were not moved after placement, and each treatment was replicated five times.

MHA plates with antibiotic disks were incubated at 37°C for 16–18 h. Following incubation, the inhibition zones surrounding the disks were observed and measured using a caliper and expressed in millimeters. The inhibition zone was defined as the diameter of the clear area where no bacterial growth occurred, indicating the inhibitory effect of the tested antibiotics on bacterial culture (CLSI, 2023).

Data analysis

Data were processed and analyzed using the Statistical Product and Service Solutions (SPSS) software. Normality and homogeneity tests were conducted, and if assumptions were met, One-Way ANOVA followed by post-hoc analysis was applied to determine significant differences among treatment groups.

Results

pH levels of media solvents

The solvents used in this study exhibited varying pH values (**Table 1**). Both distilled water and bottled water showed a neutral pH of 7.0, RO water was slightly acidic with a pH of 6.0, and PDAM tap water was slightly alkaline with a pH of 8.0.

Table 1 pH of solve nts used in Mueller Hinton Agar media preparation

Solvent	pH	Category
Distilled water	7.0	Neutral
Bottled water	7.0	Neutral
RO water	6.0	Acidic
PDAM tap water	8.0	Alkaline

RO: reverese osmosis

Culture and biochemical test results of *S. aureus*

The bacterial cultures showed good growth of *S. aureus*, as indicated by turbidity in the TSB medium (**Figure 1A**). On blood agar, *S. aureus* formed round colonies 1–2 mm in diameter, with an off-white to cream color (**Figure 1B**). The presence of β-hemolysis was evident in the clear zones surrounding the colonies. However, some colony morphologies indicate potential contamination. To avoid introducing contaminants into MSA medium, colonies were selected from areas distant from the suspected contamination zones.

Upon subculturing on MSA, *S. aureus* displayed mannitol fermentation activity, as indicated by a color change in the medium from red to yellow (**Figure**

1C). Gram staining revealed Gram-positive, purple-stained cocci arranged in clusters (**Figure 1D**), which is consistent with the typical morphology of S. aureus. The cultures also showed positive results for both catalase and agglutination tests, confirming their identification as *S. aureus*.

Antibiotic susceptibility test

This study focused on evaluating solvents other than distilled water for the preparation of MHA in inhibition zone assays. The results demonstrated that PDAM tap water produced significantly different inhibition zones ($P<0.05$) compared with the other solvents for both tetracycline and gentamicin (**Table 2**, **Figure 2**).

For tetracycline, the mean inhibition zone diameters for media prepared with distilled water, bottled water, RO water, and PDAM water were 23.8 ± 0.99 mm, 23.4 ± 0.37 mm, 23.0 ± 0.43 mm, and 17.8 ± 1.41 mm, respectively. Based on the CLSI (2023) standards, inhibition zones from distilled water, bottled water, and RO water were categorized as sensitive, whereas PDAM water yielded intermediate sensitivity results.

For gentamicin, the mean inhibition zones for the respective solvents were 11.7 ± 0.37 mm (distilled), 12.7 ± 0.77 mm (bottled), 9.8 ± 0.18 mm (RO), and 17.6 ± 1.19 mm (PDAM). According to CLSI guidelines, the results from distilled, bottled, and RO water were categorized as resistant, whereas PDAM water was categorized as sensitive.

Discussion

S. aureus growth in TSB medium was confirmed by turbidity, which results from the high bacterial density scattered light passing through the medium (Ishii *et al*., 2015). This turbidity confirmed that the bacterial culture was viable and was ready for further testing. On blood agar, *S. aureus* exhibited β-hemolytic activity, characterized by clear zones surrounding bacterial colonies. These zones result from hemolysins produced by *S. aureus* that lyse red blood cells in the medium (Ridder *et al*., 2021).

Colonies displaying a whitish color and β-hemolysis were subsequently subjected to catalase and coagulase testing. Due to the contamination observed in the initial cultures, subculturing was performed using the streak plate method to obtain isolated, pure colonies. This technique facilitates separation of bacterial cells over successive streaks, allowing isolated colonies to emerge in the final quadrant. The plates were then incubated at 35–37°C for 24–48 h (Kamaruddin, 2020). Morphologically appropriate colonies were further tested by Gram staining and biochemical assays to confirm their identity as *S. aureus*.

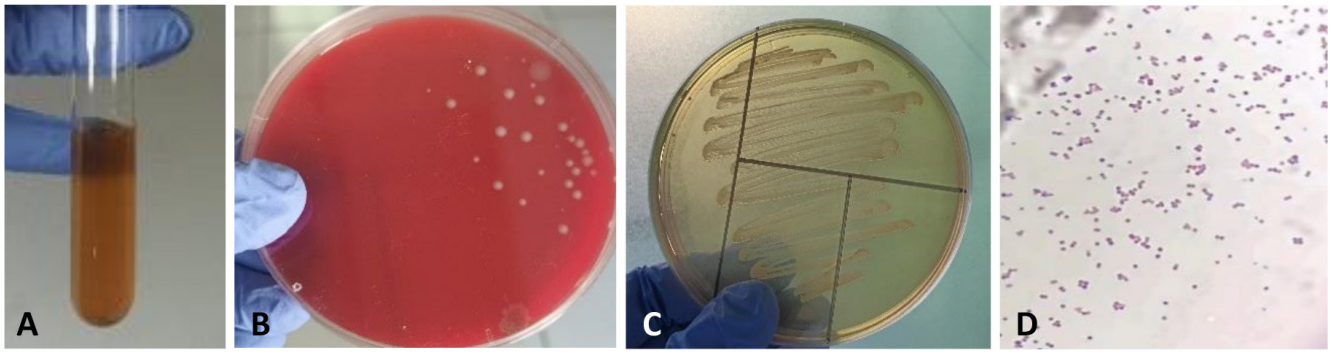

Figure 1 Confirmation of Staphylococcus aureus culture through growth on tryptic soy broth (TSB) (A), blood agar (B) indicating colony selection from uncontaminated areas, mannitol salt agar (MSA) (C), and Gram staining showing Gram-positive, clustered cocci (D) (objective lens magnification 100×).

Table 2 Inhibition zone diameters of antibiotics against *Staphylococcus aureus* using different media solvents for Mueller Hinton Agar

Antibiotic	Solvent	Inhibition zone diameter (mm)					Mean ± SD	Category*
		1	2	3	4	5		
Tetracycline	Distilled water	24.0	25.0	24.0	22.0	24.3	23.8 ± 0.99[a]	Sensitive
	Bottled water	23.5	23.0	23.0	24.0	24.0	23.4 ± 0.37[a]	Sensitive
	RO water	23.4	23.3	22.4	22.7	23.5	23.0 ± 0.43[a]	Sensitive
	PDAM tap water	17.6	19.7	19.0	15.6	17.5	17.8 ± 1.41[b]	Intermediate
Gentamicin	Distilled water	11.8	12.0	12.3	11.2	11.6	11.7 ± 0.37[a]	Resistant
	Bottled water	12.0	13.8	12.0	13.5	12.3	12.7 ± 0.77[a]	Resistant
	RO water	9.6	9.6	10.0	9.9	10.0	9.8 ± 0.18[a]	Resistant
	PDAM tap water	16.0	19.6	17.6	18.0	17.0	17.6 ± 1.19[b]	Sensitive

RO: Reverse osmosis. Different superscript letters (a, b) in the same column within the same antibiotic group indicate statistically significant differences ($P<0.05$). *Sensitivity interpretations based on CLSI (2023) criteria.

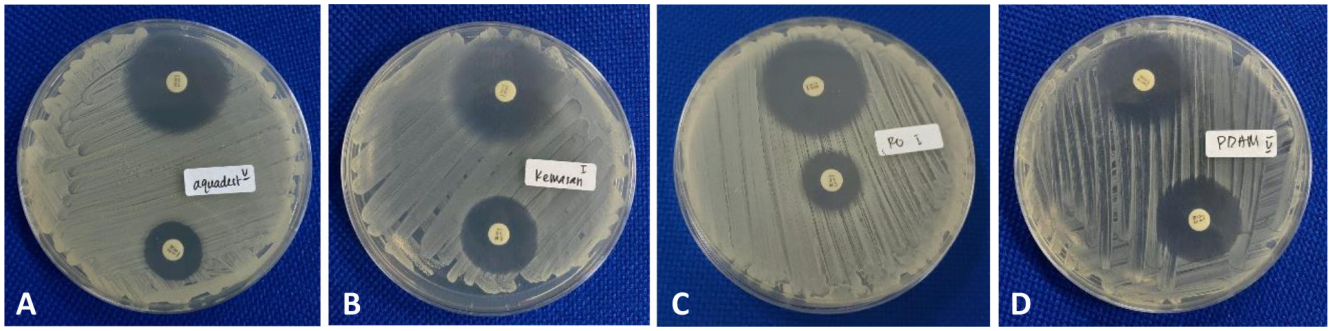

Figure 2 Inhibition zone of antibiotic susceptibility assays using tetracycline (top disk) and gentamicin (bottom disk) against *Staphylococcus aureus* on Mueller Hinton Agar prepared with distilled water (A), bottled water (B), reverse osmosis water (C), and PDAM tap water (D).

The identification of MSA confirmed *S. aureus* as a mannitol fermenter, as evidenced by the color change from red to yellow. MSA contains 7.5% NaCl, making it selective for salt-tolerant species such as *Staphylococcus spp*. (Darmawi, 2019). Positive catalase results were confirmed by the formation of oxygen bubbles resulting from the breakdown of hydrogen peroxide by catalase. Coagulase positivity was evidenced by the formation of visible clumps due to the conversion of fibrinogen into fibrin by *S. aureus* coagulase enzyme, independent of thrombin (Khusnan & Kusmanto, 2018). Collectively, these tests confirmed that the isolates used were *S. aureus*.

The solvents used for medium preparation demonstrated variable effectiveness. An ideal solvent should fully dissolve the medium, be non-toxic, not interfere with media components, support bacterial growth, and have a pH range of 7.5–8.5 suitable for most microorganisms. Acidic or alkaline pH values can alter the media chemistry and microbial growth (Fajar *et al*., 2022). In this study, bottled water, reverse osmosis (RO) water, and PDAM tap water had distinct pH values (**Table 1**), which likely influenced the results of antibiotic sensitivity testing.

Medium pH significantly affects bacterial growth and antibiotic performance by affecting cell membrane permeability, efflux mechanisms, and biofilm formation. Maintaining an appropriate pH is essential to ensure accurate and clinically relevant antibiotic sensitivity results (Hassan *et al*., 2024). In this study, PDAM tap water with a pH of 8.0 produced significantly larger inhibition zones with gentamicin, possibly due to enhanced antibiotic stability and diffusion under alkaline conditions.

The presence of minerals, such as calcium and magnesium, can also influence antibiotic activity. Unregulated mineral concentrations may affect inhibition zones and lead to misinterpretation of bacterial resistance or susceptibility (Høiby *et al*., 2024). This effect is particularly evident in PDAM tap water, which likely contains higher mineral levels, making it a critical variable alongside pH (Dong *et al*., 2022).

The variation in the solvent types used in the preparation of MHA media in this study affected the diameter of the antibiotic inhibition zones against S. aureus (**Table 2**). PDAM water resulted in a significantly different inhibition zone compared with the other solvents. This discrepancy may be attributed to the differences in mineral content found in the PDAM water, which can influence antibiotic stability within the medium (Høiby et al., 2024). PDAM water contains higher levels of calcium and magnesium, which contribute to enhanced antibiotic diffusion within the agar, thereby affecting the effectiveness of bacterial growth inhibition (Jorgensen & Ferraro, 2009).

Tetracycline and gentamicin both target the 30S ribosomal subunit to inhibit protein synthesis, but operate via distinct mechanisms. Tetracycline blocks aminoacyl-tRNA binding to the A-site of the ribosome, halting peptide chain elongation (Li et al., 2024; Barrenechea et al., 2021), whereas gentamicin induces mRNA misreading, leading to aberrant protein synthesis and bacterial cell death (Webster & Shepherd, 2022). Tetracycline can enter bacterial cells via passive diffusion or active transport, which is often influenced by pH gradients (Pearson et al., 2025), whereas gentamicin requires oxygen-dependent active transport (Chaves & Tadi, 2023). These differences may explain the variations in antibiotic diffusion and effectiveness.

The observed differences in the inhibition zones across different antibiotics and solvents indicated differential bacterial sensitivity. However, PDAM tap water consistently produced significantly divergent results for both antibiotics, suggesting that this deviation is primarily due to the solvent properties rather than the antibiotics themselves. These findings indicate that bacterial response differences are inherently related to the mode of action of each antibiotic (Barrenechea et al., 2021; Webster & Shepherd, 2022).

The inhibition zone assay used in this study adhered to international standards established by the Clinical and Laboratory Standards Institute (CLSI, 2023). These findings align with previous research by Dong *et al*. (2022), who demonstrated that media solvents with high mineral content can alter the efficacy of antibiotics against specific bacterial species. Given the influence of the solvent pH and mineral content on antibiotic activity and diffusion, it is critical to avoid non-standard solvents for agar preparation, as this could lead to inconsistent or inaccurate results.

Conclusion

Variations in the type of water used as a solvent for MHA media significantly affected the inhibition zones of the antibiotics tested against Staphylococcus aureus. The use of PDAM tap water altered the test results, whereas bottled water and reverse osmosis (RO) water produced inhibition zones that remained acceptable under specific conditions. Nevertheless, CLSI guidelines clearly mandate the use of distilled water as the standard solvent for antibiotic susceptibility testing. Therefore, the use of nonstandard solvents may result in inconsistent outcomes and should be avoided in laboratory practice.

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Author contribution FNH: Conceptualization, Investigation, Data curation, and Writing – original draft. HIS: Supervision, Methodology, Formal analysis, and Writing – review & editing. IK: Supervision, Methodology, Formal analysis, and Writing – review & editing. ZR: Supervision, Methodology, Formal analysis, and Writing – review & editing.

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