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Effect of The Temperature on The Size of Inhibition Zone of the Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim Activity Against *Staphylococcus aureus* ATCC 25923

Sistiyono[1a], Marta Atik Martsiningsih[1b], Menik Kasiyati[1c], Muji Rahayu[1d], Rita Rena Pudyastuti[1e], Suyana[1f], Ully Rahmawati[1g], Rahmad[2h], Zulfikar Husni Faruq[1i], Budi Setiawan[1,3j*]

[1] Department of Medical Laboratory Technology, Poltekkes Kemenkes Yogyakarta, Yogyakarta, Indonesia
[2] UPTD Health Laboratory of East Kalimantan Province, Samarinda, East Kalimantan, Indonesia
[3] Center of Excellent of Innovation Applied Technology on Public Health, Poltekkes Kemenkes Yogyakarta, Yogyakarta, Indonesia

[a] Email address: sistiyono@gmail.com
[b] Email address: atikmartsiningsih@gmail.com
[c] Email address: rifani.2010@gmail.com
[d] Email address: hayuningpuji@gmail.com
[e] Email address: ritapudyastuti1968@gmail.com
[f] Email address: suryaniajeng.2014@gmail.com
[g] Email address: ullya.rahmawati@poltekkesjogja.ac.id
[h] Email address: sky_lights43@yahoo.com
[i] Email address: faruq@poltekkesjogja.ac.id
[j] Email address: budi.setiawan@poltekkesjogja.ac.id

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Abstract
Antibiotic sensitivity testing is essential for determining bacterial susceptibility to antibiotics. In disc diffusion testing, several technical factors influence the diameter of the inhibition zone, including incubation temperature, which must be carefully controlled to ensure the validity of test results. This study aims to determine the mean, difference, and analyze the diameter of the inhibition zone of the antibiotics, namely Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim against Staphylococcus aureus on Mueller-Hinton agar media with incubation temperatures of 33°C, 34°C, 35°C, 36°C and 37°C for 18 hours. This research is observational, with a cross-sectional design. The primary data is 100 data on the diameter of the antibiotic inhibition zone, obtained by measuring the diameter of the inhibition zone with different incubation temperatures. The selection of antibiotics is based on the mechanism of action of antibiotics inhibiting bacteria, namely the cell wall or cell membrane that surrounds the bacterial cell; the pieces of machinery that make the nucleic acids DNA and RNA, and the machinery that produces proteins (the ribosome and associated proteins) with a range of inhibition zones based on Internal Quality Control CLSI. The data and the repeated measure statistical test will be processed univariately to determine the significance of the difference in the diameter of the formed inhibition zone using the ANOVA test. The measurement of the inhibition zone diameter on the incubation temperature variation showed a significant difference with a p-value of 0.000 for Levofloxacin, Tetracycline, and Trimethoprim, while the p-value of Clindamycin is 0.010. For the other antibiotics, Levofloxacin, Tetracycline, and Trimethoprim antibiotics, the higher the incubation temperature, the average diameter of the inhibition zone is condensed, while for Clindamycin the higher the incubation temperature, the higher the average diameter of the inhibition zone is the same. Incubation temperature and volume affect the diameter of the antibiotic inhibition zone in the disc diffusion method for the antibiotic sensitivity test. It can be concluded that incubation temperature affects the diameter of the antibiotic inhibition zone in disc diffusion tests. It is recommended for future standardized and precise testing conditions to ensure accurate and reliable antibiotic sensitivity results.

Keywords: Incubation Temperature, Inhibition Zone, Sensitivity Test, Disk Diffusion.

Corresponding Author:
Budi Setiawan
Department of Medical Laboratory Technology, Poltekkes Kemenkes Yogyakarta, Yogyakarta, Indonesia
Email: budi.setiawan@poltekkesjogja.ac.id

1. INTRODUCTION

The primary health problem that occurs in developing countries is infectious diseases. Microorganisms that cause human disease are called pathogenic microorganisms, one of which is pathogenic bacteria (Novard et al., 2019). *Staphylococcus aureus* is a Gram-positive commensal bacterium that is an opportunistic pathogen. These bacteria live in about 30% of the healthy adult population and are mostly found on the skin and mucous membranes. Although *Staphylococcus aureus* is a commensal bacterium, it can potentially to cause various diseases with varying degrees of severity (Jenul & Horswill, 2019).

Bacterial infections can be treated using antibiotics that are bactericidal or bacteriostatic. In order to treat patients appropriately and adequately, data on sensitivity tests of bacteria that cause infection are needed for various antibiotics available on the market (Nadjamuddin et al., 2023). The antibiotic sensitivity test using a disc diffusion method for *Staphylococcus sp* bacteria can use Mueller-Hinton media with a turbidity standard of 0.5 McFarland. Bacterial growth on the media will be optimal at 35°C with ambient air for 16-18 hours of incubation (CLSI, 2020).

According to several research results on sensitivity testing, technical factors that affect the diameter or size of the inhibition in the disc diffusion method are inoculum density, disc installation time, incubation temperature, incubation time, plate size, and thickness of agar medium and media composition (Lenggu et al., 2020). Research on the effect of incubation temperature on the inhibition zone has also been carried out on bacteria originating from water, which was carried out by incubation at various temperatures and times, including at a temperature of 35°C for 16 hours, 28°C, and 22°C for 24 hours, 22°C. C for 48 hours, and <19°C for >96 hours. The study's disc method on inhibition zone data precision in this study decreased significantly as the incubation temperature decreased and the time increased (Smith et al., 2018).

The selection of antibiotics in this study was based on the function of the mechanism of action of antibiotics in killing or inhibiting the growth of *Staphylococcus aureus* bacteria, including, Clindamycin and Tetracycline, which work by inhibiting protein synthesis, Levofloxacin which works by inhibiting nucleic acid synthesis, and Trimethoprim which acts as an antimetabolite. In addition, the selection of these four antibiotics was also based on their use as an Internal Quality Control strain of pure bacteria *Staphylococcus aureus* ATCC 25923 (Murray et al., 2021; CLSI, 2020).

Laboratory personnel's main targets in the clinical microbiology are the rapid and accurate identification of pathogenic microorganisms from clinical specimens and accurate interpretation of the results of the sensitivity test of pathogenic bacteria. The accuracy of antibiotic sensitivity test results will help the healing process of patients infected with bacterial diseases (Willey et al., 2017). For this reason, laboratory personnel must continue to perform internal quality stabilization, one of which is equipment maintenance, to ensure the quality of the inspection results.

The microbiological incubator is one of the important instruments in the process of culturing microorganisms or testing for antibiotic sensitivity. This instrument must be periodically tested for performance and calibration so that the quality of the results of laboratory examinations is maintained. However, laboratory workers still do not perform routine maintenance of microbiological equipment, including incubators. Incubators that are not maintained properly will cause high-temperature deviations. The threshold value for electrical safety, the allowable deviation value at the performance output of the maintenance incubator, is ±1 C. The easiest way to monitor the incubator temperature is to compare the temperature on

screen with a calibrated thermometer. If the temperature deviation exceeds 2°C, it is necessary to reset the incubator or calibration (Siregar et al., 2018).

A temperature that is not optimal will affect the formation of bacterial inhibition zones in the bacterial sensitivity test (Nadjamuddin et al., 2023). The fluctuatuin in mains voltage, unmonitored equipment perfomance, frequent opening and closing of the incubator door, and a high media load within the incubator can lead to innacurate temperature regulation. The importance of the incubation temperature variable in the antibiotic sensitivity test is the basis for researching the Effect of Incubation Temperature on the Diameter of the Inhibitory Zone in the Antibiotic Sensitivity Test of Staphylococcus aureus. This study aims to determine the mean, difference and analyze the difference in the diameter of the inhibition zone of the antibiotics Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim against *Staphylococcus aureus* on Mueller-Hinton agar media with incubation temperatures of 33°C, 34°C, 35°C, 36°. C and 37°C for 18 hours.

2. RESEARCH METHOD

The type of research used in this research is observational research with a cross-sectional study. This type of research is conducted without intervention on the research subject (Notoatmodjo, 2005). This study was conducted to observe whether there was a difference in the diameter of the antibiotic inhibition zone against *Staphylococcus aureus* bacteria incubated at different temperatures. The data collection technique used in this study was to measure the diameter of the inhibition zone using the disc diffusion method of the antibiotic sensitivity test. The purity test of *S. aureus* bacteria on blood agar plate media can be seen in Figure 1 below.

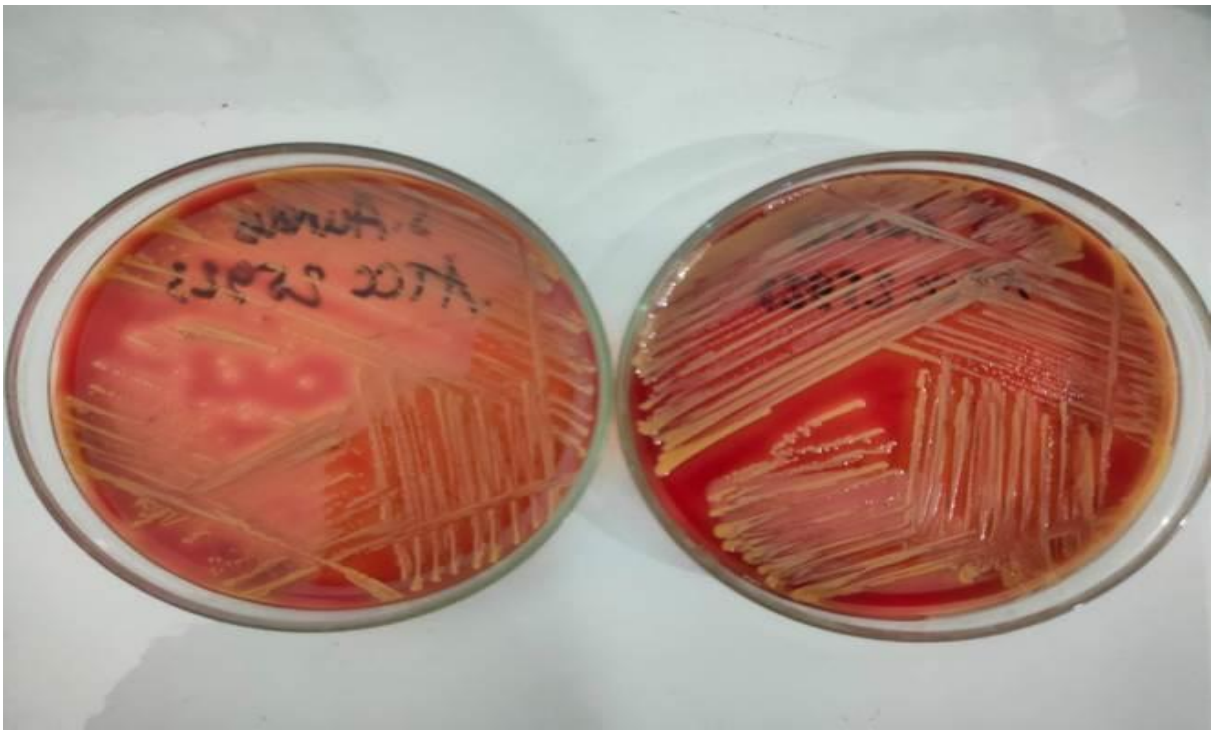


Figure 1. Purity Test of *S. aureus* Bacteria on Blood Agar Plate Media

A pure bacterial strain of *Staphylococcus aureus* ATCC 25923 was obtained from the microbiology laboratory at Health Laboratory East Kalimantan Province. The bacterial strain of *Staphylococcus aureus* ATCC 25923 was enriched for 24 hours in Brain Heart Infusion (BHI) media. The cloudy BHI media indicated bacterial growth, then inoculated on an inclined.

Nutrient Agar medium is used for antibiotic sensitivity testing, and inoculated on two Blood Agar Plate media to identify the purity of the bacteria. All inoculated media were

incubated at 35°C for 24 hours. *Staphylococcus aureus* bacteria was indentified using an automatic device brand BD Phoenix 50i by reacting a 0.5 McFarland bacterial suspension to the PMIC panel substrate reagent.

Collecting samples with Consecutive sampling is done by selecting and making a standard of bacterial turbidity (0.5 McFarland). The diameter of the inhibition zone on the antibiotics Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim was measured against the pure bacterial strain of *Staphylococcus aureus* ATCC 25923, which was formed after incubation at 33°C, 34°C, 35°C, 36°C, and 37°C. The antibiotic sensitivity test was repeated 5 times so that the number of plates examined for five variations in temperature was 25 times. One plate can be filled with four types of antibiotics so that with five repetitions can produce 100 data on the diameter of the antibiotic inhibition zone.

The instrument validity test is carried out utilizing periodic maintenance and calibration of the equipment to strenghten the laboratory's internal quality. One of the tools used in this research is an incubator; this tool is well maintained and calibrated regularly every year at the microbiology Laboratory, Health Laboratory of East Kalimantan Province, by the Calibration Testing Agency. For other tools, such as the Nephelometer, the instrument's validity can also be measured by testing the performance of the tool against the turbidity standard available on the brand of the tool. Other validity tests that were also carried out were the Mueller-Hinton media sterility test and the *Staphylococcus aureus* bacteria purity test. Mueller-Hinton media were prepared according to standard procedures, taken as much as 5%, and incubated without inoculation at 35°C for 18-24 hours. After 24 hours of incubation, the colony growth was observed on Mueller-Hinton media. If there is no bacterial growth, then all media can be continued for antibiotic sensitivity testing. However, if there is bacterial growth, then all media in one batch cannot be continued for testing.

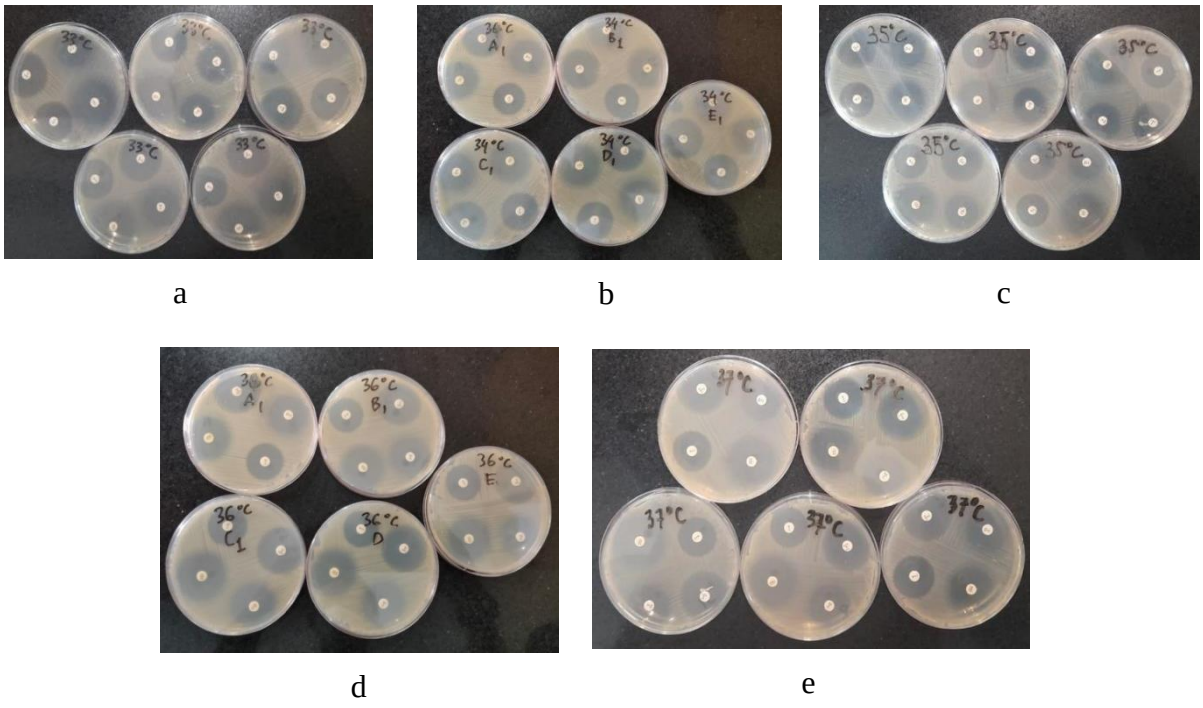
Data were collected by measuring the diameter of the inhibition zone on the antibiotic sensitivity test that had been determined using the disc diffusion method after being incubated at various temperatures. The data collected were analyzed descriptively and statistical analysis using the Repeated Measures ANOVA test with a degree of error (α) of 5%.

3. RESULTS AND DISCUSSION

This study used a pure strain of *Staphylococcus aureus* bacteria. The antibiotics used were Clindamycin 2µg, Levofloxacin 5µg, Tetracycline 30µg, and Trimethoprim 5µg. The results of the study in the form of the diameter of the antibiotic inhibition zone against *Staphylococcus aureus* bacteria were analyzed according to the research objectives, namely, to see the mean and difference in the mean of each independent variable and to see the significance of the difference in the diameter of the inhibition zone formed after incubation at 33°C, 34°C, 35 °C, 36°C, and 37°C.

Table 1. Average diameter of antibiotic inhibitory zone against *staphylococcus aureus* at variation of incubation temperature

Antibiotic	**Average Diameter of Antibiotic Inhibitory Zone (mm)**				
	33°C	34°C	35°C	36°C	37°C
Clindamycin	23.33	25.71	24.28	23.85	23.43
Levofloxacin	28.29	27.41	25.87	26.91	25.30
Tetracycline	28.13	27.58	228.80	25.01	25.23
Trimethoprim	25.77	25.59	23.40	23.26	23.32



a b c

d e

Noted: *a=33℃; b=34℃; c=35℃; d=36℃; e=37℃*

Figure 2. Inhibition zone at various incubation temperatures.

Table 2. The difference in mean diameter of antibiotic inhibitory zone at variation of incubation temperature to optimal temperature

Antibiotic	**Average Inhibitory Zone Diameter at Variation of Incubation Temperature**		
	Temperature Variation	**Optimal Temperature**	**Deviation**
Clindamycin	33°C	35°C	0.95
	34°C		1.43
	36°C		0.43
	37°C		0.85
Levofloxacin	33°C	35°C	2.42
	34°C		1.54
	36°C		1.04
	37°C		0.57
Tetracycline	33°C	35°C	0.67
	34°C		1.22
	36°C		3.79
	37°C		3.57
Trimethoprim	33°C	35°C	2.37
	34°C		219
	36°C		0.14
	37°C		0.08

Table 1 shows the mean diameter of the different inhibition zones of the four types of antibiotics. It can be seen that for Levofloxacin, Tetracycline, and Trimethoprim antibiotics, the higher the incubation temperature, the average diameter of the inhibition zone is smaller, while for Clindamycin, the higher the incubation temperature, the higher the average diameter

of the inhibition zone is the same, macroscopically this can be seen in Figure 2. Table 2 shows the difference in the mean diameter of the inhibition zones of each antibiotic. The most minor mean difference is 0.08 (mm), and the largest is 3.79 (mm).

The distribution of the observed data was the diameter of the inhibition zone from each variation of the incubation temperature, and the overall data were normally distributed with a significance value of p (0.05). The data then continued with the homogeneity tes; all data had sig values. (Mauchly's Test of Sphericity s) 0.05 so that all data are homogeneous

Table 3. Statistical test of repeated ANOVA

Antibiotic	**Sig. (Sphericity Assumed)**
Clindamycin	0.010
Levofloxacin	0.000
Tetracycline	0.000
Trimethoprim	0.000

Table 3 shows that a comparative test of Repeated ANOVA, shows that sig. (Sphericity Assumed) < 0.05, so it can be concluded that H0 is rejected. This result means that there is a difference in the diameter of the inhibition zone of each antibiotic (Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim) after incubation at 33°C, 34°C, 35°C, 36°C, and 37°C for 18 hours.

Table 4. Pairwise comparison test results of temperature variations to an optimal temperature

Antibiotic	**Incubation Temperature**	**p-value**
Clindamycin	Inhibition zone diameter 33°C	0.137
	Inhibition zone diameter 34°C	0.162
	Inhibition zone diameter 36°C	0.668
	Inhibition zone diameter 37°C	0.219
Levofloxacin	Inhibition zone diameter 33°C	0.005
	Inhibition zone diameter 34°C	0.015
	Inhibition zone diameter 36°C	0.213
	Inhibition zone diameter 37°C	0.322
Tetracycline	Inhibition zone diameter 33°C	0.175
	Inhibition zone diameter 34°C	0.009
	Inhibition zone diameter 36°C	0.000
	Inhibition zone diameter 37°C	0.001
Trimethoprim	Inhibition zone diameter 33°C	0.001
	Inhibition zone diameter 34°C	0.010
	Inhibition zone diameter 36°C	0.735
	Inhibition zone diameter 37°C	0.780

In Table 4, it can be observed that the difference in the average variation of incubation temperature to the optimal temperature occurs in Levofloxacin antibiotics at 33°C and 34°C, in Tetracycline antibiotics at 34°C, 36°C, and 37°C. In contrast, a significant difference was seen at 33°C and 34°C on the antibiotic Trimethoprim to the optimal temperature.

DISCUSSION

Microbes can grow everywhere but are still influenced by environmental factors. Temperature is one of the ecological factors that affect microbial growth. The environment can control microorganisms using various processes or physical means. Bacterial growth depends

on chemical reactions, and since the rate of these reactions is affected by temperature, bacterial growth is strongly influenced by temperature (Pelczar, 2008).

Every organism has the lowest temperature limit and the highest temperature, microbes need an optimum temperature for their growth and development. Bacteria grow at temperatures above 35°C; each species has a maximum and minimum temperature limit for growth. The optimum temperature is closer to the maximum temperature, while at the minimum temperature, the growth is slower. If the temperature is higher than the maximum temperature, the growth of bacteria will decrease rapidly. This outcome illustrates that temperature mainly affects enzymes. The higher the temperature, the faster the enzyme activity. If the temperature is too high, the enzyme will be denatured, so that the cell will die (Winarwi, 2006).

Staphylococcus aureus is a gram-positive bacterium with a diameter of 0.5-1.0 mm, in the form of a series of grapes, does not form spores, does not move, groups, pairs, and sometimes has short chains. Some strains of this bacterium have a capsule (Karimela et al., 2017). *Staphylococcus aureus* contains polysaccharide antigens, protein, and other important substances in the cell wall structure. Peptidoglycan, a polysaccharide polymer containing subunits joined together to form a rigid exoskeleton of the cell wall. Peptidoglycan can be damaged by strong acids or exposure to lysozyme. *Staphylococcus aureus* grows well on various bacteriological media under aerobic or microaerophilic conditions. It grows rapidly at 37°C, but the best pigment formation is at room temperature (20-35°^{C}) (Mietzner et al., 2019).

The four types of antibiotics used in this study, namely Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim can be used to monitor the quality and purity of *Staphylococcus aureus* strains (CLSI, 2020). The selection of these four types of antibiotics is also based on the mechanism of action of antibiotics in inhibiting the growth of *Staphylococcus aureus* bacteria.

Clindamycin is the first lincomycin group of antibiotics to be introduced for clinical use, and its mechanism of action is very similar to that of erythromycin. Clindamycin belongs to a group of narrow-spectrum antibiotics used for anaerobic and aerobic gram-positive bacteria. The antibiotic inhibits bacterial protein synthesis by reversibly binding to the 50S ribosomal subunit, thereby blocking the transpeptidation reaction or translocation of susceptible bacteria (Fanayoni et al., 2019).

Levofloxacin is a quinolone antibiotic that helps treat diseases caused by bacterial infections, such as pneumonia, sinusitis, prostatitis, conjunctivitis, urinary tract infections, and skin infections. This fluoroquinolones class of antibiotics works by inhibiting the activity of bacterial topoisomerase (DNA gyrase) which is important in bacterial replication (Toy, 2008).

Tetracycline is a broad-spectrum antibiotic used to treat many infectious diseases. This antibiotic works by inhibiting bacterial protein synthesis by inhibiting aminoacyl-tRNA binding to the bacterial 30s ribosome (Toy, 2008). Trimethoprim is a bacteriostatic class of antibiotics, but trimethoprim has a broad spectrum effect with activity against Gram-positive and Gram-negative organisms. This antibiotic works by binding to dihydrofolate reductase, a process that blocks the reduction of dihydrofolic acid to tetrahydrofolic acid. Interrupting the synthesis of tetrahydrofolic acid is important because these compounds play a central role in the pathway of thymidine synthesis and the synthesis of thymidine, which plays a role in bacterial DNA synthesis. In other words, by binding to dihydrofolate reductase, trimethoprim causes reduced bacterial folate synthesis (Toy, 2008).

The clear zone on the agar layer formed is caused by antimicrobial compounds diffusing into the agar layer and inhibiting the growth of microorganisms (bacteria). This area is called the inhibition zone, while the agar layer overgrown with microorganisms will appear cloudy. Antimicrobial compounds interact with bacterial cell walls, impairing permeability o and facilitating antimicrobial compounds to diffuse into bacterial cells. Diffusion that occurs will

disrupt of a series of growth processes of bacteria to inhibit their growth (bacteriostatic) or provide other effects, namely by killing bacteria (bacteriocidal). In addition, antimicrobial compounds can also penetrate cell membranes and interact with genetic material from bacteria so that bacteria can undergo mutations (Perdana & Setyawati, 2016).

This study obtained the results that to get optimal growth, incubation must be carried out at a temperature of 35°C. Temperatures less than 35°C were found to be able to make most antibiotics form a wider diameter of the inhibition zone. This discrepancy can be fatal in concluding and determining the choice of antibiotics that can be used for patient therapy. Resistance can be read susceptible if it is incubated at a low temperature. As a result, patient therapy is inadequate and the chances of developing antibiotic resistance are high (Nadjamuddin et. al., 2023). Incubating stacked media plates can lead to uneven temperature distribution, with the central plates receiving insufficient heat. As a result, the temperature as the stack's core may remain below 35°C. The higher the incubation temperature (> 35 ° C), the more germs can grown and become fertile. The diameter of the inhibition zone formed is getting smaller, so it could be that antibiotics that should be used for patient therapy are reported to be resistant because the inhibition zone becomes smaller. In addition, high temperatures can also cause the diffusion of antibiotics into the Mueller-Hinton medium to be less good.

The bactericidal activities of ciprofloxacin and levofloxacin against Staphylococcus aureus and epidermidis were significantly reduced when incubated at lower temperatures. Levofloxacin was more effective against both bacteria, suggesting it may be a more suitable option for staphylococcal infections, particularly at skin and soft-tissue sites (Parte & Smith, 1994). Another study found that isoniazid and rifampicin's bactericidal activities decreased at lower temperatures, while TMC207 was immediately bactericidal at 37°C. Pyrazinamide exhibited increased bactericidal activity at lower temperatures and in dormant seed cultures. This result suggests that low temperatures make bacteria more susceptible to TMC207's blocking of ATP synthesis and hindering the export of pyrazinoic acid (Coleman et al., 2011). Some research examined the impact of temperature on the adhesion and disinfection properties of Ag+-doped BiVO4 coatings. The study found that the monoclinic scheelite phase remained unchanged after annealing at 450-650 °C. The silver-modified samples showed good disinfection activity, with the best adhesion and complete killing of Escherichia coli, Staphylococcus aureus, Shigella, and Salmonella after 2 hours of visible-light irradiation (Zhang et al., 2022).

The inhibition of bacterial growth by antibiotics can be seen from the clear zone formed around the antibiotic disc paper. The inhibition of the growth of bacterial colonies can be caused by damage to the structural components of the bacterial cell membrane. Damage to cell membranes disrupts nutrient transport (compounds and ions) so that bacterial cells experience a lack of nutrients needed for growth (Leboffe & Pierce, 2019). The time to kill *Escherichia coli* inoculum increased significantly after preservative dilution or temperature reduction, with a concentration exponent of 3 and a temperature coefficient of 2.3 (Lusher et al., 1984). The addition of sodium chloride and temperature significantly enhanced the bactericidal activity of spirit vinegar. The time required for a 3-log decrease in viable cell numbers was shortened by 5% sodium chloride and temperature rise. The study suggests that vinegar solution containing sodium chloride may be a valueable method to prevent food poisoning by reducing the number of viable cells (Lusher et al., 1984).

The study shows that even at a low pH (4.4), ATSSB spores can grow without organic acids, but acetic and lactic acids have significant antibacterial activity. The study also found that combining these acids inhibits growth from *B. subtilis* and *B. velezensis*, making ambient storage of low-acid pasteurized sauces feasible (Sun et al., 2021). High-temperature aging of

copper-bearing 2205 duplex stainless steel (Cu-2205 DSS) improved its corrosion resistance, mechanical properties, and antibacterial activity. Results showed increased γ phase in microstructure and formation of new σ phase and copper-rich precipitates. The mechanical properties were significantly enhanced, and aging increased uniform corrosion resistance but slightly affected pitting resistance. The antibacterial performance was improved due to the increased release of copper ions, indicating potential applications in marine engineering (Khan et al., 2022). The antibacterial activity of SPR19, a Brevibacillus sp. strain, was evaluated using atmospheric and room temperature plasma (ARTP) mutagenesis. The 469 mutants were screened and confirmed, with M285 showing the highest activity. M285 was stable and tolerant to various conditions, retaining over 90% of its antibacterial properties. This result suggests that M285 is a potential antibacterial source (Songnaka et al., 2022). Another study reveals that microbial competition affects the synthesis of antimicrobial compounds by psychrophilic micromycetes, particularly in summer when higher temperatures increase total fungal activity. The most promising strain, Penicillium vulpinum KPB F-290, showed maximum antimicrobial activity against test cultures. It exhibited high antimicrobial activity against opportunistic strains and B. subtilis ATCC 6633, as well as phytopathogenic bacteria *Pectobacteria carotovorum a*nd *Pseudomonas savastanoi.* The optimal cultivation method for maximum antibiotic production is stationary, with ethyl acetate being the best extractant from the culture liquid (Kuvarina et al., 2022).

A multifunctional temperature-triggered antibacterial hemostatic fluoropolymer aggregate coating, consisting of fluoropolymer and quaternary ammonium salt, was developed. This coating has antibacterial and hemostatic properties, promoting biocompatibility and anti-adhesion performance. It also has rapid coagulation, low blood loss, minor secondary bleeding, and minimal bacteria infiltration, making it potential for biomedical applications (Li et al., 2022).

In addition to the incubation temperature factor, several other factors can affect the formation of the diameter of the inhibition zone in the antibiotic sensitivity test, including the turbidity of the bacterial suspension, the time of absorption of the bacterial suspension into the MH media, the incubation time, the thickness of the MH media, the distance between the drug discs and the potency of the drug disc. In this study, control of other factors was carried out following standard procedures, such as the bacterial suspension was measured on a nephelometer with a turbidity of 0.5 McF, did not allow bacterial infiltration in MH media to exceed 15 minutes, the incubation time was not more than 18 hours, the thickness of the medium MH is only 4 mm or 20 ml of MH media is poured into the plate, the distance between the discs does not match because only four antibiotics are used which are permanently stored at -20°C so as not to reduce the quality of the antibiotic potency (Hudzicki, 2009). Another research found that the antimicrobial activity of the MgO-NPs increased significantly ($p \leq 0.05$) with increased temperature, pH and NaCl concentration in TSB (Yoon et al., 2022).

The four types of antibiotics that have been selected are based on different mechanisms of action; besides based on the CLSI the four selected antibiotics, namely Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim can be used for Internal Quality Control of *Staphylococcus aureus* ATCC 25923 (CLSI, 2020). The yield range of the diameter of the QC inhibition zone for Clindamycin is 24-30 mm, Levofloxacin 25-30 mm, Tetracycline 24-30 mm, and Trimethoprim 19-26 mm (CLSI, 2020). Using the agar well diffusion method, the synthesized GONs demonstrated significant antibacterial activity against for bacterial strains. They also showed 60% higher radical scavenging activities (RSA) than Gallic acid, a standard used in the study (Goyat et al., 2022).

The results of the average diameter of the inhibition zone at temperatures of 33°C, 34°C, 35°C, 36°C, and 37°C for all tested antibiotics were still within the allowable range. Hence, the results of the Quality Control of *Staphylococcus aureus* were acceptable. Clinicians need

appropriate antibiotic sensitivity testing to provide antibiotic treatment therapy to patients. To guarantee sensitivity test results, routine QC testing using pure bacterial strains is essential in clinical microbiology. When selecting Quality Control strains and determining QC ranges, care is required to reproduce test results, determine starains stabilty, and so on (Hong et al., 2015).

The findings from this study demonstrate that incubation temperature significantly affects the diameter of the antibiotic inhibition zones in disc diffusion tests for Staphylococcus aureus. Specifically, the study observed that the inhibition zone diameters for Clindamycin, Levofloxacin, Tetracycline, and Trimethoprim varied with temperature changes. For instance, the inhibition zone for Levofloxacin generally decreased as the incubation temperature increased, with significant reductions at 33°C and 34°C compared to the optimal temperature. Tetracycline showed a marked decrease in inhibition zone diameter at higher temperatures, particularly at 36°C and 37°C, indicating a strong temperature dependence. These temperature-related variations were statistically significant, as confirmed by repeated ANOVA tests ($p < 0.05$ for all antibiotics), highlighting the importance of maintaining precise incubation conditions.

Due to their dual functions and diverse oligomerization patterns, the high-temperature requirement A (HtrA) serine protease family is a promising target for antibacterial therapeutics. A redesigned HtrA production method produces cleaner preparations with high yields by overexpressing and purifying target proteins under denaturing conditions. This method retains proteolytic and chaperone activity, allowing for higher production quantities and application in various protein purification strategies (Ronzetti et al., 2022).

The implications of these findings are profound for antibiotic sensitivity testing protocols. To ensure consistent and reliable results, laboratories must standardize incubation temperatures, as deviations can lead to significant differences in the measured effectiveness of antibiotics. This standardization is crucial for accurate diagnosis and appropriate treatment decisions in clinical settings, where inconsistent testing conditions could result in inappropriate antibiotic choices, potentially impacting patient outcomes. Furthermore, these results emphasize the need for rigorous quality control measures and updated laboratory guidelines to meticulously monitor and regulate incubation temperatures. Ensuring that all personnel are trained on the importance of precise incubation conditions can reduce variability and improve the reliability of antibiotic sensitivity testing across different settings.

4. CONCLUSION

There is a difference in the diameter of the antibiotic inhibition zone at various incubation temperatures. Based on these differences, it can be concluded that incubation temperature affects the diameter of the antibiotic inhibition zone in the antibiotic sensitivity test of Staphylococcus aureus by the disc diffusion method. A high incubation temperature (above the optimal temperature) can make the average diameter of the antibiotic inhibition zone in the sensitivity test smaller. In contrast, a low incubation temperature (below the optimal temperature) widens the antibiotic inhibition zone's average diameter in the sensitivity test. In addition, the temperature of ± 2°C from the optimal temperature of 35°C did not significantly affect the interpretation of the diameter of the antibiotic inhibition zone for the Internal Quality Control of *Staphylococcus aureus*. The research is limited by its focus on a single bacterial strain (*Staphylococcus aureus*) and a narrow range of antibiotics and temperatures, which may not fully represent the variability across different bacteria, antibiotics, and broader temperature ranges, potentially affecting the generalizability and robustness of the findings. Additionally, conducting the study in a single laboratory setting and maintaining a constant incubation period

without exploring the potential interaction with time also limits the comprehensiveness of the results.

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