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Differences in Diameter of the Growth Inhibition Zone of *Klebsiella pneumonia* Bacteria After Incubation at 37°C and 25°C

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Abstract

Pneumonia is an infection that causes the largest death in children worldwide (South Sumatra Province), which occupies the 6th position. One of the laboratory re-identification of *Klebsiella pneumonia* is a multi-antibiotic testing. The high sensitivity antibiotic that is still used is amikacin. The uniqueness of the diameter of the inhibition zone using the antibiotic amikacin is that it is active against most gram-negative bacilli. But one of the several factors that affect the diameter of the inhibition zone is the incubation temperature**.** The optimum temperature for pathogenic bacteria is 37^{o}C by using an incubator; however, several factors in the use of instruments such as frequent instability and disruption of installation lead to a need of incubation at 25^{o}C. The study aimed to determine the difference in the diameter of the growth inhibition zone of *K. pneumoniae* after incubation at 37^{o}C and 25^{o}C. This research is an experimental research conducted at the Microbiology Laboratory of IKesT Muhammadiyah Palembang. The sample is *K. pneumoniae* which will be subjected to gram staining, biochemical tests, followed by a sensitivity test on Mueller Hinton media which is given an amikacin antibiotic disk and incubated at 37^{0}C and 25^{0}C in order to calculate the diameter of the zone of inhibition for the growth of *K. pneumoniae* bacteria. The data was analyzed using the alternative Wilcoxon test which obtained a *p* value of 0.014. The results of this investigation showed that *K. pneumoniae* incubated at 37°C and 25°C had a significantly different diameter of the growth inhibition zone.

Keywords

Growth Inhibition Zone, Inhibitory Zone Diameter, *Klebsiella pneumonia*.

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INTRODUCTION

Indonesia is a developing country that has a tropical climate where microbes can thrive. Infectious diseases are the main health problem in developing countries, one of which is Indonesia. According to Riskesdas (Indonesia Basic Health Research) 2018, one of the main causes of infectious disease in Indonesia was transmission through the air, as 44.0% was transmitted by respiration (1).

Especially in South Sumatra Province in 2018, it was found that the number of cases of pneumonia was in the 6th position with 12.707 cases or 39.24%. The highest percentage coverage of pneumonia sufferers based on the existing target of discovery is achieved by Muara Enim Regency with 1.997 cases (88.97%) (2). Pneumonia is an infectious disease that causes the largest death in children worldwide. One of the bacteria that cause pneumonia is *Klebsiella pneumonia* (3).

K. pneumoniae is a type of *Klebsiella* group of bacteria that infects humans a lot. These bacteria are short gram-negative (-) rods, which has a size of 0.5-0.5 x 1.2µm, has a capsule, but does not form spores. The species *K. pneumoniae* showed mucoid growth, large and non-motile colonies (4).

K. pneumoniae laboratory diagnostics include bacterial isolation, gram staining, biochemical assays, and sensitivity tests to antibiotics. Antibiotic that is still widely used in the treatment of respiratory tract infections that have high sensitivity is amikacin (6,7).

Antibiotic susceptibility test could be carried out using the Kirby Bauer diffusion method by attaching the antibiotic to Mueller Hinton (MHA) media using tweezers and then incubating it at 37°C for 24 hours. During incubation, antimicrobial compounds will diffuse into the agar medium. The results were observed by measuring the drag diameter (mm) and compared it with Kirby Bauer's CLSI standard (8).

One of the factors that affects the sensitivity test of bacterial growth was incubation temperature. Temperature is the most important environmental factor that can affect microorganisms. Therefore, the temperature can affect the growth of bacteria (9). The optimum temperature for pathogenic bacteria is 37°C using an incubator, but there are constraint factors in the use of the tool, such as frequent instability and disturbances in electrical installations and lack of an incubator in a laboratory at a certain temperature, namely an ambient temperature that can grow bacteria, namely at 25°C (10).

According to research conducted by Saïd1 et al., (11) the effect of temperature on the sensitivity test of *Escherichia coli* HO25 antibiotics with the diffusion method in order to state that at temperatures ranging between 25°C and 44°C can be used with the same results as the standard. Based on the description above, the researchers found several problematic factors in the use of an incubator at a temperature of 37°C, one of

which is a laboratory test that requires an incubator for bacterial growth at a temperature of 37°C. Therefore, in this work, we compared the diameter of the growth inhibitory zone of *K. pneumoniae* bacteria at incubation temperatures of 37°C and 25°C.

MATERIALS AND METHODS

This study used true experimental design. It was conducted on December, 2021 at the Microbiology Laboratory of Institut Ilmu Kesehatan dan Teknologi Muhammadiyah Palembang, Indonesia. The sample used was the bacterium *K. pneumoniae* ATCC 10031 using Posttest-Only Control. This study begins with the process of testing a pure strain of bacteria *K. pneumoniae* ATCC 10031 with Gram staining for biochemical test IMVIC (Indole, Methyl Red, Voges-Proskauer, and Citrate), Triple Sugar Iron Agar (TSIA), glucose, lactose, maltose, mannitol, sucrose, and decarboxylases test), macroscopic colony readings followed by instrument sterilization, working procedures for glassware sterilization processes, followed by manufacturing media by weighing 38g of Mueller Hinton Agar and putting it in an Erlenmeyer 1000 mL, then distilled water was added and then heated on a hotplate. Furthermore, the media was sterilized using an autoclave at 121^{o}C for 15 minutes and poured into a petri dish (12).

Then, the manufacture of a bacterial suspension; one cycle of bacterial culture from the pure culture of the test bacteria was suspended in 0.9% of NaCl solution in a sterile test tube. Moreover, the bacterial suspension was vortexed until it became homogeneous and the turbidity was obtained according to the standards of Mc Farland 0.5 (13).

The sensitivity test for the growth of *K. pneumoniae* bacteria was carried out using Kirby Bauer method by taking the bacterial suspension and then inoculating into the Mueller Hinton media using a sterile swab, waiting a few minutes for the inoculated media to allow the bacterial suspension to seep into the media. Then, an antibiotic disk amikacin was prepared, after that each antibiotic was attached to the Mueller Hinton media using tweezers and incubated at 37°C and ambient temperature at 25°C for 24 hours. The diameter measurement of the inhibition zone used a Vernier caliper (11).

The study was conducted to determine the difference in the diameter of the growth inhibition zone of *K. pneumoniae* bacteria after incubation at 37°C and 25°C. Based on the Federer formula, there were 16 repetitions of each solvent on Mueller Hinton Agar (MHA) media which was incubated at 37°C and 25°C, and the difference in the diameter of the inhibition zone was identified after the media was incubated at 37°C and 25°C for 24 hours.

The diameter of the inhibition zone (mm) and the data obtained from laboratory

examinations are processed using the SPSS program. Due to the small amount of data (less than 50), the Shapiro-Wilk test was utilized for the normality test. The results obtained were seen from the sig value; if the value of sig was > 0.05 then the data was declared normally distributed, while if sig < 0.05 then the data was declared not normally distributed. If the results were normally distributed then it would be proceeded with *a paired t-test* (14). If the results obtained were not normally distributed, the data was transformed, the analysis carried out depends on the distribution and variance of the transformation results. If the distribution was not normal, the analysis was continued with the *Wilcoxon test* (15).

RESULTS

The identification test of *K. pneumoniae* bacteria was carried out before the research in order to ensure the purity of the strain to be used. The result of the macroscopic test of *K. pneumoniae* bacteria was large colonies (about 4 to 6 mm), gray, opaque, and slightly mucoid. These results can be seen after incubation at 37°C for 24 hours.

The identification of *K. pneumoniae* in the IMVIC test (Indole, Methyl Red, Voges-Proskauer, and Simmons Citrate) obtained negative results on the indole and methyl red tests, then positive results on the Voges Proskauer and Simmons Citrate tests. In the TSIA test, the results were A/A, H_2S (-), Gas (+). The urease test was positive, then the sugars (glucose, lactose, maltose, mannitol and sucrose) were positive. The decarboxylases tests (arginine, lysine, and ornithine) were negative and the phenyl alanine test was negative.

Figure 1 shows the results of the average value of measurement of the inhibitory zone diameter in *K. pneumonia* bacteria on the media Mueller Hinton incubated at a temperature of 37°C, which is 21 mm and Figure 2 shows the measurement of the inhibitory zone diameter of *K. pneumonia* bacteria on the media Mueller Hinton incubated at a temperature of 25°C, which is an average yield of 20 mm.

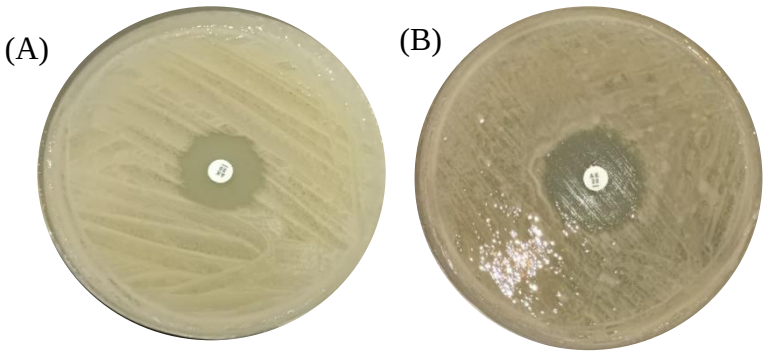

Figure 1. Diameter of the inhibition zone of *K. pneumoniae* bacteria growing at incubation temperature. (A) at 37°C, (B) at 25°C.

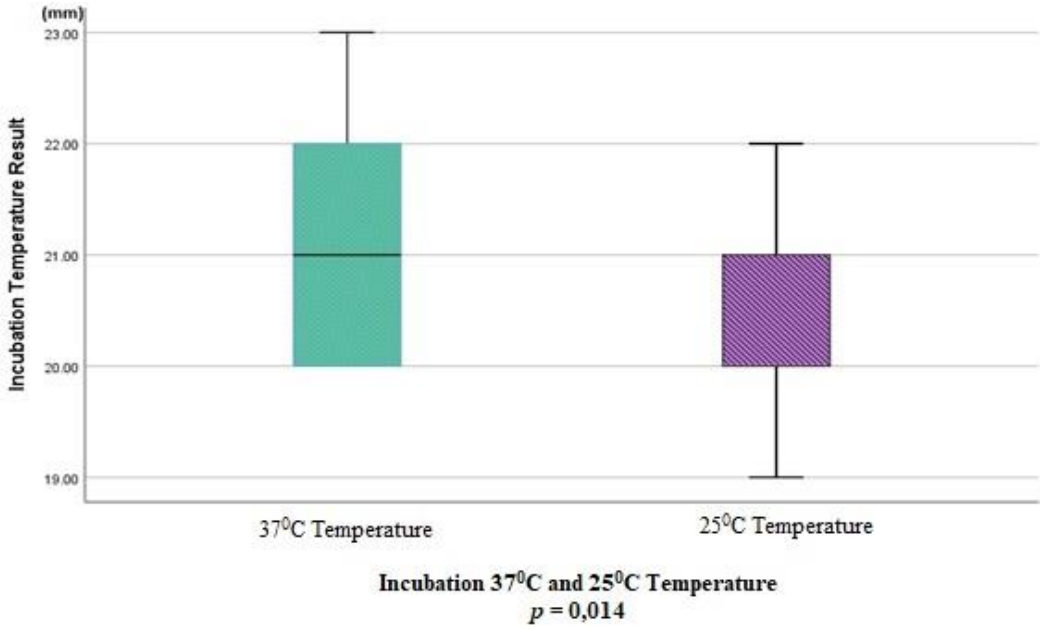

Figure 2. Diameter of the inhibitory zone of bacteria *K. pneumonia* after incubating at the 37°C and 25°C temperatures.

The study has found that *K. pneumoniae* bacteria incubated at 37^0C and 25^0C had different diameters of inhibition zones (mm). The results of the analysis of the Shapiro Wilk Normality Test showed that the diameter of the inhibition zone of *K. pneumoniae* bacteria incubated at 37°C was sig 0.014, while the diameter of the inhibition zone of *K. pneumoniae* bacteria incubated at 25°C was 0.046. Because the value obtained was sig < 0.05 based on these results, the normality of the data was not normally distributed, followed by data transformation.

Data transformation test was carried out to convert the original data into another form. The results of the analysis showed that the results of the data transformation test on the diameter of the inhibition zone at 37°C and 25°C were 0.014 (Table 1). Therefore, it can be inferred that the data are not normally distributed from the results of the statistical data transformation test of the diameter of the growth inhibition zone of amikacin antibiotics of K. pneumoniae bacteria after incubation at 37°C and 25°C. As a result, the nonparametric Wilcoxon test was conducted. (16).

Table 1. Result of SPSS Statistical Program

	p *Normality*	*P* *Transformation*	*p* *Wilcoxon*
37°C temperature	0.014	0.014	0.014
25°C temperature	0.046	0.049	0.014

p value (Sig 2 Tailed) = 0.05

Wilcoxon test is an alternative test and the Wilcoxon test showed p value (Sig 2 Tailed) = 0.014, p < therefore a significant HI value was obtained, namely there is a difference in the diameter of the inhibition zone at a temperature of 37°C and 25°C.

DISCUSSION

Incubation at a temperature of 25°C has a diameter of growth inhibition zone using amikacin antibiotic that is significantly different from incubation at an optimum temperature of 37°C. The findings of this study diverge from those of studies by Smith et al., (17), which used precision disc diffusion antimicrobial sensitivity test data carried out at 35°C, 28°C, 22°C. As a result, incubation at constant room temperature was to blame for the diameter of the inhibitory zone in the 2018 investigation in the Alzair region. The temperature instability in the microbiology laboratory for the incubation of *K. pneumoniae* is likely one of the reasons for the disparity in results between our study and Smith et al., (17). Condition at 25°C produced by excessive ventilation and variable weather that significantly alters the diameter of the inhibitory zone. The ambient temperature will rise during the day, and at night it will drop once more.

The measurement of the diameter of the inhibition zone shows that the test conditions have a significant influence on the accuracy of the data obtained. The accuracy of the inhibition zone diameter data decreased as the temperature decreased and the incubation time increased so that the inhibition zone diameter measurement was disrupted. Temperature greatly affects enzyme activity when catalyzing a reaction; the higher the temperature the more active the enzyme activity. Enzyme activity increases at the rate of reaching the optimum. An increase in temperature exceeding the optimum temperature causes weak bonds in the enzyme (17,18).

According to the research conducted by Smith et al., (17) temperature conditions have a significant effect on the accuracy of the data set obtained for the precision of measuring the diameter of the inhibition zone.

Our research is in line with Saïd1 et al., (11), which states that temperature greatly affects the antibiotic sensitivity test with the agar diffusion method because room temperature shows the most significant effect compared to other temperatures, namely at 37°C and 30°C, while in our study there was a significant difference in the diameter of the inhibition zone incubated at 37°C and 25°C.

Temperature is one of the environmental factors that affect microbial growth. Each microbe has a certain optimum temperature range for its growth. Microorganisms will develop less rapidly at temperatures that are below or beyond their optimal range. The optimum temperature for pathogenic bacteria was 35±2°C. Pathogenic bacteria that grow

best in the middle of this range are referred to as mesophilic, which include all human and opportunistic pathogens (19).

The results of the study are the same as previous studies by Saïd1 et al., (11). This can occur because temperature can affect the growth of the diameter of the inhibition zone. Temperatures with optimum conditions are needed for the speed of cell growth. There are several obstacles faced during the research, namely the occurrence of room temperature instability due to laboratory conditions that are often open. This greatly affects the measurement of the diameter of the inhibition zone of the bacteria on Muller Hinton (MH) media. Each bacterium has an optimal temperature where they can grow very fast and has a temperature range in which they can grow (20).

CONCLUSIONS

Based on the research, with regard to the average diameter of the amikacin antibiotic inhibition zone on *K. pneumoniae* bacteria, there was a significant difference after incubation at a temperature of 37°C and 25°C, and laboratory personnel can use incubation at a temperature of 25°C as an alternative temperature by adjusting the temperature in a certain area.

AUTHOR CONTRIBUTIONS

Bastian: conceptualization, methodology, drafting, monitoring and editing. Dewi Hartati: revision, data curation, and validation. Juwy Trianes: Data processing and data retrieval.

CONFLICT OF INTEREST

There is no conflict of interest in the process of carrying out the research until completion.

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