Antibiotic Sensitivity Test Gentamicin in Bacteria *Staphylococcus aureus* with Incubation Temperature 33ºC and 35ºC

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Keywords: Sensitivity Test, antibiotic Gentamicin, *Staphylococcus aureus*

Abstract: *Staphylococcus aureus* is one of the bacteria that causes infection but is very resistant to various antibiotics Many factors affect the results of this antibiotic sensitivity test, one of which is the temperature factor. According to CLSI (2014) the incubation temperature for the sensitivity test used was 35 ± 2ºC, whereas according to WHO (2004) the temperature used for this test was 35ºC temperature, temperatures higher than 35ºC of the culture seemed sensitive, whereas at temperatures lower than 35ºC resistant colonies will grow inside the inhibition zone. This type of research is analytic observation. The research sample used was a pure strain of *Staphylococcus aureus* ATCC 25923. The study sample was taken after fulfilling the inclusion and exclusion criteria. *Staphylococcus aureus* bacteria that have been inoculated were planted on the Mueller Hinton Agar media and antibiotics were added by the Kirby-Bauer method, then incubated at 33ºC and 35ºC respectively. The inhibition zone yield is measured in mm. The measurement data then tested the hypothesis by the Wilcoxon test with a 0.05 consecutive confidence level. The results showed the mean zone of inhibition at 33ºC gentamicin 21.67 mm while at 35ºC gentamicin 21.67 mm. These data were analyzed and showed no difference in the zone of inhibition of *Staphylococcus aureus* at 33°C and 35°C incubation temperatures. Based on the results of the study it can be concluded that the difference in incubation temperature of 33ºC and 35ºC can be done in the examination of the Susceptibility Test because there is no difference between the results of the zone of inhibition of *Staphylococcus aureus* bacteria with variations in incubation temperature.

1 INTRODUCTION

The bacteria *Staphylococcus aureus* is a bacterial pathogen that can cause infections and disorders of the skin include impetigo and folliculitis (Radji, 2010). The spread of *Staphylococcus aureus* bacteria in the world is known as *Methycillin Resistant Staphylococcus aureus* (MRSA). MRSA is an infection of the bacterium *Staphylococcus aureus* which is very resistant to various antibiotics that causes new problems in the world of health. The last overall report of the prevalence of *Staphylococcus* and MRSA in Indonesia in 2006 reached 23.5% (Ramadhani, 2014). Bacterial sensitivity test is a method to determine the level of bacterial susceptibility to antibacterial substances and to find out the pure, antibacterial activity. The principle of this method is inhibition of the growth of microorganisms, which is a zone of obstacles will be seen as a clear area around discs containing antibacterial substances (antibiotics). The Diameter of the bacterial growth barrier zone indicates bacterial sensitivity to antibacterial substances. Furthermore, the wider diameter of the zone of obstacles formed by the bacteria is increasingly sensitive (Waluyo, 2008).

The sensitivity test result, as reported to the Clinisi, is the classification of these microorganisms into one of two or more sensitivity categories. The simplest system consists of only two categories that are sensitive and resistant. This classification has many advantages for statistical and epidemiological purposes, too rigid to be used by Clinilians. Therefore, often used classification of three categories. The Kirby-Bauer method and its modifications are often used to determine the sensitivity of the bacteria using three categories of sensitivity, clinicians and laboratory workers must understand the exact definition and clinical significance of those categories (WHO, 2003). Many factors affect the results of this antibiotic sensitivity

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test, including environmental factors. Environmental factors that affect growth include abiotic and biotic factors. Abiotic factors include temperature, osmose pressure, drying, and ions from electricity. Biotic factors are among the factors found in the bacteria itself (Gultom, 2015). Temperature is the most important factor in influencing the rate of bacterial growth. Sometimes temperature is not much noticed in growth and of bacterial identification. Laying bacterial isolation at room temperature when incubators are being used for the safety of other bacteria often done. Temperature analytic procedures should be observed to diagnosis in the breeding of bacteria is acceptable. Influence incubation temperature may result in an error analytic procedure to fault inhibition zone results (Safety, 2015).

The optimum growth temperature for *Staphylococcus aureus* is 35-37ºC. This sensitivity test was incubated at 35ºC for optimal growth. If the temperature is lowered, the time needed for effective growth will lengthen and produce a wider zone. At temperatures of 35ºC or lower, colonies can grow inside the inhibition zone. The incubation time is 16-18 hours (for rapid diagnosis), but you should use a conventional 24 hour time for optimum results (Vandepitte *et al*., 2010). The bacterial temperature metabolism *Stapylococcus aureus* is when the temperature rises, the metabolic rate in the form of macromolecules such as proteins, nucleic acids, in Stapylococcus aureus bacteria will suffer damage permanent (Abrar *et al*., 2013). When lowered, the metabolic rate descending (Brooks *et al*., 2005). According to Madigan *et al* (2012) the bacterial growth temperature can be divided into psychophilic bacteria (15ºC-20ºC), mesophilic bacteria (20ºC-40ºC) and thermophilic bacteria (50ºC-60ºC). *Staphylococcus aureus* bacteria whose growth is optimal in temperatures of 35-37ºC are included in mesophilic bacteria (Vandepitte *et al*., 2010). According to WHO on Basic Laboratory the incubation temperature used for the optimal sensitivity test is 35ºC with a time of 16-18 hours or 24 hours(WHO, 2003). CLSI on *Performance Standards for Antimicrobial Suceptibility* suggests using a temperature of 35 ± 2ºC (CLSI, 2014). Previous research stated that temperatures of 37ºC and 40ºC were effective for the growth of *Staphylococcus aureus* toxins and enterotoxins (Vandenbosch *et al*., 1973). Other studies on the isolation and characterization of *Staphylococcus aureus* of Milk of Ettawa Crossbred Goat, were inoculated on the Mueller Hinton media in temperature 37 ºC to obtain intermediet results (Purnomo, 2006). Antibiotic sensitivity test according to CLSI uses a temperature of 35 ± 2ºC (33ºC and 37ºC). According to WHO on *Basic Laboratory the* temperature used is 35ºC, at temperatures higher than 35ºC the culture appears sensitive, whereas at temperatures lower than 35ºC the resistant colony will grow inside the inhibition zone (WHO, 2003).

2 MATERIAL AND METHODS

The study was conducted at the Microbiology Laboratory at Grandmed Lubuk Pakam Hospital. This Research uses the Wulcoxon Test statistical analysis. In this research the tools and materials used include: Ose, Autoclave, Bunsen, sterile cotton swab, test tube, ruler/ calipers, Nutrient Agar, Mueller Hinton Agar, Antibiotic Gentamicin, NaCl 0.9% sterile, Incubator. Media MRVP, Media Lactose, Media Maltose, H_2O_2 3%, Coagulase Test, DHO, Mac-Farland 0,5, Gentian Violet, Lugol/Iodium, Alkohol 96%, and Safranin.

The first step in this research is sterilize the tools and materials. The tools used must be sterile. The tool is sterilized using a *Dry Heat Oven* (DHO) at a temperature of 160ºC for 2 hours. Then, the materials used for testing and bacterial growth after being made in accordance with the specified composition are then sterilized using an autoclave at 121ºC for 15 minutes. The next step is to do sterility and quality test of the media. Steps to do the media sterility test is put the media Mueller Hinton Agar, Nutrient Agar, Lactose, Maltose to the incubator at 35ºC for 18-24 hours. Then proceed witd testing the quality of the media is create *Staphylococcus aureus* bacterial suspense with Mac Farland turbidity level 0.5, the do 10.000 dilution suspensions. Then obtained 10^2 dilutions or equivalent to the number of colonies from 100-200. The media to be tested is 10% of the amount of media. After obtaining the media to be tested then take 100 µL suspense that has been diluted then inoculated in the media using the spread plate method. Incubate for 18-24 hours at 35ºC. The next steps is making the suspension bacteria, take 1 ose of bacterial suspense. Scratch the Nutrient Agar media evenly with the Streak method. Incubate for 18-24 hours at 35ºC.

2.1 Bacteria Standard Test

Catalase Test. Procedure catalase test (Figure 1) is Apply one drop of H_2O_2 3% on a object glass, then transfer the *Staphylococcus aureus* bacterial colony with a loop to the H_2O_2 solution, then mix. Catalase positif reaction:evident by immediate effervescence (air bubble formation), and then catalase negatif

reaction: no bubble formation (no catalase enzyme to hydrolyze the hidrogen peroxide. The catalase test is primarily used to distinguish among gram positive cocci: members of the genus *Staphylococcus* are catalase positif, and members of genera *Steptococcus* and *Enterococcus* are catalase negatif (Tankeshwar, 2013).

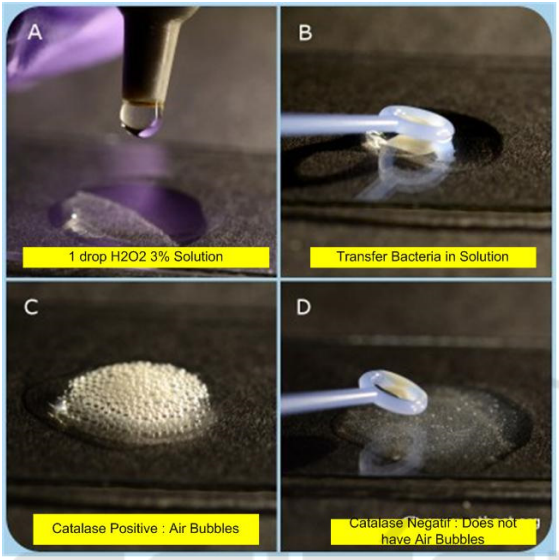

Figure 1: Catalase Test (Vetbatch, 2017)

Microscopic Test. Make bacterial suspense from bacterial colonies that grow on agar nutrient media. Make preparations slide of bacteria, then do gram staining. The steps of gram staining (Figure 2) : (a) fixation of the slide, (b) apply a crystal violet stain for 1 minute, (b) wash slide in a gentle and indirect stream of tap water for 2 seconds, (c) flood slide with the iodine wait 1 minute, (d) wash slide in a gentle and indirect stream of tap water for 2 second, (e) flood slide with decolorizing agent (alcohol decolorizer) wait 10-15 second or add drop by drop to slide until decolorizing agent running from the slide runs clear, (f) flood slide with a counterstain with safranin, wait 30 second to 1 minute, (g) wash slide in a gentile and indirect stream of tap water until no color appears in the effluent and then blot dry with absorbent paper, (h) observe the result of the staining procedure under oil immersion using a microscope (Tankeshwar, 2015). The result of gram staining is gram negatif bacteria will stain pink/red and gram positif bacteria wil stain blue/purple.

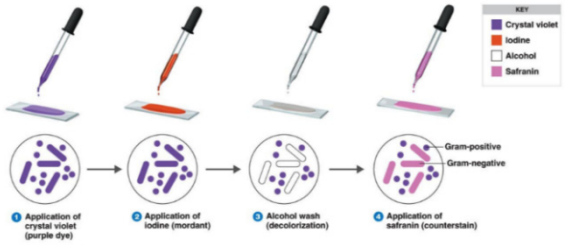

Figure 2: Gram Staining (Prajapati et al., 2018)

The next step of bacteria standart test is biochemical characterization. Potential isolate were characterized by different biochemical methods like lactose and maltose test, MR (Methyl red) and VP (Voges Proskauer) test, and Coagulase test. Sugar Test (Lactose and Maltose) : Take 1 ose of bacterial suspense and put it in a sugar medium (lactose and maltose). Mix evenly. Incubate at 35ºC for 18-24 hours. Methyl Red (MR) Test : Take 1 ose bacterial suspense, insert into MR media, mix evenly, incubate at 35ºC for 18-24 hours. After incubating to find out the reaction, add the Methyl red reagent. Positive results are indicated by a change in color from yellow to red. Voges Proskauer Test (VP) : Take 1 ose bacterial suspense, insert into the VP media, mix evenly, incubate at 35ºC for 18-24 hours. After incubating to find out the reaction, add the KOH and α-Naphthol reagents. Positive results are indicated by a change in color from yellow to red. Coagulase Test : This test is used to determine the presence of free coagulase by means of 200 µL plasma aseptically inserted into a sterile test tube. Take 3-5 bacterial colonies of *Staphylococcus aureus* , put them in the test tube, mix carefully. Incubate at 35ºC for 18-24 hours.

The next step is prosedure of antibiotic sensitivity test. The first Making the Inoculum (Suspension) : Take 3-5 ose *Staphylococcus aureus* bacteri colonies of the same size in the media using an ose (loop) that has been incanded above Bunsen. Then insert the colony into a test tube that contains sterile 0.9% NaCl solution, then homogeneous. Compare this with turbidity suspension of *Staphylococcus aureus* with 0.5 Mac-Farland comparison solution. NB: Turbidity Bacterial suspension should be equivalent to Mac-Farland 0.5. And then, step of Antibiotic Sensitivity Test (Susceptibility Test) : Inoculation using the Kirby-bauer method and place the antibiotic in the middle of the media surface. Incubate the media at 33ºC and 35ºC for 18-24 hours. And then, record and measure each zone formed around the antibiotic (disc paper). The resulting inhibition is measured in mm (millimeters), show procedure in Figure 3.

3 RESULT

Identification of *Staphylococcus aureus* bacteria can be microscopic and biochemical test of bacteria. On microscopic examination found Gram (+) cocci (+). These bacteria are round like a ball or spherical, clustered and purple. The results obtained are the same as the results of microscopic examination by Holt *et al* (1994) which states that the *Staphylococcus aureus* bacteria are gram-positive coccus bacteria that are spherical in shape like a ball. These bacteria are purple because it is the first absorbing dye is gentian violet. The result of microscopic test show in Figure 4 below.

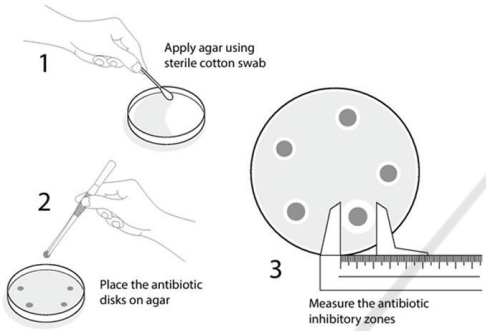

Figure 3: Step Antibiotic Test (ACS, 2020)

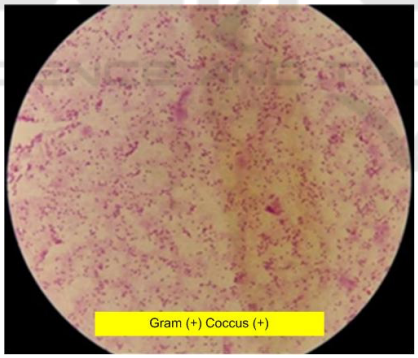

Figure 4: Microscopic Test

Biochemical tests used to test further identification of *Staphylococcus aureus* bacteria. This test is carried out on confectionery media namely lactose and maltose, Methyl Red (MR), Voges Proskauer (VP), Catalase Test and Coagulase Test. The results can be seen in Table 1 and Figure 5. The Result of *Staphylococcus aureus* bacterial biochemistry test carried out in confectionery media, namely lactose and maltose, showed positive results with a marked change in green to yellow, indicating that the bacteria were able to ferment lactose and maltose. Patty research states that the test of lactose and maltose sugars of the bacterium *Staphylococcus aureus* get positive results that are marked by a yellow discoloration, this shows that the bacteria tested are able to ferment the type of sugars tested (Patty *et al*., 2016).

In the Methyl Red and Voges Proskauer tests, positive results were obtained with a change from the deep red yellow which indicates the presence of acetoin in the solution produced by bacteria. Dewi's research states that the positive results of the Methyl Red and Voges Proskauer tests are characterized by a change in color from yellow to red indicating the presence of glucose fermentation in *Staphylococcus aureus* from the formation of acetilmetilcarbinol (acetoin) (Dewi, 2013). Catalase test is a test to distinguish between groups of *Staphylococcus aureus* or *Steptococcus* bacteria. The results obtained in the catalase test are positive which is marked by the presence of air bubbles (O_2). Toelle's research states that the *Staphylococcus aureus* bacterial catalase test with positive results because these bacteria produce enzymes capable of hydrolyzing hydrogen peroxide (H_2O_2) into water (H_2O) and air bubbles (O_2) (Toelle & Viktor, 2014). On the examination of the coagulase test positive results were indicated by the presence of lumpy white jelly. Purnomo's research states that *Staphylococcus aureus* can agglutinate blood, bacause it has a coagulase reacting factor, the role of coagulase produced by germs is used as a determinant of *Stapylococcus aureus* species that make white clots like jelly that show positive results (Purnomo, 2006).

Table 1: Test Results for Identification of *Staphylococcus aureus* Bacteria

Identification Test	Results
Lactose Test	(+) Positive yellow
Maltose Test	(+) Positive yellow
Metyl Red Test	(+) Positive red color
Voges Proskauer	(+) Positive red color ring
Catalase Test	(+) Positive air bubbles

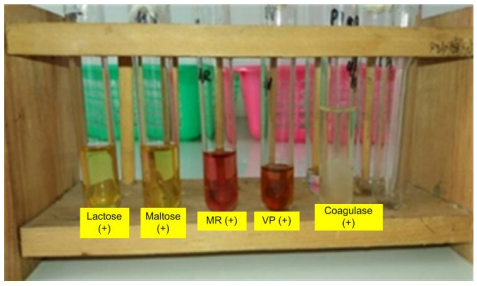

Figure 5: Biochemical Test

Sterility test in this study was carried out on Nutrient Agar Media and Muller Hinton Agar Media. This test is performed by non-cultivation of *Staphylococcus aureus*. The media standard will be tested by means incubated at 37^0C for 24 hours. The purpose of the media sterility test is to determine whether there is growth of bacteria and fungi of other microorganisms that can affect the results of research. The results of sterility media test can be seen in Table 2 and Figure 6.

Table 2: Media Sterility Test

Media	Test result	Sterile / Not steril
Nutrient Agar	Does not grow	Sterile
Mueller Hinton Agar	Does not grow	Sterile

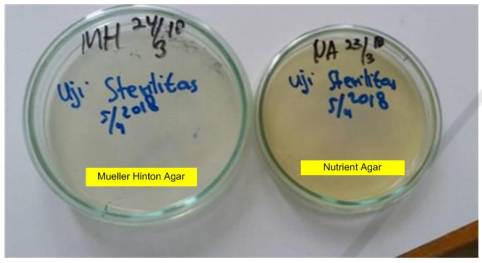

Figure 6: Nutrient Agar Media and Mueller Hinton Agar Media for media sterility test result

Media quality test performed to determine the growth of bacteria on a medium which will be used, whether the media can be overgrown with fertile bacteria or not. This test is performed on Nutrient Agar and Mueller Hinton Agar. The results can be seen in Table 3, Figure 7 and Figure 8.

Table 3: Media Fertility Test

Media	Test result (Colonies)	Good/ Not Good
Nutrient Agar	Grow	Good
Mueller Hinton Agar	Grow	Good

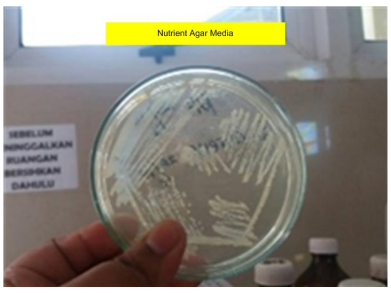

Figure 7: The results of the agar nutrient media quality test

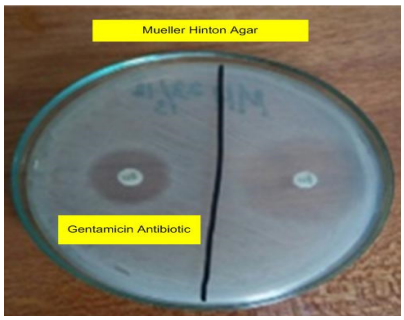

Figure 8: The results of the agar Mueller hinton media quality test (*Mueller Hinton Agar* with Gentamicin Antibiotic Media is shown in the image on the left

3.1 Results of Gentamicin Antibiotic Sensitivity Test (Susceptibility Test)

Susceptibility test or antibiotic sensitivitty test is used to determine the inhibitory zone produced from an antibiotic. Inhibition zone was used as the diagnosis result if the antibiotics are sensitive or resistant to these bacteria. The results of the *Staphylococcus aureus* bacteria inhibition zone on Gentamicin antibiotics with an incubation temperature of 33ºC (Figure 9) and 35ºC (Figure 10) for 24 hours are presented in Table 4.

Table 4: Results of inhibition zones of *Staphylococcus aureus* bacteria on Gentamicin antibiotics with an incubation temperature of 33ºC and 35ºC.

No	Gentamicin Antibiotic Zone (mm)		Information
	Temperature of 33ºC	Temperature of 35ºC	
1	21	22	Sensitive
2	22	22	Sensitive
3	21	21	Sensitive
4	21	22	Sensitive
5	22	22	Sensitive
6	21	21	Sensitive
7	22	22	Sensitive
8	22	22	Sensitive
9	23	21	Sensitive
Average	21.67	21.67	Sensitive

Description :
Sensitive ≥ 15mm; Resistance <12mm

Figure 9: The result of inhibition zone of antibiotic gentamicin at a temperature 33ºC

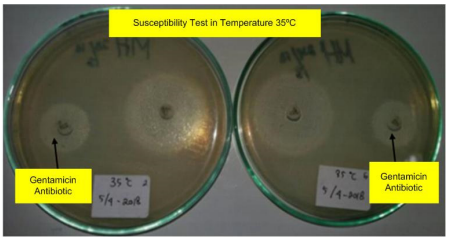

Figure 10: The result of inhibition zone of antibiotic gentamicin at a temperature 35ºC

3.2 Data Analysis

The results of the examination of the zone of gentamicin antibiotic inhibition of bacteria *Staphylococcus aureus* that have been collected, then processed and then presented in tabular form and analyzed the data through several stages, namely normality test and hypothesis testing.

3.2.1 Normality Test

In this study the normality test was carried out using the Kolomogorov-Smirnov test because the number of samples used was less than 50 with a significance level of 95% (α=0.05). The following are normality test results presented in Table 5 below:

Table 5: Normality Test Results

	Kolomogorov-Smirnov		
	Statistic	Df	Sig
Temp33	,272	9	,054
Temp35	,414	9	,000

Based on Table 5 the p value (sig) obtained for the temperature of 33ºC gentamicin antibiotic is 0.054, for temperature 35ºC gentamicin antibiotic is 0,000. From the values above there is 1 data that is normally distributed at 33ºC, while at 35ºC the data is not normally distributed. Because there is one data that is not normally distributed, it is followed by a non-parametric statistical test.

3.2.2 Hypothesis Testing

The Wilcoxon Test is a test used to test differences in sample pairs in 2 groups (Dahlan, 2014). The results of data analysis are obtained as shown in Table 6.

Table 6: Wilxocon Test Results

	Temp 33 - Temp 35
Z	,000
Asymp. Sig.	1,000

From the analysis of the data obtained, it can be seen that the significant value in the Table above is 0,000. Based on the specified conditions if it is significantly greater than the alpha value of 0.05 then Ho is accepted, whereas if it is significantly smaller than the alpha value of 0.05, Ho is rejected. So it can be seen that the asymp.sig value obtained is 1,000 is greater than the alpha value of 0.05 then Ho is accepted. Thus, it can be concluded that "There is no difference in the inhibition zone in the Gentamicin Antibiotic Sensitivity Test for *Staphylococcus a ureus* Bacteria for Incubation Temperature Variations of 33ºC and 35ºC".

4 DISCUSSION

In this study, a number of preliminary tests were carried out including sterility tests, quality tests, and bacterial standard tests. Sterility tests are carried out with the aim of ensuring that the media used are not contaminated with bacteria or other microorganisms. Fertility test or media quality test is a test used to see that the media used is good for bacterial growth. Bacterial standard tests are also used to identify or confirm tests that the bacteria used are *Staphylococcus aureus* bacteria.

Identification of *Staphylococcus aureus* bacteria can be done with microscopic tests and bacterial biochemical tests. On microscopic examination, gram (+) coccus (+) was obtained. These bacteria are round like a ball or spherical, clustered and purple. The results obtained are the same as the results of microscopic examination by which states that the *Staphylococcus aureus* bacteria are gram-positive positive coccus bacteria that are spherical in shape like a ball. This bacterium is purple because it absorbs the first dye, gentian violet (Holt *et al.*, 1994). *Staphylococcus aureus* bacterial biochemistry test carried out on confectionery media, namely lactose and maltose, showed positive results with a marked change in green to yellow, indicating that the bacteria

were able to ferment lactose and maltose. In the *Methyl Red* and *Voges Proskauer* tests positive results were obtained with a change in color from yellow to red mangosteen which showed the presence of acetoin in the solution produced by bacteria. Catalase test is a test to distinguish between *Staphylococcus* and *Streptococcus* bacteria groups , in this test positive results are indicated by marked bubbles when dripped with H_2O_2. In the examination of the coagulase test obtained positive results in the presence of lumpy white jelly. The results obtained are the same as the results of Dewi's research (Dewi, 2013).

In this study the results of gentamicin antibiotic inhibition zones, each incubated at 33ºC and 35ºC, showed no difference because the temperature range used in the growth temperature range of *Staphylococcus aureus* bacteria was either used or commonly called mesophilic temperature. According to the research of James H. Jorgensen *et al* the optimum growth of *Staphylococcus aureus* at 35ºC for 16-24 hours with vancomysin, daptomycin, and linezolid antibiotics (James *et al*., 2009). According to the Clinical and Laboratory Standars Institute (2014) sensitivity test or antimicrobial susceptibility test using temperatures of 35 ± 2ºC (33ºC, 35ºC and 37ºC). Meanwhile, according to WHO on *Basic Laboratory, it is* recommended that the temperature sufficiency test be used for 35 ° C for 24 hours, because at the most optimal temperature it can reduce the risk of contamination of other bacteria that will grow (WHO, 2003).

Analysis of the data used in this study is Wilxocon. Based on Wilxocon test results, it is known that there is no difference in the zone of inhibition of *Staphylococcus aureus* bacteria on gentamicin antibiotics with incubation temperatures of 33ºC and 35ºC. The results obtained are seen from the value of sig. 1,000 is greater than the alpha value (1,000> 0.05).

5 CONCLUSION

Based on the results and discussion carried out it can be concluded as follows;

a. The average zone of inhibition of the antibiotic test for gentamicin in *Staphylococcus aureus* with an incubation temperature of 33ºC was 21.67 mm
b. The average zone of inhibition of the antibiotic test for gentamicin in *Staphylococcus aureus* with an incubation temperature of 35ºC was 21.67 mm
c. From the results of gentamicin antibiotic inhibition zones that have been carried out statistical tests, it can be concluded that there is no difference in the zone of inhibition in the antibiotic sensitivity test of *Staphylococcus aureus* bacteria to the incubation temperature variations of 33ºC and 35ºC.

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