Biomedical & Pharmacology Journal, March 2024. Vol. **17**(1), p. 409-416

Identification of vanA gene on Vancomycin-Resistant *Staphylococcus aureus* from Diabetic Ulcer Isolate at Lampung Province

Tri Umiana Soleha[1]*, Sutyarso[2], Asep Sukohar[3], Sumardi[2] and Sutopo Hadi[4]

[1]Doctoral Program, Faculty of Mathematics and Natural Sciences, University of Lampung, Bandar Lampung 35145, Indonesia.
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, University of Lampung, Bandar Lampung 35145, Indonesia.
[3]Department of Pharmacology, Faculty of Medicine, University of Lampung, Bandar Lampung 35145, Indonesia.
[4]Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Lampung, Bandar Lampung 35145, Indonesia.
*Corresoponding Author E-mail:dr.triumiana.unila@gmail.com

https://dx.doi.org/10.13005/bpj/2868

(Received: 03 October 2023; accepted: 23 January 2024)

Staphylococcus aureus is a type of bacteria that causes an increasing number of infections in hospitals, particularly in diabetic ulcers. Over the last few decades, there has been an alarming increase in the prevalence of pathogen strains of Staphylococcus aureus resistant to antibiotics such as Vancomycin. This study aimed to identify the presence of the vanA gene, which is responsible for the mode of Vancomycin resistance in Staphylococcus aureus Lampung isolate. Ulcer swab was collected from 32 patients with ulcer complications from surgical Installation, Internal Medicine, Home Diabetes Wound Clinic and Diabetes Wound Center in Bandar Lampung. Among the total ulcer swab, 12 samples of S. aureus were identified and subjected to the Minimum Inhibitory Concentration test to identify Vancomycin Resistant Staphylococcus aureus and evaluated the vanA gene by Polymerase Chain Reaction. To detect the presence of the vanA gene, a Polymerase Chain Reaction was performed on Staphylococcus aureus, Vancomycin-Resistant Staphylococcus aureus, and Vancomycin-intermediate Staphylococcus aureus using a specific primer arrangement. There were two samples of Vancomycin- Resistant Staphylococcus aureus and one sample of Vancomycin-intermediate Staphylococcus aureus from the Minimum Inhibitory Concentration test but only one sample tested positive for the vanA gene on Polymerase Chain Reaction. There is Staphylococcus aureus resistance to Vancomycin in Lampung isolate and the vanA gene was detected in some resistant isolates. The arrangement of the vanA gene in the Lampung isolate is different from that of the vanA gene in other places isolates.

Keywords: Resistant; S. aureus; vanA gen; Vancomycin.

The most common bacteria isolated from diabetic foot infections are Gram-positive cocci and members of the Gram-negative Enterobacteriaceae. Gram-positive bacteria such as *Staphylococcus aureus*, Enterococcus, and Gram-negative bacteria such as *Pseudomonas aeruginosa, Escherichia coli*, *Klebsiella sp*, and *Proteus sp*, can cause ulcer infection. *S aureus* is a major human pathogen that was first identified as the causative agent of suppurative abscesses more than 130 years ago[1,2]

Using antibiotics such as Penicillin and Methicillin in the mid-20th century was initially shown to be effective against *Staphylococcus aureus*. However, *S. aureus* quickly developed resistance to these antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) is becoming increasingly difficult to treat and makes this type of bacteria a major threat all over the world. Meanwhile, in the United States, Vancomycin is the drug of choice for handling MRSA[2,3]

Vancomycin is currently the antibiotic of choice for treating MRSA infections. The rate of MRSA infection continues to rise globally, and as a result, so does the consumption of Vancomycin. In Japan, the first case of Vancomycin intermediate *Staphylococcus aureus* (VISA) was reported in 1997. Since then, cases of Vancomycin resistant *Staphylococcus aureus* (VRSA) began to emerge, particularly in developing countries[4]. In Indonesia, research on the *vanA* gene (please add some explanation to relate *the vanA* gene VRSA) is still very rare, especially in Lampung province.

The purpose of this study was to analyze the suitability of Minimum Inhibitory Concentration (MIC) values to detect the presence of the novel *vanA* gene and find a new primer arrangement for the *vanA* gene from diabetic ulcer isolates in Lampung province. The vanA operon, which encodes the Tn1545 transposon and is part of the conjugative plasmid of Vancomycin-resistant enterococci, was used to generate the complete VRSA strain (MIC 16 mg/ml) (VRE). Vancomycin resistance is mediated by the glycopeptide-specific *vanA* gene. The presence of *vanA* causes an exchange within the terminal goal of D-alanyl-D-alanine to D-alanyl-D-lactate or D-alanyl-D-serine, resulting in a negative binding to Vancomycin due to the loss of the important factor for hydrogen bonding.[5,6]

MATERIALS AND METHODS

Antibiotic Susceptibility Test

In this study, 32 samples were collected from patients with diabetic foot ulcers in Lampung province. The bacterial samples were placed on liquid nutrient agar media. After 24 hours of incubation, one colony was streaked into Mannitol Salt Agar (MSA) medium and incubated at 37°C for 48 hours to obtain the *S. aureus* strain[7]. Then the strain was suspended in 3 ml of physiological NaCl solution and its turbidity was compared to the McFarland standard of 0.5. In a petri dish, 100 µl of pure culture suspension was spread on the surface of Mueller Hinton Agar (MHA). The test medium was left at room temperature for five minutes to allow the bacteria to adapt to the medium. Subsequently, antibiotic disks containing Amoxycillin, Cefotaxime, Cefoxitin, and Vancomycin were placed on the medium. The mixture was then incubated at 37°C for 24 hours. A caliper was used to measure the diameter of the inhibition zone across the colony. Data from the clear zone were compared to those available in Clinical and Laboratory Standard Institute (CLSI) literature[8]

Minimum Inhibitory Concentration (MIC)

MIC for the strains collected from diabetic ulcers was determined by agar dilution as recommended by the National Committee for Clinical Laboratory Standards. Standard quality control strains were included in each run. Cultures were incubated for 20 to 24 h in ambient air. Drugs were obtained from their respective manufacturers. MIC was determined using the tube dilution method with Mueller Hinton Broth (MHB) at concentrations ranging from 4, 8, 16, 32, and 64 mg/mL. To test for the presence of VRSA, 1 mL of 0.5 MacFarland bacterial colony suspension was added to 1 mL of MHB and Vancomycin at various concentrations. The tube with the highest concentration, where bacterial growth was still visible, was scraped again on Nutrient Agar media and incubated for 24 hours to see if the bacteria, Vancomycin-resistant *S. aureus*, grew. The MIC concentration for whole vancomycin resistance in *S. aureus* was 16 mg/ml[8,9]

Primer Design

The primers were created using the National Center for Biotechnology Information's BLAST software (NCBI). The primer sequence was created based on the DNA sequence that has been isolated or amplified. Primer comes in two varieties: forward primer and reverse primer. The primer acts as a limiter for the region to be read in the PCR reaction. A good primer design is critical to the success of the PCR reaction.[10,11]

Polymerase Chain Reaction for *vanA* Gene

PCR was used to amplify 1-3 µL of DNA in 100 µL of reaction mixture solution consisting

of PCR buffer (10 m MTris-HCl pH 9.0), 50 mMol KCl, 1.5 mM $MgCl_2$, 0.1 percent Triton X-100, 0.2 mg/ml bovine serum albumin, 50 M deoxynucleoside triphosphate, 2 primer pairs each, and 2 Taq polymerase enzyme (Qbiogene). The amplification was completed in 3 minutes at 94°C, and 30 amplification cycles consisting of 1 minute at 94°C, 1 minute at 54°C, 1 minute at 72°C, and 7 minutes at 72°C as the final step. This DNA fragment was examined using 0.5xTBE (Tris-Borate-EDTA) electrophoresis on a 1% agarose gel stained with ethidium bromide.[12,13]

This study has received approval from the Studies Ethics Committee College of Medication Lampung University with ethical clearance number 1616/UN26.18/PP.05.02.00/2021.

RESULTS

S. aureus Antibiotic Susceptibility Test

Amoxicillin (Amx.), Cefotaxime (Ctx.), Cefoxitin (Fox.), and Vancomycin were used in bacterial susceptibility testing of isolates from patients of diabetic ulcers (Va.). Bacteria grown on Muller Hinton agar media were incubated for 24 hours at 37°C, after which the inhibition zone formed around the antibiotic was measured and classified as Resistance (R), Intermediate (I), or Sensitive (S) based on CLSI criteria, 2014. The results of antibiotic sensitivity tests are shown in Table 1.

Based on the data in Table 1, 100% of *S. aureus* samples were resistant to the antibiotic Amoxicillin; 50% of samples were resistant to Cefotaxime antibiotics, 33% were Cefotaxime intermediates, and 8.3% were sensitive to Cefotaxime. 50% of samples were resistant to the antibiotic Cefoxitin, while 41,6% were not. 50% of samples were resistant to the antibiotic Vancomycin, 8.3% were intermediate, and 41.7% were sensitive. It was discovered that 6 out of 12 test samples that met the inclusion criteria were resistant to Vancomycin or exhibited Vancomycin-resistant *S. aureus* (VRSA), while 7 out of 12 patients exhibited MRSA or Methicillin-resistant *S. aureus* (MRSA) or were multidrug resistant. As

Table 1. Susceptibility Profile of *S. aureus* Lampung Isolate with Antibiotics Penicillin, Cephalosporin and Glycopeptide

Sample Code	Antibiotic AMX	CTX	Fox	VA
S1	R	R	R	R
S2	R	R	R	R
S3	R	R	R	R
S4	R	I	S	R
S5	R	R	S	R
S6	R	I	R	S
S7	R	R	R	S
S10	R	R	R	S
S14	R	I	S	S
S17	R	R	S	S
S18	R	S	S	R
S19	R	I	R	I

Note : AMX (R<21;122-27;S≥28), CTX (R≤14;115-22;S23), FOX (R21;121-22; S22), VA (R9;110-11;S12)
AMX = Amoxicillin, CTX = Cefotaxim, FOX = Cefoxitin, VA = Vancomycin

Table 2. Minimum Inhibitory Concentration (MIC) of Vancomycin Resistant *Staphylococcus aureus (*VRSA) Lampung Isolate

Sampel	4 mg/mL	8 mg/mL	16 mg/mL	32 mg/mL	64 mg/mL	Kind (MIC)	Disk Diffusion (VA)
S1	-	-	-	-	-	VSSA	VRSA
S2	+	+	+	+	-	VRSA	VRSA
S3	+	+	-	-	-	VISA	VRSA
S4	+	+	+	+	-	VRSA	VRSA
S5	-	-	-	-	-	VSSA	VRSA
S18	-	-	-	-	-	VSSA	VRSA

Note : (+) still growing (cut off point 16mg/mL)
(-) not growing

a result, there is little difference in the prevalence of MRSA and VRSA.

MIC of Vancomicyn-resistant *Staphylococcus aureus* (VRSA)

Six *S. aureus* isolates (Table 2) that were resistant to the antibiotic Vancomycin were tested for the presence of VRSA, and it was discovered that two samples have MIC = 32 g/mL that were VRSA, one sample has MIC = 8 g/mL that was Vancomycin Intermediate *Staphylococcus aureus* (VISA), and the other three samples were still susceptible to Vancomycin antibiotics.

Primer Design for vanA Gene of Vancomycin Resistant *Staphylococcus aureus* (VRSA)

The complete VRSA strain (MICe"16 µg/ml) was obtained from the vanA operon

Fig. 1. MIC on Vancomycin Resistant *Staphylococcus aureus* (VRSA)

vanA 1 ATGAATAGAATAAAAGTTGCAATAC
vanA 2 CCCCTTTAACOCTAATACGAT

Fig. 2. Primer arrangement of *vanA* gene (5' to 3')

encoding transposon Tn1545 which is part of the conjugative plasmid of Vancomycin resistant enterococci (VRE). The primers were created with the help of the National Center for Biotechnology

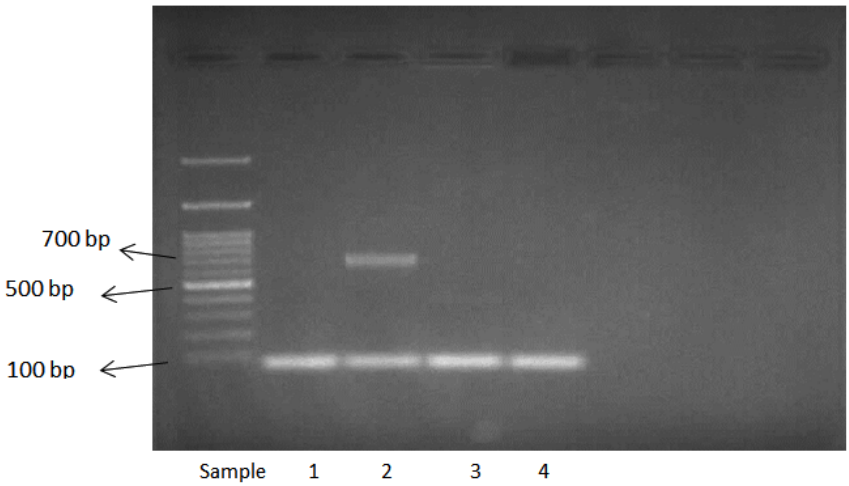

Fig. 3. Sample 1. VSSA 2. VRSA 3. VISA 4. VRSA

Information's BLAST software (NCBI). The following candidates received the most votes:
(5' 3')
vanA_Foward :
AGGAGACAGGAGCATGAATAG
vanA_Reverse : CAATACCGCACAACCGAC

In addition, a preliminary analysis was performed through the website https://sg.idtdna.com/calc/analyzer to obtain primary data in accordance with predetermined criteria. The results of primer analysis obtained are percentage of nucleotides in primer candidates (%GC Forward = 47.62%, %GC Reverse = 55.56%).

In another study in Tehran, a *vanA* gene primer pair was found with the arrangement shown in Figure 2.[4]

The primer pair is slightly different from the primers designed in this study, in terms of the percentage of guanine-cytosine and the size of the PCR product. Primer sequences were created from the isolated or amplified DNA sequence. Primer is classified into two types: forward primer and reverse primer. A primer serves as a separator for the region of the sequence to be read in a sequencing reaction. A good primer design is critical to the success of the PCR reaction. [6,14] Specific primers were developed in this study to detect Staphylococcus aureus strains from diabetic ulcer isolates in Indonesia.

Polymerase Chain Reaction

Using independently designed primers, PCR can detect Vancomycin-resistant *Staphylococcus aureus* (VRSA) and compare it to Vancomycin-sensitive *Staphylococcus aureus* (VSSA). VSSA is sample one, VRSA is sample number two and four, and VISA is sample number three. The results obtained are shown in the image in Figure 3.

Only sample No. 2 has the *vanA* gene because it is VRSA, whereas sample No. 1 does not have the *vanA* gene. After all, it is *S. aureus*, which is still susceptible to Vancomycin. Meanwhile, no *vanA* gene was detected by PCR examination in samples No. 3 (VISA) and No. 4 (VRSA) based on the MIC value. This is due to several factors, including the determination of VRSA based on the MIC value, which is the phenotype of resistant bacteria, whereas the genotype can be carried by genes other than *vanA*, such as *vanS, vanR,* and *vanH*. Another possibility is that the primer length used in sample No. 3 does not match the *vanA* gene.[15,16]

To clarify, the PCR process was repeated on sample No. 2, and the results of DNA amplification were obtained with an amplicon length of 673 bp, as shown in Figure 3.

DISCUSSION

The *S. aureus* is the causative agent of the high incidence of infection and is responsible for 80% of suppurative diseases with a natural habitat on the surface of the skin so that its presence can be suppressed with antibiotics so that increased morbidity rates lead to reduced mortality and human survival can be maintained longer. Diabetic ulcer is a chronic ulceration that occurs on the feet of patients with diabetes mellitus. Diabetic ulcers are mostly caused by *S. aureus* and *Pseudomonas spp*.[17,18]

Treatment of *S. aureus* bacterial infection in diabetic ulcers is difficult due to the ability of the bacteria to develop resistance to various antibiotics. For several decades, there has been an increase in the prevalence of pathogens and resistant strains to antibiotics. Methicillin was created in response to increased penicillin resistance. With the isolation of the first strain of Methicillin-resistant *Staphylococcus aureus* (MRSA) in 1961, infection by *S. aureus* can be well controlled using methicillin.[19,20]

Vancomycin is the last antibiotic resistance test in this study. The cultured isolates from diabetic ulcer patients in Lampung province revealed that six of the twelve isolates identified by the *S. aureus* bacteria are resistant to Vancomycin antibiotics, one is in the intermediate group and the remaining five samples are still in the sensitive group. Based on these findings, we can see that the sensitivity of vancomycin antibiotics has decreased, even though it is still a positive control of each antibiotic sensitivity test and the most effective control for the presence of MRSA.[21,22]

The number of VRSA obtained from the results of the Vancomycin antibiotic susceptibility test using the disc diffusion method differs from the number of VRSA obtained from the results of the Vancomycin antibiotic susceptibility test using the dilution method, where the number of VRSA in the disc diffusion method is 6 samples and the

MIC method is 3 samples. This is because the Vancomycin antibiotic is more evenly dispersed into the bacterial suspension in the MIC test, and Vancomycin is more likely to inhibit the growth of *S. aureus*.[9,23] Because the disc diffusion technique for antibiotic susceptibility testing is not a quantitative method, the data obtained from the antibiotic susceptibility test based on the MIC value as recommended by Clinical and Laboratory Standard Institute (CLSI).[7]

When the *vanA* gene primer from VRE was used, no PCR products were produced from the 4 samples. This is because the low percentage of Guanine Cytosine (GC) reduces the efficiency of the PCR process due to the weaker bonding of the primers so that they are easily released and the primers do not recognize the sample. Good Cytosine Guanine binding in the primer is 40% – 55%[10]. Next, a new primer using the BLAST primer design method from NCBI. [24,25]was designed. Primers are usually designed to isolate specific fragments of genomic DNA by PCR. The primer sequence is made based on the DNA sequence to be isolated or amplified. Good primer design is essential for the success of a PCR reaction. In this research, only the vanA gene can be detected in one isolate, this happens because only 69% of VRSA can detect the the *vanA* gene. [26]The primers produced in this study are specific for the *vanA* gene derived from diabetic ulcers in Lampung province. For VRSA detection, the discovery of the *vanA* gene cannot yet be used as a standard examination, so further research is needed.

The discovery of the *vanA* gene in diabetic ulcer isolates indicates that the process of Staphylococcus aureus bacteria resistance to Vancomycin antibiotics in Indonesia, particularly in the province of Lampung, is confirmed and this should be monitored. Because the findings of the v*anA* gene and specific new primers are very important to detect the presence of VRSA in various regions so that it can prevent further bacterial resistance, similar studies need to be carried out with a larger number of samples.[27,28] Not only do VRSA from different regions have different genetic features, but even from 1 patient, several VRSA strains can be obtained. [29,30]It is necessary to consider more rational use of antibiotics in the cases of severe infections such as diabetic ulcers to avoid the occurrence of antibiotic resistance.

CONCLUSION

VRSA that was found in Lampung isolate has a MIC value of 32 mg/mL and based on the PCR electrophoresis images it turned out that a new band with a size of 673 bp was found in VRSA sample No.2 which indicated the presence of the vanA gene carrying resistance to the antibiotic Vanomycin Lampung isolate. This band appears in the PCR electrophoresis image using the new primer arrangement as follows

vanA_Foward : AGGAGACAGGAGCATGAATAG

vanA_Reverse : CAATACCGCACAACCGAC.

There is *S. aureus* resistance to Vancomycin in Lampung isolate and the vanA gene was detected in some resistant isolates. The emergence of VRSA must be taken care of because it is an indicator of widespread bacterial resistance.

ACKNOWLEDGEMENT

None.

Conflict of Interest

The authors declare no conflict of interest to other parties.

Funding Source

None.

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