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DETECTION OF PANTON-VALENTINE LEUKOCIDIN (PVL) GENE AGAINST METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) IN DIABETIC ULCER PATIENTS

DETEKSI GEN PANTON-VALENTINE LEUKOCIDIN (PVL) TERHADAP METHICILLIN-RESISTANT STAPHYLOCOCCUS AUREUS (MRSA) PADA ULKUS PASIEN DIABETES MELITUS

Research Report
Penelitian

Prisma Anjarlena*, Suliati, Wisnu Istanto, Retno Sasongkowati

Department of Medical Laboratory Technology, Health Polytechnic Ministry of Health Surabaya, Indonesia

A B S T R A C T

Background: *Diabetes Mellitus (DM) patients are susceptible to wound infections brought on by S. aureus bacteria, in dealing with infections caused by bacteria, antibiotic treatment was conducted. Nevertheless, misapplication of antibiotics might lead to drug resistance or MRSA. Panton-Valentine Leukocidin (PVL) is a crucial factor of S. aureus pathogenicity. Skin and soft tissue infections can be caused by the PVL gene.* ***Purpose:*** *To detect PVL gene in ulcer swabs of diabetic mellitus patients against MRSA strains.* ***Method:*** *Employing descriptive quantitative data analysis of PVL gene detection observations on ulcer swab of diabetes mellitus patients using the RT-PCR method. Ulcer swab samples were collected at Dharmahusada and Banyu Urip Diabetes Wound Specialist Hospital, Surabaya, East Java, Indonesia. The research was conducted at the Department of Medical Laboratory Technology, Health Polytechnic Ministry of Health Surabaya, Bacteriology Laboratory for the isolation and identification of MRSA and Molecular Biology Laboratory for the detection of the PVL gene in April–May 2022.* ***Result:*** *From 30 samples of ulcer swabs, 8 samples were positive for MRSA and 22 samples were negative for MRSA. After that, the MRSA positive samples were detected using the RT-PCR method to detect the PVL gene. The results of PVL detection were obtained from 6 samples (75%) positive for detecting the PVL gene and 2 samples (25%) negative for detecting the PVL gene.* ***Conclusion:*** *The results of the study showed that ulcer swabs from diabetes mellitus patients had the PVL gene against MRSA.*

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Correspondence:
Prisma Anjarlena

E-mail :
anjarprisma@gmail.com

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A B S T R A K

Latar belakang: Pasien Diabetes Melitus (DM) rentan terkena infeksi luka yang disebabkan oleh bakteri *Staphylococcus aureus*, dalam menangani infeksi yang disebabkan oleh bakteri yaitu dengan pengobatan antibiotik. Penyalahgunaan antibiotik menyebabkan resistensi obat atau MRSA. *Panton-Valentine Leukocidin* (PVL) merupakan komponen penting dari patogenisitas *S. aureus*. Infeksi pada kulit dan jaringan lunak dapat disebabkan oleh gen PVL. **Tujuan:** Mendeteksi gen PVL terhadap strain MRSA pada swab ulkus pasien diabetes melitus. **Metode:** Deskriptif kuantitatif analisis data observasi deteksi gen PVL pada swab ulkus pasien diabetes melitus dengan metode RT-PCR. Pengumpulan sampel swab ulkus dilakukan di Rumat Spesialis Luka Diabetes Cabang Dharmahusada dan Banyu Urip, Surabaya, Jawa Timur, Indonesia. Penelitian dilakukan di Jurusan Teknologi Laboratorium Medis Poltekkes Kemenkes Surabaya, Laboratorium Bakteriologi untuk isolasi dan identifikasi MRSA dan Laboratorium Biologi Molekuler untuk deteksi gen PVL pada bulan April – Mei 2022. **Hasil:** Dari 30 sampel swab ulkus, diperoleh sebanyak 8 sampel positif MRSA dan 22 sampel negatif MRSA. Setelah itu pada sampel positif MRSA, dilakukan deteksi gen PVL dengan metode RT-PCR. Hasil diperoleh sebanyak 6 sampel (75%) positif terdeteksi gen PVL dan 2 sampel (25%) negatif atau tidak terdeteksi gen PVL. **Kesimpulan:** Hasil penelitian ini menunjukkan bahwa swab ulkus pasien diabetes melitus memiliki gen PVL terhadap MRSA.

Kata kunci:
Swab ulkus diabetes melitus, MRSA, Gen PVL

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INTRODUCTION

Recently *Diabetes Mellitus* (DM) is still a severe health issue. According to data from the East Java Provincial Health Office in 2020, DM patients in Surabaya amounted to 94.624 people, while DM patients who received health services in accordance with the standard amounted to 96.900 people with a percentage of 102.4%. Diabetes mellitus, if not treated properly, can increase the risk of serious disease complications (American Diabetes Association, 2018). According to statistical data from the Diabetes Wound Specialist Hospital in 2015–2020, the total number of patients treated was 22.654 people, while in 2020 alone there were 7.056 people. Uncontrolled diabetes mellitus can cause *peripheral neuropathy*. Complications of *peripheral neuropathy* cause loss of pain in the legs, so it has a risk of injury that if left unchecked can become a diabetic ulcer (Bandyk, 2018). Various complications occur in the first diagnosis of DM. Diabetic ulcers affect 15-25% of diabetic patients and approximately 85% of DM patients were amputated because they previously had foot ulcers that developed into serious gangrene or infections (Wang *et al*., 2021). Ulcers occur due to superficial infections of the skin of people with DM caused by bacteria. One of the bacteria that causes wound infection for people with diabetic ulcers is *S. aureus* (Sugireng and Rosdarni, 2020).

In dealing with an infection caused by bacteria, the right antibiotics is given to inhibit the growth of bacteria. However, in its use, *S. aureus* bacteria experience drug resistance or called *Methicillin-Resistant Staphylococcus Aureus* (MRSA). Bacterial mutations and antibiotic abuse in recent times have caused drug resistance in *S. aureus* to gradually increase. MRSA infections recently have a more global impact, making clinically effective MRSA anti-infective therapy more challenging (Guo *et al*., 2020). Research on diabetic foot infection patients conducted by Rinaldo and Farhanah (2017) showed *S. aureus* bacteria were found in 6 (7.1%) of 85 specimens. Meanwhile Garoy *et al*. (2019) in his study found 59 (72%) MRSA positive samples in patients with abscesses of 15 (62.5%), burns of 12 (60%), diabetic ulcers of 11 (78.6%) and surgical wounds of 21 (87.5%).

One of the main virulent factors that *S. aureus* generates is *Panton-Valentine Leukocidin* (PVL). This cytotoxin that forms beta-sheets damages leukocyte membranes and induces tissue necrosis. *S. aureus* infections and invasive disorders of the skin and soft tissues are all brought on by the PVL gene. Infected skin is pus-filled, red, and irritated (Shettigar and Murali, 2020). A total of 400 *S. aureus* isolates were studied by Bhatta *et al*. (2016), 139 of those isolates were discovered to be antibiotic-resistant (MRSA) and 79 isolates (56.8%) were discovered to have the PVL gene. Most of the samples used in this study were isolated from pus samples. In a study conducted by Ahmad *et al*. (2020) which involved 90 MRSA positive samples, as many as 18 (20%) isolates detected PVL genes.

The PVL gene in *S. aureus* and MRSA isolates is still detected in studies conducted in Indonesia (Santosaningsih *et al*., 2014). For example, a study conducted in Indonesia by Rasita (2017), who took samples at Dr. Soetomo Hospital, Surabaya, found that as many as 85 isolates of *S. aureus* and as many as 20 isolates (23.5%) were strains of MRSA bacteria, but only 1 isolate (5%) has the PVL gene. Another study, also conducted on ICU patients at Cipto Mangunkusumo Hospital, discovered that only 2 (8.3%) of 24 MRSA isolates produced the PVL gene (Linosefa *et al*., 2016). Other previous studies conducted in Indonesia have revealed the presence of the PVL gene in MRSA strains from samples of hospital patients.

Recent data on this study are rarely found, so further research is needed. Based on these problems, the purpose of this study was to identify a preventive measure against the increased risk of complications in patients with diabetes mellitus caused by the PVL gene, which played a role in increasing the virulence of *S. aureus*. The present study was expected to provide the latest information related to the presence of the PVL gene in the MRSA strain from patients with diabetes mellitus ulcers.

MATERIAL AND METHOD

Types of research design

The present study employed a descriptive quantitative analysis of observational data for PVL gene detection in ulcer swabs of diabetes mellitus patients in the Diabetes Wound Specialist Hospital with the R*eal Time* PCR (RT-PCR) method.

Sample and population

The population of this study was all patients with diabetes mellitus ulcers in the Diabetes Wound Specialist Hospital, which was 32. The study sample was a diabetes mellitus ulcer swab accompanied by pus and 30 samples were calculated using the slovin formula.

Tools and materials

The tools used for the culture of MRSA bacteria included erlenmeyer, ose needle, bunsen fire, object glass, incubator, anticoagulant tube, measuring cup, beaker cup, oven, autoclave, and microscope. While the tools used for DNA extraction and PCR process were biosafety cabinet, vortex, micropipette, dry bath, spectrophotometer, centrifuge, refrigerator, eppendorf tube, yellow tip, blue tip, micro tip, and PCR tool.

In this study we used a swab of diabetes mellitus ulcer isolates taken from the Diabetes Wound Specialist Hospital in Surabaya. Other materials used in this study were bacterial growth medium *Blood Agar Plate* (BAP), *Mannitol Salt Agar* (MSA), and *Mueller Hinton Agar* (MHA), H_2O_2 3%, plasma citrate, NaCl, fuchsin, gentian violet, lugol, water, and cefoxitin antibiotic discs 30 µg. Materials for extraction were *nucleic lysis solution*, cell *lysis solution*, protein precipitation solution, isopropanol absolute, alcohol 70%, DNA rehydration, and material for PCR was a PCR mastermix (BIO-RAD SsoFast™ EvaGreen® Supermix, Cat. #172-5200) and primer (Macrogen Singapore).

Procedure

Isolation of MRSA bacteria

The diabetic ulcer swab sample was cultured on BAP medium, then incubated at 37^{0}C for 24 hours. Colonies growing with a rounded shape and a gray to golden yellow color were taken 1 ose and grown into MSA medium, then incubated for 24 hours at 37^{0}C. The medium that turned yellow indicated that the growing bacteria was *S. aureus*. After that, Gram staining was carried out and observed on a microscope. The coagulase test was continued by adding one drop of citrate plasma and putting one ose culture on the glass object. Positive coagulase was characterized by the presence of agglutination on the glass object and a catalase test using a 3% solution of H_2O_2 to which one culture was added grown on MSA media. Positive catalase occurred when foam was formed on the glass object. The *S. aureus* colony was grown on MHA medium, then an inoculum suspension was carried out. In this case, the colony was moved onto a tube containing 3 mL of sterile NaCl and turbidity was regulated according to McFarland standard 0.5. After that, a sensitivity test was carried out with the antibiotic cefoxitin 30 µg and incubated for 16–18 hours at 33-35^{0}C. Positive MRSA was characterized by the formation of inhibition zone ≤21 in MHA medium.

DNA extraction

DNA extraction was performed by adding 1 mL of MRSA bacterial culture to an eppendorf tube, pipetting a *nucleic lysis solution* of 300 µL into a tube, then vortexing for 10–60 seconds. Next, add the 300 µL *nucleic lysis solution* to the tube and vortex for 10 seconds. Add 100 µL cell *lysis solution*, and then incubate for 3 hours at 55^{0}C. At this stage, a vortex of 30 seconds is carried out every hour. The total of 200 µL of precipitation protein was added to the tube, which then vortexed at high speed for 20 seconds. The following stage was centrifuged at 13.000 rpm for 9 minutes after being placed on ice for 5 minutes. The supernatant result was moved to a new eppendorf tube. Add 600 absolute isopropanol (at this stage there would be visible DNA threads, which means it had a high level of DNA purity), then for 10 minutes, centrifuge at 12.000 rpm. In a new eppendorf tube, add 600 mL of 70% ethanol to the supernatant and centrifuge at 15.000 rpm for 5 minutes. The eppendorf tube was turned over and disposed of the supernatant to obtain a precipitate. Add up to 75% DNA rehydration and incubate for 1 hour at 65^{0}C. Store DNA isolates at 2–8^{0}C (Promega, 2019).

DNA purity test

A spectrophotometer set to a wavelength ratio of 260 nm was used to check the quality of DNA samples obtained from the extraction findings. Sterile aquadest blanks were used to calibrate each spectrophotometers wavelength. At these wavelengths, the absorbance of DNA samples was evaluated with the *formula Optical Density* (OD) 260/280, if the result was 1.8-2.0 then the purity level of the resulting DNA was high and could be used further. The interplay of the results of the OD comparison value of 260/280 nm was as follows OD ratio of 260/280 <1.8 indicated contaminated with protein, OD ratio of 260/280 + 1.8 – 2.0 indicated high level of DNA purity, and OD ratio of 260/280 > 2.0 indicated RNA contamination (Wasdili, 2019).

PVL gene identification by real-time PCR method

The primers used to detect PVL genes were LukS-PV forward primers (5' CAG GAG GTA ATG GTT CAT TT 3') and LukF-PV reverse primers (5' GCA TCA AGT GTA TTG GAT AGC AAA AGC 3') with readings of 35 cycles. The PCR conditions used for identification were by pre-denaturation at a temperature of 95^{0}C for 5 minutes, continued by denaturation at 95^{0}C for 30 seconds, annealing at 59.2^{0}C for 30 seconds, extension at 72^{0}C for 5 minutes, and finally the last extension at 72^{0}C for 5 minutes were repeated 35 cycles in total.

RESULT

The results of the study from 30 diabetic ulcer swab samples from diabetic wound specialists obtained the growth of bacterial colonies in 12 samples. The identification results are found in Table 1. The results of the identification of colonies that grew in BAP had a round shape, gray to golden yellow and were β-hemolysis. The results in the catalase test obtained 12 positive samples that O_2 formation occurred. The results of the coagulase test obtained 12 positive samples formed agglutination. As a result of gram staining, there were clustered round shaped-gram-positive colonies after microscopic examination. A total of 12 samples on MSA could ferment mannitol by changing yellow color on medium. Susceptibility testing (Table 2) using a cefoxitin disk found 8 samples with an inhibition zone ≤21 mm on antibiotic cefoxitin and 4 samples with an inhibition zone ≥22 mm on antibiotic cefoxitin. Thus, as many as 8 positive samples of MRSA were found.

Table 1. Characteristics of respondents

Sample code	BAP medium identification	Catalase test	Coagulase test	Gram staining	MSA medium identification
A01	β-haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A02	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A03	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A04	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation fermentation
A05	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A06	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A07	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A08	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A09	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A10	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A11	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A12	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A13	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A14	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A15	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A16	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A17	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A18	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A19	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A20	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A21	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A22	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A23	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A24	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	Acid /Mannitol fermentation
A25	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A26	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A27	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid
A28	β- haemolysis	Positive (+)	Positive (+)	Cocci in clusters, Gram-positive	
A29	γ- haemolysis	Negative (-)	Negative (-)	Negative (-)	Acid /Mannitol fermentation
A30	α- haemolysis	Negative (-)	Negative (-)	Negative (-)	Negative (-) Acid

Table 2. Susceptibility test results of *S. aureus*

Sample code	Inhibition zone (mm)	Result
A01	17	Resistant
A03	16	Resistant
A04	19	Resistant
A08	23	Sensitive
A12	22	Sensitive
A14	18	Resistant
A19	28	Sensitive
A20	16	Resistant
A22	19	Resistant
A23	17	Resistant
A24	26	Sensitive
A28	18	Resistant

Table 3. The results of PVL gene detection using the real-time PCR method

Sample code	CT value	Results
A01	10.48	Positive
A03	9.33	Positive
A04	34.47	Positive
A14	32.30	Positive
A20	9.76	Positive
A22	9.90	Positive
A23	N/A	Negative
A28	N/A	Negative
Positive control (+)	11.97	Positive
Control negative (-)	N/A	Negative
A24	26	Sensitive
A28	18	Resistant

Description: Positive control (+) using colony of MRSA, Negative control (-) using sterile PZ

The detection results in samples that were positive for the PVL gene had a CT value ≤35 and samples that did not have the PVL gene had a CT value ≥35 and would be displayed as N/A or negative (Table 3). The percentage of PVL genes showed (Figure 1) the results from 8 MRSA positive samples, as many as 6 (75%) samples had PVL genes and 2 (25%) samples did not have PVL genes.

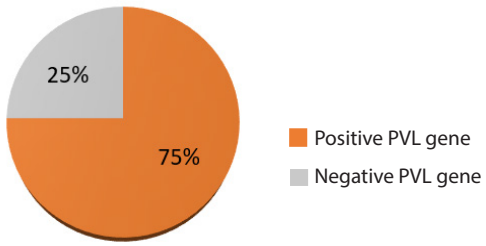

Figure 1. The percentage of PVL gene detection against MRSA in diabetes mellitus ulcers

DISCUSSION

This study has produced as many as 8 positive samples of MRSA and 22 negative samples of MRSA. The number of MRSA positive samples was found to be less because the samples came from patients at the Diabetic Wound Specialist Hospital who had received wound care and antibiotic treatment. The results of the identification of bacteria with MRSA positive, followed by the amplification stage used the real-time PCR method to determine the presence or absence of PVL genes. The amplification results on the real-time PCR indicated as many as 6 (75%) MRSA samples had a CT value of ≤35 meaning that the positive sample had a PVL gene, while 2 (25%) MRSA samples had a CT value of ≥35 meaning a negative sample of the PVL gene or N/A .

The results of the study revealed that 75% of the samples were PVL gene positive with limited antibiotic options, the combination of hypervirulent factors and the potential for *S. aureus* to develop antibiotic resistance can lead to serious infections and complicate treatment (Rasita, 2017). Patients who are positive for PVL genes must be more frequently monitored to carry out routine control of wound care, so that infections in the wound can be monitored properly and declared cured. According to a study done on patients at the Ciptomangunkusumo National Central General Hospital (RSUPNCM), as many as 2 isolates (8.3%) of MRSA produced the PVL gene. In Rasita's study (2017), who took samples at Dr. Soetomo Hospital Surabaya, as many as 20 isolates (23.5%) were strains of MRSA bacteria, only 1 isolate (5%) had the PVL gene.

In the amplification process, the amount of DNA is measured each cycle with fluorescent dyes that will increase the signal to coincide with the increase in the number of copy products (amplicons). When a fluorescent signal is detected at the threshold, it will exit the *Cycle Threshold* (CT) value. CT values are essential for obtaining accurate data and relating to the quantity of target DNA sequences. The CT value will be known sooner if there are many target DNA sequences in the initial stages of the reaction (Hewajuli and NLPI, 2014). The collected signal data will be translated into amplification plots and processed through PCR software (ThermoFisher, 2015). *Penicillin-binding protein* (PBP), PBP2a, and peptidoglycan transpeptidase, which are produced by mecA genes with a low affinity for beta-lactam, facilitate methicillin resistance, resulting in MRSA's resistance to beta-lactam group drugs (Nawrotek *et al*., 2018). MRSA can survive and grow even though the bacteria are grown on a medium containing high concentrations of beta-lactam due to a low affinity for the compound (Boswihi and Udo, 2018).

The existence of MRSA has become a serious public health problem. *S. aureus* strain's pathogenicity and treatment resistance are both influenced by a number of factors. Infection rates are rising globally due to the emergence of medication resistance, and it

is getting harder to treat resistant strains of infections clinically, particularly when the infection is brought on by leukocidin (Zeouk *et al*., 2021). By causing necrosis, rapid apoptosis, and the death of polymorphonuclear and mononuclear cells that affect morbidity and cell death, PVL can make *S. aureus* more dangerous (Bhatta *et al*., 2016). Through the LukS and LukF proteins, MRSA generates PVL toxins that damage cell defense membranes and result in tissue necrosis (Iliya *et al*., 2020).

One of *S. aureus* bacteria's virulent factors is called PVL which can influence the immune system and ultimately lead to leukocyte cell lysis by making pores in the mitochondrial membrane. PVL causes neutrophils to produce IL-8, macrophages and monocytes to produce IL-1b. The factor works closely with PVL to cause inflammation (Perret *et al*., 2012).

A pathogenic member of the typical skin flora is *S. aureus*. Bacteria enter the body through the skin barrier to start the illness, which spreads to the bloodstream and destroys the body's defense cells (neutrophils). These bacteria infect by spreading widely in host cells and tissues (Cheung *et al*., 2021). Keratinocytes produce antibacterial peptides that are directly active against *S. aureus*. Neutrophils circulate in the blood and contribute to the body's defense against *S. aureus*. and reaches the site of infection. By generating chemotactic factors, which are largely produced by keratinocytes, T cells, PMN, and macrophages. Neutrophils respond to infections of the skin and soft tissues. The accumulation of neutrophils leads to the lysis of necrotic cells that are involved in the pathological mechanisms of SSTIs (Jayanthi *et al*., 2020). The number of neutrophils will be reduced due to apoptosis, but the reduced number of neutrophils is also caused by the production of virulent factors, namely PVL toxins, *Phenol-Soluble Modulins* (PSM) and alpha toxins (Kurniyanto *et al*.,2018).

Based on the description of the research results, it was discovered that 6 MRSA samples were positive for having the PVL gene. It is necessary to have further assessment of virulent factors produced by MRSA bacteria from patients with untreated diabetes mellitus ulcers that have the PVL gene, so as to reduce the increased risk rate and become a preventive and treatment measure against infections caused by MRSA.

CONCLUSION

In this study, it was concluded that a PVL gene against MRSA was detected in the ulcer swab of diabetes mellitus patients. In the identification of MRSA from 30 ulcer swab samples of diabetes mellitus patients, 8 (26.6%) positive samples of MRSA bacteria and 22 (73.4%) negative samples of MRSA bacteria were obtained. PVL gene detection using the real-time PCR method indicated that results from 8 MRSA positive samples, as many as 6 (75%) samples were detected positive for PVL genes and 2 (25%) PVL gene negative samples. It is necessary to develop further research on uncontrolled ulcers of diabetes mellitus patients with a larger number of samples and a wider research site, accompanied by an examination of leukocyte type calculations to determine for sure neutrophil levels against neutrophil lysis caused by the PVL gene.

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