ANTIBIOTIC RESISTANCE PATTERNS AND MOLECULAR CHARACTERISTICS OF METHICILLIN-RESISTANT *Staphylococcus aureus* ISOLATED FROM ACNE OUTPATIENTS IN CAN THO CITY, VIETNAM

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ABSTRACT

This study aimed to characterize the methicillin-resistant *S. aureus* strains circulating on the skin of individuals with acne. *S. aureus* isolates were screened for methicillin resistance by methods including, oxacillin agar (OA), cefoxitin disk diffusion (CDD), and PCR for the *mec*A gene. The *mec*A-positive methicillin-resistant *S. aureus* (MRSA) strains were examined for the presence of the *Panton-Valentine leukocidin* (*PVL*) gene and typing based on the Staphylococcal cassette chromosome *mec* (SCC*mec*). Thirty-seven *S. aureus* strains were isolated from 78 skin swab samples. Eighteen isolates were identified as positive for the *mec*A gene. The OA and CDD methods showed lower sensitivity than PCR in MRSA screening. Sixteen MRSA isolates were recorded as multidrug-resistant. Isolates showed a high proportion of phenotype resistance to clindamycin, gentamycin, erythromycin, and linezolid. Eight strains of MRSA carried the PVL fusion gene. In the molecular epidemiology of MRSA, the most predominant were SCC*mec* types II and III. The rest was SCC*mec* type IV. Consequently, the spread of multidrug-resistant MRSA with genetic diversity could raise public health concerns. This study indicated the urgency of determining antibiotic resistance and epidemiological characteristics of MRSA in treating and controlling the spread of this pathogen.

MÔ HÌNH KHÁNG KHÁNG SINH VÀ ĐẶC ĐIỂM PHÂN TỬ CỦA *Staphylococcus aureus* KHÁNG METHICILLIN PHÂN LẬP TỪ CÁC BỆNH NHÂN BỊ MỤN NGOẠI TRÚ Ở THÀNH PHỐ CẦN THƠ, VIỆT NAM

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TÓM TẮT

Nghiên cứu này nhằm xác định đặc điểm của các chủng *S. aureus* kháng methicillin lưu hành trên da của những người bị mụn. Các chủng *S. aureus* được sàng lọc tính kháng methicillin bằng các phương pháp bao gồm, oxacillin agar (OA), khuếch tán đĩa cefoxitin (CDD) và PCR phát hiện gen *mec*A. Các chủng MRSA dương tính với *mec*A được kiểm tra sự hiện diện của gen Panton-Valentine leukocidin (PVL) và phân loại dựa trên Staphylococcal cassette chromosome *mec* (SCC*mec*). Ba mươi bảy chủng *S. aureus* được phân lập từ 78 mẫu phết da. Mười tám chủng được xác định là dương tính với gen *mec*A. Các phương pháp OA và CDD cho thấy độ nhạy thấp hơn PCR trong sàng lọc MRSA. Mười sáu chủng MRSA phân lập được ghi nhận là đa kháng thuốc. Các chủng phân lập cho thấy tỷ lệ cao về kiểu hình kháng clindamycin, gentamycin, erythromycin và linezolid. Tám chủng MRSA mang gen tổng hợp PVL. Trong dịch tễ học phân tử của MRSA, chiếm ưu thế nhất là SCC*mec* loại II và III. Các chủng còn lại là SCC*mec* loại IV. Do đó, sự lây lan của MRSA đa kháng thuốc với sự đa dạng di truyền có thể gây lo ngại về sức khỏe cộng đồng. Nghiên cứu này chỉ ra tính cấp thiết của việc xác định tính kháng kháng sinh và đặc điểm dịch tễ của MRSA trong điều trị và kiểm soát sự lây lan của mầm bệnh này.

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1. Introduction

The human skin is a vital defense organ, protecting the body from external factors. The skin microbiota is diverse, mutualistic, or pathogenic on the skin [1]. The skin microbiome varies between sites on the skin and is changed by the environment and individual genetics [2]. *S. aureus* could exist as part of the skin microbiome in up to half of the population [3]. In the community, skin and soft tissue infections caused by *S. aureus* are common infectious diseases [4]. The overuse of antibiotics has led to the development of antibiotic-resistant strains of *Staphylococcus aureus* [5]. MRSA is one of the common pathogens leading to treatment failure of skin infections caused by *S. aureus*, with an increased risk of invasive disease [6], [7].

The Staphylococcal cassette chromosome *mec* (SCC*mec*) carries either the *mec*A or *mec*C genes, which encode a novel penicillin-binding protein (PBP2a) that confers resistance to beta-lactams of MRSA [8]. Acquisition of SCC*mec* through horizontal gene transfer led to the emergence of new clones of MRSA [9], [10]. MRSA can carry *PVL* genes. PVL toxin induces necrosis and apoptosis in leukocytes [11], causing many difficulties in treatment.

Besides being effective in treating bacterial infections, some antibiotics can have side effects on patients. As an example, tetracycline is used to treat MRSA infections [12]. However, tetracycline has significant side effects that can lead to morbidity and mortality [12]. The detection of bacteria from the wound is required for the determination of antibiotic susceptibility in the area [13]. At the same time, typing methods can help to study the molecular epidemiology, spread, and evolutionary pathways of MRSA clones [14]. Antibiotic therapy to achieve therapeutic efficacy and avoid adverse effects is essential for clinicians [15]. This study was carried out to evaluate the extent of MRSA infection in people with acne on the face, the proportion of antibiotic resistance, and the molecular characteristics of MRSA isolates.

2. Materials and methods

2.1. Isolation of S. aureus

Ethical approval. The study protocol was approved by the scientific committee of Instute of Food and Biotechnology, Can Tho University, Can Tho city, Vietnam (668/QĐ-ĐHCT). The study did not involve clinical interactions with volunteers. Ethical approval was not required. The study received informed consent from volunteers.

Collecting skin swab samples. The volunteers with acne appeared on the face and agreed to participate in the study. The participants lived in Can Tho city, Vietnam, and the ages ranged from 18 to 30. Sterile cotton swabs moistened with sterile physiological saline were gently swabbed thoroughly in the desired area (15-30 seconds). The swab head was inserted into tubes containing sterile physiological saline solution. Collected samples were kept at 4°C and rapidly transported to the laboratory.

Isolation and identification of *S. aureus*. Samples were vortexed evenly for 5 min, and 100 µL of each was spread on mannitol salt agar (MSA) plates. Plates were incubated at 37°C for 24-48 h. Yellow-colored colonies and surrounding red-to-yellow media were transferred to Muller Hinton Agar (MHA). The cell shape and motility of the isolates were observed on an optical microscope (Olympus, Japan) at 1000× magnification. The Gram test was performed according to the description of the Gram staining kit (Himedia). The ability to produce endospores was carried out according to the instructions of Schaeffer-Fulton's Spore Kit (Himedia). A catalase test was performed with 3% H_2O_2 (Merck, Germany). The oxidase test is performed with the test strip Bactident® oxidase (Merck, Germany). The coagulase test was performed with coagulase kit (Himedia).

2.2. Investigating the level of oxacillin resistance by culture methods

Oxacillin agar screen: Mueller Hinton Agar with 4% NaCl was supplemented with oxacillin sodium salt (Sigma-Aldrich) to a final concentration of 6 µg/mL. The medium was dispensed into

Petri dishes and allowed the medium to be set. The isolates were streaked on the prepared plates, and incubated at 30°C for 24 h. If any growth was detected, the test strain was considered MRSA [16].

Cefoxitin disk diffusion: *S. aureus* suspension was prepared in sterile 0.85% NaCl solution and adjusted turbidity to the equivalent of a 0.5 McFarland. The bacterial suspension was swabbed evenly on the MHA plates with a sterile cotton swab. Three cefoxitin discs (30 µg/disc) (Oxoid™) and one control plate (without cefoxitin) were placed on MHA plates spread with the isolates. Plates were incubated at 37°C for 24 h. The inhibition zone size is compared and explained as described by CLSI [17].

2.3. Investigate the presence of the mecA gene in isolates of S. aureus

DNA extraction. A bacterial colony was added to Eppendorf tubes containing 200 µL of TE buffer (pH 8). Five µL of lysozyme (Merck, Germany) (10 mg/mL) was added to the tubes, and incubated at 37°C for 30 min. Then, the extraction procedure was performed as described by Chen & Kuo [18].

Oligonucleotides. Two pairs of primers were used to investigate the presence of the *mec*A gene in isolates of *S. aureus*. Primers mecA1 (5'- GTAGAAATGACTGAACGTCCGATAA-3') and mecA2 (5'- CCAATTCCACATTGTTTCGGTCTAA-3') were used to amplify the *mec*A gene in *S. aureus* [16]. The primer pairs SauF (5'-TCAAGCAAATCCCATAAAGT-3') and SauR (5'-TACAAACTCTCGTGGTGTGA-3') were designed based on the 16S rDNA gene region of *S. aureus* ATCC 25923 (Genbank: U02910.1). The amplification product with two primer pairs mecA1-mecA2 and SauF-SauR has a size of 310 bp and 154 bp, respectively.

Amplification process. The PCR reaction was performed with a total volume of 25 µL containing the following ingredients: 14.5 µL sterile double-distilled water, 5 µL MyTaq DNA polymerase buffer 5X (Bioline, United States), 0.25 µL BSA (10 µg/µL) (Promega, United States), 1 µL of each primer mecA1 (10 µM) and mecA2 (10 µM), 0.5 µL of each primer SauF and SauR (10 µM), 0.25 µL MyTaq DNA polymerase (Bioline, United States), and 2 µL bacterial DNA. The PCR reaction was performed in an ABI 9800 Fast Thermal Cycler, with the following temperature cycles: 94°C for 4 min, followed by 30 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s, and ending at 72°C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel supplemented with SafeView™ Classic (ABM, Canada). A ladder (100 bp Hyperladder, Bioline, United States) was used to determine the product size. The gel slides were illuminated under ultraviolet light on a Bio-Rad UV 2000 gel reader and imaging system (BioRad, USA).

2.4. Investigation of the antibiotic susceptibility of MRSA strains

The antibiotic sensitivity of *mec*A-positive *S. aureus* was determined as described above. The antibiotics (Oxoid™) used in the study included gentamycin (10 µg/disc), erythromycin (15 µg/disc), tetracycline (30 µg/disc), ciprofloxacin (5 µg/disc), clindamycin (2 µg/disc), trimethoprim-sulfamethoxazole (1.25/23.75 µg/disc), and linezolid (30 µg/disc).

2.5. Investigation of the presence of the pvl gene in MRSA

PCR amplification of *lukS-PV* and *lukF-PV* genes involved in PVL toxin synthesis was performed as described by Jarraud [19]. The primers were used as follows:

PVL-1 5'-ATCATTAGGTAAAATGTCTGGACATGATCCA-3'

NPVL-2 5'-GCATCAASTGTATTGGATAGCAAAAGC-3'

The PCR reaction was performed with a total volume of 25 µL containing the following ingredients: 15.75 µL sterile double-distilled water, 5 µL My*Taq* DNA polymerase buffer 5X (Bioline, United States), 1 µL of each primer PVL-1 (10 µM) and NPVL2 (10 µM), 0.25 µL My*Taq* DNA polymerase (5 U/µL) (Bioline, United States), 2 µL bacterial DNA. The PCR reaction was performed in ABI 9800 Fast Thermal Cycler, with the following conditions: 94°C

for 5 min, then repeated 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final step was maintained at 72°C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel supplemented with SafeView™ Classic (ABM, Canada). A ladder (100 bp Hyperladder, Bioline, United States) was used to determine the product size. The product amplified with primer pairs PVL-1 and NPVL-2 had a size of 433 bp.

2.6. Characterization of MRSA isolates by SCCmec typing

The pairs of primers and the analysis of the results were described by Boye (Table 1) [20]. The PCR reaction was performed with a total volume of 25 µL including the following components: 14.65 µL sterile double-distilled water, 5 µL MyTaq DNA polymerase buffer 5X (Bioline, United States), 0.5 µL for each primer β (10 µM) and α3 (10 µM), 0.625 µL for each primer crrCF (10 µM) and ccrCR (10 µM), 0.2 µL for each primer 1272F1 (10 µM) and 1272F2 (10 µM), 0.25 µL for each primer 5RmecA (10 µM) and 5R431 (10 µM), 0.25 µL MyTaq DNA polymerase (5 U/µL) (Bioline, United States), and 2 µL bacterial DNA. The PCR reaction was performed in an ABI 9800 Fast Thermal Cycler, with the following conditions: 94°C for 4 min, then 30 cycles were repeated of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The stabilization phase was maintained at 72°C for 4 min. The final step was maintained at 72°C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel supplemented with SafeView™ Classic (ABM, Canada). A ladder (100 bp Hyperladder, Bioline, United States) was used to determine the product size.

Table 1. *Primers used for PCR and the resulting analysis patterns of SCCmec typing*

Primer	Primer sequence (5' → 3')	Product size	Target	Type of SCC*mec*				
				I	II	III	IV	V
β	ATTGCCTTGATAATAGCCYTCT	937 bp	ccrA2-B		X		X	
α3	TAAAGGCATCAATGCACAAACACT							
crrCF	CGTCTATTACAAGATGTTAAGGATAAT	518 bp	ccrC			X		X
ccrCR	CCTTTATAGACTGGATTATTCAAAATAT							
1272F1	GCCACTCATAACATATGGAA	415 bp	IS1272	X			X	
1272F2	CATCCGAGTGAAACCCAAA							
5RmecA	TATACCAAACCCGACAACTAC	359 bp	mecA–IS431					X
5R431	CGGCTACAGTGATAACATCC							

2.7. Statistical analysis

The correlation between antibiotic resistance phenotypes was assessed through bivariate analysis, using MINITAB 16 statistical packages (Minitab, Inc.). The correlation was considered statistically significant when the p-value was less than 0.05.

3. Results

3.1. Prevalence of S. aureus

Of 78 swab samples of volunteers with acne, 37 were positive for *S. aureus*. Thirty-seven strains produced yellow or pale-yellow colonies on MSA, and around the colonies showed a red to-yellow color change of the medium. The cells of isolates were spherical, bound together in clusters, and non-motile. The isolates belonged to Gram-positive, non-endospore formers, oxidase-negative, catalase-positive, and coagulase-positive. Characterization of the isolates was homologous to the description of Schleifer and Bell [21].

3.2. Detection and characterization of MRSA

In the oxacillin agar test, 25/37 strains were able to grow on a medium supplemented with oxacillin. These strains were identified as MRSA in this test. Eleven strains were classified as MRSA, and the rest were MSSA when examined with the cefoxitin disc diffusion method.

Eighteen strains were positive for the *mec*A gene in the PCR method. Eleven isolates were MRSA in all 3 tests, 7 in oxacillin agar and PCR, and 6 in oxacillin agar test only. The PCR method showed higher accuracy than the other two. The sensitivities of the OA and CDD methods to PCR were 0.72 and 0.61, respectively.

3.3. Antibiotic resistance profile of MRSA isolates

Eighteen MRSA isolates were resistant to clindamycin (100%). The isolates showed a high rate of resistance to gentamycin (88.9%), erythromycin (77.8%), and linezolid (66.7%). The isolates exhibited a low prevalence of resistant phenotype with tetracycline (44.4%), trimethoprim/sulfamethoxazole (44.4%), and ciprofloxacin (27.8%). Eleven resistance phenotypes were observed (Table 2). Sixteen strains (88.9%) were resistant to at least three antibiotics. Seven isolates were no longer susceptible to all seven antibiotics. The two isolates exhibited phenotypes resistant to all seven antibiotics examined. By bivariate analysis, a highly significant correlation was observed between resistance patterns of gentamycin and erythromycin (Pearson correlation coefficients, $\rho = 0.661$, $p \leq 0.01$), gentamycin and linezolid ($\rho = 0.500$, $p \leq 0.05$), and erythromycin and trimethoprim/sulfamethoxazole ($\rho = 0.478$, ≤ 0.05).

Table 2. *Phenotypic resistance, PVL gene, and SCCmec profile of MRSA isolates*

Isolate	Resistance patterns	Occurrence of PVL gene	Type of SCC*mec*
HS7	GEN-E-TE-CL-TS-L	-	III
HS13	GEN-E-CL	+	II
HS22	CL	+	IV
HS39	GEN-TE-CL-L	-	III
HS46	GEN-E-CL-L	+	IV
HS47	GEN-CL-L	-	III
HS49	GEN-E-TE-CL-TS-L	-	IV
HS52	GEN-E-TE-CIP-CL	-	III
HS57	GEN-E-CIP-CL-TS	-	II
HS58	GEN-E-TE-CIP-CL-TS-L	+	IV
HS60	CL	-	II
HS61	GEN-E-CIP-CL-TS	+	II
HS63	GEN-E-TE-CIP-CL-TS-L	+	II
HS64	GEN-E-TE-CL-TS-L	+	II
HS73	GEN-E-CL-L	+	II
HS74	GEN-E-TE-CL-L	-	III
HS75	GEN-E-CL-L	-	II
HS76	GEN-E-CL-TS-L	-	III

GEN, gentamicin; E, erythromycin; TE, tetracycline; CIP, ciprofloxacin; CL, clindamycin; TS, trimethoprim/sulfamethoxazole; L, linezolid.

3.4. Occurrence of pvl gene and typing based on SCCmec

Of 18 MRSA isolates, eight carried the PVL gene. All MRSA isolates were classifiable by multiplex PCR. Type II and III SCC*mec* were the majority (7 strains for each type). The remaining belonged to SCC*mec* type IV. The detail results were presented in Table 2.

4. Discussions

In this study, the rate of skin infections caused by *S. aureus* was high in the community in the survey area. *S. aureus* originates from the skin and mucous membranes but can cause sepsis, lung infections, fulminant purpura, and post-viral toxic shock syndrome [22]. *S. aureus* is considered a "superbug" carrying many virulence factors, resistant to most antibiotics, and capable of generating many new clones [14]. In particular, the management of MRSA infections is difficult due to their multidrug-resistant properties. Therefore, identification of pathogens should be done first, which can help control their spread in the population.

Screening for MRSA infections is central to controlling the spread of this pathogen. In our study, the level of oxacillin resistance of isolates of *S. aureus* was different between the three tests of oxacillin agar, cefoxitin disk diffusion, and PCR for *mec*A. CDD detected the least MRSA, and OA showed the highest number of MRSA strains. PCR was a highly accurate, time-saving method for detecting MRSA. However, the high cost was the disadvantage of this method. Regulating the expression of mechanisms can lead to differences between MRSA detection methods. MRSA encoded a protein with a low affinity for methicillin, the penicillin-binding protein (PBP), PBP-2a. The activity of PBP-2a allowed bacteria to grow in the presence of antibiotics [23]. PBP2a is encoded by the *mec*A gene on SCC*mec*, the expression of which is regulated through a sensor (*mec*R1) and an inhibitor (*mec*I) protein [24]. The regulatory activity of *mec*R1 and *mec*I leads to different methicillin-resistant phenotypes in MRSA [23]. In addition, some borderline oxacillin-resistant *Staphylococcus aureus* strains are resistant to oxacillin non-*mec*A, usually due to the overproduction of β-lactamases [25].

MRSA can cause different clinical syndromes, and varied in antibiotic susceptibility [4]. Compared with some other studies, the research results show that antibiotic resistance characteristics in MRSA strains are different in terms of isolated origin and geographical area. *S. aureus* strains isolated from acne patients were resistant to doxycycline and tetracycline [26]. MRSA isolated at Masovian district hospital in Poland were resistant to the following antibiotics with rates as follows: levofloxacin (83.9%), ciprofloxacin (83%), erythromycin (77.7%), clindamycin (72.3%), tetracycline (10.7%), amikacin (14.2%), gentamicin and trimethoprim/sulfamethoxazole (8.0%), linezolid and teicoplanin (0.0%) [27]. MRSA infections prolonged antibiotic therapy and reduced the success rate of antibiotic therapy [28], [29]. Transmission, penetration, and virulence of MRSA are likely to increase when they are exposed to antibiotics such as beta-lactams (especially cephalosporins), quinolones, and macrolides [9]. Antibiotic resistance in *S. aureus* can develop through de novo mutations in chromosomal genes or the acquisition of resistance determinants through horizontal gene transfer [30]. The *erm*A gene, resistance to gentamicin, ciprofloxacin, and tetracycline are reported to facilitate biofilm formation in erythromycin-resistant MRSA strains [31]. Acquisition of the *cfr* gene through horizontal gene transfer by transposons and plasmids played an important role in the formation of linezolid resistance in MRSA [32]. Therefore, the drug resistance profile of MRSA in each region needs to be evaluated to improve treatment effectiveness and control its spread.

The PVL toxin is an important virulence factor related to the pathogenicity of *S. aureus*. The Panton-Valentine leukocidin (PVL) is encoded by the genes lukS-PV and lukF-PV [11]. Similar to our results, the presence of PVL gene in MRSA strains has been reported in many studies. Community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) isolates USA300 and USA400, and their methicillin-sensitive clones commonly produce the Panton-Valentine leukocidin [22]. A high proportion of hospital-acquired methicillin-resistant *Staphylococcus aureus* carrying PVL was observed to be associated with skin and soft tissue infections but not with pneumonia or sepsis [33]. A total of 51.8% of atopic dermatitis cases in children in Brazil had the presence of *S. aureus*, of which 41.6% were positive for *S. aureus* as MRSA. More than 70% of isolates of *S. aureus* were positive for the PVL gene [34].

MRSA isolated from skin swab samples mainly belonged to SCC*mec* types II and III, and the low prevalence belonged to type IV. Results from several other studies showed different distribution characteristics of SCC*mec* in MRSA in each region. Davis reported that CA-MRSA infection was most common in the skin/soft tissue group [28]. Among the CA-MRSA isolates, 71% were SCC*mec* type IV, 29% were SCC*mec* type II, and 54% had the PVL gene [28]. In India, CA-MRSA mainly belonged to the genotypes ST22 (SCC*mec* IV), ST772 (SCC*mec* V), and ST672 (SCC*mec* V) [35]. SCC*mec* type III predominated among CA-MRSA strains isolated in Malaysia [36]. SCC*mec*-based typing can help investigate the molecular epidemiology of MRSA [37]. SCC*mec* consists of two important gene groups, the *mec*-gene complex (encoding

methicillin resistance) and the cassette chromosome recombinase (*ccr*)-gene complex (encoding one or two site-specific recombinases for the movement of the element) [38]. SCC*mec* elements can be as large as >50 kb (SCC*mec* type I, II, III) or as small as in SCC*mec* IV (21 to 24 kb) or V (27 kb) [30]. Comparative genetic analyzes have shown that despite the wide range of genotypes, outbreaks of MRSA strains are limited to certain genotypes, some of which are also limited by geography [35]. Differences in local infection control practices and pathogen-specific characteristics of circulating clones can lead to varying degrees of severity of outbreaks of MRSA infections [10].

5. Conclusions

In conclusion, this study points to the spread of MRSA in the community, especially among individuals with facial acne. MRSA strains are phenotypically resistant to many commonly available antibiotics. These strains may carry genes involved in the synthesis of the PVL toxin, indicating a worrisome public health risk. SCC*mec* typing showed differences in the origin of drug resistance in MRSA strains. Our study demonstrates the urgency of characterizing antibiotic resistance to provide appropriate therapy and control the spread of MRSA in the population.

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