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Original Article

Prevalence of vancomycin resistant *Staphylococcus aureus* (VRSA) in methicillin resistant *S. aureus* (MRSA) strains isolated from burn wound infections

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ABSTRACT

Objectives: The increase in resistance of methicillin resistant *Staphylococcus aureus* (MRSA) strains to vancomycin has been perceived as a formidable threat in the therapeutic fields. The present study investigated the vancomycin resistance traits of MRSA isolates [vancomycin resistant *S. aureus* (VRSA)] collected from burn patients.

Materials and Methods: Twenty-nine of 40 isolates of *Staphylococcus* spp. were identified as *S. aureus* which were further tested against 20 commercially available antibiotics to determine antibiotic susceptibility patterns.

Results: Imipenem was the most potential antibiotic resulting in 90% sensitivity, followed by netilmicin, clindamycin, and nitrofurantoin (80% sensitivity). All isolates were found to be resistant to penicillin. Approximately 75% of them were found to be resistant to methicillin, oxacillin, azithromycin, ciprofloxacin, and tetracycline. Approximately 45% isolates exhibited resistance to amikacin, chloramphenicol, gentamycin, and tobramycin. Twenty-one of the 29 strains of *S. aureus* were MRSA, of which 11 were resistant to vancomycin when employing the disc diffusion method. However, when the broth microdilution procedure was used to measure the minimum inhibitory concentration (MIC) of vancomycin, eight isolates were resistant to vancomycin, six with an MIC of 32 µg/mL and two with an MIC of 64 µg/mL.

Conclusion: A significant fraction of VRSA was found among MRSA strains in this study, revealing the necessity for new and effective drugs against MRSA.

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1. Introduction

Over the past few decades, there has been an alarming increase in the prevalence of antibiotic resistant pathogens and strains in serious infections [1–11]. The occurrence of bacterial infection had decreased with the discovery of penicillin in 1940 until *Staphylococcus aureus* began producing β-lactamase, which destroys the penicillin β-lactam core ring [11,12]. This increase in resistance towards penicillin drove the development of methicillin drugs, which are virtually resistant against many genetic variations of the

β-lactamase enzyme. Infection by *S. aureus* was well controlled using methicillin until the isolation of the first strain of methicillin resistant *S. aureus* (MRSA) in 1961 [1,12,13]. Since then, MRSA has become endemic in hospitals and nursing homes worldwide [1,3,7,14].

Burn patients are susceptible to infection, especially skin and soft tissue infections such as burn wound impetigo, burn wound cellulitis, and invasive types infection, because of their impaired immune system. Hence hospital-associated strains of MRSA have become a great concern, mostly due to treatment failure [7]. *S. aureus* was noted in the skin and mucosa of up to 40% of all burn patients of which 30% had severe cases of toxic shock [4,15,16]. In one study, the frequency of *S. aureus* infection reached 47% in burn patients and the prevalence of MRSA was up to 45% [15]. Although vancomycin has been the most reliable therapeutic agent against infections caused by MRSA, there has been an alarming emergence

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of vancomycin-resistant *S. aureus* (VRSA), possibly due to: (1) the widespread use of vancomycin to treat infections caused by MRSA; (2) a patient's immune status; (3) surgical procedures; and (4) involvement of healthcare workers infected with MRSA [17–23]. With the increasing resistance of *S. aureus* as well as the emergence of multidrug resistant strains, the choice of medication remains one of the most challenging concerns in the burn management unit.

Although nosocomial infections are associated with remarkable morbidity and mortality both in developed and developing countries, information concerning such infections in Bangladesh in the international literature is limited [4,7]. The detection rates of MRSA in hospitals in different cities in Bangladesh were recently reported to be 32–63%, which is high compared with the United States and European countries [18]. In this context, information on the antimicrobial susceptibility patterns of MRSA could help in the selection of appropriate treatment. Based on this rationale, the current study investigated the occurrence of MRSA strains in burn patients and the susceptibility patterns of these strains against various antibiotics used to treat hospitalized patients in Bangladesh.

2. Materials and methods

2.1. Study area, sampling, and sample processing

The experiment was carried out in the Microbiology Laboratory of the Department of Microbiology, Stamford University Bangladesh, Dhaka, Bangladesh from April 23, 2012 to January 15, 2013. A total of 40 wound samples from patients with tertiary burns of partial or full thickness (deep reticular dermis) were collected aseptically with a sterile cotton swab by a clinician wearing gloves (US Safety & Supply Co.) and a mask in the burn unit of Dhaka Medical College Hospital, Dhaka, Bangladesh [7,24]. The patients were under treatment with antibiotics including trimethoprim–sulfamethoxazole, methicillin, and ceftriaxone (Oxoid, UK). All patients were men aged between 20 years and 45 years from a lower middle-class community. The samples were inoculated on Mannitol salt agar (MSA) (HiMedia, India) plates immediately after sample collection for isolation of *S. aureus* and transported to the laboratory as early as possible [19,23,25].

2.2. Isolation and identification of *Staphylococcus aureus*

All MSA plates were incubated for 24 hours at 37°C. After incubation, isolated colonies suspected to be *Staphylococcus* were allowed to grow on nutrient agar plates (HiMedia, India) and then identified microscopically, biochemically, and serologically [19,20]. For microscopic observation, a pure colony was selected and subjected to Gram staining. Then the shape, arrangement, and Gram reactions of the isolates were observed under a light microscope (Max-plank- Ring 21 D-65205, Wiesbaden, Germany) (at a magnification of 100×) [25]. Required confirmatory biochemical tests including catalase and triple sugar iron agar tests were performed to identify suspected *S. aureus* following standard protocols [25].

2.3. Hemolytic activity and coagulase test

The hemolytic activity of *S. aureus* isolates was tested using blood agar plates containing 5% defibrinated sheep blood. An isolated colony from a nutrient agar (NA) plate was inoculated on blood agar and incubated at 37°C for 24 hours. The hemolytic zones were characterized as α (partial hemolysis), β (complete hemolysis), and γ (no hemolysis) depending on the extent of each colony [25]. A coagulase test (Becton Dickinson Microbiology Systems, USA) was performed to differentiate the hospital-acquired isolates. For this purpose, 10 μ L of the antiserum was placed on the slide and

a suspension of the organism was added. Agglutination was observed against light and the results were recorded [25].

2.4. Assay of antibacterial susceptibility

A standard agar-disc diffusion (Kirby–Bauer) assay using Mueller–Hinton agar (MHA) (HiMedia, India) plates was conducted to determine the susceptibility of the isolated *S. aureus* to different antibiotics [26–28]. A suspension of the test organism was prepared by adjusting the turbidity of the broth in phosphate buffer saline by comparing with that of the McFarland standard solution of 0.5 [27,28]. By means of a sterile cotton swab, a uniform lawn of bacterial growth was prepared on the MHA plates. A total of 20 antibiotic discs including amikacin (30 μ g), azithromycin (15 μ g), ceftriaxone (30 μ g), ceftioxin (30 μ g), cephadrine (30 μ g), chloramphenicol (30 μ g), ciprofloxacin (5 μ g), clindamycin (2 μ g), erythromycin (15 μ g), gentamycin (10 μ g), imipenem (10 μ g), methicillin (5 μ g), netilmicin (30 μ g), nitrofurantoin (300 μ g), oxacillin (1 μ g), penicillin (10 μ g), tetracycline (30 μ g), tobramycin (10 μ g), trimethoprim/sulfamethoxazole (25 μ g), and vancomycin (30 μ g) were applied aseptically on the surface of the inoculated plates in an appropriate spatial arrangement using a sterile needle. The plates were incubated at 37°C for 12–18 hours and examined for zones of inhibition (mm) [26,29].

2.5. Identification of MRSA

For the detection of MRSA, oxacillin (1 μ g) and methicillin (5 μ g) were introduced on the MHA plates against the growth of *S. aureus*. For this purpose, a bacterial suspension was prepared in sterile saline by selecting colonies produced by overnight incubation on NA agar plates. After 5–7 hours of incubation, the cell turbidity was adjusted to 0.5 McFarland standards [27,28]. Subsequently, the suspensions were inoculated onto MHA plates and the antibiotic discs were then placed onto the plates [20,21]. All plates were incubated for 24 hours at 37°C to observe for oxacillin and methicillin resistant *S. aureus*.

2.6. Identification of VRSA through disc diffusion methods

MHA plates were inoculated with the bacterial suspension which was previously adjusted to 0.5 McFarland standards. Afterward, a 30 μ g vancomycin disc and a blank disc as a control were aseptically placed over the surface of the MHA plates at a distance of 5 mm to observe the range of the zone diameter for the detection of strains of VRSA [20,21].

2.7. Determination of vancomycin resistance by minimum inhibitory concentration test

The minimum inhibitory concentration (MIC) of vancomycin (Oxoid, UK) was determined by the tube dilution method [7,30–32]. Muller–Hinton Broth was prepared with 4–512 μ g/mL of vancomycin. By using a direct colony suspension method, 0.5 McFarland equivalent bacterial inoculums were prepared in normal saline after culturing for 24 hours on an agar plate. The suspension was further diluted to achieve the desired inoculum concentration. All strains were spotted onto Muller–Hinton plates containing different concentrations of vancomycin. The plates were incubated for 24 hours at 37°C and checked for any visible growth [26].

3. Results

In recent years, *Staphylococcus aureus* has become one of the most dangerous pathogens due to its increased resistance to

Table 1
Biochemical determination of the presumptively selected *Staphylococcus aureus*.^a

Isolates ID	Catalase test	TSI		Hemolytic activity	Coagulase test
		Slant	Butt		
S-1	+	A	A	β	+
S-2	+	A	A	β	+
S-3	+	A	A	β	+
S-4	+	A	A	β	+
S-5	+	A	A	β	+
S-6	+	A	A	α	+
S-8	+	A	A	β	+
S-9	+	A	A	β	+
S-10	+	A	A	β	+
S-11	+	A	A	β	+
S-12	+	A	A	β	+
S-14	+	A	A	β	+
S-16	+	A	A	β	+
S-18	+	A	A	β	+
S-19	+	A	A	β	+
S-20	+	A	A	β	+
S-21	+	A	A	β	+
S-22	+	A	A	β	+
S-23	+	A	A	β	+
S-24	+	A	A	β	+
S-25	+	A	A	β	+
S-26	+	A	A	β	+
S-27	+	A	A	β	+
S-28	+	A	A	α	+
S-29	+	A	A	β	+
S-30	+	A	A	β	+
S-31	+	A	A	β	+
S-32	+	A	A	β	+
S-33	+	A	A	β	+

A = acidic reaction; S = *Staphylococcus aureus* isolates; TSI = Triple Sugar Iron.
^a All the experiments were performed in triplicates and the results were reproducible.

β-lactam antibiotics and vancomycin [33–35]. Studies showed that MRSA is a causative agent of hospital-acquired infection and an incipient community pathogen in many geographical regions [13,36–38]. In the present study, the isolation rate of *S. aureus* (72.5%) from burn wound patients was high, as the microorganism was confirmed in 29 of the 40 isolated strains of *Staphylococcus* spp. based on cultural, biochemical, and coagulase properties (Table 1). In addition, imipenem was found to be the most effective antibiotic against the isolates with 90% of strains exhibiting sensitivity to this drug (Fig. 1). Most of the isolates (80%) were also sensitive to netilmicin, clindamycin, and nitrofurantoin. For vancomycin, 62% of isolates showed sensitivity. Almost 55% of isolates were sensitive to amikacin, chloramphenicol, gentamycin, and tobramycin. However, 26 of 29 strains of *S. aureus* were resistant to penicillin G and 75% of isolates were resistant to azithromycin, ciprofloxacin, methicillin, oxacillin, and tetracycline. Approximately 65% of isolates exhibited resistance to erythromycin, and trimethoprim–sulfamethoxazole (Fig. 1).

Twenty-one of the 29 *S. aureus* isolates studied were found to be MRSA (Table 2). The prevalence of methicillin resistance among staphylococci isolated from burn patients in our hospital has not been determined accurately to date. In this study, the prevalence of MRSA was 72% (Table 2), which varied from findings in other studies in other countries. In three separate studies in Iran, 56%, 72%, and 58% of staphylococci were identified as methicillin resistant [39–41]. Interestingly, a study in Korea in 2001 showed that the incidence of MRSA in burn cases could be as high as up to 98% [42]. A study in the United States in 2006 showed the rate of MRSA in a burn center was 33% [43]. Therapeutic strategies for severe MRSA infections are indeed limited to a few antibiotics including vancomycin. Thus the acquisition of high-level vancomycin

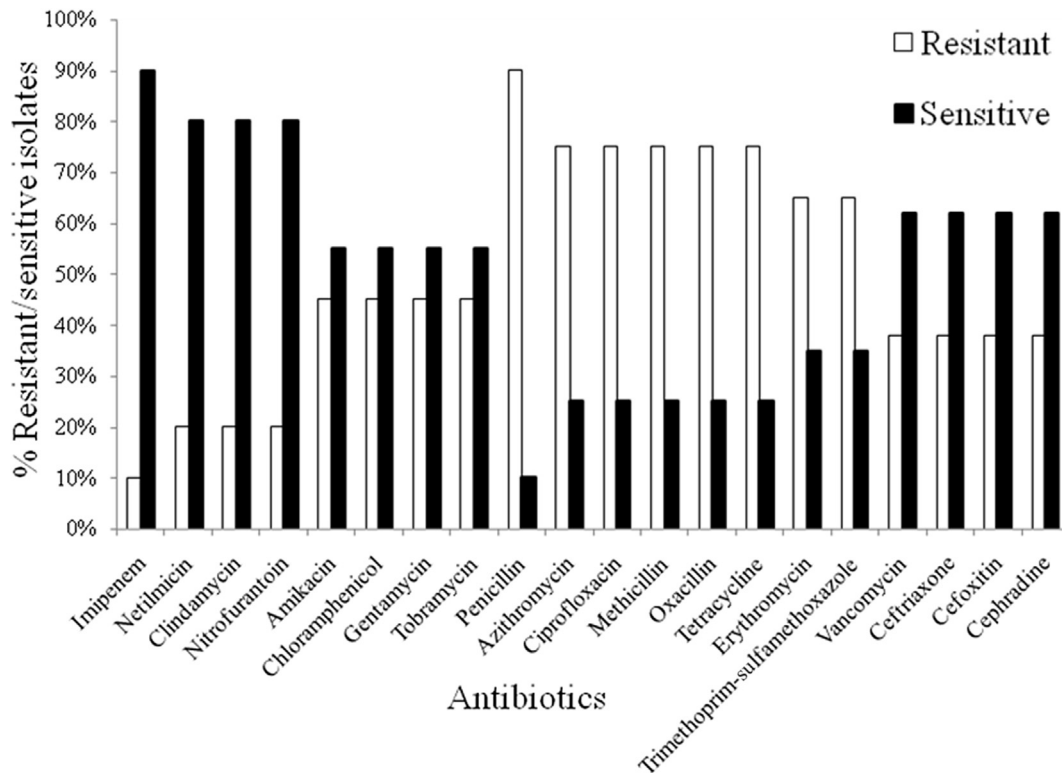


Fig. 1. Resistance and susceptibility patterns of *Staphylococcus aureus* towards commonly used antibiotics including amikacin (30 μg), azithromycin (15 μg), ceftriaxone (30 μg), cefoxitin (30 μg), cephadrine (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), methicillin (5 μg), netilmicin (30 μg), nitrofurantoin (300 μg), oxacillin (1 μg), penicillin (10 μg), tetracycline (30 μg), tobramycin (10 μg), trimethoprim/ sulfamethoxazole (25 μg), and vancomycin (30 μg). Among 20 tested antibiotics, imipenem was found to be the potent antibiotic against all isolated *S. aureus*. However, 90% of strains exhibited resistance to penicillin G.

Table 2
Detection of methicillin resistant *Staphylococcus aureus* (MRSA).^a

No of isolates <i>n</i> = 21 (out of 29)	Antibiotics Oxacillin & methicillin	Resistant presence (%)
S-2	R	72%
S-3	R	
S-4	R	
S-5	R	
S-6	R	
S-8	R	
S-9	R	
S-10	R	
S-12	R	
S-14	R	
S-21	R	
S-23	R	
S-24	R	
S-25	R	
S-26	R	
S-27	R	
S-28	R	
S-29	R	
S-31	R	
S-32	R	
S-33	R	

R = resistant.

^a All the experiments were performed in triplicates and the results were reproducible.

resistance by MRSA is a major health concern. Genomic studies have provided information on the evolutionary history of VRSA and identified genetic features that may focus on the acquisition mechanism of vancomycin resistance genes [37–44].

To determine vancomycin resistance among isolated MRSA in the current study, *S. aureus* strains were further tested using both agar disc diffusion and broth microdilution procedures (MIC). A total of 11 of the MRSA isolates (S-8, S-9, S-14, S-21, S-23, S-25, S-26, S-27, S-28, S-31, and S-33) were resistant to vancomycin with the disc diffusion method (Table 3). Subsequently, MIC assay showed that eight strains of *S. aureus* (28%) were resistant to vancomycin. Two of these strains had MIC values of 64 µg/mL and the other six strains had MIC values of 32 µg/mL, which were defined as VRSA in accordance with the laboratory breakpoints published by the Clinical and Laboratory Standards Institute [45]. Interestingly, among 29 samples 16 strains were noted to be vancomycin intermediate *S. aureus* (VISA), eight strains with MIC values of 8 µg/mL and another eight strains with MIC values of 16 µg/mL, and five samples were vancomycin sensitive *S. aureus* (VSSA) with MIC

Table 3
Identification of vancomycin resistant *Staphylococcus aureus* (VRSA) among MRSA through disc diffusion method.^a

No of isolates <i>n</i> = 11 (out of 21)	Antibiotic Vancomycin	Resistant presence (%)
S-8	R	52%
S-9	R	
S-14	R	
S-21	R	
S-23	R	
S-25	R	
S-26	R	
S-27	R	
S-28	R	
S-31	R	
S-33	R	

R = resistant.

^a All the experiments were performed in triplicates and the results were reproducible.

values of 5 µg/mL (Table 4). We assume the MIC values of vancomycin for these 21 MRSA isolates varied because of different levels of expression of the *vanA* gene in these isolates or other mechanisms [46,47].

In the past few years, several antibiotics have been noted to be less effective in the context of disease mitigation worldwide, as an array of pathogenic microorganisms are gradually becoming resistant to these therapeutic agents [48]. This raises the possibility of greatly increased mortality from simple infections and treatment-mediated failures. Along with multi-drug resistant (MDR) and extensively-drug resistant *Mycobacterium tuberculosis*, the MRSA strains, VRSA strains, coagulase-negative staphylococci, glycopeptide intermediate sensitive *S. aureus*, vancomycin-resistant *Enterococcus* species, penicillin-resistant *Streptococcus pneumoniae*, and the extended-spectrum β-lactamase producing bacteria are highly prominent [48]. In Bangladesh, recent studies of burn patients revealed huge growth in aerobic viable bacteria including *Pseudomonas* spp., *S. aureus*, and *Klebsiella* spp, *Enterobacter* spp. and *Escherichia coli* of which most were found to be MDR [7,14]. The current findings on the prevalence of VRSA strains among MRSA isolates further demonstrates the necessity for research emphasis on the microbiology of burn injuries, which in turn, could enhance the overall treatment of burns [7,14].

The major drawback of this study was the lack of molecular characterization of the isolates and detection of virulent genes, which could be investigated in future research. Such study may help physicians generate new treatment policies as well as to develop new drugs against the resistant properties of isolates.

Table 4
Determination of vancomycin susceptibility pattern (VRSA, VISA & VSSA) of *Staphylococcus aureus* through minimal inhibitory concentration (MIC).^a

<i>S. aureus</i> strain	Vancomycin MIC (mg/mL)	Vancomycin phenotype	Resistant presence (%)		
S-21	32	VRSA	28%		
S-23	32				
S-25	64				
S-26	32				
S-27	64				
S-28	32				
S-31	32				
S-33	32				
S-1	8			VISA	55%
S-2	8				
S-3	8				
S-4	16				
S-6	16				
S-8	16				
S-10	8				
S-11	8				
S-12	16				
S-14	16				
S-16	8				
S-19	8				
S-29	16				
S-30	16				
S-32	16				
S-33	8				
S-5	5	VSSA	17%		
S-9	5				
S-18	5				
S-20	5				
S-22	5				

MIC = minimal inhibitory concentration, VISA = vancomycin intermediate *Staphylococcus aureus*, VRSA = vancomycin resistant *Staphylococcus aureus*, VSSA = vancomycin sensitive *Staphylococcus aureus*.

^a All the experiments were performed in triplicates and the results were reproducible.

The current study revealed a high percentage of VISA isolates compared with VRSA and VSSA in isolated MRSA, highlighting the necessity for local or country-based investigations to characterize and monitor MRSA and to develop strategies that will accelerate MRSA management and control. In our study, imipenem was found to be the most effective drug against MRSA. In addition, the application of antibiotic combination therapy against VISA and VRSA, and maintenance of proper hygiene by hospitalized patients and staff could effectively reduce the rate and dissemination of such cases. Further molecular studies are required to identify resistance-conferring genes.

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