Molecular Detection of Methicillin Resistant *Staphylococcus aureus* Strains (MRSA) Isolated from Wound Infections

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Abstract

Methicillin resistant *S. aureus* (MRSA) has become a major public health problem worldwide. The major mechanism of methicillin resistance in *S. aureus* is the acquisition and expression of the *mecA* gene that encodes penicillin-binding protein 2a. Detection of methicillin resistance with phenotypic methods is still a problem especially because of heterogenous expression of mecA gene. The aim of this study was to determine the prevalence of (MRSA) in wound infections among hospitalized patients and to detect the mecA gene and species specific gene for the *S. aureus*. To compare conventional phenotypic methods in routine laboratory practice (Standard disc diffusion method) with an established molecular method. 66 samples of wound swabs were collected from patient, with septic wounds in Khartoum teaching hospital, Soba hospital and Al-ribat hospital. All the samples were gram stained, cultured in blood agar, mannitol salt agar and subjected to further conventional microbiological methods for identification. Antibiotic sensitivity testing was performed for the confirmed S. aureus isolates using disc diffusion technique to 5 antibiotics: Methicillin, Erythromycin, Vancomycin, Kanamycin and Chloramphenicol. PCR technique was used to identify *S. aureus* isolates and to detect methicillin resistant genes. Sixty two isolates identified as *S. aureus* showed positive reaction to the biochemical tests besides the species specific gene by PCR. 48.4% of the isolates were resistant to methicillin, 45.2% to kanamycin, 33.9% to erythromycin. 96.8% were sensitive to chloramphenicol and 87.1% to vancomycin. PCR results revealed that from the 30 strains culture confirmed as MRSA by disc diffusion method, 14 (46.7%) were *mecA* gene negative, 8 (25%) of the 32 MSSA were mecA gene positive.

Keywords: Molecular Detection, of Methicillin Resistant *Staphylococcus aureus*, mecA gene

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Introduction

Staphylococcus aureus is a versatile pathogen capable of growth and infection under diverse conditions. It has an extremely plastic genome capable of high variability and is known to acquire genes from closely related species. It is also a medically important pathogen. It causes a wide spectrum of afflictions (Sivaraman et al., 2009). It is the leading cause of skin and soft tissue infections such as folliculitis and furunculosis, and it is the pathogen most often implicated in wound infections resulting from surgery or trauma. Thus, *S. aureus* is of major significance in both community-onset and healthcare-associated infections (Huebner and Goldmann, 1999). *Staphylococcus aureus* continues to be one of the most difficult pathogens to treat because of its resistance to antibiotics. The most resistant strains have typically been found in hospitals, particularly in intensive care units, where antibiotics are extensively used. The widespread availability of penicillin in the 1940s offered hope that modern medicine had defeated this human scourge. Yet by 1960, about half of *S. aureus* strains were resistant to the antibiotic. Fortunately, a new antibiotic, methicillin, could treat most of these infections. Widespread resistance to methicillin eventually developed and, by 1996, about one-third of *S. aureus* strains were no longer susceptible to this drug (Patrick, 2007). Methicillin-resistant strains of *S. aureus* are those that have acquired the ability to grow in the presence of methylpenicillins and derivatives, including methicillin, oxacillin, and nafcillin (Hartman and Tomasz, 1984). However, the term MRSA persists to describe *S. aureus* isolates resistant to all currently available beta- lactam antibiotics, including cephalosporins and carbapenems. Resistance to methicillin in staphylococci is mediated by the gene *mecA*. The *mecA* gene encodes a penicillin-binding protein, PBP2a (also known as PBP2′), which has low affinity for beta-lactam antibiotics (Reynolds and Brown, 1985). The *mecA* gene is carried on an element termed staphylococcal cassette chromosome *mec* (SCC*mec*). SCC elements without a *mec* determinant have been found in coagulase negative staphylococci (Mongkolrattanothai et al., 2004). In addition to *mecA*, SCC*mec* contains two site-specific recombinase genes: cassette chromosome recombinases A and B (called *ccrA* and *ccrB*) (Katayama et al., 2000). The recombinase genes encode enzymes that mediate precise excision and site and orientation-specific insertion of SCC*mec* in the *S. aureus* chromosome. This is postulated to play a role in the horizontal transmission of *mecA* within and among staphylococcal species (Hanssen et al., 2004). The major mechanism of methicillin resistance in *S. aureus* is the acquisition and expression of the *mecA* gene that encodes penicillin binding protein 2a (PBP2a) (Wenchi et al., 2010). The targets of the antibiotic methicillin in sensitive strains of *S. aureus* are the penicillin-binding proteins (PBPs), essential enzymes that catalyze transpeptidation cross linking of peptidoglycan in the bacterial cell wall. Inhibition of this reaction with methicillin results in the arrest of cell wall biosynthesis, triggering death of the organism through induction of the autolytic response (Wise and Park, 1965). The *mec*A gene is the gold standard for the detection of MRSA (Dominguez et al., 1997). The phenotypic methods such as broth microdilution test for minimal inhibitory concentration (MIC), oxacillin disk agar diffusion (ODD) and oxacillin salt screening test (OSS) are widely used in routine microbiological laboratory. The problem with phenotypic methods is that they can be influenced by culture condition such as temperature, medium pH and NaCl content in the medium (Taweeporn et al., 2002). Several PCR methods have been developed to detect the *mecA* gene (Tokue et al., 1992). The application of genetic techniques to the recognition of determinants of resistance will clearly enhance our understanding of the epidemiology of antimicrobial resistance and improve therapy during the early stages of infection (Kim Lewis et

al., 2002). Methicillin resistant *S. aureus* (MRSA) has become a major public health problem worldwide (Jarvis et al., 2007). In the developing world, mortality associated with severe *S. aureus* infections far exceeds that in developed countries (Nickerson et al., 2009). Recent studies have identified *S. aureus* as the main etiological agent of many infections in sub-Saharan Africa (Nantanda et al., 2008). Cohort studies of patients with MRSA bacteremia have reported increased morbidity, longer hospital length of stay, and higher costs compared with patients with methicillin sensitive *S. aureus* (MSSA) bacteremia (Lodise et al., 2005). Prevention of *S. aureus* infection and reduction of the spread of virulent and resistant strains are therefore of great importance (Wertheim et al., 2005). Rapid diagnostic surveillance is becoming necessary in health care institutions to reduce infection rates and to better understand the disease (Lance and Peterrson, 2008).
In this study we used PCR technique for the identification of *S.aureus* isolates (specific genes) and detection of methicillin resistance genes (mecA) to compare between phenotypic and genotypic methods.

Materials and methods

Sixty six samples of wound swabs were collected from patients with septic wounds in Khartoum teaching hospital, Soba hospital and Al-ribat hospital.
Isolation attempts were made on all samples on the same day of collection, at the laboratory each sample was direct-plated onto blood agar and mannitol salt agar, incubated at 37 ˚C for 24 h. *S.aureus* was identified by Gram stain, catalase, slide and tube coagulase test and DNase tests according to procedures described by Cowan and Steel's (Barrow and Feltham, 2003).

Antibiotics susceptibility testing of isolated bacteria

Sensitivity of the *Staphylococcus aureus* isolates to five antibiotics (methicillin, vancomycin, erythromycin, kanamycin and chloramphenicol) was performed by the Standard disc diffusion method on Mueller-Hinton agar according to criteria of the national committee for clinical laboratory standard (NCCLS, 2004).

Molecular analysis by using PCR method

PCR technique was used to identify *Staphylococcus aureus* isolates and to detect methicillin resistant genes in all Staphylococci isolates.

DNA extraction

We performed a widely-practiced procedure that involves alkaline lysis of cells. This protocol, often referred to as a plasmid "mini-prep," yields fairly clean DNA quickly and easily.

PCR amplification

The oligonucleotide primers used in this study were synthesized and purchased from Vivantis Technologies Sdn Bhd, Malaysia. DNA polymerase, dATP, dCTP, dGTP, dTTP, MgCl2 and other chemicals were purchased from the same company.

Table (1): Characteristics of the primers used in the study

Primer pair	Target gene	Sequence (5---→3)	Ampilcon Size bp
MecA1 MecA2	MecA	AAA ATC GAT GGT AAA GGT TGG C, AGT TCT GCA GTA CCG GAT TTG C	532
Sau1 Sau2	S.aureus specific	AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA	107

A touchdown PCR assay targeting *S. aureus* species specific gene (Sau), and Methicillin resistance gene (mecA) was used in our study. Amplification was performed using the thermal cycler It was carried out as follows: A PCR mixture (20 µl) contained 1µl of each forward and reverse primers, 0.5 µl of 100 µmol/L dNTP, 1Uof *Taqpolymerase*, 2 µl of *Taq* buffer (2×),and 2µLof template DNA (50 ng). PCR conditions included heated lid 110 °C, temperature 94.0 °C for 00.05 touchduwn 70.0 °C to 52.0 °C cycles 14, start cycle ABL 35 times , initial denaturation (94 °C for 00.30sec), annealing (52 °C for 00.30 sec), and extension (72 °C for 00.45sec), and final extension (72 °C for 5 min) store 4.0°C.

Detection of PCR products

The PCR products were analyzed on a 2% agarose gel. The gel electrophoresis separation method was used. A 2% agarose gel stained with 1µl ethidium bromide was prepared. 5µl of the PCR product is mixed with 3µl bromophenol blue dye. 1.5µl of a 100bp DNA molecular weight marker was transferred into the first well. In the following wells DNA samples products were transferred followed by –ve template. Gel electrophoresis was performed at 85 v for 45 min. and the analysis was done by using an automated gel photo documentation system.

Results

The total samples collected were 66 samples. The total number of Gram positive bacteria isolated was 62, they were *Staphylococcus aureus*. The biochemical reactions of the organism were similar to those described by Cowan & Steels (Barrows & Feltham, 2003). 48.4% of the isolated bacteria were resistant to methicillin, 45.2% to kanamycin, 33.9% to erythromycin. 96.8% were sensitive to chloramphenicol and 87.1% to vancomycin (Table 2), Figure (3).

PCR technique was used to identify *S. aureus* isolates and to detect methicillin resistant genes. All bacterial isolates showed positive reaction to the biochemical tests besides the species specific gene by PCR. From the 30 strains culture confirmed as MRSA by disc diffusion method, 14 (46.7%) were *mecA* gene negative by PCR, 8 (25%) of the **32** MSSA were mecA gene positive (Table 3), Figures (1,2).

Table (2) Results of antibiotic sensitivity testing

Antibiotic	Disk Potency	Résistant		Sensitive	
		No.	%	No.	%
Chloramphnicol	30 mcg	2	3.2	60	96.8
Erythromycin	15 mcg	21	33.9	41	66.1
Methicillin	10 mcg	30	48.4	32	51.6
Kanamycin	30 mcg	28	45.2	34	54.8
Vancomycin	30 mcg	8	12.9	54	87.1

Table 3: Results of molecular detection of mecA gene by PCR technique

mecA	Résistant MRSA		Sensitive MSSA		Total
	No.	%	No.	%	No.
mecA +ve	16	53.3	8	25	24
mecA -ve	14	46.7	24	75	38
Total	30	48.4	32	51.6	62

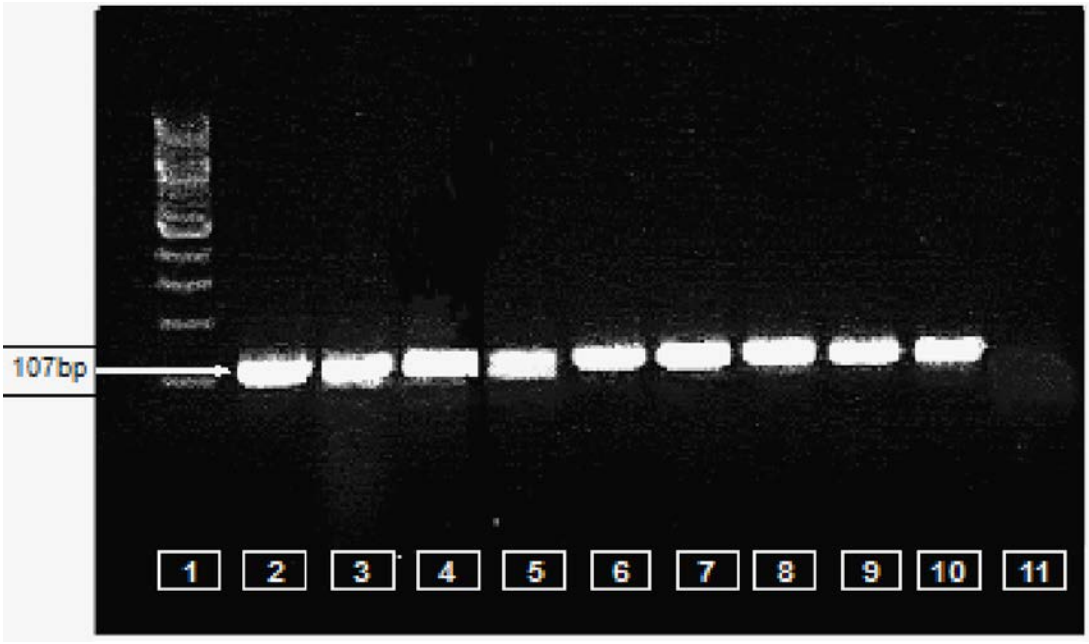

Figure (1): PCR amplification products for S.aureus specific gene. Lane 1 marker, 2-10 *S.aureus* and Lane 11 negative control.

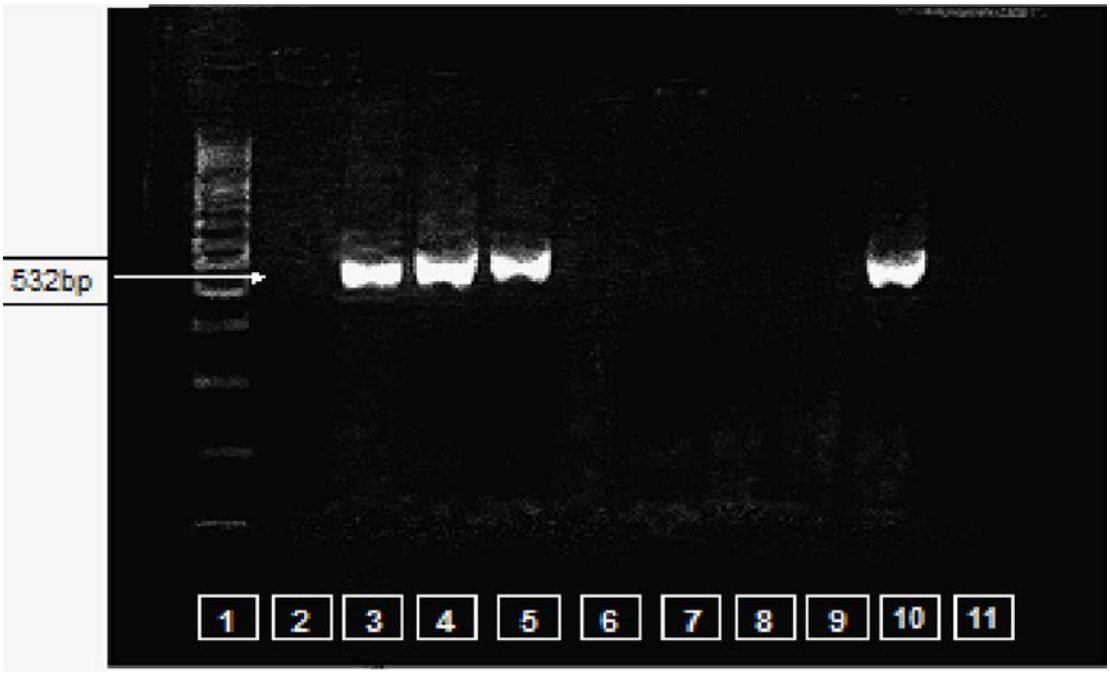

Figure (2): PCR amplification products for mecA gene. Lane 1: marker, Lane 11: negative control Lane 3,4,5 and 10 mecA +ve samples (methicillin resistance gene).

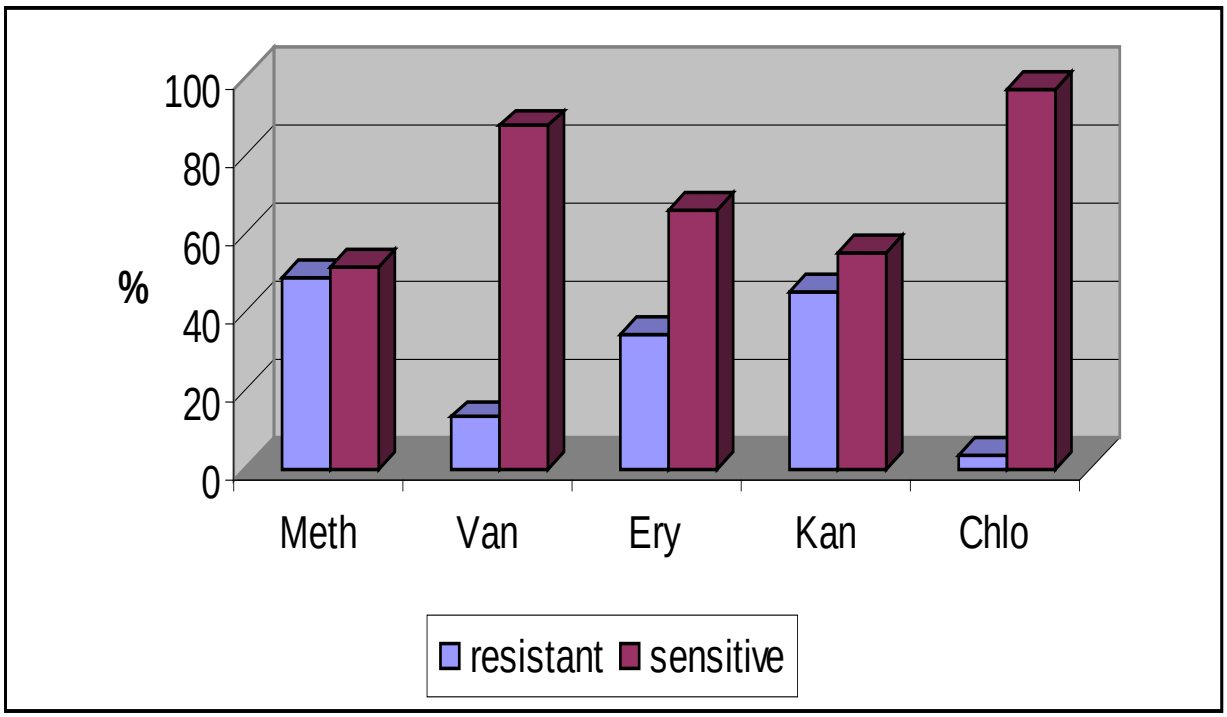

Figure (3): Results of antibiotic sensitivity testing (Meth=Methicillin, Van=Vancomycin, Ery=Erythromycin, Kan= Kanamycin, Chlo= Chloramphnicol).

Discussion

Detection of methicillin resistance with phenotypic methods is still a problem especially because of heterogenous expression of mecA gene. Although mecA gene determination by polymerase chain reaction is considered as the gold standard method, molecular tests are not easily applied in all routine laboratories (Özel et al., 2011).

In this study we evaluated the efficiency of the disk diffusion method and PCR for detection of methicillin resistance. 62 *Staphylococcus aureus* strains were isolated and identified from wound swabs culture. All bacterial isolates showed positive reaction to the biochemical tests besides the species specific gene by PCR. The antibiotic susceptibility by disc diffusion method revealed that 48.4% of the isolates were resistant to methicillin. From the 30 strains culture confirmed as MRSA by disc diffusion method, 14 (46.7%) were *mecA* gene negative by PCR. On the other hand, 8 (25%) of the 32 MSSA were mecA gene positive. Discrepant findings observed in this study was also reported by other workers, Azfar Qureshi et al., (2012) in their study found that out of 98 isolates 78 isolates were classified as methicillin resistant based on disk diffusion method, while 20 were classified as MSSA. The mecA gene was found positive in 89 (MRSA & MSSA) isolates (87.22%), 20 out of 98 isolates which were categorized as MSSA by disk diffusion method 11 showed amplification of mecA gene by PCR. Wielders et al., (2002) in their study reported that the *mecA* gene, is carried by 95% of the isolates that display a phenotype of

methicillin resistance and was detected in all multiresistant *S. aureus* isolates. These discrepancies might be explained by some other mechanism rather than the absense of the *mec*A gene. Three major mechanisms of resistance have been associated with the resistant phenotype: 1) *mec*-encoded resistance, 2) overproduction of penicillinase and 3) modifications of normal penicillin-binding proteins (Lorian,1996).

Phenotypic expression of methicillin-resistance can vary depending on the growth conditions (e.g., the temperature or osmolarity of the medium, pH and NaCl content in the medium), making susceptibility testing of MRSA by standard microbiological methods potentially problematic (Chambers,1997) (Taweeporn et al., 2002) (Sabath,1982). As a result, methicillin resistance is established slowly and may only appear after 48 hr on methicillin-containing plates, making these strains appear initially falsely susceptible at 24 hr (Berger-Bachi,1997). The mechanism of heteroresistance in *S. aureus* is poorly understood but is believed to involve the interaction of PBP 2a and various gene products such as those encoded by *fem* (factor essential for methicillin resistance) genes that are involved in cell wall peptidoglycan synthesis (Maranan et al.,1997). Despite the standardized recommendations for susceptibility testing of MRSA given by the National Committee for Clinical Laboratory Standards (NCCLS, 2000), a small percentage of isolates that carry *mecA* are phenotypically susceptible to methicillin. These isolates represent extremely heteroresistant isolates in which less than 1 in 10^8 of the population is highly resistant to methicillin (Smyth et al., 2001). It is known that the heterogeneous resistance phenotype of *mecA*-positive MRSA strains progresses to homogeneous resistance upon incubation with methicillin (Chambers,1997). Furthermore, since *mecA*-positive, phenotypically methicillin-susceptible *S. aureus* strains likely represent strains with an extremely heteroresistant methicillin resistance phenotype, one would suspect that the use of beta-lactams would select for highly resistant bacteria in the population, ultimately leading to the failure of therapy (Martineau et al., 2000).

Vancomycin, a glycopeptide antibiotic, became available in the mid-1950s, has been used as a last resort weapon to fight MRSA strains. Vancomycin inhibits synthesis of cell wall peptidoglycan by binding to the C-terminal of the cell wall precursor pentapeptide. After 40 years of use, the first isolate of *S. aureus* with reduced susceptibility to vancomycin was isolated from a child in Japan (CDCP, 1997) (Weigel et al., 2003).

In this study a low level of S. aureus resistance is seen against vancomycin (12.9%). Intermediate levels of resistance were reported from a number of countries around the world (Hiramatsu, 1998). The exact mechanism by which vancomycin intermediate S. aureus isolates become resistant to vancomycin remains unclear, but it is most likely that it involves a thickening of the cell wall due to the accumulation of cell wall fragments capable of binding vancomycin extra-cellularly and changes in several metabolic pathways that slow cell growth (Cui et al., 2000). The *vanA* gene complex, which mediates high-level glycopeptide resistance in enterococci, was detected in the vancomycin resistant *S. aureus* (VRSA) isolates confirmed to date (CDCP, 2002). The VRSA isolates detected to date in the United States are methicillin resistant and contain the *mecA* gene. In *S. aureus*, the *vanA* gene complex appears to confer changes in the cell wall similar to those seen in vancomycin-resistant enterococci, and *mecA* and *vanA* seem to function independently (Severin et al., 2004). To date, VRSA strains have been susceptible to other antibiotics, including linezolid, minocycline, quinupristin–dalfopristin, and

trimethoprim/sulfamethoxazole. Person-to-person transmission of VRSA has not been identifi ed, and the isolates do not appear to be epidemiologically linked. In at least one patient, VRSA arose in the absence of vancomycin selective pressure (Whitener et al., 2004).

In our study 33.9% of *Staphylococcus aureus* isolates were resistant to erythromycin. Erythromycin was introduced in 1952 as the first macrolide antibiotic. Unfortunately, within a year, erythromycin-resistant (Emr) staphylococci from the United States, Europe, and Japan were described (Zhang et al., 1992). Resistance to erythromycin in staphylococci is usually associated with resistance to other macrolides (Tarek Zmantar et al., 2011). Three related erythromycin ribosomal methylase genes, *ermA*, *ermB*, and *ermC*, alter the ribosomal target site and confer MLSB resistance (Leclercq and Courvalin, 1991). *S. aureus* strains with the inducible MLSB phenotype have traditionally been considered resistant to macrolides that induce *erm* expression (Weisblum, 1995). *Staphylococcus aureus* develops resistance very quickly and successfully to different antimicrobials over a period of time. The level of resistance of *Staphylococcus aureus* bacteria to the tested antibiotics revealed in this study,(methicillin48.4%, kanamycin 45.2% and erythromycin33.9%) could be associated with earlier exposure of these drugs to isolates which may have enhanced development of resistance. There is high level antibiotic abuse in our country arising from self-medication which is often associated with inadequate dosage and failure to comply to treatment, and availability of antibiotics to consumers across the counters with or without prescription.

Conclusion

There is an obvious need for more effective antibiotic therapy for infections with MRSA. Reports describing treatment failure of vancomycin for multi–drug-resistant MRSA infections have raised concern for the emergence of strains of MRSA for which there will be no effective therapy. However, new therapeutic agents alone will not provide a long-term solution, and our attention to prevention must remain constant. Strict adherence to hospital infection-control practices, as well as appropriate use of antibiotics and improved surveillance systems to track the emergence of resistance patterns, are of primary importance as we look to the future usefulness of antibiotic therapy against this extremely adaptive organism.
culture-based methods are slow--they can take days between when the patient first is seen by a doctor and the time the results come back from the clinical lab. For this reason, molecular methods are much quicker than the culture-based methods and detection of the gene responsible for methicillin resistance, *mec*A, has been the gold standard for identifying MRSA, even beyond phenotypic methods. Caution must be used when using susceptibility testing as the criterion for MRSA, as some testing methods can overestimate methicillin resistance.

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