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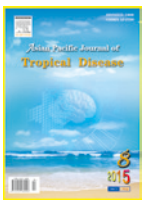

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Threat of multidrug resistant *Staphylococcus aureus* in Western Nepal

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ABSTRACT

Objective: To determine the prevalence of methicillin resistant *Staphylococcus aureus* (MRSA) and antimicrobial susceptibility patterns of the isolates from Manipal Teaching Hospital, Pokhara, Nepal.

Methods: This study was conducted over a period of 11 months (September 2012–August 2013) at the Manipal Teaching Hospital, Pokhara, Nepal. A total of 400 isolates were collected from various clinical specimens including hospital units (operation theaters and intensive care units). Antibiotic susceptibility testing was performed by Kirby-Bauer disc diffusion method. Primary screening for MRSA was performed using disc diffusion test by cefoxitin (30 μg) and oxacillin (1 μg) disc, further confirmation was done by detection of *mecA* gene using PCR.

Results: Out of 400 *Staphylococcus aureus* strains, 139 (34.75%) were found to be MRSA. Among the MRSA isolates, 74 (53.2%) were from inpatient departments, 58 (41.7%) of the isolates were from outpatients and 7 (5.0%) isolates were from hospital units (operation theaters and intensive care units). Majority of MRSA (73.38%) isolates were multidrug resistant while less than 15% were resistant to amikacin, clindamycin and tetracycline. None of the isolate was resistant to vancomycin. Inducible clindamycin resistance was found in 54 (25.47%) isolates.

Conclusions: This study showed a high prevalence of MRSA in our hospital. There is need of regular surveillance of antibiotic resistance, standardization of laboratory methods for detecting methicillin resistance and performing antibiotic susceptibility testing in developing countries like Nepal. Hospital acquired infections including prevalence of MRSA can be minimized by appropriate hygienic measures in patient care and management and by antibiotic stewardship. Screening of erythromycin resistant isolates would minimize clinical failures associated with clindamycin therapy.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most common and clinically significant human pathogen. It is responsible for various human infections, severity ranges from mild skin infection to severe life threatening infections like septicemia, meningitis, pneumonia and endocarditis[1,2]. *S. aureus* is responsible for both community–associated and hospital–associated infections. Infection due to *S. aureus* also imposes a high and increasing burden on health care resources as well as increasing morbidity and mortality[2].

S. aureus forms commensal microflora of skin and anterior nares. It can survive from hours to weeks, or even months, on dry environmental surfaces, depending on strain[3]. The presence of *S. aureus* does not always indicate infection but can be a pathogen under certain circumstances.

Drug resistance among staphylococci is a global problem and has posed serious therapeutic challenge for clinicians. Methicillin resistant *S. aureus* (MRSA) was first identified in 1961, since then these strains have become widespread in hospitals and intensive care units (ICUs)[4]. Resistance to β-lactams in MRSA is mediated by the acquisition of the *mecA* gene encoding penicillin binding protein. It has low affinity for β-lactam antibiotics and enables bacteria to assemble the cell wall in presence of the drug[5]. More recently, a divergent form of the *mecA* gene, known as *mecC* (previously $mecA_{LGA251}$), was identified in isolates from both animals and humans[6].

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Global scenario of MRSA showed its increasing prevalence[7]. Studies from various parts of Nepal reported prevalence of MRSA from 39% to 69%[8,9]. A considerable variation was reported in a number of clinical infections among hospitals, countries and among individual isolates. Recent data from India revealed that, MRSA blood stream infections are associated with a high mortality rate of 31%[10]. The burden of MRSA infections in Asia is high, and approximately 13% cases of nosocomial pneumonia in Asia are caused by MRSA[11].

Healthcare workers and hospital instruments are likely to be colonized by MRSA and play an important role in its transmission in hospital environment. Early detection of MRSA and formulation of effective antibiotic policy in tertiary care hospitals is of great importance from the epidemiological point. The present study was conducted to determine the prevalence and antimicrobial susceptibility profiles of *S. aureus* isolates, from patients and hospital environment in order to disseminate the information among the clinicians and formulate antibiotic policy for appropriate control measures.

2. Materials and methods

This study was conducted at Microbiology Department of Manipal Teaching Hospital, a 825 bedded tertiary care hospital of western region of Nepal. The samples were collected in sterile containers by clinicians/nurses using aseptic technique and immediately transported to the laboratory. All the isolates were identified as *S. aureus* using standard techniques, including slide and tube coagulase test, DNase, phosphatase test and mannitol fermentation test[12]. A total of 400 strains of *S. aureus* were isolated from various clinical specimens, pus (from abscess, drainage, ear discharge, wound swab, *etc*.), sputum, blood, body fluids, urine and from hospital environment during the study period (between September 2012 to August 2013).

Antibiotic susceptibility testing of all the isolates was performed by modified Kirby-Bauer disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines[13,14]. Following antibiotic discs (concentration) were tested: penicillin (10 IU), amoxicillin-clavulanic acid (20/10 μg), gentamicin (10 μg), erythromycin (15 μg), cefazolin (30 μg), tetracycline (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), vancomycin (30 μg) and trimethoprim sulfamethoxazole (1.25/23.75 μg).

Primary screening of MRSA was performed by oxacillin (1 μg) and cefoxitin (30 μg) discs. Test plates were incubated for 24 h at 35 ℃. The diameter of the zone of inhibition was interpreted as susceptible or resistant according to the criteria of CLSI. *S. aureus* isolates were considered methicillin resistant when the zone of inhibition was ⩽ 10 mm with the oxacillin disc and 21 mm with the cefoxitin disc[13,14].

Possibility of vancomycin resistant *S. aureus* and vancomycin intermediate *S. aureus* were ruled out by performing vancomycin minimum inhibitory concentration test using CLSI guidelines[13,14], as disc diffusion methods are not reliable. All the isolates were stored in semi-solid media and preserved at 4 ℃ for further testing. Periodic subcultures were undertaken to maintain their viability. *S. aureus* ATCC 43300 and *S. aureus* ATCC 25923 were used as MRSA and methicillin sensitive *S. aureus* (MSSA) quality control strains. *S. aureus* showing resistance to at least one agent in three or more antimicrobial categories are labelled as multidrug resistant[15].

Hospital and community associated *S. aureus* isolates were categorized based on following parameters. Isolates cultured from clinical specimens that were obtained after 72 h of admission of the patients or from patients with a history of hospitalization within 6 months were considered as hospital acquired *S. aureus* strains. Isolates which were cultured within 72 h of hospitalization, from outpatient department (OPD) or patients with no history of hospitalization within 6 months were categorized as community strains. The details of the patients were obtained from the medical record.

2.1. MecA gene detection by PCR

Extraction of DNA of the MRSA isolates was performed by chloroform: phenol extraction method by Sambrook *et al*.[16]. Monoplex PCR was used for detection of *mecA*. Primers used for *mecA* gene were MecA1 (5'-GTA GAA ATG ACT GAA CGT CCG ATA A) and MecA2 (5'-CCA ATT CCA CAT TGT TTC GGT CTA A) with 310 bp amplicon, as described earlier by Geha *et al*.[17]. The thermocycler was programmed for initial denaturation at 94 ℃ for 4 min; 30 cycles of amplification (denaturation at 94 ℃ for 45 s, annealing at 56 ℃ for 45 s, and extension at 72 ℃ for 30 s); and a final extension at 72 ℃ for 2 min. To visualize, 10 μL of the PCR amplicon was loaded with dye in 1.2% agarose gel containing ethidium bromide followed by electrophoresis at 100 V for one hour and visualized by using UV transillumination at 310 nm. Images of the test were obtained by gel documentation system. DNA fragments of 310 bp corresponded to *mecA* gene.

2.2. Detection of inducible clindamycin resistance

Detection of inducible clindamycin resistance was performed by D-test. All the isolates resistant to erythromycin were subjected to D-test as per CLSI guidelines[14]. The erythromycin disc was placed at distance of 15 mm (edge to edge) from clindamycin disc on Mueller-Hinton agar plate for standard disc diffusion test. A flattening of the zone of inhibition in the area between the discs with D shaped appearance after 18–24 h of incubation was considered to give an indication of inducible clindamycin resistance.

Data analysis: percentage resistance against various antibiotics amongst MSSA and MRSA groups was compared by using Pearson's *Chi*-square test. A *P*-value of < 0.05 was considered as statistically significant. Similarly pattern of resistance amongst OPD *vs.* ward isolates were compared using above statistical methods.

2.3. Ethical clearance

All the samples included in this study were from routine clinical specimens received at the microbiology laboratory for daily testing. None of the sample included in this study was collected separately for the study purpose from the patients. Permission to conduct the study was taken from the institutional ethical committee.

3. Results

A total of 400 *S. aureus* strains were isolated from various clinical specimens. Details of clinical specimens and relative distribution of MRSA are shown in Table 1. Primary screening of MRSA performed by oxacillin and cefoxitin disc diffusion method detected 128 and 141 MRSA, respectively. Out of 400 isolates 139 (34.75%) were *mecA* positive and confirmed as MRSA. Figure 1 shows *mecA* gene (310 bp) detection by PCR. Out of 139 MRSA, 71 (51.1%) were isolated from male, 61 (43.9%) from female patients and the remaining 7 (5.0%) were isolated from environmental samples. The majority of isolates (MRSA and MSSA) were isolated from pus samples received from the surgery department. A total of 74 (53.2%) of the MRSA isolated from ward patients, 58 (41.7%) from OPD patients and the remaining 7 (5.0%) isolates were from hospital units [operation theaters (OT) and ICUs]. Out of 139 MRSA isolates, 56 (40.3%) were found to be hospital associated MRSA and the remaining 83 (59.7%) were community associated MRSA according to the criteria based on patient information which were described above. The majority of the MRSA isolates were found resistant to erythromycin, ciprofloxacin and cotrimoxazole. Table 2 shows overall resistance pattern of *S. aureus* with comparison between resistance pattern of MRSA and MSSA isolates. Our study revealed that, the majority of MRSA (73.38%) isolates were multidrug resistant. Resistance to penicillin was highest (93.7%) followed by erythromycin (53.0%), ciprofloxacin (48.0%), cotrimoxazole (44.0%) and amoxicillin–clavulanic acid (35.4%). Resistance pattern of MRSA and its relative distribution in ward and OPD patient is shown in Table 3. The MRSA isolates from ward patients showed significantly higher resistance as compared to OPD patients.

Table 1
Frequency of *S. aureus* and MRSA in various specimens. *n* (%).

Specimen	Isolates (*n* = 400)	MRSA (*n* = 139)
PWS	288 (72.00)	98 (70.5)
Blood	46 (11.50)	14 (10.0)
Urine	28 (7.00)	12 (8.6)
Sputum	16 (4.00)	6 (4.3)
OT/ICU	15 (3.75)	7 (5.0)
Throat swab	5 (1.25)	0 (0.0)
Body fluids	2 (0.50)	2 (1.4)

PWS: Pus and wound swab.

Table 2
Antibiotic resistance pattern of MSSA and MRSA isolates. *n* (%).

Antibiotic	RI (*n* = 400)	RI MSSA (*n* = 261)	RI MRSA (*n* = 139)	*P*-value
Penicillin	375 (93.7)	236 (90.4)	139 (100.0)	< 0.001
Erythromycin	212 (53.0)	110 (42.1)	102 (73.4)	< 0.001
Ciprofloxacin	192 (48.0)	80 (30.6)	112 (80.5)	< 0.001
Cotrimoxazole	176 (44.0)	83 (31.8)	93 (66.9)	< 0.001
ACA	142 (35.4)	40 (15.3)	102 (73.4)	< 0.001
Cefazolin	52 (13.0)	11 (4.2)	41 (29.5)	< 0.001
Gentamicin	86 (21.5)	20 (7.6)	66 (47.5)	< 0.001
Clindamycin	17 (4.2)	2 (0.8)	15 (10.8)	< 0.001
Amikacin	15 (3.7)	1 (0.4)	14 (10.0)	< 0.001
Tetracycline	15 (3.7)	5 (1.9)	10 (7.2)	0.008
Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)	–

ACA: Amoxycillin-clavulanic acid; RI: Resistant isolates. Comparison between MSSA and MRSA isolates was done.

Table 3
Antibiotic resistance pattern of MRSA and its distribution in OPDs and wards. *n* (%).

Antibiotic	RI (*n* = 139)	RI OPD (*n* = 58)	RI ward (*n* = 81)	*P*-value
Penicillin	139 (100.0)	58 (100.0)	81 (100.0)	–
Erythromycin	98 (70.5)	31 (53.4)	67 (82.7)	< 0.001
Ciprofloxacin	112 (80.5)	38 (65.5)	74 (91.3)	< 0.001
Cotrimoxazole	93 (66.9)	35 (60.3)	58 (71.6)	0.164
ACA	102 (73.3)	37 (68.9)	65 (80.2)	0.023
Cefazolin	41 (29.5)	11 (18.9)	30 (37.0)	0.021
Gentamicin	66 (47.5)	22 (37.9)	44 (54.3)	0.041
Clindamycin	15 (10.8)	6 (10.3)	9 (11.1)	0.886
Amikacin	14 (10.0)	4 (6.9)	10 (12.3)	0.293
Tetracycline	10 (7.2)	3 (5.1)	7 (8.6)	0.435
Vancomycin	0 (0.0)	0 (0.0)	0 (0.0)	–

ACA: Amoxycillin-clavulanic acid; RI: Resistant isolate. Comparison between OPD and ward isolates was done.

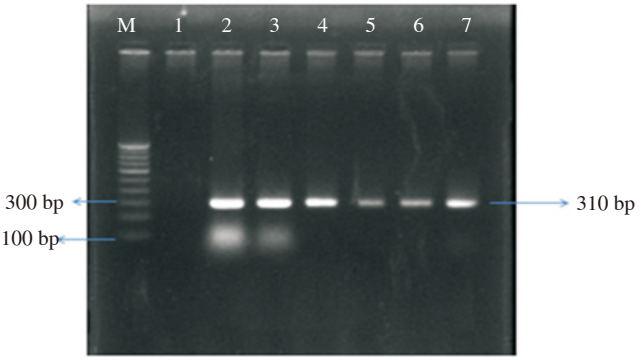

Figure 1. Monoplex PCR for detection of *mecA* gene (310 bp).
M: Marker (100 bp); 1: Negative control; 2: Positive control; 3-7: Test isolates positive for *mecA* gene.

Out of 212 erythromycin resistant *S. aureus* isolates, inducible clindamycin resistance was found in 54 (25.47%) isolates by D-test. Out of 54 D-test positive isolates, 35 isolates were MRSA while remaining 19 were MSSA.

4. Discussion

S. aureus is one of the most common human pathogen and significantly associated with pyogenic infection. Increasing drug resistance among bacterial pathogen and decreased availability of newer antimicrobial is worrisome. Among Gram positive bacteria, *S. aureus* is notorious for resistance against various antimicrobial agents. In this study of 400 *S. aureus* isolates, 288 (72.00%) were isolated from pus samples indicating their key role in pyogenic soft tissue and wound infections.

Antimicrobial resistance is a global threat and MRSA has emerged as an important human pathogen with wide range of antibiotic resistance. Global scenario of MRSA is not uniform and great variation in its prevalence has been observed throughout the world. Earlier reports of MRSA from Nepal reported prevalence of 15.4%–26.0%[18,19]. Newer studies from various hospitals of Nepal reported higher prevalence of 26%–69%[8,9,20,21]. Most of the MRSA related studies conducted in Nepal, used only cefoxitin and/or oxacillin for screening MRSA[8,9,20,21]. In our study, methicillin resistance status was confirmed by detecting *mecA* gene. As per our results, cefoxitin gives satisfactory results when zone diameter is < 19 mm. If zone

diameter is between 19–22 mm, then results are difficult to interpret and need confirmation by better methods like *mecA* gene detection. This may be one of the possible reasons of comparatively less percentage of MRSA in our study as compared to other studies from Nepal[22,23]. Prevalence of MRSA in our hospital was 34.75% which is comparable with study from Chitwan, Nepal (MRSA prevalence 39.6%). MRSA prevalence in our study is comparable with the study from India by Tsering *et al.* and Joshi *et al*.[24,25].

A small percentage of the MSSA isolates (9.6%) were found susceptible to penicillin. Significant difference in antibiotic resistance pattern was found among MRSA and MSSA isolates ($P \leqslant 0.05$)reflecting increased ability of MRSA to develop resistance against various antimicrobials. The majority of MSSA strains were sensitive to antibiotics like ciprofloxacin, erythromycin, cotrimoxazole, and amikacin except penicillin. Comparative study of resistance pattern of MRSA in ward and OPD patients showed higher resistance in ward isolates. Manipal Teaching Hospital, being referral hospital, majority of the ward patients were referred from other hospitals after admission of variable duration and primary treatment. Prolonged hospital stay and prior exposure to antibiotics could be one of the possible reasons associated with higher resistance among ward isolates as compared to OPD. Resistance to ciprofloxacin, amoxicillin-clavulanic acid and gentamicin was significantly higher among MRSA isolates from wards as compared to OPD ($P \leqslant 0.05$).

Vancomycin was the only drug to which 100% isolates were susceptible. However, the possibility of emergence of vancomycin resistance should always be kept in mind. Although, no vancomycin resistant *S. aureus* was found in our study, yet vancomycin should never be considered as first line drug, in view of the possibility of emergence of resistance. Thus other drugs like clindamycin and amikacin which were found quite effective against MRSA in the present study would be better options for the management of such infections.

Overall resistance of *S. aureus* to antibiotics likes ciprofloxacin, erythromycin, co-trimoxazole and amoxicillin-clavulanic acid was found high. These antibiotics being cheaper and easy to administer were extensively used in past few years which have now been slowly replaced by newer antibiotics like cefixime and cefpodoxime. Use of expensive and injectable antibiotics like gentamicin and amikacin was less in small clinics and reflects on higher percentage of sensitive isolates. Clindamycin being more expensive and not much in use in past years also showed relatively high sensitivity against MRSA isolates. Higher rates of resistance to various antimicrobials in western region of Nepal may be attributed to the low socio-economic status of the patients, lack of appropriate medical facilities, partial treatment and prescription of antibiotics without susceptibility testing, besides easy access to antibiotics across the counter. Indiscriminate use of antibiotics and delay in seeking medical treatment could be other reason for high rate of resistance. Our hospital is a tertiary care center of the Western Nepal, many patients take the initial treatment in primary health care centers or in small pharmacy clinics before reporting to us. Patients usually come to us when the disease becomes chronic, this could be another possible reason for higher percentage of drug resistance in our hospital. Due to lack of trained staff and unavailability of basic infrastructure, there are challenges in the healthcare facilities to perform antibiotic susceptibility testing in adequate manner. This could be one of the important factors responsible for indiscriminate use of antibiotics ultimately contributing to drug resistance.

In vitro susceptibility testing results provides valuable information to clinician for the treatment of microbial infections but sometimes *in vitro* tests may not reflect *in vivo* effectivity. One of the major problems with therapeutic use of clindamycin in staphylococcal infection is the possibility of presence of inducible resistance to clindamycin and possibility of clinical failure despite *in vitro* susceptible report. The prevalence of inducible clindamycin resistance in our study was 25.47%. The D-test is simple laboratory test for detection of inducible clindamycin resistance, therefore all erythromycin resistant isolates of *S. aureus* should be subjected to the D-test to rule out inducible clindamycin resistance and prevent the clinical failures.

Isolation of MRSA from various units of the hospital is worrisome. There is the possibility of transmission of MRSA from hospital units (OT, ICUs) to patients, patients to health care professionals and vice versa during patient care, various diagnostic and therapeutic procedures. Therefore, regular surveillance, disinfection and/or fumigation with suitable agent at regular interval would minimize the colonization and transmission of MRSA. Performing routine bacteriological examination of hospital equipment and environment before and after disinfection on regular basis also provides valuable information about prevalence of bacterial pathogens in a particular unit as well as effectiveness of fumigation and disinfection. Determination of antibiotic resistance profile of the pathogens isolated from various units guides clinician to start empirical therapy in suspected cases of hospital acquired infections.

Cefoxitin disc diffusion method was found reliable marker for primary screening of MRSA isolates with 100.0% sensitivity and 98.6% specificity. When considering *mecA* gene results oxacillin disc diffusion was found less sensitive (92.0%). Therefore, oxacillin disc diffusion method is no more recommended for screening of MRSA. Cefoxitin disc diffusion method is highly sensitive and specific when the zone diameter is < 19 mm. Isolates with zone diameter between 19–22 mm sometimes create confusion whether to report it as MRSA or MSSA. In our study, out of 15 doubtful cases of MRSA by cefoxitin disc diffusion method (zone diameter between 19–22 mm), only 11 were found *mecA* positive. Out of four *mecA* negative isolates, two were found cefoxitin sensitive on subsequent retesting while two were found resistant. These isolates may be associated with issues with disc diffusion method and need for standardization or the resistance mechanism other than *mecA* and may indicate the possibility of *mecC* mediated resistance. Therefore, doubtful cases of MRSA need re-testing further confirmation by PCR for both *mec* genes in order to get accurate results. In this way, cefoxitin, being cheaper, easily available everywhere and easy to perform, should be the method of choice for screening of MRSA especially in smaller laboratories.

The prevalence of MRSA in the studied isolates was found high. Monitoring antibiotic sensitivity pattern of MRSA on regular basis, optimization of MRSA detection methods, implementation

of preventive measures for MRSA spread, dissemination of data to clinicians and formulation of definite antibiotic policy may be helpful in reducing the incidence of MRSA infection and emergence of vancomycin resistant *S. aureus* as well. Screening of erythromycin resistant isolates by D-test would minimize clinical failures associated with clindamycin therapy.

Limitation of the study: although we have isolated MRSA from various hospital units, the nosocomial transmission of these isolates could not be demonstrated. Detection of *mecC* gene and further characterization were not performed.

Conflict of interest statement

We declare that we have no conflict of interest.

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