Brief Original Article

Phenotypic characterization of nosocomial isolates of *Staphylococcus aureus* with reference to MRSA

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Abstract

Background: Apart from being a major cause of mortality, nosocomial infections due to *Staphylococcus aureus* have been imposing a burden on patients, hospitals and health care systems. The present study was designed to determine the prevalence of methicillin resistant *S. aureus* (MRSA) among nosocomial isolates along with their phenotypic characterization.

Methodology: MRSA and methicillin sensitive *S. aureus* (MSSA) were determined by performing four different tests viz: disc diffusion, oxacillin screen agar test, MRSA latex agglutination test, and MIC of oxacillin by E test.

Results: Of the 149 *S. aureus* nosocomial isolates, 44.9% were MRSA, which included 82.1% of homogeneous MRSA and 17.9% of heterogeneous MRSA. Association of MRSA infection was found to be significantly higher in skin and lower respiratory tract infections. Of the MRSA isolates, 65 were multiresistant oxacillin resistant *Staphylococcus aureus* (MORSA) and 2 were nonmultiresistant oxacillin resistant *Staphylococcus aureus* (NORSA). D tests performed on 136 isolates showed that Inducible macrolide-lincosamide-streptogramin B (MLS_B) and constitutive MLS_B resistance were found to be associated with MRSA. On the contrary, susceptibility to both erythromycin and clindamycin was found to be associated with MSSA. However, MS_B (macrolide-streptogramin B) resistance was not found associated either with MRSA or MSSA. Furthermore, both inducible and constitutive MLS_B were found to be associated with only homogenous MRSA.

Conclusion: D tests may be made mandatory in all *S. aureus* isolates as inducible MLS_B resistance cannot be detected in routine susceptibility test unless erythromycin and clindamycin are placed 15-26 mm apart.

Key Words: Nosocomial infections; MRSA; inducible and constitutive macrolide-lincosamide-streptogramin B resistance

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Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) has been a major cause of nosocomial infection worldwide, causing high mortality and placing a great burden on patients, hospitals, and health care systems. Nosocomial infection due to *Staphylococcus aureus* constitutes a major part of the total annual nosocomial infections, i.e., 2 million. Since 1980, MRSA has become endemic in American hospitals. The prevalence of MRSA has since then been on the rise and its prevalence has increased from 14.3% in 1987 to 39.7% in 1997 [1]. By 2006, MRSA prevalence approached 50-60% [2]. The treatment cost of patients infected with MRSA has been reported to be $65,000, when much higher compared to MSSA infection at $24,500 [3].

Staphylococci developed resistance to erythromycin in 1956, a few years after the drug's introduction in therapy. The resistant strains were found in France, the United Kingdom (UK), and in the United States of America (USA) [4]. Since then macrolide, lincosamide and streptogramin (MLS) antibiotic cross-resistance has been observed in *Staphylococcus* species due to the modification of drug targets [5].

The target modification mechanism, also known as macrolide-lincosamide-streptogramin B (MLS_B) resistance, confers resistance to erythromycin, clindamycin, and streptogramin B. This MLS_B resistance mechanism can either be constitutive or inducible. In constitutive resistance, rRNA methylase is produced constitutively; in inducible resistance, methylase is produced only in the presence of an inducer, that is, erythromycin. Erythromycin is an effective inducer and clindamycin is a weak inducer [6]. *S. aureus* with constitutive resistance is resistant to both erythromycin and clindamycin. On the other hand, inducible resistant strains are resistant to erythromycin but appear susceptible to clindamycin in the absence of erythromycin as an inducer. *S. aureus* with inducible resistance are positive in the D test for the detection of induction of resistance to

clindamycin in the presence of erythromycin. *S. aureus* with macrolide-streptogramin B (MS_B) resistance are resistant to erythromycin and susceptible to clindamycin and they are negative for D test. Susceptible *S. aureus* are susceptible to both erythromycin and clindamycin.

The present study was aimed to determine the prevalence of nosocomially acquired *S. aureus* infections in a tertiary care hospital in Kathmandu, Nepal, and to detect different susceptibility patterns to clindamycin in both MRSA and MSSA**.** The study population was comprised of patients admitted to one of the largest tertiary care hospitals in Kathmandu, where patients from all over Nepal seek treatment. In addition, patients from other hospitals (referral cases) also constituted a large part of the study population.

Methods and materials

Case selection and sample processing

Clinical samples of admitted patients submitted to the microbiology laboratory of a Kathmandu based teaching hospital in Kathmandu, Nepal, for culture were processed by following the standard protocols [7]. Gram positive cocci occurring in clusters/short chains, catalase positive, oxidase negative, fermentative, Voges Proskauer positive, mannitol fermenter, clumping factor positive, DNase positive, coagulase positive and Staphytect plus latex agglutination (Oxoid, UK) positive were identified as *S. aureus*. The history was taken of all patients whose sample *S. aureus* had been isolated, and only those isolates from nosocomial infections were identified as nosocomial isolates. The *S. aureus* isolates were categorized in four groups on the basis of infection sites (Table 1).

Samples such as pus, abscess drainage, ear discharge, wound swab, and bed sore swab were included in the skin infection group. Similarly, samples from lower respiratory tract infections, such as sputum and tracheal aspirate, and from invasive devices such as endotracheal tubes, were put together in the lower respiratory tract infection group. In the same way, urine and the urinary catheter were included in the urinary tract infection group. Samples such as blood, body fluid, high vaginal swab, tissue, and ulcer were separately grouped as "Others."

All the nosocomial isolates were processed for antibiotic susceptibility/resistance by disc diffusion by following the Kirby Bauer method [8]. The following antibiotic discs were used: Penicillin (10 units), oxacillin (1 μg), cefoxitin (30 μg), vancomycin (30 μg), teicoplanin (30 μg), cotrimoxazole (1.25/23.75 μg), rifamycin (5 μg), gentamicin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), clindamycin (2 μg) and norfloxacin (10 μg), nitrofurantoin (300 μg). For urine and urinary catheter isolates, erythromycin, clindamycin, chloramphenicol and ciprofloxacin were not used. Instead, novobiocin (5 μg for identification), norfloxacin and nitrofurantoin were used. Isolates with colony/colonies inside the zone of inhibition of oxacillin and cefoxitin that matched with the susceptible criterion were regarded as heterogeneous MRSA.

The oxacillin screen agar (OSA) test was also performed on the same isolates, following CLSI guidelines [8]. Isolates with confluent visible growth in OSA were identified as homogeneous MRSA. Those with scanty growth after 24 hours' incubation that transformed to perfectly visible growth at 48 hours were identified as heterogeneous MRSA.

The MRSA latex agglutination test was taken as a basis for the characterization of the isolate as MRSA. All MRSA were tested for the detection of PBP2a (product encoded by *mecA* gene) by using the MRSA latex agglutination test (Denka Seiken, Japan) by strictly following the manufacturer's instructions. The organism was suspended in four drops of Extraction Reagent 1 and heated at 97º C for three minutes. After cooling, one drop of Extraction Reagent 2 was added, mixed, and centrifuged at 1500X g for 5 minutes. Fifty microliters each of the supernatant dispensed on two separate circles on a card was tested with control and test latex. Agglutination with test latex but no agglutination with control was regarded as positive for the MRSA latex agglutination test.

For the identification of homogeneous or heterogeneous MRSA, Oxacillin MIC was considered as a standard method. For the determination of MIC for oxacillin, a 0.5 McFarland turbidity standard matched suspension of the test organism was swab-inoculated onto MHA containing 2% sodium chloride and an oxacillin E test strip (AB Biodisk, Sweden) was placed over the inoculated medium and incubated at 35ºC. Isolates whose MICs were > 100 μg/ml were noted as homogeneous MRSA; those with ≥ 4 and < 100 μg/ml and with a colony inside the zone of inhibition were regarded as heterogeneous MRSA.

For the D test, 0.5 McFarland turbidity standard matched suspensions of all isolates were swab-inoculated onto MHA. Erythromycin (15 μg) and

Table 1. Isolation of homogeneous and heterogeneous MRSA.

Infection group	Clinical samples	MRSA					
		Homogeneous			Heterogeneous		
		No.	Total	%	No.	Total	%
Skin infection	Pus	34	38	82.6	7	8	17.4
	Abscess	1			-		
	Wound Discharge	2			1		
	Bed Sore	1			-		
Lower respiratory tract	Sputum	8	12	85.7	2	2	14.3
	Endotracheal Tube	2			-		
	Tracheal Aspirate	2			-		
Urinary tract	Urine	1	3	75	1	1	25
	Urinary Catheter	2			-		
Others	Body Fluid	-	2	66.7	1	1	33.3
	High Vaginal Swab	1			-		
	Ulcer	1			-		
Total			**55**			**12**	

clindamycin (2 μg) (Oxoid UK) discs were placed 15 mm apart from each other and incubated at 35ºC for 18 hours [8]. Isolates susceptible to erythromycin and clindamycin were termed as susceptible. Isolates resistant to erythromycin and susceptible to clindamycin (with no induction of resistance to clindamycin in the presence of erythromycin, that is, D test negative) were identified as MS_B resistance phenotype. Isolates resistant to both erythromycin and clindamycin were identified as constitutive MLS_B resistance phenotype, and isolates resistant to erythromycin and having a flattened zone of clindamycin near the erythromycin disc (D test positive), were regarded as inducible MLS_B resistance phenotype.

S. aureus ATCC 43300 and *S. aureus* ATCC 25923 were used as MRSA and MSSA reference strains.
Data was analyzed by using the statistical tool X^2 test.

Results

Of the 149 *S. aureus* nosocomial isolates, 44.9 % (n = 67) were identified as MRSA by the disc diffusion test. The disc diffusion test correlated perfectly with the OSA test result. Both homogeneous and heterogeneous MRSA were also identified by the MRSA latex agglutination test and E test for the determination of oxacillin MIC. The MRSA latex agglutination test also correlated with the disc diffusion test and the oxacillin MIC E test. Of the 67 MRSA isolates, 82.1% (n = 55/67) were homogeneous MRSA and 17.9% (n = 12/67) were heterogeneous MRSA, clearly showing the greater occurrence of homogeneous MRSA ($X^2 = 27.59$, $P < 0.05$).

In the skin infection group, 82.6% (n = 38/46) of the isolates were homogeneous MRSA and 17.4% (n = 8/46) were heterogeneous MRSA, showing significant association of homogeneous MRSA with skin infections ($X^2 = 34.04$, $P < 0.05$). Similarly, association of homogeneous MRSA was significantly higher, 85.7% (12/14) in respiratory tract infections ($X^2 = 7.14$, $P < 0.05$). In the urinary infection and in the Others groups, homogeneous MRSA occurrence was greater than heterogeneous MRSA (Table 1).

Susceptibility pattern

A uniform pattern of antibiotic susceptibility was observed among the MRSA isolates. Thirty-eight isolates (37 homogeneous and one heterogeneous MRSA) comprised of 26 pus, 2 wound swab, 5 sputum, one endotracheal tube, one tracheal aspirate, and 3 urine isolates uniformly exhibited resistance to co-trimoxazole, ciprofloxacin, gentamicin and tretracycline, and susceptibility to rifamycin and chloramphenicol. None of the MRSA isolates were susceptible to ciprofloxacin and penicillin. In the remaining MRSA isolates, there were certain deviations from the uniform pattern of susceptibility to either one or two antibiotics. Ten isolates were susceptible to cotrimoxazole, 13 to tetracycline and 4 to gentamicin. On the other hand, 10 isolates were resistant to rifamycin and 5 were resistant to chloramphenicol. All isolates were susceptible to glycopeptides.

Table 2. Resistance phenotypes among *S. aureus*.

Resistant and Susceptible phenotype	Erythromycin	Clindamycin	D test	*S. aureus* No. (%)	MRSA No. (%)	MSSA No. (%)
Inducible MLS_B	R	S	D+	28 (20.6)	28 (44.4)	0
Constitutive MLS_B	R	R	-	27 (19.9)	25 (39.7)	2 (2.7)
MS_B	R	S	D-	17 (12.5)	7 (11.1)	10 (13.7)
Susceptibile/ no resistance	S	S	-	64 (47.1)	3 (4.7)	61 (83.6)
Total				**136**	**63**	**73**

Sixty-five MRSA isolates were multi-resistant oxacillin resistant *Staphylococcus aureus* (MORSA) exhibiting resistance to more than three non-β lactam antibiotics and 2 isolates (one each from pus and sputum) were non-multi-resistant oxacillin resistant *Staphylococcus aureus* (NORSA) that were resistant to ≤ 2 non-β lactams). Both NORSA isolates, one resistant only to ciprofloxacin and another to ciprofloxacin and gentamicin, were heterogeneous MRSA. Of the 12 heterogeneous MRSA, 8 isolates were susceptible to cotrimoxazole and tetracycline, a deviation from the uniform susceptibility pattern exhibited by other 38 MRSA isolates.

D test

Of the 67 MRSA isolates, 4 were isolated from urine and urinary catheter culture in which the susceptibility test for erythromycin and clindamycin was not performed. Hence the number of MRSA isolates taken for the study of constitutive MLS_B and inducible MLS_B resistance phenotypes was 63. Similarly, the number of MSSA isolates was 73.

Among 136 *S. aureus* isolates, inducible MLS_B resistance, constitutive MLS_B, MS_B and susceptibility was found in 20.6% (n = 28), 19.9% (n = 27), 12.5% (n = 17) and 47.1% (n = 64) respectively (Table 2).

Of the 63 MRSA isolates, 44.4% (n = 28/63) had inducible MLS_B resistance, of which 85.7% (n = 24/28) was contributed by the skin infection group especially by pus isolates 82% (n = 23/28). Constitutive MLS_B was observed in 39.7% (n = 25/63) of MRSA isolates. Similar to the inducible MLS_B, the skin infection group again contributed to 60% (n = 15/25) of constitutive MLS_B and pus isolates made up 48% (n = 12/25) of the total constitutive MLS_B resistance. In 11.1% (n = 7/63) of isolates, MS_B resistance was observed and only in 4.7% (n=3/63) susceptibility to both erythromycin and clindamycin was observed (Table 3).

Of the 73 MSSA isolates, 61 were susceptible to both erythromycin and clindamycin, two isolates (both from pus) had constitutive MLS_B resistance, and 10 isolates (nine from pus and one from abscess) had MS_B resistance. Inducible MLS_B was not observed in any of the MSSA isolates (Table 3). Both inducible MLS_B and constitutive MLS_B was found associated with MRSA ($X^2 = 40.8559$, $P < 0.05$ and $X^2 = 29.0047$, $P < 0.05$ respectively). Susceptibility to both erythromycin and clindamycin was found to be associated with MSSA ($X^2 = 84.2819$, $P < 0.05$), whereas association of MS_B was not found with either type.

Of the 28 MRSA isolates having inducible MLS_B resistance, the majority (n = 26) were homogeneous MRSA showing association with inducible MLS_B resistance ($X^2 = 20.56$, $P < 0.05$) compared to that of heterogeneous. Similarly, constitutive MLS_B (n = 24/25) was also found to be associated with homogeneous MRSA ($X^2 = 21.16$, $P < 0.05$). On the contrary, 71.4% and 100% of MS_B resistance and susceptibility to erythromycin and clindamycin respectively, were observed in greater occurrence among the heterogeneous MRSA.

Discussion

MRSA has been associated mainly with nosocomial infections. A high occurrence of MRSA was expected in nosocomial infections as the organisms develop resistance in the closed environments of hospitals and health care facilities due to selection pressure and their convenience in spreading from patient to patient via the health care workers and the instruments, etc.

The findings of the present work of 44.9% prevalence of MRSA are almost in the range of prevalence in US hospitals, 50-60% [2]. There are many reports from around the world on the prevalence of *S. aureus* and MRSA in admitted patients inclusive of the out patients. In India, MRSA

Table 3. Infection sites and clindamycin resistance type in MRSA

Infection site group	**Infection sites**	**MSSA Resistance or Susceptibility**						**MRSA Resistance or Susceptibility**					
		$IMLS_B$	**$CMLS_B$**	**MS_B**	**Susceptible/No resistance**	**Sub Total**	**Total**	**$IMLS_B$**	**$CMLS_B$**	**MS_B**	**Susceptible/No resistrance**	**Sub Total**	**Total**
Skin infection	Pus		2	9	44	55	**61**	23 (2)	12	5 (4)	1 (1)	41 (7)	**46 (8)**
	Bed sore								1			1	
	Abscess				3	3		1				1	
	Wound swab				2	2			2		1 (1)	3 (1)	
	Ear discharge				1	1							
Lower respiratory tract	Sputum			1	3	4	**4**	3	6 (1)		1 (1)	10 (2)	**14 (2)**
	Endotracheal tube								2			2	
	Tracheal aspirate							1	1			2	
Others	Body fluid				1	1	**8**			1 (1)		1 (1)	**3 (1)**
	High vaginal swab				4	4				1		1	
	Ulcer								1			1	
	Blood				1	1							
	tissue				2	2							
Total			**2**	**10**	**61**		**73**	**28 (2)**	**25 (1)**	**7 (5)**	**3 (3)**		**63 (11)**

numbers in parenthesis indicates heterogeneous MRSA.

prevalence in the hospital isolates has been reported in the range of 20-39.5% [9-12]. Similarly, there are few reports on prevalence of MRSA in hospital isolates from Nepal with prevalence in the range of 15.4-29% [13-15]. However, no report on nosocomial isolates of *S. aureus* and MRSA in Nepal could be found.

All MRSA isolates were resistant to penicillin and ciprofloxacin. Ciprofloxacin and penicillin derivatives are the commonly used antibiotic in Nepalese hospitals and the organisms that are resistant to these antibiotics tend to become the cause of nosocomial infections such as MRSA [16]. The uniform multi-resistance pattern of the MRSA isolates obviously indicates that infections by these isolates are difficult to treat.

Inducible MLS_B was found in 20.6% of the *S. aureus* isolates. Such an occurrence is quite low compared to published reports [17-19]. Similarly, the occurrence of constitutive MLS_B (19.9%) obtained in the present study was low compared to another report [19]. Such differences could be due to the varied occurrence of different resistance patterns among clinical Staphylococcal isolates according to patient group, hospital, and geographical locations [20].

In the present study, higher incidence of inducible MLS_B and lower occurrence of constitutive MLS_B in MRSA is not in accordance with other reports [19, 21-24]. However, one report has stated high inducible MLS_B among MRSA [25].

The significantly lower occurrence of constitutive and inducible MLS_B among MSSA, compared to MRSA, obtained in present study is in concordance with other reports [19, 21-24]. Others have also reported association of MRSA with inducible MLS_B [26]. They have stated that clindamycin resistance emerge readily and is common in MRSA. On the contrary, certain reports suggest a remarkably greater occurrence of inducible MLS_B among MSSA [23, 27]. Significantly small occurrence of MS_B in both MRSA and MSSA is concordant with other reports [21, 24].

Clindamycin is one of the useful antibiotics in serious infections caused by *S. aureus*, as it has excellent tissue penetration and accumulation in the abscesses. Good oral absorption and no requisition of renal dosing adjustment make it an important therapy. It is especially important in people with penicillin allergy and also has been an important therapy in MRSA and MSSA infection. The increasing frequency of staphylococcal infection and changing patterns of antimicrobial resistance have led to the interest in the use of clindamycin therapy in the treatment of staphylococcal infections [28]. However, the existence of constitutive MLS_B resistance among MRSA has raised a question necessitating the D test in *S. aureus* isolates. In MRSA expressing inducible MLS_B, use of clindamycin is a matter of debate because of the ability of *S. aureus* expressing inducible MLS_B resistance to develop clindamycin resistance *in vitro* during therapy [29]. Similar observations are also made with MRSA [30]. Despite these reports, there are reports of successful clindamycin treatment of infection by MRSA expressing inducible MLS_B resistance [30]. Therefore, elimination of a useful antibiotic such as clindamycin is not desirable, especially for the treatment of MRSA [31]. Therefore, the D test should be included in the routine susceptibility test and clinicians should be informed of the possible failure of clindamycin therapy in infections caused by MRSA expressing inducible MLS_B resistance

Conclusion

Prevalence of MRSA among nosocomial *S. aureus* was found to be 44.9%. Most of the strains were homogeneous MRSA which were predominantly MORSA. Regarding clindamycin resistance and susceptibility patterns, MRSA was associated with inducible and constitutive MLS_B, whereas MSSA was associated with susceptibility to both erythromycin and clindamycin. MS_B was not found to be associated with either type.

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