Prevalence and Antimicrobial Susceptibility Pattern of Methicillin-Resistant *Staphylococcus aureus* and Coagulase-Negative Staphylococci in Rawalpindi, Pakistan

Irum Perveen[1*], Abdul Majid[2], Sobia Knawal[3], Iffat Naz[1], Shama Sehar[1], Safia Ahmed[1] and Muhammad Asam Raza[4]

[1]*Department of Microbiology, Quaid-i-Azam University, Islamabad, 45320, Pakistan.*
[2]*Department of Internal Medicine, Military Hospital, Rawalpindi, Pakistan.*
[3]*Department of Animal Sciences, Quaid-i-Azam University, Islamabad, 45320, Pakistan.*
[4]*Department of Chemistry, Hafiz hayat Campus, University of Gujrat, Gujrat, Pakistan.*

Authors' contributions

This work was carried out in collaboration between all authors. Author IP designed the study, carried the experiments. Authors AM and SK searched out the literature. Authors SA and IN did the analysis and wrote the first draft of the manuscript. Authors SS and MAR contributed to the study design, the analysis of the results and writing the manuscript. All authors read and approved the final manuscript.

Research Article

ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase negative staphylococci (MRCoNS) are the important nosocomial infectious agents. There is a growing concern about the rapid rise in the resistance of *Staphylococcus aureus* to presently available antimicrobial agents. The aim of this study was to evaluate the prevalence rate of MRSA and MRCoNS and their rate of resistance to different antistaphylococcal antibiotics used broadly for treatment. Out of the total 350 staphylococcal isolates from different clinical specimens 148 isolates (60.40%) were identified as MRSA by oxacillin screen agar method, and 46 isolates (43.80%) were screened as MRCoNS. All the MRSA and MRCoNS isolates were tested for antibiotic resistance pattern by disc diffusion method for 16 different antibiotics. All the isolates of

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*Corresponding author: Email: irum.mederapharma@gmail.com;

MRSA and MRCoNS were multi-drug resistant. Antibiotic resistance pattern of these isolates was high against penicillin. All the MRSA strains were resistant to penicillin and oxacillin (100%), followed by cephalothin and nalidixic acid (89.18%), cotrimoxazole (86.48%), erythromycin (85.81%), cephalaxin and cephradine (83.10%), levofloxacin (80.40%), imipenem (77.70%), gentamicin (76.35%), tetracycline (59.45%), ciprofloxacin (44.59%), chloramphenicol (18.24%) and rifampicin (10.13%). The MRCoNS strains also showed closely similar drug resistance pattern with 97.82% isolates being resistant to penicillin, followed by oxacillin (95.65%), cephalothin (86.95%), cephradine (82.60%), levofloxacin and nalidixic acid (80.43%), erythromycin, cephalaxin and imipenem (78.26%), cotrimoxazole (73.91%), gentamicin (69.56%), ciprofloxacin and tetracycline (63.04%), chloramphenicol (13.04%) and rifampicin (6.52%). However, all the MRSA and MRCoNS isolates, even those with very high oxacillin MIC (>130 µg/ml) were uniformly susceptible to vancomycin. Chloramphenicol and rifampicin also showed excellent activity against methicillin-resistant isolates. Overall, data presented in this study indicated a slightly higher methicillin resistant rate in MRSA compared to MRCoNS strains. Multi-drug resistance rates in our MRSA and MRCoNS isolates were, 58.10 and 32.60%, respectively. Application of ß-lactamase production method revealed that 84% of MRSA and 87% of MRCoNS strains tested positive for the ß-lactamase production. This study indicated a high level prevalence of MRSA and MRCoNS strains resistance against widely used antimicrobial agents. An appropriate knowledge on the current antibiotic susceptibility pattern of MRSA and MRCoNS is essential for appropriate therapeutic regime determination.

1. INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin resistant coagulase negative *staphylococci* (MRCoNS) are prevalent worldwide [1,2]. These are considered as the most important cause of hospital-acquired infections (HAI) and community-acquired infections (CAI), resulting in increased morbidity and mortality in the hospital settings [3]. Methicillin was first introduced in human medicine in 1960s for the treatment of infections caused by penicillin's resistant *S. aureus* [4] however first methicillin resistant *S. aureus* emerged in 1961 in England [5]. The widespread use of antimicrobial agents to treat staphylococcal infections has resulted in the emergence of resistant forms of these organisms. To date most MRSA have become resistant to number of antimicrobial agents like ß-lactams [6]. Emergence of MRSA worldwide has led to the overuse of glycopeptides antibiotics and to the emergence of vancomycin-resistant *S. aureus* [7].

Presently MRSA isolates have been uniformly susceptible only to glycopeptides. But recently, number of isolates resistant to glycopeptides has been reported [8,9]. MRSA strains are frequently resistant to many different classes of antibiotics [10]. Clinical isolates of MRSA with reduced susceptibility to glycopeptide were first described in Japan in 1997 [11]. To date, three types of reduced susceptibility to glycopeptides have been described in *S. aureus*: vancomycin-resistant *S. aureus* (VRSA), glycopeptide-intermediate *S. aureus* (GISA), and hetero-GISA (hGISA) [12]. Multidrug-resistant bacteria, such as MRSA, are endemic in healthcare settings in the United States and many other countries of the world. Nosocomial transmission of MRSA serves as a source of hospital outbreaks, and

recent reports of vancomycin resistant *S. aureus* strains in the United States emphasize the need for better control of MRSA and other resistant bacteria within healthcare settings [13].

Power of morbidity and simultaneously resistance to other antibiotics in MRSA strains is higher than methicillin sensitive *S. aureus* (MSSA). Regarding to increased prevalence of these strains in recent years and pathogenesis of *S. aureus*, accurate and early identification of these strains is very important [14,15]. MRSA strains can grow in the presence of 16µg/ml or more of methicillin while sensitive strains are inhibited [16]. At present, MRSA has become an endemic pathogen worldwide (Kluytmans et al.,1997) and multi drug resistant [17]. Therefore, the knowledge about the prevalence of MRSA, MRCoNS and their antibiotic susceptibility pattern has become fundamental in the selection of appropriate treatment especially in a hospital setting.

Although MRSA infections were traditionally limited to hospitals, community-associated cases of MRSA (CA-MRSA) were reported starting in the late 1990s [18]. The epidemiological success of CA-MRSA strains is believed to stem from the combination of antibiotic resistance at low fitness cost [19,20] with extraordinary virulence, allowing these strains to infect otherwise healthy individuals and spread sustainably in the population [21]. CA-MRSA infections, which were first described in small series of adult and pediatric patients presenting with skin and soft tissue infections (SSTIs), pneumonia, or bacteremia [22,23] have become a significant public health threat in the United States and abroad [24]. In the United States, a single clone of CA-MRSA (USA 300 ST-8) has become the most prevalent cause of staphylococcal SSTI acquired in the community [25] and has moved into the inpatient setting, causing not only SSTIs but also invasive diseases [18,20,26] .

Considering the increasing rate of infections caused by MRSA, performance of reliable, accurate and rapid testing for detection of MRSA is essential for both antibiotic therapy and infection control measures [27]. In the present study we determined the prevalence level of MRSA and MRCoNS strains in different clinical specimens and there *in vitro* susceptibility pattern towards various antibiotics to record the current status of MRSA and MRCoNS response against commonly used anti-staphylococcus antibiotics.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of Clinical Specimens

A total of 350 *Staphylococcus* isolates were obtained from various sites of infection including blood, wound swabs, nasal and ear swabs, pus and urine collected from five major Government hospitals in Rawalpindi during April, 2011 to June 2012. The isolates collected from various clinical specimens submitted at the microbiology laboratory were processed and all *Staphylococcus* isolates were included in this study. *S. aureus* identification was performed based on standard tests such as Gram's staining, catalase, DNase, growth on manitol salt agar, slide and tube coagulase [28]. Strains positive for these tests were labeled as *S. aureus*.

2.2 Antibiotic Susceptibility Testing

The antibiotic susceptibility pattern of all the confirmed *S. aureus* strains was determined by Kirby Bauer disc diffusion method (1966) [29] against the following antibiotics: penicillin (60 µg), oxacillin (1 µg), gentamicin (10 µg), erythromycin (15 µg), cotrimoxazole (25 µg),

ciprofloxacin (5 µg), vancomycin (30 µg), cephalaxin (30 µg), cephalothin (30 µg), cephradine (30 µg), imipenem (10 µg), levofloxacin (5 µg), tetracycline (30 µg), rifampicin (5 µg), nalidixic acid (30 µg) and chloramphenicol (30 µg), purchased from Oxoid-UK. All tests were performed on Muller- Hinton agar (Oxoid-UK), and were interpreted after incubation for 24 h at 37ºC. The zone diameters measured around each disk were interpreted on the basis of guidelines published by the Clinical and Laboratory Standards Institute [12].

2.3 Screening Test for MRSA

Screening was performed following NCCLS guidelines using oxacillin agar. Briefly, a suspension equivalent to MacFarland 0.5 was prepared from each strain. Then a swab was dipped and streaked on the surface of Muller-Hinton agar (Oxoid-UK) supplemented with 6 µg/ml oxacillin and 4% NaCl, growth was observed after incubation for 24 h at 35ºC [12], if any growth was detected, the isolate was considered oxacillin or methicillin resistant.

2.4 MRSA Screening for Decreased Vancomycin Susceptibility

Further, vancomycin resistance was tested by vancomycin agar screening test whereby MRSA isolates were spot inoculated into the Muller-Hinton agar (Oxoid-UK) supplemented with 6 µg/ml of vancomycin from 0.5 McFarland standard suspensions. The plates were incubated at 35ºC for 24 h as recommended by the CLSI (2006) [12]. Any isolate growing two or more colonies on this agar would be considered as positive.

2.5 Determination of Minimum Inhibitory Concentration (MIC)

Micro dilution broth method, using Muller Hinton broth (Oxoid-UK) was used to determine the lowest concentration of antimicrobial agents (MICs) required inhibiting the growth of microorganism against methicillin, vancomycin, tetracycline, rifampicin and gentamicin. Bacterium inoculations of 5×10^5 cfu and incubation at 35ºC for 24 h was done according to Clinical and Laboratory Standards (CLSI) guidelines [15,16].

2.6 Detection of ß-lactamase Production

ß-lactamase production was determined by iodometric strip method, benzyl penicillin was dissolved in 0.2% starch solution; the mixture was soaked in Whatman No. 1 filter paper. When the filter papers were saturated, they were dried and cut into strips; these strips were stored at -20ºC until use. Prior to test, strips were put in desiccators and brought to room temperature. Strips were moisturized with iodine and 2-3 colonies of bacteria were smeared. If the color of the strip changed in 5 min, the bacteria were ß-lactamase positive [30].

3. RESULTS AND DISCUSSION

MRSA is a major nosocomial pathogen causing significant morbidity and mortality [31]. RSA were gradually reported [32], whereas MRCoNS have become the predominant pathogen in hospitalized patients with the number of infections caused by these pathogens increased dramatically [33-35]. Presently, we isolated a total of 350 *Staphylococcal* isolates from different clinical specimens collected from patients. The highest percentage of these isolates was collected from urine samples and the least number of isolates were recovered from blood samples (Table 1).

Table 1. Distribution of *staphylococcus* from various clinical specimens

Clinical specimens	Number of isolates	Percentage (%)
Pus	78	22.28
Urine	88	25.14
Wound swabs	75	21.42
Nasal/eye swabs	50	14.28
Blood	34	9.71
Sputum	25	7.14
Total	350	100

Prevalence and antibiotic susceptibility pattern of various MRSA isolates obtained from different clinical specimens of patients was determined. Out of 350 isolates tested, 245 (70%) were coagulase positive *staphylococci* and 105 isolates (30%) were coagulase negative *staphylococci*. Among the 245 coagulase positive *staphylococci* strains, 148 (60.40) were methicillin resistant *S. aureus* (MRSA), and out of 105 coagulase negative *staphylococci*, 46 (43.80%) were methicillin resistant (Table 2). 55% of isolates of *S. aureus* were found to be methicillin resistant which shows that the prevalence of methicillin resistance is higher than previous reports in Pakistan. The major claim is of 35% MRSA prevalence in Pakistan [36,37]. Whereas Qureshi et al., reported a 28.4% occurrence of MRSA in Rawalpindi [38]. In another multicenter study conducted by Hafiz et al. [39], the prevalence of MRSA strains in various cities of Pakistan was found to be 42%, highest seen in Lahore (61%), closely followed by Karachi (57%), Rawalpindi Islamabad (46%), Peshawar (36%), Azad Kashmir (32%) and Quetta (26%) while minimum resistance was seen in Sukkur (2%).

Table 2. Prevalence of MRSA and MRCoNS

Bacterial isolates	Resistance to methicillin (%)		
	Resistant	Intermediate	Susceptible
MRSA	148 (60.40)	21 (8.57)	76 (31.02)
MRCoNS	46 (43.80)	13 (12.38)	46 (43.80)
Total	194	34	122

The majority of MRSA strains were recovered from wound swabs (39.18%) whereas the MRCoNS strains were isolated from urine samples (34.78%) (Table 3).

Table 3. Frequency of MRSA and MRCoNS in clinical specimens

Specimens	Frequency of MRSA and MRCoNS			
	MRSA	MRSA (%)	MRCoNS	MRCoNS (%)
Pus	31	20.94	12	26.08
Urine	23	15.54	16	34.78
Wound swab	58	39.18	7	15.21
Nasal/eye swab	18	12.16	5	10.86
Blood	6	4.05	3	6.52
Sputum	12	8.10	3	6.52
Total	148	100	46	100

The antimicrobial susceptibility pattern of MRSA and MRCoNS isolates against agents of different classes (Table 4). The drug resistance patterns of MRSA isolated from clinical specimens was found to be highly variable. All the 148 MRSA strains were resistant to penicillin and oxacillin (100%), followed by cephalothin and nalidixic acid (89.18%), cotrimoxazole (86.48%), erythromycin (85.81%), cephalaxin and cephradine (83.10%), levofloxacin (80.40%), imipenem (77.70%), gentamicin (76.35%), tetracycline (59.45%), ciprofloxacin (44.59%), chloramphenicol (18.24%) and rifampicin (10.13%). The MRCoNS strains also showed closely similar drug resistance pattern with 45 isolates out of 46 (97.82%) being resistant to penicillin, followed by oxacillin (95.65%), cephalothin (86.95%), cephradine (82.60%), levofloxacin and nalidixic acid (80.43%), erythromycin, cephalaxin and imipenem (78.26%), cotrimoxazole (73.91%), gentamicin (69.56%), ciprofloxacin and tetracycline (63.04%), chloramphenicol (13.04%) and rifampicin (6.52%). However, all MRSA and MRCoNS strains tested in this study were recorded sensitive to vancomycin (100%).

Table 4. Antibiotic resistance pattern of MRSA and MRCoNS

Antibiotics	Percent of isolates resistant to antibiotics			
	MRSA (n=148)	MRSA (%)	MRCoNS (n=46)	MRCoNS (%)
Penicillin	148	100	45	97.82
Oxacillin	148	100	44	95.65
Gentamicin	113	76.35	32	69.56
Erythromycin	127	85.81	36	78.26
Cotrimoxazole	128	86.48	34	73.91
Ciprofloxacin	66	44.59	29	63.04
Vancomycin	0	00	0	00
Cephalaxin	123	83.10	36	78.26
Cephalothin	132	89.18	40	86.95
Cephradine	123	83.10	38	82.60
Imipenem	115	77.70	36	78.26
Levofloxacin	119	80.40	37	80.43
Tetracycline	88	59.45	29	63.04
Rifampicin	15	10.13	03	6.52
Nalidixic acid	132	89.18	37	80.43
Chloramphenicol	27	18.24	06	13.04

Thus, we found all isolates of MRSA resistant to multiple antibiotics tested. Isolates exhibited resistance towards various antibiotics such as cephalosporins, tetracycline and gentamicin which is almost similar to previous reports [40-42]. In another study James and Reeves [43], found MRSA strains resistant to first, second, third and fourth generation of cephalosporins.

Presently, 83% of the MRSA and 82% MRCoNS isolates showed resistance against cephradine. Mahmood et al. [44] reported 29% resistance of *S. aureus* against first generation cephalosporins. Gentamicin is an amioglycoside and is most often prescribed because of its low cost and synergistic activity with ß-lactum antibiotics. In the present study 76.35% of MRSA and 69.56% of MRCoNS showed resistance towards gentamicin which is higher than reported earlier 30% [45]. Rifampicin is a drug considered suitable for treatment of MRSA infection [14,46,47]. In this study MRSA resistance to rifampicin is found 10.13% and that of MRCoNS is found 6.52%. Yameen et al. [48] reported MRSA resistance of 14% towards rifampicin. In this study 85.81% of MRSA were resistant to erythromycin, which is comparable to previous reports [9,38]. Among fluoroquinolones, ciprofloxacin and

levofloxacin tested, the percentage resistance found in MRSA and MRCoNS was 44.59 and 63.04% for ciprofloxacin and 80.40 and 80.43% for levofloxacin respectively. Previously reported resistance of ciprofloxacin shows the similar type of pattern [49,50]. Since the emergence of methicillin resistant *S. aureus*, the glycopeptides vancomycin has been the only effective treatment for MRSA infections [51]. In the present study all the MRSA and MRCoNS isolates were susceptible to vancomycin. These results coincide with the findings of Mitchell et al. [52].

Multi-drug resistance in this study was taken as resistance to three or more of the twelve antimicrobial drugs tested. Of the 148 MRSA strains isolated, 86 strains (58.10%) were found to be multidrug resistant, whereas 15 (32.60%) out of total 46 MRCoNS strains were found to be multidrug resistant (Table 5). Application of ß-lactamase production method revealed that 84% of MRSA and 87% of MRCoNS strains tested positive (Table 5). Multi-drug resistance rates in our MRSA and MRCoNS isolates were, 58.10 and 32.60%, respectively. Other published reports have indicated a closely similar or higher percentage of resistance [53-55]. In our study the ß-lactamase production rates found were 84 and 87% for MRSA and MRCoNS respectively, these results are similar to the findings of Paradisi et al. [51], Ang et al. [56] and Olowe et al. [57].

Table 5. Detection of multi-drug resistance and ß-lactamases production

Bacterial isolates	No. of isolates and percentage of multi-drug resistance			ß-lactamases detection (percentage)
	Total	MDR	Percentage	
MRSA	148	86	58.10	84
MRCoNS	46	15	32.60	87
Total	194	101	-	-

The MIC values of MRSA were determined against antibiotics includes vancomycin, tetracycline, rifampicin and gentamicin. The MIC of vancomycin for MRSA isolates ranged from 1-5 µg/ml; 88% isolates were inhibited at concentration of ≤2 µg/ml, and 12% at 5 µg/ml. These ranges of vancomycin MICs are higher than the previously reported ranges. Denis et al. (2006) reported 100% inhibition at concentration of 1 µg/ml and Lozniewski et al. (2001) reported MRSA inhibition at concentration of 0.5-1 µg/ml. Higher MICs for vancomycin against MRSA is an alarming sign of development of infections with vancomycin resistant *S. aureus* (VRSA) [58,59]. For tetracycline the MIC ranged from 8-131 µg/ml; 37% isolates were inhibited at ≤20 µg/ml, 16% at 32 µg/ml and 47% at ≥65 µg/ml. For rifampicin the MIC value ranged from 1-38 µg/ml; 62% isolates were inhibited at 1 µg/ml, 23% at <10 µg/ml, and 15% at 38 µ/ml. Denis et al. [58], showed 99% of isolate inhibition at concentration of 2µg/ml. For gentamicin the MIC value ranged between 0.5-61 µg/ml; 21% of isolates were inhibited at concentration 0.5 µg/ml, 63% at <30 µg/ml, and 16% were inhibited at 61 µg/ml. (Table 6). Denis et al. (2006) reported 95% of MRSA inhibition at 0.5 µg/ml and total 4% of isolates inhibition at a concentration of ≥32 µg/ml. Higher MICs observed in our study is an alarming sign to resistance of pathogen for available options [58].

Table 6. MICs of different antibiotics for MRSA isolates

Antibiotics	Range (µg/ml)	% inhibited	MIC (µg/ml)
Vancomycin	1-5	88	≤2
		12	5
Tetracycline	8-131	37	≤20
		16	32
		47	≥65
Rifampicin	1-38	62	1
		23	<10
		15	38
Gentamicin	0.5-61	21	0.5
		63	<30
		16	61

4. CONCLUSION

The emergence of drug resistance in MRSA and MRCoNS is worrisome in the present therapeutic scenario. The percentage of drug resistant isolates of both *Staphylococcus aureus* and Coagulase negative *Staphylococcus* were seen to be higher than in other countries. Although all strains were sensitive to vancomycin the minimum inhibitory concentration for this drug was also higher than previously reported ranges. Regular surveillance of hospital associated infection including monitoring antibiotic sensitivity pattern of MRSA and MRCoNS is mandatory to controlling the spread in the hospital and strict drug policy are of importance or else the threat will increase and even vancomycin resistant strains may emerge. In conclusion most of the clinical isolates of MRSA were resistant to cephalosporins, gentamicin, fluoroquinolones and even to imipenem, so these are less effective in the treatment of MRSA infections, vancomycin use should be limited to those cases where they are clearly needed. The rise in resistance in Pakistani hospitals is clearly due to irrational use of antibiotics and non existence of protocols and guidelines for treatment of Methicillin resistant organisms based on local data and resistance patterns.

CONSENT

All authors declare that verbal informed consent was obtained from the patient for publication of this case report.

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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