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Isolation and identification of Methicillin and Vancomycin Resistance *Staphylococcus aureus* From Pus Samples of Injured Skin Patients in Lahore, Pakistan

Wasif Ghias[1], Musarrat Sharif[1]*, [1]Maham Yazdani[1], Farheen Ansari[1], Masood Rabbani[2]

[1]Institute of Molecular Biology and Biotechnology, The University of Lahore, Defense Road, Lahore, Pakistan
[2] University Diagnostic Laboratory, University of Veterinary and Animal Sciences, Lahore, Pakistan

Abstract
Staphylococcus aureus especially methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Staphylococcus aureus* (VRSA) is a major health problem recognized as the most important nosocomial pathogen often causing respiratory tract infection, gastrointestinal infection, urinary tract infection, surgical site infection, blood stream infection and soft tissue infection etc. This study was designed to identify MRSA and VRSA from the pus of injured skin of hospitalized patients. A total of 200 pus samples were collected from four hospitals of Lahore city *i.e.* Mayo Hospital, Services Hospital, Ganga ram Hospital and Jinnah Hospital. *Staphylococcus aureus* were isolated from samples by culturing on nutrient agar, mannitol salt agar and identified by morphological characteristics on selective media and biochemical characterization. For MRSA screening, 1µg oxacillin and 30µg cefoxitin discs were used. Resistance isolates was screened by vancomycin agar screening method and confirmation of resistance to vancomycin was done by measuring MIC. It was concluded that hygiene is not sufficiently adequate. To eliminate MRSA and MRSA complete surface sanitation and hand hygiene are necessary.
Keywords: Nosocomial infection, antibiotic, resistant, MRSA, VRSA.

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***Correspondence:** Musarrat Sharif, **Email** musarratsharif388@gmail.com, **Contact**: +92-3104008714

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Introduction

Staphylococcus genus is a heterogeneous group of bacteria consisting of 30 species. *Staphylococcus aureus* are Gram-positive cocci bacteria when seen under microscope they look grape like clusters. They show hemolysis when grown on blood agar plates [1, 2]. It has been found to be the most clinically important species with broad presence in nature. It is part of the normal flora of human body and commonly carried on the skin or in the nose of healthy individuals which makes it easy to be transmitted by air or fomites from patients or carriers [3, 4].

The main reservoirs of *S. aureus* are the mucous membrane and skin of human and animals. These bacteria able to produce enterotoxin that cause harm to human. Therefore *S. aureus* is classified as bacterial pathogen. Under new selective pressure, it has evolved and developed resistance to many antibiotics. The evolution of *S. aureus* is associated with serious community-acquired and nosocomial infections [5].

Methicillin resistant *Staphylococcus aureus* (MRSA) has been isolated and recognized more than 50 years ago. MRSA is a specific strain of the *Staphylococcus aureus* which is resistant to methicillin and β-lactams. Later use of an alternative to methicillin in susceptibility tests resulted in the term oxacillin resistant *Staphylococcus aureus* (ORSA) which is resistant to numerous antibiotics, invasive infections caused by *Staphylococcus aureus* have often been fatal [6]. Currently, vancomycin has been accepted worldwide as the last choice against MRSA infections [7]. Rarely, clinical isolates of vancomycin resistant *Staphylococcus aureus* (VRSA) have been reported recently. The emergence of *Staphylococcus aureus* isolates resistant to vancomycin and other antibiotics have been elevated MRSA into multidrug resistant making it more and more dangerous than ever in a hospital environment and also recently in the healthy community [8, 9].

Hospital acquired infection also known as nosocomial infection that spreads through hospitals and patients gets the infection from the hospital during the stay. Infections are said to be hospital acquired if they occur within 48 hours after admission to the hospital or within 30 days after discharge of the patient. These infections have many complications which increase the morbidity and mortality rate [10].

The spread rate of hospital acquired infection varies from country to country health care system (e.g. wards vs. intensive care units, ICU) disciplines (medical vs. surgical) and different sites (e.g. respiratory tract infection, gastrointestinal infection, urinary tract infection, surgical site infection, blood stream infection and soft tissue infection etc. [11]. The main causes of all these infection are invasive procedures, alternate drug therapies and complicated diseases. Hospital acquired infection are mostly related to drug resistant microorganisms e.g. methicillin-resistant *Staphylococcus aureus* (MRSA) and extended spectrum B-lactamase (ESBL) producing Gram negative bacteria and there prevalence increases in hospitals and communities [12].

Microorganisms that cause most common hospital acquired infections include *Staphylococcus aureus*, Methicillin resistant *Staphylococcus aureus*, *Candida albicans*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, *E. coli*, Vancomycin resistant Enterococcus [13]. Resistance to antibiotics is the major cause of disease in humans and due to the misuse or overuse of antibiotics. Antibiotic resistance is due to the mutation in genes of bacteria present in plasmid. *S. aureus* causes many skin infections like pimples, impetigo, boils and abscesses. It also causes pneumonia, meningitis, osteomyelitis, endocarditis and sepsis. It is one of the most common causes of hospital acquired infection [14].

Macrolide, lincosamide and streptogramin are the antibiotics that widely used to treat staphylococcal infections [15]. However, the resistance ability of *S. aureus* increased due to the widespread use of these antibiotics. The integration of Staphylococcus cassette chromosome mec (SCCmec) element into *S. aureus* changed it into methicillin resistant *Staphylococcus aureus* (MRSA) The MRSA contains the ability of resistance to macrolides, aminoglycoside, lincosamide, tetracycline and other antimicrobial drugs [16].

The aim of the present study was to determine the prevalence, antimicrobial susceptibility patterns, Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *S. aureus* in patients of different hospitals of Lahore, Pakistan.

Materials and methods

2.1 Collection of samples

A total of 200 samples (50 from each hospital) were collected from different injured skin patients of 4 hospitals (Mayo Hospital, Services Hospital, Ganga ram Hospital and Jinnah Hospital) of Lahore city with a brief history of patient age, gender, date of admission in hospital, nature of infections before or during hospital stay, type of surgery performed category of wound, duration of preoperative and postoperative hospital stay and also the detail of antibiotic therapy given to the patient in near past. Samples were taken aseptically from the wound exudates after cleaning any remnants of ointments by using sterile cotton swabs. Each sample swab was properly labeled before transportation. Swabs were placed in a sterile plastic bags maintaining in cold chain (at 4^oC) and immediately transferred to University Diagnostic Laboratory (UDL), University of Veterinary & Animal Sciences (UVAS) Lahore, Pakistan.

2.2 Sample processing

Pus collected from wounds exudates were directly inoculated on nutrient agar plates for purification of single colonies then streak pure colonies on Mannitol salt agar (MSA), a selective as well as differential media and on blood agar for *S. aureus.* Plates were incubated aerobically at 37^o C for 24 hours. This selective and differential media only allows Staphylococcus to grow and furthermore differentiate the growth of *S. aureus* from other Staphylococcus species attributable to change in media color from pink to yellow because of Mannitol fermentation [17]. Mannitol fermentation is a specific activity of *S. aureus* and growth with no color change indicates any other Staphylococcus species while growth of all other bacteria expected in the pus sample was repressed. Confirmation of colonies was done by Gram staining and biochemical profile (catalase and coagulase test) [18].

2.3 Identification of MRSA

All coagulase positive *S. aureus* isolates were subjected to detect methicillin resistance by checking their susceptibility to oxacillin and cefoxitin. Identification of MRSA was done by Kirby Bauer disc diffusion method on Mueller-Hinton agar (MHA) (Oxoid Limited, Hampshire England) plates. Suspensions of freshly revived bacterial culture were prepared in sterilized normal saline and turbidity was adjusted by comparing with 0.5 McFarland turbidity standards. A sterile swab was dipped in suspension and was swabbed thoroughly on Mueller-Hinton agar plates. Discs of $1\mu g$ oxacillin and $30\mu g$ cefoxitin were placed in equal distance and plates were incubated at 37^oC for 24 hours. After incubation zones of inhibition were measured. The results were

interpreted according to CLSI guidelines. Isolates showing no zone of inhibition or zone ≤10mm and ≤ 21mm for oxacillin and cefoxitin respectively were affirmed as MRSA [19].

Colonies of positive MRSA isolates were inoculated in nutrient broth and incubated at 37° C for 18 hours. After incubation samples were preserved in cry vials containing 500μl bacterial suspension and 500μl 40% glycerol solution. Each isolate was properly labeled with its unique sample ID and preserved at -80° C (New Brunswick Scientific) temperature till further use. Interpretive standards for antibiotics are shown in **Table 1**.

Table 1: Interpretive Standards for Oxacillin and Cefoxitin antibiotics

Antibiotic Disc Tested	Code	Disc quantity	Zone of inhibition (mm)		
			S	I	R
Oxacillin	OX	1µg	≥13	11-12	≤ 10
Cefoxitin	FOX	30µg	≥ 22	__	≤ 21

Note: S= susceptible I= Intermediate R= Resistant

2.4 Minimum Inhibitory concentration (MIC) of MRSA

MRSA isolates grown were further processed to recover MRSA by performing MIC of Oxacillin by broth microdilution test [20]. All the steps were performed according to CLSI approved standards and recommendations. Oxacillin powder was aseptically weighed and dissolved in sterile distilled water to prepare a stock with the final concentration of 50mg/ml. After complete dissolution of powder, small volumes of the sterile stock solutions were dispensed into sterile cry vials and stored at -80° C freezers until further use.

Micro dilution test was carried out in 96 well round bottom trays (Kartel). Trays were opened aseptically in safety cabinet and dispensed with Mannitol salt Broth (MSB) 100 μl per well of the tray. Stock solution of Oxacillin was thawed to prepare working solution with a concentration of 4.096 mg/ml. 100μl of this working solution was added in 1st well of each row and diluted two fold up to 11th well while 12th well was kept as growth control and help in detection of color change in the well and button formation.

Cultures of MRSA isolates were revived on nutrient agar plates and pure colonies were suspended in freshly prepared sterile normal saline to prepare the inoculum by "Direct Colony Suspension Method". Turbidity was adjusted equivalent to 0.5 McFarland turbidity standards. This results in a suspension containing approximately $1x10^8$ CFU/ml. This suspension was further diluted by 1:200 to yield required concentration of $5 x10^5$ CFU/ml (Standard number of cells for Micro broth dilution test). Finally 100μl of this ultimate suspension was inoculated within 15 minutes in all wells of 96 well trays except 1st well which was kept as control for Oxacillin. 1st well verify the sterility of Oxacillin working solution and rule out the button formation due to any un-dissolved Oxacillin contents. With the addition of 100μl inoculum, contents of each well were diluted two fold as each well contains 100μl broth used to dilute Oxacillin. Microdilution trays were covered with the lids to prevent drying and incubated at 37° C for 24 hours in ambient air.

The MIC is the lowest concentration of Oxacillin that completely inhibits the growth of *S. aureus* in the microdilution wells. Since mannitol salt broth was used in the wells as growth medium therefore the results interpretation was done easily by the color change of broth from pink to yellow. The well which show no color change and no button formation as compared to control well were recorded as positive and the concentration of Oxacillin in this well was the end point or MIC value. All isolates which had shown the MIC value of ≥4μg/ml was considered as MRSA according to CLSI interpretation standards of Oxacillin antibiotic.

2.5 Vancomycin agar screening test

For vancomycin screening agar preparation, one liter Heart Infusion agar (BHI) was mixed well and dissolved by heating until complete dissolution. Sterilization was done by autoclaving at 121° C for 15 minutes. Media was cooled to 45-50° C and 6.0mg (final concentration of 6μg/ml) of Vancomycin was added aseptically followed by gentle homogenization and pouring into Petri dishes. The prepared medium was stored at 4° C [21].

Preserved MRSA isolates were revived in broth and then grown on nutrient agar plates and pure colonies were directly suspended in sterilized normal saline to obtain the turbidity equal to 0.5 McFarland turbidity standard. 10μl of this suspension was spread on vancomycin containing BHI agar plates. All the isolates were inoculated in duplicates on vancomycin agar screening plates with positive and negative control strains. Plates were incubated for 24 hours at 37° C in ambient air. After incubation results were recorded watchfully. Plates containing more than one colony or a thin film of growth or growth similar to positive control strain were taken as positive and isolates showing positive result on both inoculated plates were considered as confirmed VISA [22].

VISA isolates grown on 6μg vancomycin containing agar were further processed to recover VRSA by performing MIC of vancomycin by broth microdilution test [20]. All the steps were performed

according to CLSI approved standards and recommendations [23]. Detailed procedure of each step included in broth microdilution method is given below.

Vancomycin powder was aseptically weighed and dissolved in sterile distilled water to prepare a stock with the final concentration of 50mg/ml. After complete dissolution of powder, small volumes of the sterile stock solutions were dispensed into sterile cry vials and stored at -80^{o} C freezers until further use.

Microdilution test was carried out in 96 well round bottom trays. Trays were opened aseptically in safety cabinet and dispensed with Mannitol salt Broth (MSB) 100μl per well of the tray. Stock solution of vancomycin was thawed to prepare working solution with a concentration of 4.096 mg/ml. 100μl of this working solution was added in 1st well of each row and diluted two fold up to 11th well while 12th well was kept as growth control and help in detection of color change in the well and button formation.

Cultures of VISA isolated were revived on nutrient agar plates and pure colonies were suspended in freshly prepared sterile normal saline to prepare the inoculum by "Direct Colony Suspension Method". Turbidity was adjusted equivalent to 0.5 McFarland turbidity standards. This results in a suspension containing approximately 1x 10^{8} CFU/ml. This suspension was further diluted by 1:200 to yield required concentration of 5 x10^{5} CFU/ml. (Standard number of cells for Micro broth dilution test). Finally 100μl of this ultimate suspension was inoculated within 15 minutes in all wells of 96 well trays except 1st well which was kept as control for vancomycin. 1st well verify the sterility of vancomycin working solution and rule out the button formation due to any un-dissolved vancomycin contents. With the addition of 100μl inoculum, contents of each well were diluted two fold as each well contains 100μl broth used to dilute vancomycin. Microdilution trays were covered with the lids to prevent drying and incubated at 37^{o} C for 24 hours in ambient air.

The MIC is the lowest concentration of vancomycin that completely inhibits the growth of *S. aureus* in the microdilution wells. Since MSB was used in the wells as growth medium therefore the results interpretation was done easily by the color change of broth from pink to yellow. The well which show no color change and no button formation as compare to control well were recorded as positive and the concentration of vancomycin in this well was the end point or MIC value. All isolates which had shown the MIC value of $\geq$16μg/ml was considered as VRSA according to CLSI interpretation standards of vancomycin antibiotic. MIC calculation in broth dilution is shown in **Table 2**.

2.6 MIC and MBC Calculation of Antibiotic

Linezolid, Clindamycin and Moxifloxacin were tested in vitro against five VRSA isolates. MIC was performed using standard broth micro dilution test adapting similar procedure mentioned above for vancomycin MIC calculation, with the final inoculums of 5 x 10^{5} colony forming units (CFU)/ml in each well of the 96-well plate according to the CLSI guidelines and incubated at 37º C for 24 hours. MBC was determined by culturing 50µl of the suspension on MSA plates from the wells where no visible growth and color change was found. Plates were properly labeled with the well number and incubated for 24 hours at 37º C. After incubation note the minimum concentration showing no growth on inoculated plate. MBC was the minimum concentration of antibiotic which kill the bacteria within well, consequently reveals no growth on subsequent inoculation.

Results

3.1 Isolation and identification of S. aureus from Pus Samples

S. aureus were isolated from wound exudates of hospitalized patients and characterized by biochemical tests. Out of 200 samples, 110 isolates were presumably recognized as *S. aureus* on them being coagulase positive, which further were characterized upon their biochemical profile. Remaining isolates contain coagulase negative Staphylococcus (CONS) and based upon the absence of Mannitol fermentation character was considered as *Staphylococcus epidermidis.* Colony character, morphological features and biochemical profile of the *S. aureus* isolates are displayed in **Figure 1** shows the Mannitol fermentation character by the *S. aureus* culture.

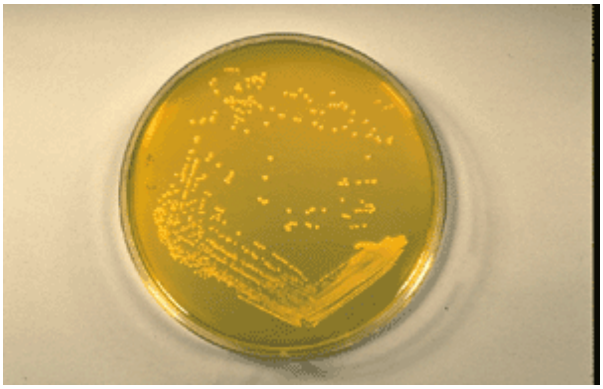

Figure 1: Yellow colonies of *S. aureus* on Mannitol Salt Agar

3.2 Prevalence of MRSA among S. aureus

MRSA were detected by their sensitivity to oxacillin and cefoxitin as performed according to the CLSI guidelines. Interpretive standards for disc diffusion breakpoints of oxacillin and cefoxitin antibiotics given by CLSI are explained in **Table 4** It was observed that out of 200 samples, 110 (55%) were coagulase positive *S. aureus* isolates, 51(25.5%) isolates were MRSA and remaining 56 (50.92%) isolates were MSSA and 3 (2.73%) isolates were methicillin intermediate *S. aureus.* Prevalence of MRSA was recorded. 18%. 38%, 22%, 24%, in Services Hospital, Mayo Hospital, Jinnah Hospital, Ganga Ram Hospital, respectively. Prevalence of MRSA and VRSA according to each hospital.

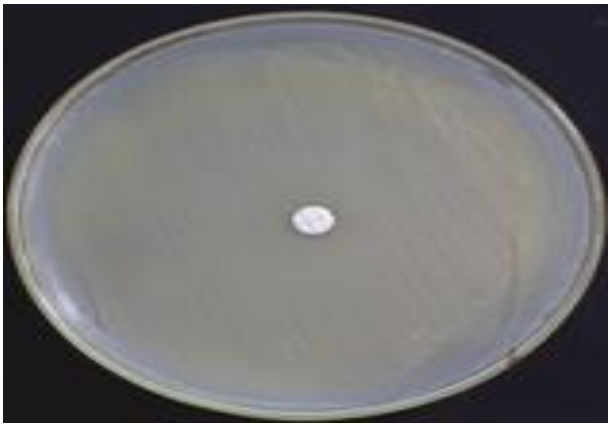

Figure:-2 Oxacillin disc showing zone less than ≤ 10mm

Table 2: Sketch of 96 Well Plate used for MIC Calculation in Broth Micro dilution Test

Well #	Isolate ID	1	2	3	4	5	6	7	8	9	10	11	12
1	A	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
2	B	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
3	C	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
4	D	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
5	E	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
6	F	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
7	G	2048 SC	1024	512	256	128	64	32	16	8	4	2	0
8	H	2048 SC	1024	512	256	128	64	32	16	8	4	2	0

Note: 2 Fold Serial Dilution of Antibiotic in µg/ml starting from well # 1 up to well #11. Well Seeded with Inoculum starting from well # 2 up to well # 12, **Key: GC:** Growth Control, **SC:** Sterility Control.

Table 3: Microscopic, Macroscopic and Biochemical Characteristics *S. aureus*

Bacterial specie	Gram stain	Colony characters on blood agar	Morphology	Growth on high salt conc.	Catalase test	Mannitol Fermentation	Coagulase test
S. aureus	Gram +ve	Large, Round golden to yellow colonies, often with hemolysis	Cocci arranged in clusters	+	+	+	+

Table 4: Prevalence of MRSA and VRSA in Various Hospitals of Lahore City

Name of Hospital	Sample #	*S. aureus* (%)	MRSA (%)	VISA (%)	VRSA (%)
Services Hospital	50	20 (40%)	9 (18%)	3 (6%)	1 (2%)
Mayo Hospital	50	34 (68%)	19 (38%)	10 (20%)	2 (4%)
Jinnah Hospital	50	25 (50%)	11 (22%)	2 (4%)	0 (0%)
Ganga Ram Hospital	50	31 (62%)	12 (24%)	7 (14%)	2 (4%)
Total	200	110 (55%)	51 (25.5%)	22 (11%)	5 (2.5%)

3.3 Total Recovery of VISA by Agar Screening Test

Vancomycin agar screening test of 51 MRSA isolates yielded 22 VISA which were resistant to 6µg/ml and showed growth on vancomycin screening agar plates. Recovery rate of VISA was 11%of the total MRSA isolates, while in each hospital the prevalence was recorded as 6%, 20%, 4%, 14%, in Services Hospital, Mayo Hospital, Jinnah Hospital, Ganga Ram Hospital, respectively. These 22 VISA were subjected to MIC calculations for the recovery of VRSA isolates. 22 VISA isolates were tested for MIC measurement and out of these 5 showed MIC value ≥ 16 µg/ml and were considered as VRSA.

Table 5: Categorization of *S. aureus* Isolates into VISA and VRSA Based upon Their Minimum Inhibitory Concentration Values

Sr #	Isolate ID	MIC value (µg/ml)	Result
1	2	8	VISA
2	5	8	VISA
3	6	8	VISA
4	8	8	VISA
5	11	64	VRSA
6	13	8	VISA
7	16	8	VISA
8	21	32	VRSA
9	22	8	VISA
10	25	32	VRSA
11	26	64	VRSA
12	29	8	VISA
13	33	8	VISA
14	37	4	VISA
15	38	8	VISA
16	42	8	VISA
17	43	8	VISA
18	45	16	VRSA
19	46	8	VISA
20	48	8	VISA
21	49	8	VISA
22	51	8	VISA

Note: VISA= Vancomycin intermediate S*taphylococcus aureus,* VRSA= Vancomycin-resistant *Staphylococcus aureus*

3.4 MIC Calculations

all 22 VISA isolates were tested for MIC measurement and out of these 5 (2.5%) showed to have MIC value ≥ 16 µg/ml and were considered as VRSA. The recovery rate of VRSA among MRSA strains from Services Hospital, Mayo Hospital, Jinnah Hospital and Ganga Ram Hospital was 1%, 2%, 0% and 2%, respectively.

Linezolid (LZD), Moxifloxacin (MFX) and Clindamycin (CD) were tested for susceptibility against five VRSA isolates by broth micro dilution method. It was observed that all 5 (100%) isolates were susceptible to Linezolid and Clindamycin antibiotic, while 4(80%) out of 5 isolates were susceptible to Moxifloxacin and only one isolate showed resistance to this particular antibiotic. MIC and MBC values are written in **Table 6.**

Discussion

In near past an obvious increase in the number of cases of hospital acquired MRSA infections have been recorded from different parts of the world. According to general surveillance, there are wide variations in the prevalence of MRSA cases from time to time in various areas, hospitals and health care centers around the year [24].

Table 6: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Various Antibiotics against VRSA Isolates

Sr. No	Isolate ID	MIC value in µg/ml			MBC value in µg/ml			Result		
		LZD	MFX	CD	LZD	MFX	CD	LZD	MFX	CD
VRSA 1	11	2	0.25	0.5	8	1	2	S	S	S
VRSA 2	21	2	0.25	0.25	8	1	0.5	S	S	S
VRSA 3	25	4	8	0.5	16	8	2	S	R	S
VRSA 4	26	2	0.25	0.25	8	0.5	0.5	S	S	S
VRSA 5	45	2	0.5	0.25	8	1	1	S	S	S

Note: Key: LZD: Linezolid, MFX: Moxifloxacin, CD: Clindamycin

In current study similar observation were reflected, where prevalence of MRSA is different in different hospitals located in Lahore city of Pakistan. *S. aureus* is becoming the most threatening pathogen among hospital setups and its prevalence is increasing globally day by day. We recovered *S. aureus* from 55% of the pus samples collected from a variety of wounds of hospitalized patients which is in line with a previous finding of Pakistan in which 72% of total isolated organism from skin infections were found to be *S. aureus* [25].

Data of present study shows that prevalence of MRSA within *S. aureus* isolates recovered from Lahore city is 25.5%. The frequency of MRSA reported from different parts of the world shows that it is highly variable among countries. Past data also clearly specified a greatly inconsistent and a rising trend of MRSA occurrence. Surveillance made in Europe on prevalence of MRSA infection reported it up to 65% [26]. In past prevalence was reported as 37.2% in Iran [27], while 42.5% and 54.7% of the isolates were recorded as MRSA by a study in United Kingdom and Ireland, respectively [28]. A report from USA showed 59% isolates among *S. aureus* .were resistant to methicillin [29]. A study from India reported high frequency of MRSA in hospital set up and its rate was reported as 71% from wounds/pus [30]. These figures highlight the fact that frequency of MRSA is variable in different parts of the world. Pakistan has also been observing an uneven trend in MRSA infections. Prevalence of MRSA has been reported to be up to 42 % on an average in Pakistan [31]. According to a recent multi-center study, it is estimated that frequency of MRSA in Pakistan varies

between 2-61 %, with highest frequency seen in major cities of country [19]. Different studies conducted in different localities and regions at various time showed inconsistent recovery rates with a substantial factor of increasing incidence over time. A study done in Sargodha during 1999 showed 23 % MRSA in various clinical samples [32]. In 2001, 38.5% MRSA were isolated from clinical samples in Mayo hospital Lahore [33]. Another study done in 2005 in Karachi on clinical samples showed that 43% isolates were MRSA out of 190 *S. aureus* strains [34]. Research conducted in 2008 recovered 40 % MRSA from pus samples in hospital of Lahore, whereas in present study recovery rate of MRSA from pus samples is 46.36% in Lahore. This high frequency and varied prevalence of MRSA in different hospitals is due to self-medication and constant ill-advised use of antimicrobial agents. This may be either due to specifically high frequency of MRSA among the patients admitted in these hospitals or due to different control measures, variable prevention strategies, dissimilar environmental conditions and distinct screening programs. Nevertheless some studies from different countries reported very low prevalence of MRSA isolates. A report from Sweden showed that only 2.1 % isolates were MRSA, similarly in Netherland it is even more or less (<1%) [35]. These figures are much lower than that in the present study. Low frequency observed in these countries may be due to controlled use of antibiotics combined with strict screening programs for MRSA.

Resistance to vancomycin has led to global concerns owing to the fact that vancomycin is well thought-out as the last successful drug to treat the Staphylococcus infections. So far the intermediate vancomycin resistance has been reported in many countries including Pakistan. Current study indicates the higher rate of VISA occurrence 11% in different hospitals of Lahore. An earlier study in Pakistan reported the presence of 13 % VISA strains [36].

Another finding claimed that vancomycin established 38% intermediate resistance among MRSA isolates [37]. Likewise different rate of VISA isolates has been reported from different parts of the world. Up to 6 % intermediate resistance to vancomycin was reported by a study done in 2007 [38], and 7.5 % was recorded in 2008 [39]. A study done in Dhaka Bangladesh on 122 clinical isolates collected from different hospitals, clinics and diagnostic centers between August 2010 and July 2011. Study did not detect any VRSA but they reported an alarming outcome that only 6.56 % *S. aureus* showed sensitivity to vancomycin and 93.44 % were intermediate [40]. Some strains of vancomycin intermediate *S. aureus* was also recovered from clinical samples in India [41].

Current work illustrates 2.5% recovery rate of VRSA isolates with a significant variation among four hospitals selected in the study. Occurrence of VRSA varies from one hospital to other which is 1% in Services Hospital, 2% in Mayo Hospital, 0% in Jinnah Hospital and 2% in Ganga Ram Hospital. These isolates belong to the MRSA phenotype as proved by their susceptibility testing. These results not only show unique percentage in hospitals but also reveal a distinctive outcome in contrast with literature existed. More recently VRSA strains have been reported from Jordan, Middle East [42]. Emergence of heterogeneous vancomycin resistant *S. aureus* strains have also been reported from several countries of Asia [43]. Some other researchers observed 4 and 1.9 % prevalence of VRSA in clinical samples during recent years while high incidence (79.6%) of MRSA was reported from the same specimen [44, 45]. Emergence of VISA and VRSA has also been observed in different parts of India. A study found two MRSA strains which were showing vancomycin resistance with MIC value of 64 µg/ml and 32µg/ml. These VRSA strains have been isolated from the pus of two different hospitalized patients. They also reported the recovery of six VISA strains isolated from the pus specimen patients admitted in post-operative ward [46].

A study conducted in Karachi, reported the emergence of VISA and VRSA from Pakistan. VRSA isolate having MIC 32µg/ml was recovered from the pus sample of 63 year old male and this isolate was found to be resistant to several other antibiotics as well [47].

Development of antibiotics resistance in the organisms is thought to be because of unjustified, irrational and irregular use of antibiotics by the human population. Most specifically the emergence of VRSA/VISA may be due to building of selective pressure of vancomycin. Uneven frequencies of VRSA among hospitals can be due to variable measures adopted for the control of infection and unsystematic use of antimicrobial agents. High vancomycin prescription rate could also be linked with the development of such antibiotics resistance, however further research is needed to completely explore the phenomenon. Due to the increasing appearance of VRSA strains, there is a need to find out a good alternative of vancomycin. Linezolid, an Oxazolidinones, is being used for hospital acquired

and community acquired complicated skin infection, post-operative and surgical infections cause by MRSA. Linezolid demonstrated broad activity against all of the Gram-positive bacteria tested, including Staphylococci, Enterococci and Streptococci [48]. Linezolid has been proved to be an ideal alternative of vancomycin and isolates which were found resistant to vancomycin remained susceptible to Linezolid. Superiority of Linezolid reported by different studies from USA and Japan as oral administration of Linezolid can reduce patient's stay in hospital and better choice than Vancomycin for multidrug resistant strains [49, 50].

In present experiment Linezolid was found effective *in vitro* against all five VRSA isolates and it demonstrated 100% susceptibility with the MIC ranges from 2-4 µg/ml. Similar results have been reported in the past where MIC of Linezolid was 2-4µg/ml against *S. aureus* with 100% susceptibility [51]. Furthermore 100% effectiveness of Linezolid has been proved by multiple researches in the past [52, 53, 19].

However, a finding demonstrated relatively higher MIC values of Linezolid (0.4-5 µg/ml) in comparison to the present study [54]. Clindamycin, a lincosamide antibiotic, was also found effective against VRSA isolates with an average MIC value of 0.25µg/ml. Similar results are shown in report with 100 % susceptibility of Clindamycin by broth microdilution test with the MIC of ≤ 0.5 µg/ml against *S. aureus* [55].

Moxifloxacin belong to 4th generation Quinolones have greater activity against Gram-positive bacteria and has excellent *in vitro* activity against *S. aureus.* Results of current study revealed an excellent *in vitro* activity of Moxifloxacin against VRSA with MIC values between 0.25-8 µg/ml. Analogous results were depicted by a study where multidrug resistant isolates were tested against Moxifloxacin and found susceptible with the MIC up to 0.12µg/ml [56].

In new Jersy Moxifloxacin was considered effective antibiotics against most of the tested bacterial strains with MIC values ranging between 0.5-2µg/ml [57]. Good activity of Moxifloxacin was reported by Farrell [54].

Conclusion

Emergence of MRSA and VRSA infections is a growing problem in our setup. Therefore there is need for early recognition of these isolates in hospitals. Implementation of strict aseptic technique and suitable antimicrobial policy may reduce the spread of MRSA in our community. This study exposed the presence of MRSA and VRSA in different hospitals. Consequently there should be an immediate response from the concerned authorities to check further emergence and spreading of these notorious VRSA strains. A strict regulation on irrational antibiotic usage might be an appropriate and effective approach in this direction. Moreover nationwide surveillance program should be carried out to map the Methicillin and Vancomycin susceptibility pattern in our community. Rational use of Methicillin and Vancomycin and the approval of adequate control measures of infection are necessary to avoid the surfacing of glycopeptides resistant microorganisms and their distribution within hospitals.

The importance of regular surveillance of hospital associated infections including monitoring antibiotic sensitivity pattern and strict drug policy for antibiotics used within and outside the hospital environments. Moreover, *in-vitro* susceptibility testing of every isolate of MRSA in the clinical laboratories may be helpful for reducing the incidence of these infections. The failure of antibiotics diverts the focus of modern science towards the discovery and application of new alternative antimicrobial agents.

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