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Molecular Identification and antibiotic susceptibility pattern of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)

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Abstract

Community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) is an emerging bacterium associated with a much higher incidence of clinical infection worldwide than any other strains of *S. aureus*. The misappropriation of non-prescribing drugs amongst university students in Pakistan has become a severe problem. The present study aims to evaluate the nasal carriage rates of *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) in university student and described the history of associated risk factors. Among the 350 samples, 300 (85.71%) were *S. aureus* positive and 50 (14.28%) were other isolates. The positive individual includes 295 (98.33%) males and 5 (1.66%) females. A total of 16 (5.3%) *S. aureus* out of the total 300 were resistant to cefoxitin (MRSA) and 284 (94.66%) isolates were MSSA (Methicillin Susceptible *Staphylococcus aureus*). The strains were confirmed by the amplification of *nuc* gene analysis as *S. aureus* and the MRSA was confirmed with *mec*A gene analysis. All the samples were catalase positive, whereas 72.33% was coagulase positive and 27.66% were found as coagulase negative. Among the coagulase positive 7.37% were resistant to methicillin. The antibiotic resistance patterns showed no MRSA isolates to be resistant to Vancomycin and Linzolids. In most cases the MRSA was less susceptible to other antibiotics. It was concluded that Vancomycin and Linzolid can be used as drug of choice as no MRSA was found resistant to them. Furthermore, molecular PCR diagnosis is suggested as rapid and sensitive technique for the identification of MRSA isolates based on their *mec*A gene analysis.

Introduction

Staphylococcus aureus is a facultative anaerobic Gram-positive bacterium which is present as normal flora in the nose and skin. Generally, 20% of human populations are carriers of *S. aureus* [1]. Methicillin-resistant *S. aureus* (MRSA) can be defined as a strain of *S. aureus* that has become resistant to beta-lactam antibiotics which include the cephalosporins and the penicillins (methicillin, dicloxacillin, nafcillin, oxacillin) etc. Community-associated methicillin-resistant *S. aureus* (CA-MRSA) is an emerging cause of infection worldwide. *S. aureus* causes various disorders for examples scalded skin syndrome, pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, abscess, pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, chest pain, bacteremia and sepsis.

The utmost therapeutic advantage afforded by antibiotics is being threatened by the emergence of increasing resistant strains of microbes. Infections caused by *S. aureus* are generally treated by β-lactam antibiotics and are reported to have a good response [2]. However, development of methicillin resistance amongst *S. aureus* isolates resulting MRSA, left very little choices for treatment. The mechanism that confers protection to bacteria against antibiotics is the production of alternative target. The common example of this mechanism is probably the production of alternative penicillin binding protein (PBP2a), in addition to the "normal" penicillin binding proteins production by methicillin resistant *S. aureus* (MRSA). The synthesis of this abnormal protein encoded by the *mec*A gene, and as PBP2a is not inhibited by antibiotics such as flucloxacillin the cell continues to synthesize peptidoglycan and hence has a structurally sound cell wall [3].

In Pakistan most of the peoples use antibiotics without indications, or an inadequate dose or for an inappropriate duration [4]. These all malpractices lead to resistance of *S. aureus* in Pakistani population. Moreover, MRSA is not limited to hospital associated infection but it also made its way in community. However, studies of the carriage rates of CA-MRSA strains in Pakistan population are lacking. The objective of the present study was to evaluate the carriage rates of *S. aureus* and methicillin resistant *S. aureus* (MRSA) in Pakistani university students and describe risk factors associated with their carriage. Further S. *aureus* (MRSA) was also molecularly identified along with conventional microbiological identification techniques.

Materials and methods

Samples collections

This study was conducted on the prevalence of Methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* (MSSA) strains in the nasal carriage of university students. Samples were collected from the students of Institute of Pharmaceutical Sciences (IPS, Pharmacy department), Kohat University of Science and Technology (KUST), Kohat, both male and female students were included in the study. The students were interviewed for previous history of antibiotic usage. Sterilized cotton swab was used for sample collection.

Culturing of isolates

Mannitol salt agar (MSA) medium containing 5.0 g/L casein, 5.0 g/L enzymatic digest of animal tissue, 1 g/L beef extract, 10.0 g/L D-mannitol, 75.0 g/L NaCl, 0.025 g/L phenol red, 15.0 g/L agar, pH 7.4 ± 0.2) was used for culturing and subsequent isolation of *S. aureus*. Sample was taken aseptically from the mucosa of individuals by impression the sterilized cotton swab inside the nostrils. Swabs were transferred to a screw-capped test tube containing 10 ml of sterile maintenance medium (0.85% NaCl and 0.1% peptone). The tubes were transported to lab at 4ºC and processed for further analysis within two hours. The swab was spread on the culture plates and incubated at 37˚C for 24 hours. Golden-yellow color *S. aureus* colonies were isolated and streaked on fresh MSA plates and incubated again at 37˚C for a period of 24 hours to obtained pure culture.

Phenotypic identification of Staphylococcus aureus

The isolates were presumptively identified as *S. aureus* by means of several simple procedures.

Gram staining

The Gram staining was carried out for the identification of the isolates as gram positive *S. aureus* by the standard procedure described by Lancette [5].

Catalase Test

The Gram-positive strains were further subjected for the identification of catalase positive *Staphylococci.*

The test was performed by simply putting few colonies from the culture in 2-3 mL of 3% H_2O_2 solution, and production of bubble was noticed.

Coagulase Test

the isolates were further tested for coagulase production. Few colonies from the culture were emulsified in a drop of distilled water on a slide and loopful plasma was added into it for the subsequent clumping of the organism within 10 second of the reaction.

Susceptibility patterns of the isolates

Antibacterial susceptibility of MRSA and MSSA isolates were determined according to the standard disk diffusion [6]. Mueller-Hinton agar (CM337-OXOID) medium was used for the growth of the isolates. Susceptibilities of MRSA and MSSA to the panel of antibiotics including Cefoxitin, Fusidic acid, Amoxycillin+ clavulanic acid, Sulphamethoxazole +Trimethoprim, Nalidixic acid, Cefixime, Amoxycillin, Oxacillin, Erythromycin, Vancomycin, Gentamycin, Pipedemic acid and Linzolid.

Inoculum preparation

Inoculum was preparation in sterile Tryptic Soya broth (CM129-OXOID) according to manufacturer's instruction in 5 mL screw capped test tubes. The inoculated was placed in incubator for 2-6 hours at 35ºC. The turbidity of broth cultures was adjusted according to 0.5 MacFarland standards by adding sterile saline against a white background with contrasting black lines.

Disk diffusion assay

A sterile cotton swab was saturated by dipping into standardized bacterial suspension. Inoculum was spread evenly over the entire surface of Mueller–Hinton agar plates by swabbing back and forth across the agar in three directions to give uniform colonies on entire surface. The plates were allowed to dry before applying discs, and within 15 minutes, discs of given potencies (**Table 1**) were applied on inoculated plates with the help of the forceps. Then plates were placed in incubator at 35ºC for 18 hours in an inverted position. After 18 hours of incubation, plates were examined and zones of inhibition were measured.

Detection of nuc and mecA genes by PCR amplification

The detection of MRSA strains was performed with the DNA amplification by standard PCR assay using Master cycler gradient (Eppendorf, Germany). DNA was extracted from *S. aureus* colonies grown overnight on blood agar plates using DNA Extraction Kit (Bioneer Co., Korea) in accordance with manufacturer's instructions.
For the amplification of the *nuc* gene primers (forward:5GCGATTGATGGTGATACGGTT3.
reverse:5AGCCAAGCCTTGACGAACTAAAGC3), and *mec*A gene primers
(forward: 5AAAATCGATGGTAAAGGTTGGC3;
reverse:5AGTTCTGCAGTACCGGATTTGC3)
were used [7]. The amplification was done in a reaction mixture of 25 µl containing 5µl 5X green, 4µl $MgCl_2$, 0.5µl PCR Nucleotide Mix, 0.5µl Taq DNA polymerase, 2.5µl forward primer, 2.5µl reverse primer, 5µl Template/ sample and 5µl PCR water. PCR amplification was performed at annealing temperatures calculated from the GC content of the primers which was 55 ºC. The PCR reaction was programmed as; initial 5 minutes' denaturation of template DNA at 95ºC, followed by 34 cycles of 95ºC for 1 min, 55ºC for 1min, and 72ºC for 1-2 minutes and final extension at 72ºC for 10 min [8]. The final fragment size for *nuc* gene is 270 bp whereas the product size for *mec*A gene is 533 bp. The amplified product was visualized by electrophoresis in a 2% Agarose gel electrophoresis stained with ethidium bromide.

Results

A total of 350 nasal swabs samples were collected from the university students. Both males and females' volunteers were included, in the study. Specimens were screened for *S. aureus* and MRSA using the standard media mannitol salt agar (MSA). Among the 350 samples, 300 (85.71%), (295 males and 5 females) were found *S. aureus* positive and 50 (14.28%) were other isolates (**Fig. 1A**). Figure 1B shows the percentage of other isolates in different group ages ranging from 20 to 25 years. A total of 16 (5.3%) *S. aureus* out of the total 300 were resistant to cefoxitin (MRSA) and 284 (94.66%) isolates were MSSA (**Fig. 2A**). All the MRSA positive students were males.
At the time of sample collection, history about past hospitalization and antibiotic usage was recorded

Table 1: Details of antibiotics used for the determination of susceptibility pattern

Sr. #	Antimicrobial Agent	Antibiotic Group	Code	Disc Potency µg
1	Cefoxitine	Cephalosporin	(FOX)	30
2	Fusidic acid	protein synthesis inhibitor	(F.A)	10
3	Amoxycillin+ clavulanic acid	Penicillin	(AMC)	30
4	Sulphamethoxazole +Trimethoprim	Sulpha Drug	(SXT)	25
5	Nalidixic acid	Quinolone	(N.A)	30
6	Cefixime	Cephalosporin	(CFM)	5
7	Amoxycillin	Penicillin	(AML)	25
8	Oxacillin	Penicillin	(OX)	1
9	Erythromycin	Macrolides	(E)	15
10	Vancomycin	Glycopeptide antibiotic	(VA)	30
11	Gentamycin	Aminoglycosides	(CN)	10
12	Pipedemic acid	Quinolone	(PIP)	20
13	Linzolid	Oxazolidinone	(LZD)	30

(**Table 2**). Among these 56.25% (9 out of 16) had current disease (that is, throat infection, common cold, URTI or fever), current use of antibiotics was found in 31.25% (5 out of 16), history of antibiotics use was present in 37.5% (6 out of 16) and hospitalization record was found to be 25% (4 out of 16) among the volunteers.

The samples were also screened for catalase and Coagulase activities. All the 300 (100%) samples were catalase positive, while 217 (72.33%) coagulase positive and 83 (27.66%) were found as coagulase negative (**Fig. 2B**). Out of 217 coagulases positive 16 (7.37%) were resistant to methicillin.

Table 2: Hospitalization and antibiotic usage history of MRSA positive samples

Sr. #	Sample No	Gender	Age (years)	Current disease	Current use of antibiotics	History of antibiotics Use (6months back)	Hospitilization record
1	39	Male	22	Throat infection	Erythrocin	Nil	Yes (For appendectomy)
2	56	Male	21	Nil	Nil	Amoxycillin	Nil
3	77	Male	20	Common cold	amoxycillin	Nil	Yes (kidney stone surgery)
4	86	Male	23	URTI	Azithromicin	Nil	Nil
5	92	Male	25	Nil	Nil	Yes	Yes (for tonsil removal)
6	99	Male	21	Chest infection	Nil	Nil	Nil
7	114	Male	21	Common cold	amoxycillin	Nil	Nil
8	130	Male	22	Nil	Nil	Doxycyclin	Nil
9	147	Male	21	Chest infection	Erythromicin	Nil	Nil
10	179	Male	23	Nil	Nil	Nil	Nil
11	208	Male	21	Nil	Nil	Amoxycillin	Nil
12	237	Male	24	Nil	Nil	Nil	Yes (accident)
13	246	Male	22	Common cold	Nil	Nil	Nil
14	265	Male	23	Fever	Nil	Nil	Nil
15	269	Male	20	Cough	Nil	Ampicillin	Nil
16	294	Male	21	Nil	Nil	Levofloxacin	Nil

Resistance and Susceptibility pattern of the MRSA isolates to various antibiotics

The antibiotic resistance patterns of MRSA isolated from carrier screening samples was found to be variable. The disc diffusion assay shows that the resistance was 100% to Cefoxitin, 87.5% to Oxacillin 75% to Erythromycin, 56.25% to Sulphamethoxazole + Trimethoprim,43.75% to Amoxycillin, 43.75% to Cefixime, 37.75% to Gentamycin, 31.25% to Fusidic acid,31.25% to Amoxycillin+ clavulanic acid, 25% to Pipedemic acid, 18.75% to Nalidixic acid,87.5% to Oxacillin and no resistance was found to Vancomycin and Linzolid.

Amplification of nuc and mecA genes

For the identification of *S. aureus* nuc gene DNA analysis was performed. The DNA was extracted for all the 16 resistance isolates (**Fig. 3**). The strains were confirmed by the PCR amplification of *nuc* gene. The amplified product showed a fragment size of about 270 bp on 2% Agarose gel which is specific to *S. aureus* (**Fig. 4**).

For the confirmation of resistance, *mec*A gene analysis was performed. The *mec*A gene is responsible for methicillin resistance in *S. aureus*. The amplification of533bp PCR product of *mec*A gene showed clear bands and confirming that all the isolates were MRSA positive. The result of the PCR revealed 16/16 isolates demonstrating resistance to methicillin and expression of *mec*A gene (**Fig. 5**).

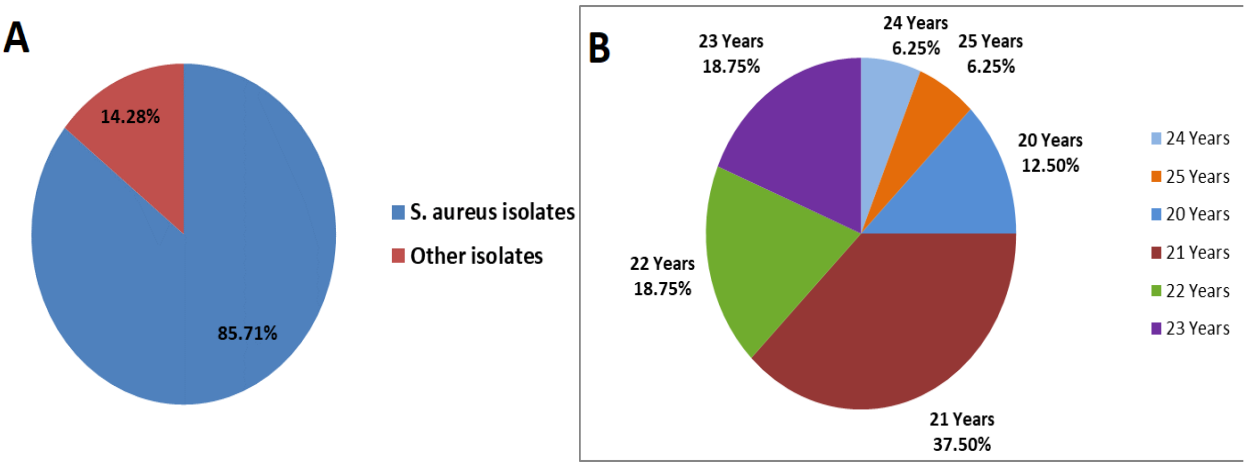

Fig.1: A. Shows prevalence of *S. aureus* in university students while **B** represents age wise distribution of MRSA positive individuals.

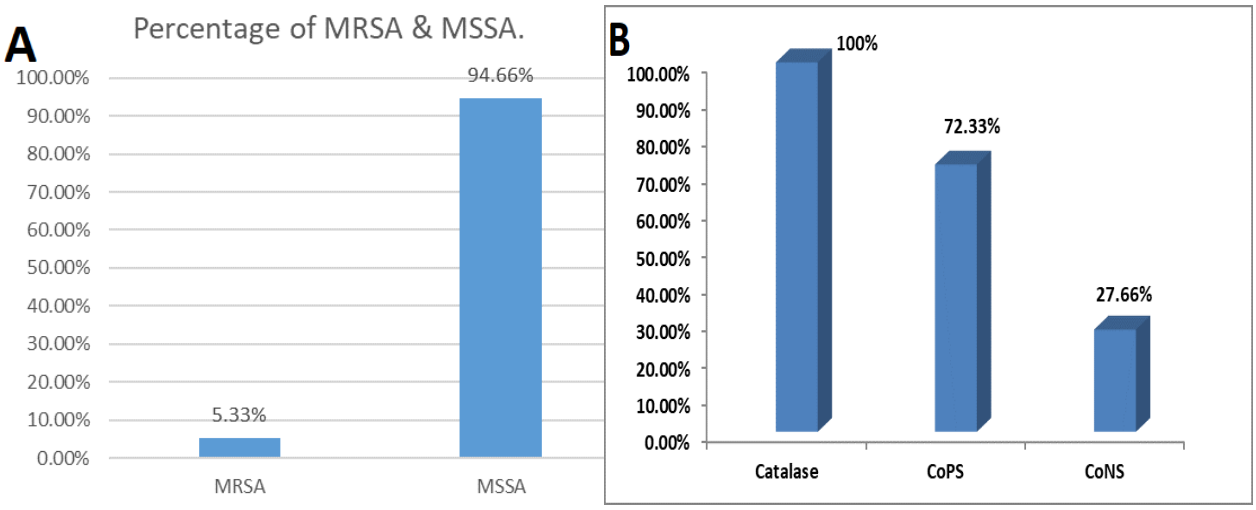

Fig. 2. **A** shows prevalence of MRSA and MSSA in university students While Fig: **B** represents percentage of catalase and coagulase positive and negative isolates among the S. aureus positive samples. CoPS: Coagulase Positive, CoNS: Coagulase Negative.

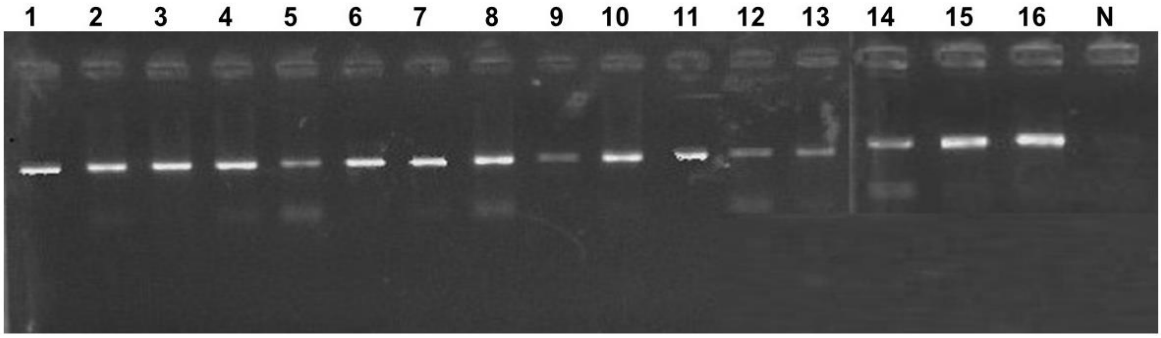

Fig. 3: Agarose gel electrophoresis for DNA extraction of 16 samples.
N: Negative control, Lane 1.sample 247, 2. Sample 237, 3.sample 208, 4. Sample 179, 5. Sample 147 , 6.sample 130, 7. Sample 114, 8. Sample 99, 9. Sample 92, 10. Sample 86, 11. Sample 77, 12. Sample 56, 13. Sample 39, Lane 14. Sample 294, 15. Sample 269, 16. Sample 265.

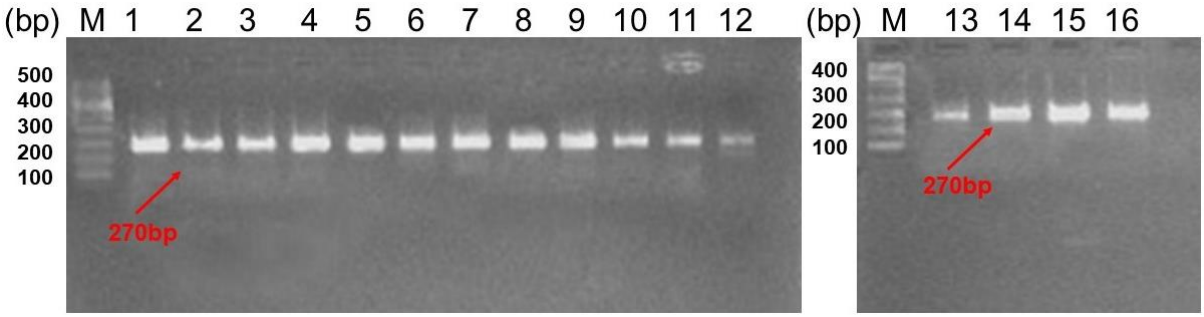

Fig:4: Agarose gel electrophoresis of PCR amplifiednuc gene for the confirmtion of S. aureus.
M. Marker, Lane 1.sample 247, 2. Sample 237, 3.sample 208, 4. Sample 179, 5. Sample 147 , 6.sample 130, 7. Sample 114, 8. Sample 99, 9. Sample 92, 10. Sample 86, 11. Sample 77, 12. Sample 56, Lane 13. Sample 39,14. Sample 294, 15. Sample 269, 16. Sample 265.

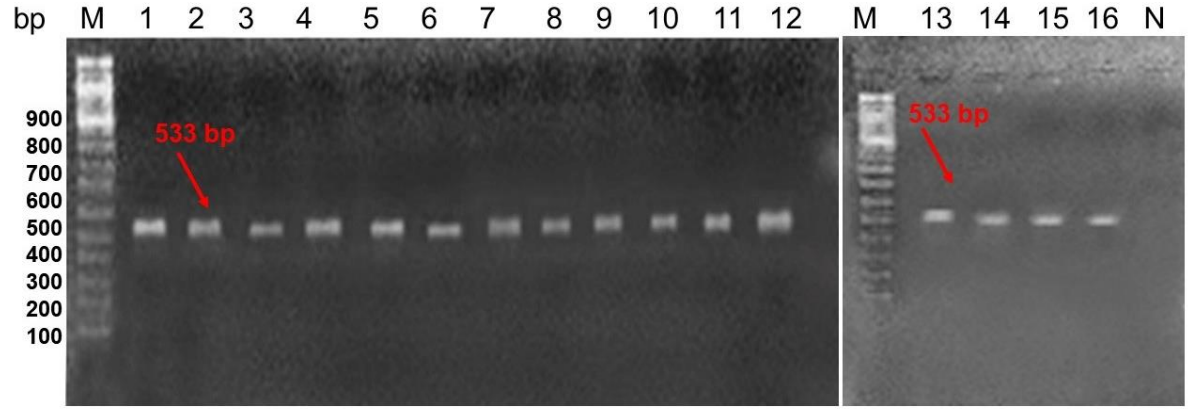

Fig:5: Agarose gel electrophoresis of PCR amplified mecA gene for the confirmation of metticiline resistance *S. aureus*.
M. Marker, Lane 1. Sample 247, 2. Sample 237, 3. Sample 208, 4. Sample 179, 5. Sample 147 , 6. Sample 130, 7. Sample 114, 8. Sample 99, 9. Sample 92, 10. Sample 86, 11. Sample 77, 12. Sample 56, 13. Sample 39,14. Sample 294, 15. Sample 269, 16. Sample 265, N: Negative control.

Discussion

S. aureus is recognized as an important pathogen in human diseases. Carriage of *S. aureus* in the nose appears to play an important role in the epidemiology and pathogenesis of infections [1]. Methicillin resistant *S. aureus* (MRSA) is a major pathogen causing significant morbidity and mortality in human [9]. A high regional variation occurs in the prevalence rate of MRSA. This is indicated in different studies conducted in Croatia (22%), Pakistan (83%), Taiwan (75- 84%), India (31-33%), and Malaysia (40%) ([10,11,12,13,14,15,16,17,18]. In USA there was progressive development of resistance to methicillin from 5% (1981) to 52 % (2005). In contrast, the prevalence rate of MRSA was found to be low in France (6%), Ireland (5%) and United Kingdom (2%). The important reservoirs of MRSA in hospitals/institutions are infected or colonized patients and transient hand carriage is the predominant mode for patient-to-patient transmission. Nasal carriage of *S. aureus* has been identified as a major risk factor for community acquired and hospital infections, affecting 20% of the population [17].

According to National Health and Nutrition Examination Survey (NHANES, 2004), the prevalence of colonization with *S. aureus* and MRSA in the civilian non-institutionalized population of the United States was 28.6% and 1.5% respectively. The prevalence of *S. aureus* carriage rate in Texas university student population was found 29.6% for *S. aureus* and 7.4% MRSA carriage [19]. In the present study, *S. aureus* carriage rate was 85.71% and MRSA carriage rate was 5.3% in Pakistani university student. This indicates a much higher prevalence of MRSA in university student. However, our current results are in close consistency with that of 5.6% [20], 5.8 % [21] and 7.4% [19] reported in different countries. A much lower prevalence1.2% [22], 1.8% [23], 1.6% [24], 1.5% [25] and 1.4% [26] has also been reported in Canada, USA, South Carolina, and Pakistan as well. Higher prevalence 9% [27], 11.1% and 21% [28] was also reported in the general population. However, the high prevalence rate in Pakistani population is due the use of non-prescribed antibiotics by the university students (self-medication), non-professional behavior of the doctors, mutual eating habit and transient nature of the student population.

Screening for catalase and Coagulase activities showed that all the 300 (100%) samples were catalase positive, whereas 217 (72.33%) coagulase positive and 83 (27.66%) were found as coagulase negative as shown in **Fig. 4**. Out of 217 coagulases positive 16 (7.37%) were resistant to methicillin.

The antibiotic resistance patterns of MRSA isolated from carrier screening samples was found to be variable. All the sixteen MRSA isolates screened were resistant to Cefoxitine, 87.5% to Oxacillin, 75% to Erythromycin, while Vancomycin and Linzolid were showing sensitivity for all. Most of the carriers of MRSA were found in persons who didn't use antibiotics as compared to those who used antibiotics in past or currently using antibiotics. It was found that the ages of those having nasal carriage of *S. aureus* fell mostly between the age group of 20-24 years. There was 37.5% *S. aureus* in the age group of 21. Similar results were reported by Prakash et al., in India [29]. Similar to our study, lower percentage of resistance was observed in lower age group students in Taiwan [30] and Turkey [31].

The *mec*A gene is a bacterial gene. The most known carrier of the *mec*A gene is the bacterium known as MRSA. The *mec*A gene allows a bacterium to be resistant to antibiotics such as methicillin, penicillin, erythromycin, tetracyclin and other penicillin-like antibiotics. Methicillin resistance is either due to expression of *mec*A gene or the synthesis of methicillinase or due to both [32]. The *mec*A gene does not allow the ring like structure of penicillin-like antibiotics to attack the enzymes that help the cell wall of the bacterium (transpeptidases) to be protected, and hence the bacteria are allowed to replicate as normal [33]. In the present study, PCR was used to amplify both the *S. aureus* specific sequence *(nuc*) gene and *mec*A gene with the amplicon size of 270 and 533 bp respectively using primers specific for the gene sequence. PCR amplification of all the isolates expressed *S. aureus* specific sequence (*nuc*) 270 bp in their PCR products [34], which confirmed the assumption that all the strains were *S. aureus*. It was also confirmed that all the isolates are resistance to methicillin and express *mec*A gene.

Conclusion

From the present study it was concluded that the degree of resistance or sensitivity of MRSA towards commonly used antibiotics is different, and the prevalence of MRSA in the university student is lower as compared to health care community. None of the MRSA isolates was found to be resistant to Vancomycin and Linzolid. Therefore, it is suggested that when vancomycin is considered for treatment,

then the *in vitro* susceptibility testing of every isolate of MRSA in the clinical laboratories is require. It is also suggested that molecular identification of MRSA (*mec*A gene) by PCR technique is a rapid, authentic and precise technique so it can be used for the rapid and accurate diagnosis of MRSA strain of *S. aureus* in diagnostic labs.

Conflict of interest

The authors declare no conflict of interest.

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