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Study of the genetic traits associated with antibiotic resistance in *Staphylococcus aureus* isolated from skin wards of Khyber Pakhtunkhwa, Pakistan

Saeed Ullah Khattak[1], Nafees Bacha[1], Ghosia Lutfullah[1], Jehan Bakht[2], Sajid Ali[3], Johar Ali[4], Abid Ali Khan[5*]

[1]*Center of Biotechnology and Microbiology, University of Peshawar, 25120, KPK, Pakistan*

[2]*Institute of Biotechnology and Genetic Engineering, The University of Agriculture, Peshawar, 25120, KPK, Pakistan*

[3]*Department of Chemistry, Bacha Khan University, Charsadda, KPK, Pakistan*

[4]*Alvi-armani, 2680 Matheson Blvd. East, Suite 102, Mississauga, ON L4W 0A5, Canada*

[5]*Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad Campus, 22060, KPK, Pakistan*

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ABSTRACT

Objective: To investigate the prevalence of *Staphylococcus aureus* (*S. aureus*) isolated from skin wards of the hospitals of Khyber Pakhtunkhwa, its resistance against various commonly and commercially available antibiotics, as well as different genetic traits of resistance and their correlations with the phenotypic visible resistance.

Methods: In the present study a simple PCR technique were used to investigate the genetic traits of resistance in *S. aureus* isolated from skin wards of two major hospitals of Khyber Pakhtunkhwa, Pakistan. A total of 100 samples were collected from both the male and female, of which 50 were from patient's site of infection and 50 from ward environment.

Results: These results demonstrated that the total prevalence of *S. aureus* both in ward as well as in patients was 48%. The *S. aureus* prevalence was the highest in female patients (50%) followed by ward environment (29%) and then male patients (21%). The antibiotic sensitivity tests revealed that the highest (91.6% isolates) sensitivity was shown to imipenem. However, the highest resistance was found to be against penicillin (100% isolates) followed by cefotaxime (75% isolates). In addition, only 29% of the isolates were found to be resistant to methicillin. PCR technique based on the previously designed primers targeting different genetic traits of resistance revealed that 13 out of the 14 isolates resistant to methicillin were positive for *mecA* gene. *blaZ* Genetic traits were found in all isolates resistant to penicillin. The multi-drug resistance traits, *vgaA* and *vgaB* each was detected only in 12.5% of *S. aureus* isolates. The phenotypic character of antibiotic resistance is highly correlated to different genetic traits of resistance.

Conclusions: Based on our findings, it is concluded that antibiotic resistance in *S. aureus* strains is increasing day by day due to self-medications and medication by non-registered medical practitioners. Therefore, for quick and fast detection, we propose next-generation sequencing be utilized to screen for antibiotic resistance.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the most intimidating pathogens commonly found on skin and mucous membranes, *e.g.* in human nose. Almost 15%-40% of all healthy human beings are found to be carriers of this opportunistic pathogen[1]. *S. aureus* is a Gram positive cocci and a facultative anaerobe which can survive in high temperature (50 ℃), salt concentrations and drying conditions[2]. Due to its formidable ability to survive in variable environmental conditions and remarkable ability to acquire resistance against antibiotics, it is considered as one of the most threatening microorganisms[3].

Different antibiotics have been used to treat some of the serious skin and other diseases including bacteremia, boils, bullous

*Corresponding author: Dr. Abid Ali Khan, Assistant Professor, Department of Chemistry, COMSATS Institute of Information Technology, Abbottabad Campus, 22060, KPK, Pakistan.

E-mail: abidkhanuop@gmail.com

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impetigo, cellulitis, endocarditis, folliculitis, food poisoning, lymphadenitis, lymphangitis, osteomyelitis, paronychia, scalded skin syndrome, septic arthritis, styes and toxic shock syndrome caused by *S. aureus*[4]. *S. aureus* has the ability to become resistant to almost all the available antibiotics and bactericidal agents, either by acquiring the resistant genes from other strains or mutating its own genes. Thus through evolution, *S. aureus* has become resistant to several antibiotics, to which it was previously susceptible. Penicillin-G has been used against *S. aureus* since 1940, but soon it became resistant to this drug by acquiring *beta-lactamase* genes. Later it adapted ways to become resistant to penicillinase-resistant penicillins. Until recently this bacterium has evolved strains resistant to macrolides, lincosamides, tetracycline and gentamycin[5]. For some time methicillin were appropriate drug against *S. aureus*. However, the first methicillin resistant *S. aureus* (MRSA) was detected in 1961, which is now quite common in hospitals all over the world. After the emergence of MRSA, vancomycin were reported to be the best choice against *S. aureus*, unfortunately due to its genetic evaluation, *S. aureus* has evolved strains that have shown intermediate level of resistance against vancomycin also. Thus the first on-record vancomycin-resistant *S. aureus* was isolated in 2002 in USA[6].

S. aureus is becoming more and more prevalent in hospitals and other communities. Its extraordinary ability of becoming resistant to approximately all the available antibiotics makes *S. aureus* a formidable challenge for researchers all over the world. Therefore, the present study was conducted with the objectives to determine the prevalence of *S. aureus* isolated from skin wards of the hospitals of Khyber Pakhtunkhwa, and also its resistance against various commonly and commercially available antibiotics, *i.e.*, cefixime, cefoperazone, cefotaxime, imipenem, methicillin, penicillin-G, streptomycin and ticarcillin. The study was further focused to investigate different genetic traits of resistance and to determine their correlations with the phenotypic visible resistance.

2. Materials and methods

The present study was conducted at Microbiology Research Laboratory, Centre of Biotechnology and Microbiology, University of Peshawar, Peshawar. A total of 100 samples were collected from skin wards of both male and female at the hospitals.

2.1. Sample collections

Commercially available sterile swabs dipped in sterile normal saline were used for the collection of samples. The samples were collected from skin ward of two local hospitals. Among these 50 samples were taken from the patient surroundings in the male and female ward while the other 50 samples were taken from patient's infection site.

2.2. Preparation of selective media

These samples were directly streaked on staphylococcus selective media. The staphylococcus selective media was prepared by dissolving the ingredients in proper proportions in distilled water according to the manufacturer's instructions. The media was autoclaved at 121 ℃ for 15 min at 15-20 psi. The media was then poured in the plates and waited till its solidification. Plates were kept in incubator for 24 h to check the sterility of the media.

2.3. Inoculation on selective media

The samples were directly streaked on the staphylococcus selective media plates and were kept for incubation of 24-48 h at 35-37 ℃.

2.4. Identification of the isolates

The isolates were identified by microscopic examinations, Gram staining and several biochemical tests including catalase, coagulase, tryptic soy agar and blood hemolysis.

2.5. Maintenance of bacterial isolates

Isolated bacterial strains were purified and preserved for further studies. For short term storage, the bacterial isolates were cultured on Muller Hinton agar slants and Petri dishes and maintained at 4 ℃. These isolates were subcultured on monthly basis for routine use.

2.6. Preparation of 0.5 McFarland turbidity standard

For making 0.5 McFarland turbidity standard, 0.5 mL of 1.175% barium chloride were dissolved in 99.5 mL of 1% sulfuric acid. This solution was stored at room temperature in dark. Using standard McFarland turbidity enables us to compare the newly prepared bacterial suspension (1.5×10^8 CFU/mL).

2.7. Determination of antibiotic sensitivity by disc diffusion method

Disc diffusion method of Kirby and Bauer was used for determination of antibiotic resistance[7]. Fresh broth culture which was incubated overnight at 35-37 ℃ was used. This culture was compared with 0.5 McFarland turbidity standard, by diluting the sample with sterile normal saline. Then the prepared broth culture was spread uniformly on the sterile Muller Hinton agar plates with the help of sterile cotton swabs. The antibiotic resistance capabilities of all the bacterial isolates were determined against various commonly used antibiotics such as cefixime, cefoperazone, cefotaxime, imipenem, methicillin, penicillin-G, streptomycin and ticarcillin[8]. Using a sterilized forceps, antibiotic discs were placed carefully on inoculated Muller Hinton agar plates. All these plates were incubated at 35-37 ℃ overnight. Next day, the zones of inhibition were measured in millimeters, and the results were classified into resistant, intermediate and susceptible according to the NCCLS guidelines (Table 1)[9].

Table 1

NCCLS guidelines for proposed discs.

Disc (Potency)	Resistant	Intermediate	Susceptible
Imipenem (10 μg)	⩽ 13 mm	14-15 mm	⩾ 16 mm
Ticarcillin (85 μg)	⩽ 14 mm	15-18 mm	⩾ 19 mm
Cefotaxime (30 μg)	⩽ 14 mm	15-22 mm	⩾ 23 mm
Cefixime (5 μg)	⩽ 15 mm	16-18 mm	⩾ 19 mm
Methicillin (5 μg)	⩽ 9 mm	10-13 mm	⩾ 14 mm
Cefoperazone (75 μg)	⩽ 15 mm	16-20 mm	⩾ 21 mm
Penicillin G (10 μg)	⩽ 20 mm	21-28 mm	⩾ 29 mm
Streptomycin (10 μg)	⩽ 11 mm	12-14 mm	⩾ 15 mm

2.8. PCR screening of determinants of antibiotic resistance

All amplification reactions were prepared up to 25 µL containing 9.7 µL PCR grade water, 2.7 µL 10× PCR buffer, 2.5 µL $MgCl_2$, 4 µL dATP, dCTP, dGTP and dTTP, 1 µL oligonucleotide primers, 0.3 µL *Taq* polymerase and 5 µL template DNA. Antibiotic resistance genes, namely, *blaZ* (penicillin resistance); *mecA* (oxacillin resistance); *tetK*, *tetM* and *tetL* (tetracycline resistance); *ermA*, *ermB*, *ermC* (erythromycin resistance) and *vgaA* and *vgaB* (streptogramin-A and lincosamides group of antibiotic resistance) were examined in all bacterial strains by using PCR techniques. Oligonucleotide primers were used for the detection of antibiotic resistance-associated genes (Table 2)[10-12]. All the amplified DNA by PCR were confirmed by running on agarose gel in separate lane.

Table 2

PCR target genes and primers used in this work.

Genetic trait	Primer pair
mecA	AACAGGTGAATTATTAGCACTTGTAAG
	ATTGCTGTTAATATTTTTTGAGTTGAA
blaZ	ACTTCAACACCTGCTGCTTTC
	TGACCACTTTTATCAGCAACC
ermA	TATCTTATCGTTGAGAAGGGATT
	CTACACTTGGCTTAGGATGAAA
ermB	CTATCTGATTGTTGAAGAAGGATT
	GTTTACTCTTGGTTTAGGATGAAA
ermC	CTTGTTGATCACGATAATTTCC
	ATCTTTTAGCAAACCCGTATTC
tetK	TCGATAGGAACAGCAGTA
	CAGCAGATCCTACTCCTT
tetL	TCGTTAGCGTGCTGTCATTC
	GTATCCCACCAATGTAGCCG
tetM	GTGGACAAAGGTACAACGAG
	CGGTAAAGTTCGTCACACAC
vgaA	AGTGGTGGTGAAGTAACACG
	CTTGTCTCCTCCGCGAATAC
vgaB	TCTCTCAATTAGAAGAACC
	TTATCTATTCGTGTTTCC

3. Results

3.1. Prevalence of S. aureus

The two largest hospitals in northwest region of Khyber Pakhtunkhwa, Pakistan were selected for studying the prevalence of *S. aureus*, which is a causal agent of various skin infections, abscesses, bacteremia, endocarditis, meningitis, myocarditis, osteomyelitis and pneumonia at different sites. A total of 100 samples were collected, in which *S. aureus* was isolated from 48 samples. However, among the 48 isolates, 14 (29%) were identified from samples taken from male and female skin wards environment, and 34 (71%) were from samples taken from patient's infection sites (Figure 1).

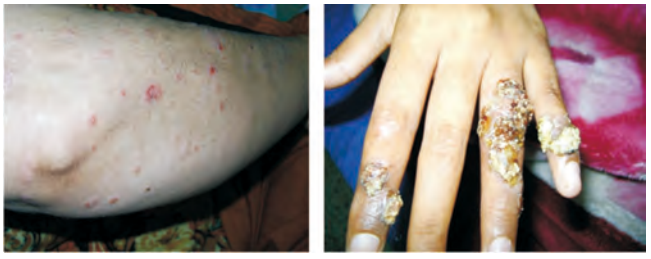

Figure 1. Infection sites of patients used for the collection of samples.

Out of the later 34 isolates, 10 were from samples taken from male patients while 24 isolates were from samples taken from female patients. These results demonstrate that the total prevalence of *S. aureus* both in ward as well as in patients was 48% (Figures 2 and 3).

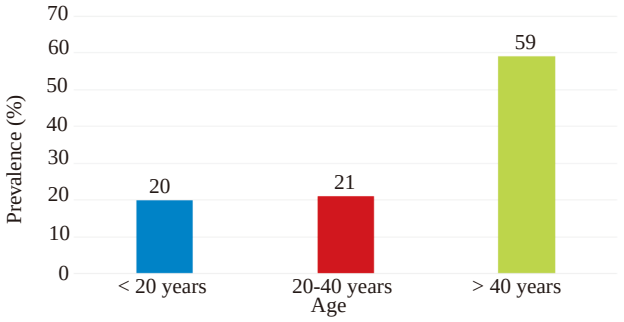

Figure 2. Prevalence of *S. aureus* in different age groups.

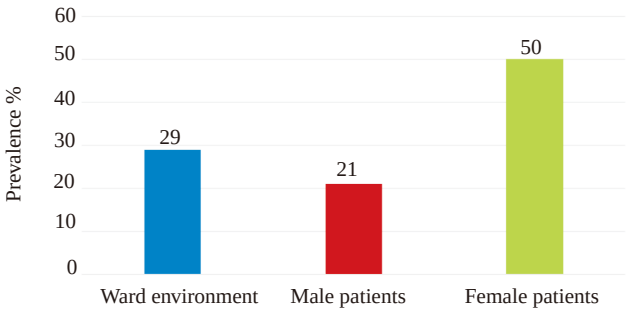

Figure 3. Occurrence of *S. aureus* in skin wards and male and female patients.

3.2. Susceptibility pattern

The results showed that several *S. aureus* strains have developed resistant to antibiotics, especially methicillin, whereas, they were previously susceptible. Therefore, we have reported the antibiotic resistance of all the 48 isolates against various commonly used antibiotics such as cefixime, cefoperazone, cefotaxime, imipenem, methicillin, penicillin-G, streptomycin and ticarcillin. The results have been shown in Figure 4, while the resistance profiles in terms of zone of inhibition of all the isolates are given in Table 3.

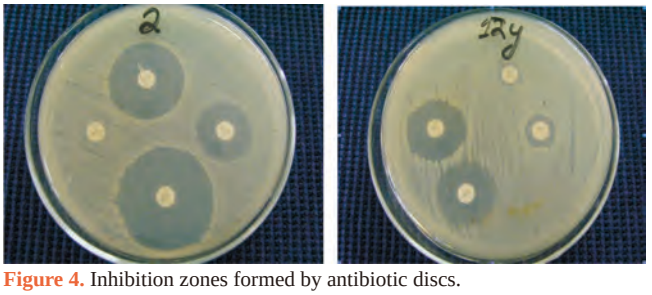

Figure 4. Inhibition zones formed by antibiotic discs.

We have further observed that all the clinical isolates were resistant to penicillin-G; however, all the isolates were susceptible to imipenem except the isolate No. 38 and 40. A total of 14 isolates were found to be resistant to methicillin, 6 isolates have shown an intermediate response and 28 were susceptible. Then a total of 36 isolates were resistant to cefotaxime while 12 isolates were with intermediate range of inhibition (15-22 mm). A total of 40 isolates

showed resistance to streptomycin, 6 isolates showed intermediate activity and only 2 isolates were found susceptible. Further a total of 46 isolates showed complete resistance while only two isolates were found to have intermediate response with the inhibition zone of 16 to 18 mm against cefixime. Cefoperazone showed more or less similar results as cefixime. Only two isolates were found to have intermediate activities and the rest of 46 were resistant. *Staphylococcus* isolates were mostly found resistant to ticarcillin. The resistant samples constituted of 79.2% of the total samples, 20.8% were found in the intermediate zone of inhibition, whereas no sample was found susceptible to ticarcillin.

3.3. Molecular screening of antibiotic resistance

Related to the respective phenotypic pattern of resistance, subsequent PCR was performed to analyze the genetic basis of resistance. These results demonstrated that 13 out of 14 isolates resistant to methicillin were positive for *mecA* gene (Table 4). One resistant isolate and 6 intermediate resistant isolates showed no sign of amplification for this gene. In addition to the phenotypic based genetic traits, we have further investigated the presence of erythromycin resistance methylase traits, *i.e.*, *ermA*, *ermB* and *ermC*, tetracycline resistance traits, *i.e.*, *tetK*, *tetL* and *tetM* and

Table 3

Zone of inhibition (in mm) of different antibiotics.

Sample	Zones of inhibition (mm)							
No.	Penicillin	Imipenem	Cefotaxime	Streptomycin	Cefixime	Cefoperazone	Ticarcillin	Methicillin
2	5	26	14	11	10	10	5	14
4	11	23	-	10	-	12	11	8
8	9	34	4	10	-	12	7	15
11	15	28	13	16	6	14	11	11
13	11	22	19	9	4	12	9	7
20	13	24	18	12	11	12	12	15
21	18	23	18	10	-	9	5	14
26	8	17	12	11	4	13	9	8
27	18	22	17	9	-	14	16	7
28	19	24	5	11	-	15	15	12
29	14	19	-	10	-	12	15	12
30	6	22	12	10	4	11	5	9
31	7	24	12	10	7	12	6	8
32	19	21	4	9	-	14	16	15
33	8	27	12	11	-	11	7	16
34	9	18	12	-	-	12	8	5
37	8	17	7	11	-	13	7	8
38	8	14	7	-	-	10	5	7
39	7	25	11	11	4	12	6	14
40	10	15	17	13	16	19	9	5
41	13	26	13	10	4	13	12	7
42	8	23	5	10	-	12	8	16
43	11	20	14	9	-	14	10	16
44	17	23	16	13	-	13	15	14
47	12	22	14	10	-	13	5	15
51	15	27	6	8	-	9	15	17
52	16	29	3	6	-	11	11	15
53	11	20	2	3	-	-	6	14
56	6	18	6	7	2	-	5	14
59	12	20	12	12	-	10	12	18
62	14	19	10	6	4	9	9	12
66	10	32	15	13	7	6	15	17
71	17	18	10	10	-	12	11	15
74	13	17	6	10	-	10	6	8
77	19	34	18	16	16	17	16	17
78	4	17	7	9	4	6	8	7
79	8	20	10	11	5	11	9	11
80	11	20	12	13	8	11	7	14
82	13	24	4	2	-	4	5	14
84	16	28	16	4	2	8	10	19
85	18	30	15	6	8	10	12	16
87	9	22	11	4	10	12	7	18
89	5	18	9	8	2	8	6	15
90	17	26	17	10	2	8	15	14
91	12	23	15	11	7	12	15	16
92	10	19	7	5	-	12	10	11
95	14	28	11	9	-	13	11	14
98	8	17	4	4	-	11	7	8

multidrug resistance plasmid, *i.e.*, *vgaA* and *vgaB* in all our 48 *S. aureus* isolates. About 79.2% of the isolates were positive for *ermA* gene, 10.4% for *ermB* gene and 25% for *ermC* gene respectively. However, 14.6% of the isolates were positive for *tetK*, and 6.25% of isolates were positive each for *tetL* and *tetM* traits respectively. The multi-drug resistance traits, *vgaA* and *vgaB* each were detected only in 12.5% of *S. aureus* isolates.

Table 4

Genotypes of antibiotic resistance.

Isolates No.	Genes									
	mec A	*blaZ*	*ermA*	*ermB*	*ermC*	*tetK*	*tetL*	*tetM*	*vgaA*	*vgaB*
2	-	+	+	-	-	+	+	+	+	+
4	+	+	+	+	+	+	-	-	-	-
8	-	+	+	-	-	-	-	-	-	-
11	-	+	-	-	-	-	-	-	-	-
13	-	+	-	-	-	-	-	-	-	-
20	-	+	+	-	+	-	-	-	-	-
21	-	+	-	-	-	-	-	-	-	-
26	+	+	+	-	-	+	-	-	-	-
27	+	+	-	-	-	-	-	-	-	-
28	-	+	+	-	+	-	-	-	-	-
29	-	+	+	-	-	-	-	-	-	-
30	+	+	+	-	-	-	-	-	-	-
31	+	+	+	-	+	-	-	-	-	-
32	-	+	+	-	-	-	-	-	-	-
33	-	+	+	-	+	-	-	+	+	+
34	+	+	+	-	-	-	-	-	-	-
37	+	+	+	-	-	-	-	-	-	-
38	+	+	+	+	+	-	+	-	+	+
39	-	+	+	-	-	-	-	-	-	-
40	+	+	+	+	+	+	-	-	+	+
41	+	+	+	-	-	-	-	-	-	-
42	-	+	+	-	+	-	-	-	-	-
43	-	+	+	-	-	-	-	-	-	-
44	-	+	-	-	-	-	-	-	-	-
47	-	+	+	-	-	-	-	-	-	-
51	-	+	+	-	-	-	-	-	-	-
52	-	+	+	-	-	-	-	-	-	-
53	-	+	+	+	+	-	-	-	+	+
56	-	+	+	-	-	-	-	-	-	-
59	-	+	+	-	+	-	-	-	-	-
62	-	+	+	-	-	-	-	-	-	-
66	-	+	+	-	-	-	-	-	-	-
71	-	+	+	-	-	+	-	-	-	-
74	+	+	+	-	-	-	-	-	-	-
77	-	+	-	-	-	-	-	-	-	-
78	+	+	+	-	-	-	-	-	-	-
79	-	+	+	-	-	-	-	-	-	-
80	-	+	+	+	+	-	+	-	+	-
82	-	+	+	-	-	-	-	-	-	-
84	-	+	-	-	-	-	-	-	-	-
85	-	+	-	-	-	-	-	-	-	-
87	-	+	+	-	+	+	-	-	-	+
89	-	+	+	-	-	-	-	-	-	-
90	-	+	-	-	-	-	-	-	-	-
91	-	+	-	-	-	-	-	-	-	-
92	-	+	+	-	-	-	-	-	-	-
95	-	+	+	-	-	+	-	+	-	-
98	+	+	+	-	-	-	-	-	-	-

In addition, some of the isolates were positive for *mecA*, *blaZ* and one of the *erm* gene cluster, also for *tetK* genes, for example, the isolate No. 4, 26, 38 and 40 were found positive for all these genes. However, for isolate No. 27, *mecA* and *blaZ* genes were present but *erm* and *tet* gene clusters were not present. In isolate No. 30 and 31 although *tet* and *vga* gene clusters were not found but *mecA*, *blaZ* and *erm* gene clusters were present. This suggests that there is a strong correlation between *mecA*, *blaZ* and *erm* genes that contributes to resistance of *S. aureus*.

4. Discussion

4.1. Prevalence of S. aureus

Various studies have been conducted to determine the resistance of different strains of *S. aureus* where the nosocomial infections cause significant patient morbidity and mortality. It has been reported in literature that prevalence of *S. aureus* varies among different populations, and is influenced by age, underlying illness, race, certain behaviors, and the environment in which the person lives or works[13]. In our study we have reported that the prevalence of *S. aureus* was 59% in patients with age of above 40 years, 21% in patients with age between 20 to 40 and 20% in patients below 20. Among these patients 70.5% were female and 29.5% were male. These results indicate that the occurrence of *S. aureus* is high in female and patients aged above 40 years.

Another study about the prevalence of *S. aureus* reported that the rate of nosocomial infections can be reduced by maintaining good hygiene in the hospital[14]. Their specific program to prevent *S. aureus* transmission had led to a significant decrease of this prevalence rate by 32%[14]. In our study, the prevalence was determined to be 29% in wards environment, which depicts the poor hygienic conditions of the skin wards.

4.2. Susceptibility pattern

All kind of bacterium especially *S. aureus* has emerged as a major clinical and epidemiological problem in hospitals. It has been previously reported that most of the methicillin resistant MRSA were not only resistant to all type of β-lactam antibiotics but also resistant to many other classes of antibiotics, *e.g.*, imipenem[15,16]. Our results clearly demonstrate that all the strains resistant to methicillin were also resistant to penicillin, *i.e.*, β-lactam, but in contrast to the literature, *S. aureus* remained susceptible to imipenem. A previous survey about the antibiotic susceptibility pattern of *S. aureus* strains from clinical and skin isolates grown at 37 and 44 ˚C respectively were carried out in Irrua Nigeria[17]. The isolates were susceptible to streptomycin (30.0%) and were resistant to penicillin. According to our study, out of 100 different specimens, 48% *S. aureus* isolates were found, in which only 4% isolates were susceptible to streptomycin, which is very much different from that of the above survey. But the results for penicillin are same in both cases. It is further concluded that the genetic diversity may result in the generation of different strains of *S. aureus*. The antibiotic resistance results indicate that *S. aureus* isolates showed greatest resistance to penicillin-G and cefixime and minimum resistance to imipenem.

4.3. Molecular screening of antibiotic resistance

For molecular screening, the pair of primers utilized for the

detection of *mecA* gene was already well known primers for the detection of methicillin resistant plasmid in *S. aureus*. It is clear from the literature that majority of the MRSA strains analyzed were *mecA* positive. While *blaZ* genetic traits were present in all *S. aureus* isolates resistant to penicillin. The *blaZ* were found to be the characteristic genetic traits responsible for resistance β-lactam group of antibiotics[10-12]. Our results further confirm the observations of correlation between penicillin and *blaZ* genetic traits. Previously it has been demonstrated that *ermA* trait is the most prevalent trait in MRSA strains[18,19], which strongly support our findings that only one strain resistant to methicillin was *ermA* negative, *i.e.* isolate No. 13. As we have mentioned previously that the same isolate No. 13 was phenotypically resistant to methicillin but was negative for *mecA* trait.

Based on our findings, it is concluded that antibiotic resistance in *S. aureus* strains is increasing day by day due to medication by non-registered medical practitioners and self-medication as well, because 100% and 29% resistance were observed against penicillin and methicillin respectively, which is a really very challenging situation. We have further concluded that the phenotypic resistance of 48 different *S. aureus* isolates is well correlated to the genetic traits of resistance as well as with the hygienic conditions. Therefore, the hygienic conditions of the hospital need improvement, in order to decrease rate of nosocomial infections caused by *S. aureus* in hospitalized patients. Further, for quick and fast detection, we propose next-generation sequencing be utilized to screen for antibiotic resistance.

Conflict of interest statement

We declare that we have no conflict of interest.

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