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Occurrence of Methicillin Resistant *Staphylococcus Aureus* in Emerging Tertiary Care Hospital, Veraval, Gujarat, India

Sujithra M[1], Vanik D[2], Sivaraman GK[3]* and Koya KM[4]

[1]Departement of biotechnology, Dr. Rangarajan Dr. Sakunthala Engineering College, India

[2]Department of Microbiology, India

[3]Department Fisheries Technology, India

[4]Department of Fisheries Resources, India

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***Corresponding author:** Gopalan Krishnan Sivaraman, Department Fisheries Technology, Principal Scientist, ICAR-CIFT, MFB Division, Matsyapuri Post, Willingdon Island, Cochin- 682029, Kerala, India, Tel: +91-484-2412384(O); Email: gkshivraman@gmail.com

Abstract

The present study was carried out to monitor the incidence of MRSA as an emerging pathogen in health care setting. In total of 36 clinical samples from men, women and children and also hand swabs of nursing staff and surrounding environment of GirSomnath District, Veraval Civil Hospital, Gujarat were collected and examined for the presence of MRSA. The collected samples were screened for the presence of MRSA and assessing their antimicrobial susceptibility testing, minimum inhibitory concentration as per CLSI (2017) guideline. The incidence rate of MRSA was found as 52.78%. The results indicate that the highest level of resistance was found to Penicillin (100%) and the least level of resistance was found to Tigecycline (4%). About 24% isolates were positive for mecA gene. The lack of health care facility (antimicrobial testing), poor hygienic practices, and awareness among the patient and healthcare workers may be plays a significant role in the presence of MRSA in the health care setting.

Keywords: Minimum Inhibitory Concentration; MRSA; Opportunistic Pathogen; Gram-Positive Bacteria; *Staphylococcus Aureus*

Introduction

Worldwide infections are the most important cause for mortality in humans and bacteria are more severe in developing and under-developed countries [1]. Pathogens are even more infectious in nature due to indiscriminate use of antibiotics. *Staphylococcus aureus* is a common inhabitant of human skin and noses of 25% of healthy

people as well as animals and do not cause infections among healthy individuals as carrier [2]. As an opportunistic pathogen *S. aureus* can cause a broad range of infections including mild skin infections such as impetigo and folliculitis to invasive diseases like wound infections, osteomyelitis, bacteraemia with metastatic complications, and toxin mediated diseases like food poisoning, toxic shock syndrome, scaled skin syndrome [3-5]. It is ubiquitous Gram-positive, catalase positive facultative anaerobic cocci [6]. The genus *Staphylococcus* comprises of 47 recognized species and 21 subspecies out of which three species viz., *S. aureus, S. saprophyticus* and *S. epidermidis* are associated with hospital-acquired infections. In addition to these three species, other staphylococcal species such as *S. warnerii, S. scuiri, S. hemolyticus and S. lugdunensis* etc., is being reported in the secondary bacterial infections either in post-operative or among immune compromised individuals [7].

Methicillin was introduced as new β-lactam antibiotics in 1950 to overcome penicillin resistance among *S.aureus*. Later in 1961, methicillin resistant *Staphylococcus aureus* (MRSA) was reported in United Kingdom which was resistant to most of the commonly prescribed antibiotics such as penicillin, cephalosporins and carbapenems [8]. Being a nosocomial pathogen [9], MRSA are frequently associated with ventilator associated infections and surgical site infections making treatment more difficult [10,11]. Emergence of MRSA has increased all over the world and is a major challenge to hospital [12]. Colonization and infection rates of MRSA are reported in 0.8% of US population with 15% increase in Alberta between 2005 and 2008 and higher rates in Canada over the past decade [6,13,14]. The incidence of MRSA is 25 % in Western part of India 50.18 % in Central India and 70% in South India and overall incidence rate was ranged between 8 and 71% [15-19]. The incidence rate of MRSA has increased in the past 20 years, accompanied by a rise in antibiotic-resistant strains. Recently vancomycin-resistant strains have also been detected around the world [20]. Infections caused by MRSA often fail to respond to standard treatment, resulting in prolonged illness, higher health care expenditures, and a greater risk of death. Poor infection control practices, inadequate sanitary conditions and inappropriate food-handling encourage the further spread of antimicrobial resistance [21]. Region-wise knowledge of antibiotic resistance patterns and resistance genes in the area is of paramount importance for surveillance and control of the spread of antibiotic resistance as well as for instituting appropriate therapy and judicious antibiotic usage. The present study was undertaken to determine the prevalence and molecular characterization of MRSA isolated from various swabs of patient's being treated in the civil hospital, Veraval, Gujarat and to establish resistance pattern among the isolates of MRSA.

Materials and Methods

Sample Collection

This study was carried for a three month period from January 2017 to March 2017 in civil hospital at Veraval, Gujarat. Different clinical samples (36) *viz*., 9 samples each of rectal swab (EC 1 W, EC 2 W and EC 3 W) hand swab (EC 1 M, EC 2 M, EC 3 M, EC 1 C, EC 2 C, EC 3 C and EC 4 C), wound swab and urine sample (Urine sample Women) were collected from patients including men, women and children. Sampling also comprised of hand swabs of Staff Nurses (HP1- HP5) and surrounding environment which encompassed operation theatre (OT1- OT5), floor sample of hospital (FS1- FS4) and toilet samples (TFS1- TFS9) were collected (Table 1). All the samples were screened for MRSA as per standard protocol using MRSA II agar plate (Difco, USA) and *mecA* gene coding for methicillin resistance and nuc gene for coagulase production were amplified by Polymerase Chain Reaction (PCR) [22].

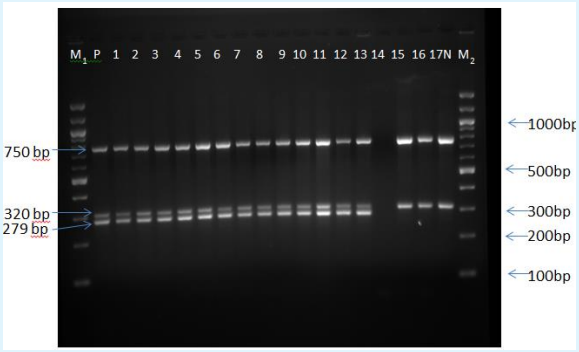
Marker	Target	Primer	Oligo sequence (5′→3′)	Primer concentration
Gene specific to *Staphylococcus* genus	16S rRNA gene	Staph756F	AAC TCT GTT ATT AGG GAA GAA CA	0.12 µM
		Staph750R	CCA CCT TCC TCC GGT TTG TCA CC	0.12 µM
Virulence gene encoding thermonuclease	*nuc* gene	*nuc*F	GCG ATT GAT GGT GAT ACG GTT	0.04 µM
		*nuc*R	AGC CAA GCC TTG ACG AAC TAA AGC	0.04 µM
Gene responsible for methicillin resistance	*mec*A gene	*mec*F	GTA GAA ATG ACT GAA CGT CCG ATA A	0.12 µM
		*mec*R	CCA ATT CCA CAT TGT TTC GGT CTA A	0.12 µM

Table 1: PCR target and primers used for the multiplex PCR.

MRSA Strains Isolation and Identification

The clinical isolates were identified as *S.aureus* using standard biochemical methods [23]. Isolation was done based on colony morphology on Baird Parker agar (Oxoid, UK) supplemented with 1 ml 50% egg yolk emulsion and 1% potassium tellurite solution with 48 hrs of incubation. Subsequently, MRSA confirmation was done on readymade plates of MRSA II (Difco) within 24 hours of incubation at 35°C. Well isolated colonies were streaked on to Tryptic Soy Agar plate for isolation and further purification. Methicillin resistance was confirmed both phenotypically by disc diffusion testing and genotypically multiplex PCR [22].

The antimicrobial susceptibility of *S. aureus* isolates to 24 antibiotics namely, penicllin-G (P), 10 µg azithromycin (AZM) 15µg, erythromycin (E) 15µg, clarithromycin (CLR) 15µg, linezolid (LZ) 30µg, cotrimoxazole (COT) 25µg, vancomycin (VA) 30µg, cefoxitin (CX) 30µg, ciprofloxacin (CIP) 5µg, gatifloxacin (GAT) 5µg,ofloxacin (OF) 5µg, clindamycin (CD) 2µg, tigecycline (TGC) 15µg, moxifloxacin(MO)5µg, gentamicin (GEN)10µg, rifampicinb (RIF)5µg, lomefloxacin (LOM)10µg, norfloxacin (NX)10µg, novobiocin (NV)30µg, teicoplanin (TEI) 30µg, nitrofurantoin (NIT) 300µg, pristinomycin (RP) 15µg ampicillin/sulbactam (A/S) 10/10µg, piperacillin/tazobactam (PIT) 100/10µg (Dodeca Staphylococci-1 and 2, HiMedia, Mumbai) were carried out by disc diffusion method (Bauer *et al*., 1966) on Mueller Hinton agar with 4% NaCl and incubated at 37°C for 18- 24 hrs (Figure 1). The inhibition zones were measured and categorized as susceptible, intermediately resistant or and resistant. Multi drug resistant (MDR) staphylococci were those isolates that were resistant to penicillin along with at least 3 other classes of antibiotics. The tests were performed and the results were interpreted as per CLSI guidelines [24].



Lane no. 3- 17 Clinical samples; P: Positive Control ATCC 43300 MRSA; N: Negative Control ATCC 25923 MSSA; M1 & M2: DNA ladder 100 bp; 278bp: *Nuc*, 320bp: *mecA* gene; 750bp: *Staphylococci*

Figure 1: Multiplex PCR assay for the confirmation of methicillin resistant Staphylococci from the clinical setting.

Minimum Inhibitory Concentration (MIC)

S.aureus strains were cultured in Brain heart infusion broth and the concentration was adjusted to 0.5Mc Farland's standard, and then the culture was spread over MHA with 4% NaCl. MIC was determined with MIC test strip (HiMedia, Mumbai) for antibiotics that are commonly used for staphylococcal infections such as methicillin A (240-0.01µg/ml), methicillin B (4-0.001 µg.ml^{-1}), penicillin (0.002-32 µg.ml^{-1}), oxacillin (0.016-256 µg.ml^{-1}) vancomycin (0.016-256 µg.ml^{-1}), gentamicin (0.016-256 µg.ml^{-1}) and ciprofloxacin (0.002-32 µg.ml^{-1}). The results were interpreted as per CLSI guidelines.

Molecular Typing of MRSA

DNA was isolated from staphylococci using GenElute bacterial genomic DNA kit (Sigma-Aldrich) according to manufacturer's instructions. Multiplex Multiplex PCR (SureCycler 8000, Agilent, USA) was set in 20 µl reaction mixture comprising 5 µl of template DNA, 2.5 mM $MgCl_2$, 0.2 mM dNTPs mix, primers (Table 1) and Taq DNA polymerase. The PCR program comprised of initial denaturation temperature of 94°C for 5 min; 10 cycles of 94°C for 40 sec, 68°C for 40 sec, and 72°C for 1 min; 25 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and the final extension at 72°C for 10 min. Agarose gel electrophoresis was performed for separation of

amplicons in 2% agarose gel containing ethidium bromide (0.5 µg.ml^{-1}) followed by capturing of images using gel documentation system (Bio Rad, USA). The positive control strain of MRSA ATCC 43300 and negative control strain of MSSA ATCC 25923 was used for quality control (Figure 2).

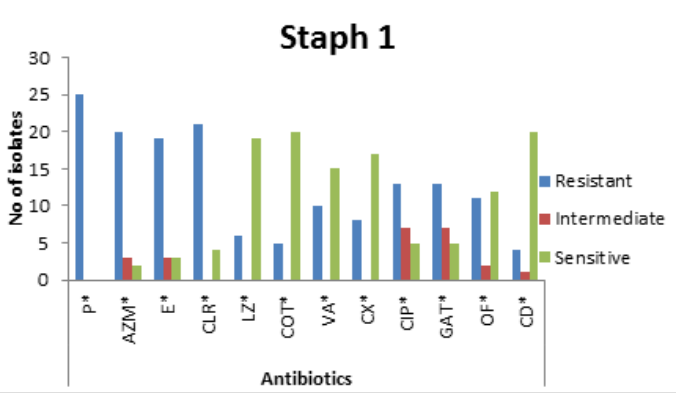


Figure 2: Antibiotic resistance pattern of the MRSA strains with Dodecca Staphylococci-1(HiMedia, Mumbai).

Results and Discussion

The present study was carried out to monitor MRSA incidence in Veraval Civil Hospital for a period of 3 months during January 2017 to March 2017. Out of total 36 samples, 19 (52.78%) samples were found positive for MRSA, 3 toilet floor samples, 7 staff hand swab, 3 operation theatre samples, 1 urine and 1 hospital sample were also positive for MRSA. Similar resistance pattern was observed by Rubali, et al. [17] wherein 58.33% coagulase positive MRSA were isolated from Tertiary care hospital in Central India. According to and Fombda, et al. [25] the prevalence rate of MRSA in a tertiary care hospital was 33% and 34.2% in Mumbai and Jammu and Kashmir, respectively, which was slightly lower than the present study (52.78%). Varied incidence rate of MRSA was reported around different part of the globe in the health care settings *viz*., 21-30% in Nigeria, Kenya and Camroon and incidence rate below 10% were reported in Algeria, Tunisia and Malta Kesah, et al. [26]. The prevalence rate in Netherland and Switzerland ranged from 2 to 15% whereas in Japan and Hong Kong the prevalence rates were as high as 70% [27]. Thus, the prevalence of MRSA varies globally and it is further suggested that the higher incidence of MRSA is due to indiscriminate use of antimicrobial agents, improper prescription of antibiotics and limited hygienic facilities available with the health care settings. Furthermore Fridkin and Gaynes, et al. [28] suggested that the rate of MRSA acquisition may be directly proportional to duration of stay in hospital.

The antimicrobial susceptibilities of confirmed MRSA isolates were evaluated against 24 commonly available antimicrobial agents as per CLSI 2015 standards (Table 2). The MRSA isolates exhibited cent percent resistance towards penicillin followed by clarithromycin, aziothromycin, pristinimycin, erythromycin and lomefloxacin. Fombda, et al. [25] also reported highest resistance of MRSA to penicillin followed by erythromycin, clindamycin and cotrimaxazole, but susceptibility towards vancomycin and gentamycin. Similarly, Vysakh, et al. [29] also reported 81% of MRSA resistant towards penicillin, followed by erythromycin and cefoxitin. In the current study, isolates showed least level of resistance to clindamycin and tigecycline. The intermediate level of resistance was found among MRSA isolates to teicoplanin, ciprofloxacin and gatifloxacin, moxifloxacin, norfloxacin, lomefloxacin and nitroflurantin, aziothromycin and erythromycin and ampicillin/sublactum. The isolates showed cent percent sensitivity to rifampicin and novobiocin followed by tigecycline, nitroflurantoin, cotrimaxazole, clindamycin, linezolid and cefoxitin.

Antibiotics	Susceptibility of *S. aureus* isolates based on disc diffusion assay		
	Resistance	Intermediate	Sensitive
Penicillin (P)	25(100.00%)	-	-
Aziothromycin(AZM)	20 (80.00%)	3 (12.00%)	2 (8.00%)
Erythromycin (E)	19 (76.00%)	3 (12.00%)	3 (12.00%)
Clarithromycin (CLR)	21 (84.00%)	-	4 (16.00%)
Linezolid (LZ)	6 (24.00%)	-	19 (76.00%)
Co-Trimoxazole (COT)	5 (20.00%)	-	20 (80.00%)
Vancomycin (VA)	10 (40.00%)	-	15 (60.00%)
Cefoxitin (CX)	8 (32.00%)	-	17 (68.00%)
Ciprofloxacin (CIP)	13 (52.00%)	7 (28.00)	5 (20.00%)
Gatifloxacon (GAT)	13 (52.00%)	7 (28.00)	5(20.00%)
Ofloxacin (OF)	11 (44.00%)	2 (8.00%)	12 (48.00%)
Clindamycin (CD)	4 (16.00%)	1 (4.00%)	20 (80.00%)
Tigecycline (TGC)	1 (4.00%)	2 (8.00%)	22 (88.00%)
Moxifloxacin (MO)	9 (36.00%)	6 (24.00%)	10 (40.00%)
Gentamycin (GEN)	12 (48.00%)	-	13 (52.00%)
Rifampicin (RIF)	-	-	25 (100.00%)
Lomefloxacin (LOM)	17 (68.00%)	4 (16.00%)	4 (16.00%)
Norfloxacin (NX)	8 (32.00%)	5 (20.00%)	12 (48.00%)
Novobiocin (NV)	-	-	25 (100.00)
Teicoplanin (TEI)	-	9 (36.00%)	16 (64.00%)
Nitroflurantin (NIT)	-	4 (16.00%)	21 (84.00%)
Pristinomycin (RP)	20 (80.00%)	-	5 (20.00%)
Ampicillin/Sublactum (A/S)	7 (28.00%)	2 (8.00%)	16 (64.00%)
Piperacillin/Tazobactum (PIT)	9 (36.00%)	-	16 (64.00%)

Antibiotic classes: P: Penicillin; Macrolides: E & CLR; Oxazolidinones: LZ; Sulfonamides: COT; Azalides: AZM; Fluoroquinilones: CIP, GAT, OF, LOM, MO & NX; Lincosamide: CD; Cephems: CX; Aminoglycosides: VA, GEN & NIT, Rifampicin: RIF; Glycylcyclines: TGC; Glycopeptides: TEI; Aminocoumarin: NV; Streptogramin: RP; Beta lactam & Beta lactam inhibitor: A/S; PIT.

Table 2: Antibiotic susceptibility of the MRSA isolates by disc diffusion method.

In this study, 63.89% MRSA strains were multidrug resistant (Figures 3 & 4) and the presence of MDR- CPS - MRSA may occur in the form of infection and or asymptomatic carrier's. The multi-drug resistance was defined as resistance to 3 or more different classes of antimicrobials in addition to the penicillin. Several studies on MRSA revealed that the hygienic- sanitary profile, personal hygienic practices and habits, raising the risk of cross contamination in the handled food [30]. *S. aureus* has developed multidrug resistance worldwide and indicates that as potential hazards to human health [31]. Recent reports revealed that MDR bacteria can be transferred from the human to environment and to animal [32]. It is further suggesting that significant awareness among the public must be made about the presence of MDR- CPS- MRSA. Tiwari, et al. [33] reported that 72.1% of MRSA were multi drug resistant in a tertiary hospital of northern India. Whereas, Styers, et al. [34] reported of multi-drug resistance of MRSA in some workers of USA. The widespread use of antibiotics has provoked an exponential increase in the incidence of antibiotic resistant and multi-drug resistant strains threaten the effective prevention and treatment of infections [35].

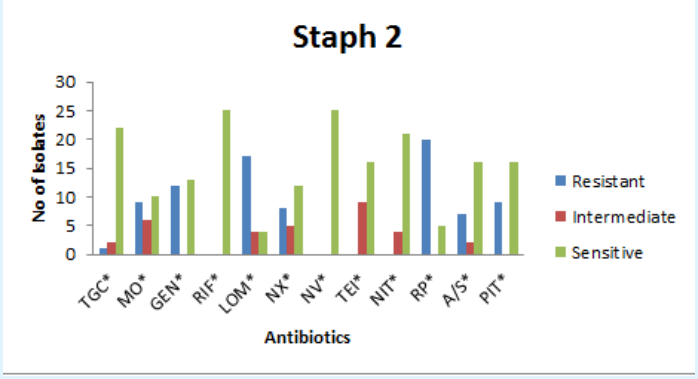


Figure 3: Antibiotic resistance pattern of the MRSA strains with Dodecca Staphylococci-2 (HiMedia, Mumbai).

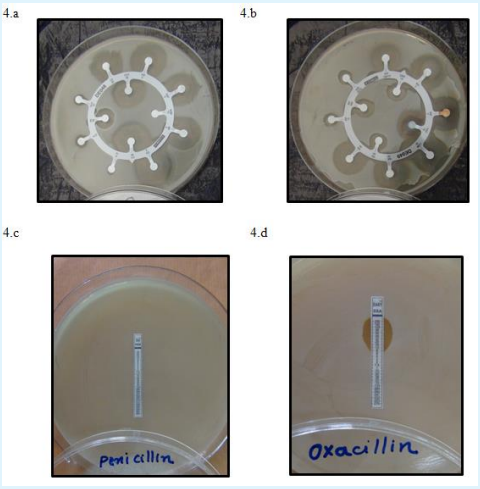


Figure 4: Antibiotic resistance of MRSA strains with Dodecca Staphylococci- 1 and 2 (4a and b) and Minimum Inhibitory Concentration using MIC strips (HiMedia, Mumbai) for penicillin (4c) and oxacillin (4d).

In the present study, values of MIC of routinely used antimicrobial agents used in tertiary hospitals were with for the test MRSA isolates and found that most of these isolates showed a higher MIC level of ≥256 μg.ml^{-1}. From the rectal swabs, hand swabs of the patients and staff and floors showed a methicillin MIC of ≥240 μg.ml^{-1}. Also shows a higher level of resistance to the methicillin B, Gentamicin, ciprofloxacin, oxacillin, vancomycin and penicillin with ≥256 μg.ml^{-1} irrespective of the types of samples examined from the patients during this study and it could pose a serious threat to public health as this affects infection control and further may also lead to dissemination MRSA strains. Hence, effective infection control measures and regular monitoring studies should be conducted in health care facilities for MRSA and other drug resistant bacteria.

Out of 19 isolates (52%), (48%) and (8%) were found to be resistant to methicillin, gentamycin and ciprofloxacin, respectively, with MIC of ≤1 μg.ml^{-1}. Exactly, 12% of isolates were resistant to oxacillin (MIC level of ≤ 2 μg.ml^{-1}). Majority of the isolates (18; 72%) were resistant to vancomycin with MIC ≤ μg.ml^{-1}. Moreover, all the MRSA isolates were resistant to Penicillin (Tables 3 & 4).

Samples	Methicillin A	Methicillin B	Gentamicin	Ciprofloxacin	Oxacillin	Vancomycin	Penicillin
Rectal swabs EC 1 W	≥240	≥4	≥256	R	R	≥256	R
EC 3 M	R	≥4	R	R	R	≥256	R
Wound swab EC 2 W	R	R	R	R	R	≥256	R
Hand swabs EC 1 C	≥240	≥4	≥256	≥256	R	≥256	R
EC 1 M A	R	R	R	R	R	≥256	R
EC 1 M B	≥240	≥4	≥256	R	R	≥256	R
EC 1 M R	≥240	≥4	≥256	R	R	≥256	R
EC 2 C	≥240	≥4	R	R	R	≥256	R
EC 4 C	≥240	≥4	≥256	≥32	≥256	≥256	R
EC 3 W	≥240	≥4	≥256	≥32	≥256	≥256	R
Toilet Floor sample FS 2 C	≥240	≥4	R	R	≥256	≥256	R
TFS 2 B	≥240	≥4	R	≥32	≥256	≥256	R
TFS 7	≥240	≥4	R	R	R	≥256	R
Staff hand swab HP 1	≥240	≥4	R	R	R	≥256	R
HP 2	≥240	≥4	≥256	R	≥256	≥256	R
HP 3 A	≥240	≥4	≥256	R	R	≥256	R
HP 3 B	≥240	≥4	R	R	R	≥256	R
HP 4 A	≥240	R	≥256	R	R	≥256	R
HP 4 B	≥240	R	≥256	R	R	≥256	R
HP 5	R	R	R	R	R	≥256	R
Operation theatre swab OT 1	≥240	R	R	R	R	≥256	R
OT 3A	≥256	≥4	≥256	R	R	≥256	R
OT 3 B	≥240	≥4	≥256	≥32	R	≥256	R
Urine Sample	R	R	R	R	R	≥256	R
Hospital bed sample HBS 2	≥240	≥4	≥256	R	≥256	≥256	R

Table 3: Minimum Inhibitory Concentrations (MICs) of antibiotic against MRSA isolates.

ISOLATES	Samples	MRSA
EC 1 W	Rectal swab from Women 1	+
EC 2 W	Rectal swab from Women 2	N
EC 3 W	Rectal swab from Women 3	+
EC 1 M	Hand swab from men 1	+
EC 2 M	Hand swab from men 2	N
EC 3 M	Hand swab from men 3	+
EC 1 C	Hand swab from child 1	+
EC 2 C	Hand swab from child 2	+
EC 3 C	Hand swab from child 3	N
EC 4 C	Hand swab from child 4	+
TFS 1	Toilet Floor sample 1	N
TFS 2	Toilet Floor sample 2	+
TFS 3	Toilet Floor sample 3	N
TFS 4	Toilet Floor sample 4	N
TFS 5	Toilet Floor sample 5	N
TFS 6	Toilet Floor sample 6	N

TFS 7	Toilet Floor sample 7	+
TFS 8	Toilet Floor sample 8	N
TFS 9	Toilet Floor sample 9	N
FS 1	Floor sample 1	N
FS 2	Floor sample 2	+
FS 3	Floor sample 3	N
FS 4	Floor sample 4	N
HP 1	Staff hand swab HP 1	+
HP 2	Staff hand swab HP 2	+
HP 3	Staff hand swab HP 3	+
HP4	Staff hand swab HP 4	+
HP 5	Staff hand swab HP 5	+
OT 1	Operation theatre swab OT 1	+
OT 2	Operation theatre swab OT 2	N
OT 3	Operation theatre swab OT 3	+
OT 4	Operation theatre swab OT 4	N
OT 5	Operation theatre swab OT 5	N
Urine Sample	Urine sample from Women	+
HBS 1	Hospital bed sample 1	N
HBS 2	Hospital bed sample 2	+

Table 4: Samples collected for the screening of MRSA.

Exactly, 6 isolates (24.0%) were amplified with the product size of 320 bp with *MecA* gene and the other isolates were of *S.aureus* except one, coagulase negative Staphylococci. Similarly, Zinzendorf, et al. [7] was able to detect *mecA* genes in 19.3% military hospital isolates. In contrast to present study, Aziz, et al. [36] was able to detect *mecA* gene in 71.05% of MRSA isolates in Iraq.

The present study reports the presence of multidrug resistant MRSA in the hospital settings could pose the serious concern over the public health issues [37]. This study further suggests that strict hygienic measures such hand wash, judicious antibiotic use and antimicrobial testing's before prescription etc., could be followed for the effective implementation of antimicrobial stewardship program for the control of AMR pathogens in the hospital settings.

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