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2

3 **TITLE:** Prevalence of methicillin-resistant *S. aureus* isolates and their antibiotic
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5

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19 **Short Running Title:** Prevalence of methicillin-resistant *S. aureus*

20

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33 **ABSTRACT**

34

35 **Aim**

36 The broad range antibiotic resistance of *Staphylococcus aureus* (SA) is a significant
37 global threat to treatment of health care and community-associated infections. This
38 study was aimed the prevalence of methicillin-resistant SA (MRSA) and the antibiotic
39 susceptibility pattern of SA isolates in tertiary care hospitals in Bangalore, India from
40 June 2012 to February 2013.

41

42 **Method**

43 Standard methods were used to identify SA from 100 clinical swab samples (45
44 wounds, 33 nasopharyngeal, and 22 high vaginal swabs), and the Kirby-Bauer disk
45 diffusion method was used to investigate their antibiotic resistance patterns.

46

47 **Results**

48 Among the 100 samples, 45 (45%) were positive for SA, including 15 high vaginal
49 swab specimens, 16 wound swab specimens, and 14 nasopharyngeal swab
50 samples. All SA isolates were screened for methicillin resistance using the ceftioxin
51 disc diffusion test and were confirmed by conventional PCR. MRSA were resistant to
52 both amoxicillin and ampicillin (100% of isolates), kanamycin (89.6%), ciprofloxacin
53 (72.4%), cotrimoxazole (65.5%), chloramphenicol (44.8%), and gentamicin (55.1%).
54 Only 3.4% of MRSA isolates were resistant to linezolid.

55

56 **Conclusion**

57 The results indicate a problematic prevalence of MRSA and multidrug-resistant
58 MRSA in Bangalore hospitals. Linezolid is recommended as the drug of choice for
59 treatment of MRSA infections in these settings.

60

61 **Keywords:** Prevalence, MRSA, multidrug resistance, linezolid,

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65 INTRODUCTION

66 Staphylococcus aureus is a facultative anaerobic gram-positive cocci. Clinically, it is
67 the most frequently isolated human bacterial pathogen [1, 2]. *S. aureus* colonizes
68 30–50% of the human body in healthy individuals; sites include the nasal cavity, skin,
69 gastrointestinal system, anus, and vagina vulva. *S. aureus* also frequently causes a
70 broad range of illnesses, ranging from minor skin infections like pimples, impetigo,
71 and boils (furuncles) to life-threatening diseases including osteomyelitis, meningitis,
72 pneumonia, endocarditis, toxic shock syndrome, sepsis, and bacteremia [2-5]. These
73 infections are caused by hospital-associated methicillin-resistant *S. aureus* and
74 community-associated MRSA. Hospital-acquired infections typically result from
75 prolonged hospitalization, burns, trauma, chronic infection, lack of awareness, and
76 contact with colonized patients [6, 7]. A survey in Taiwan in 1998 revealed that
77 MRSA strains accounted for 84% of hospital-acquired *S. aureus* isolates and 45% of
78 non-hospital acquired *S. aureus* isolates [8].

79 The prevalence of MRSA varies geographically within nations and between
80 countries. A recent multicenter study from various regions in India reported the
81 maximum prevalence of MRSA of 60-68% in tertiary care centres in central, south,
82 and east India, and 54-57% in hospitals of west, south, and north India [9]. A study
83 from Pakistan reported the highest prevalence of MRSA in Lahore (61%), followed
84 by Karachi (57%), Rawalpindi, Islamabad (46%), Peshawar (36%), Azad Kashmir
85 (32%), and Quetta (26%), with MRSA isolated from only 2% of sites in Sukkur [10].

86 The *mecA* gene, which is located on a mobile genetic element of the staphylococcal
87 cassette chromosome (SCC), is responsible for the production of an abnormal
88 penicillin binding protein designated PBP2a. PBP2a has decreased binding affinity
89 for beta (β)-lactam antibiotics, which results in resistance to methicillin and all β -
90 lactam antibiotics, including penicillin and cephalosporin [11]. The *mecA* gene
91 complex contains insertion sites for plasmids and transposons, which promote the
92 acquisition of resistance to other antibiotics. Therefore, cross-resistance is common
93 to non- β -lactam antibiotics, such as clindamycin, erythromycin, ciprofloxacin, co-
94 trimoxazole, and gentamicin [12].

95 The treatment challenge posed by MRSA has become a serious therapeutic problem
96 worldwide. These infections are a burden for patients and healthcare systems

97 because of their associated high morbidity, mortality, and increased hospitalization
98 costs [13]. Little is known about the prevalence of MRSA in Bangalore, India. The
99 aim of the present study is to determine the prevalence of MRSA and the antibiotic
100 susceptibility of *S. aureus* isolated from different clinical specimens in tertiary care
101 hospitals in Bangalore. The goal is to identify the most appropriate antibiotic for
102 treatment of *S. aureus* infections.

103

104 **MATERIALS AND METHODS**

105

106 **Sample Collection**

107 The standard technique [14] was used to collect 100 clinical swab samples, 22 high
108 vaginal swabs, 45 wounds, and 33 nasopharyngeal sites (Table 1) from patients
109 treated in tertiary care hospitals in Bangalore from June 2012 to February 2013.
110 Swab samples were taken after ethical approval and permission from each hospital.
111 Sterile cotton-tipped swabs were used to collect the samples. Each swab was placed
112 in an Amies transport medium tube and keep the medium cool by placed in a cool
113 box when it transported to the lab according to the manufacturer's instruction of
114 (Himedia Laboratories, India).

115

116 **Microbiological Identification of Isolated *S. aureus***

117 Tryptic Soy broth (TSB; Himedia Laboratories) was used for culture enrichment.
118 Each cotton tip was broken off in a tube of TSB and the broth was vortexed for 10s
119 to release bacteria from the swab into the broth. Each sample was incubated at 37°C
120 for 48 h. A fresh sterile cotton swab was dipped into the culture and then used to
121 inoculate a mannitol salt agar (MSA; Himedia Laboratories) plate. In this procedure,
122 the swab was pressed against the tube containing the TSB culture and rotated to
123 remove excess fluid prior to streaking the entire surface of the MSA. The agar was
124 then aerobically incubated for approximately 48 h at 37 °C before examination for the
125 visual indication of the fermentation of mannitol [15]. Colonies that developed were
126 used to prepare Gram stains that were examined by light microscopy for the
127 presence of Gram-positive cocci arranged in grape-like clusters, which is a hallmark
128 of *S. aureus*. These Gram-positive colonies were inoculated into blood agar (BA;

129 Himedia Laboratories) and TSA plates, which were incubated aerobically at 35 °C for
130 48 h. After incubation, the developed colonies were examined for morphological
131 characteristics e.g. smooth, convex, circular, yellow colonies) and the presence of β -
132 haemolysis. Moreover, to confirm *S. aureus*, the coagulase and catalase tests were
133 performed using samples from BA and TSA, respectively. BA colonies were not used
134 for the catalase test, because red blood cells contain catalase, leading to a false
135 positive reaction [15, 16].

136

137 **Phenotypic and Genotypic Identification of MRSA**

138 Sterile swabs were used to obtain colonies of confirmed *S.aureus* from BA. Each
139 sample was suspended in a single vial filled with 5 ml of TSB. The turbidity was
140 adjusted to a McFarland standard of 0.5, which corresponds to 1×10^8 colony
141 forming units (CFU)/ml). Then, another sterile cotton swab was dipped into the
142 suspension and swabbed on the surface of Mueller-Hinton agar (MHA; Himedia
143 Laboratories). The plates were kept at room temperature for 15 min [17].
144 Confirmed *S. aureus* strains (n=45, 45%) were phenotypically screened for *mecA* by
145 the addition of a disc containing cefoxitin 30 μ g (Himedia Laboratories) to the surface
146 of the inoculated MHA, followed by incubation at 30°C for 24 h [18]. After the
147 incubation period, the diameters of the inhibition zones were measured. The results
148 were recorded based on interpretive criteria according to the recommendations in
149 the Clinical and Laboratory Standards Institute (CLSI) guidelines [17]. Confirmation
150 was provided by PCR-amplification of *mecA*. Bacterial genomic DNA was extracted
151 using cetyl trimethylammonium bromide [19].

152 The DNA was suspended in 200 μ l TE buffer, pH 7.4 (Promega Corporation), and
153 stored at -20°C until needed for analyses. The DNA concentration in suspension was
154 measured using a Nanodrop-100 Spectrophotometer (Thermo Fisher Scientific,
155 USA) [20]. The *mecA* forward primer (5'-GTA GAA ATG ACT GAA CGT CCG ATA
156 A) and reverse primer (5'-CCA ATT CCA CAT TGT TTC GGT CTAA) were used to
157 amplify 310 bp of *mecA* MRSA by PCR [21].
158 The 25- μ l PCR reaction mixture contained 5 μ l DNA template, 2 μ l of each primer
159 (20 μ M), 2.5 μ l of 10x buffer, 2.5 μ l $MgCl_2$, 1 μ l of Taq DNA polymerase (Bangalore
160 Genei), 4 μ l of deoxynucleoside triphosphates (DNTPS), and 6 μ l distilled water. A

161 thermal cycler (Corbett Life Science, USA) was programmed with the initial
162 denaturation, 4 min at 94°C; 30 cycles with a 45-s denaturation step at 94°C, a 45-s
163 annealing step at 56°C and a 30-s extension step at 72°C and 2 min extension step
164 at 72°C and a holding step at 4°C to amplify DNA in an Eppendorf tube (Himedia
165 Laboratories) [22]. Eight microliters of each PCR product was loaded on to a 1.0%
166 agarose gel with 2 µl ethidium bromide. Resolved bands were visualized using an
167 ultraviolet illuminator.

168

169 **Antibiotic Susceptibility Testing**

170 Susceptibility testing of MRSA isolates for eight antibiotics was performed using the
171 Kirby-Bauer disk diffusion method on MHA as recommended in the CLSI protocol
172 [17]. All antibiotic discs were obtained from Himedia Laboratories. These included
173 inhibitors of cell wall synthesis [ampicillin (10 µg/disc) and amoxicillin (, 10 µg/disc)];
174 inhibitors of protein synthesis [linezolid (30 µg/disc), gentamicin (10 µg/disc), and
175 kanamycin (30 µg/disc)]; and inhibitors of DNA synthesis [ciprofloxacin (10 µg/disc),
176 chloramphenicol (30 µg/disc), and cotrimoxazole (25 µg/disc)]. Antibiotic discs were
177 placed on MHA by using sterile forceps and were gently pressed down to ensure
178 contact. Within 15 min of the discs being placed, the plates were inverted and
179 incubated aerobically at 37°C for 18 h. *S. aureus* ATCC 43300 was used as the
180 quality control strain for disc diffusion. After incubation, the diameter of the inhibition
181 zone was measured, and the results were recorded based on interpretive criteria
182 according to the recommendations in CLSI guidelines [17].

183

184 **RESULTS**

185 Of the 100 isolates, 80 (80%) were identified to be Gram-positive cocci. Of these, 72
186 showed positive results in the catalase test for *Staphylococcus* and *Micrococcus*
187 spp. Of these 73 samples, 45 were identified as *S. aureus* based on the fermentation
188 of mannitol, which was evident on MSA as a colour change from red to yellow, and
189 on the positive result in the coagulase test. Of the 45 isolates, the cefoxitin disc
190 diffusion test identified 16 as being sensitive to methicillin (i.e. MSSA) and 29 as
191 MRSA.

192 To confirm the results of the biochemical tests, all *S. aureus* samples were analysed
193 by PCR detection of *mecA* mediated resistance (Figure 1). Thus, both analyses
194 revealed 29 (64.4%) MRSA from the *S. aureus* isolates. The 29 isolates were
195 acquired from 12 high vaginal swabs, 11 wound swabs, and 6 nasopharyngeal swab
196 specimens (Table 1).

197 The antibiotic resistance patterns of the 29 MRSA isolates are summarized in
198 (Table 2) A high level of resistance (100%) was evident for the penicillin group
199 (amoxicillin and ampicillin), the fluoroquinolones group (ciprofloxacin, 72.4%), and the
200 sulfonamide and trimethoprim group (cotrimoxazole, 65.5%). A low level of
201 resistance was evident for the phenicol group (chloramphenicol, 44.8%). Moderate
202 resistance to gentamicin (55.1%) and high resistance to kanamycin (89.6%) of the
203 aminoglycoside group were observed, and resistance to linezolid was low (one
204 isolate, 3.4%). The MRSA isolates tended to display a high level of resistance to
205 diverse antibiotics, qualifying them as multi-drug resistant (MDR) MRSA.

206

207 DISCUSSION

208 In this study, 68.1% of the isolated *S. aureus* were from the high vagina region.
209 Nkwenlag et al [23] reported that 50.9% of *S. aureus* isolates in their study were from
210 the genital area of healthy persons in hospitals in Cameroon. The prior and present
211 findings may reflect the increased susceptibility of the female anatomy to bacterial
212 colonization and infection, which could be attributed to poor personal hygiene or poor
213 health education [24]. The phenotype-based detection of MRSA in the present study
214 involved the cefoxitin disc diffusion method. The method identifies MRSA with high
215 sensitivity because of the relatively better detection of PBP2a production in *S.*
216 *aureus* strains harbouring *mecA* than the previously used oxacillin disc diffusion
217 method [18, 25, 26]. The molecular (PCR) detection results of the MRSA *mecA* gene
218 of MRSA corroborated the phenotypic analysis results; however, PCR is the
219 preferred approach because of its efficiency [27, 28]. The prospective analysis of 45
220 *S. aureus* isolates revealed the highest prevalence (64.4%) of MRSA within eight
221 months in tertiary care hospitals in Bangalore, which is higher compared to the
222 previous study conducted in other regions of India, [29] and lower than the rate of

223 38.44% reported from a hospital in Delhi, [30] and the rates of 31.1% from multiple
224 sites in Tamil Nadu [31] and 80.89% in Indore [32].

225 The collective results confirm that the prevalence of MRSA varies between different
226 parts of the country and between tertiary care centres in the same geographical
227 region. For a long time, penicillin has been the main stay for the management of a
228 variety of staphylococcal infections. However, staphylococci have gradually acquired
229 penicillin resistance. This is clearly exemplified in the current study, where 100%
230 resistance was evident against ampicillin and amoxicillin. Hanumanthappa et al [33]
231 reported the same findings in 2003 from Karnataka state. The higher prevalence of
232 antibiotic resistance in India may reflect the misuse or abuse of penicillin antibiotics.
233 In the present study, 89.6% of *S. aureus* strains were resistant to kanamycin, which
234 is lower than the result reported by Shittu et al [34]. The resistance of *S. aureus*
235 isolates to β -lactam antibiotics is related to the production of β -lactamase. The
236 enzyme hydrolyses the β -lactam ring of the relevant antibiotics, destroying their
237 antimicrobial activity [2]. Presently, the *S. aureus* isolates displayed resistance rates
238 of 55.1% and 65.5% to gentamicin and cotrimoxazole, respectively. However, in a
239 tertiary referral hospital in eastern Uttar Pradesh, India, the resistance rate to these
240 antibiotics exceeded 80%, [35, 36] while Rajaduraipandi et al [31] reported
241 resistance rates of 62% and 63.2% to gentamicin and cotrimoxazole, respectively, in
242 southern districts of Tamil Nadu. Previous studies reported MRSA isolates in India
243 that harboured SCCmec type IV or V resistance to gentamicin [37, 38]. Presently,
244 55.1% of MRSA isolates were resistant to gentamicin. The collective findings
245 indicate the community dispersion of MRSA SCCmec type IV strains in Bangalore.
246 Presently, 72.4% of the MRSA isolates displayed resistance to ciprofloxacin.
247 Previous studies reported lower rates (40%-48%) of resistance to ciprofloxacin in a
248 tertiary care hospital in India [29, 39]. The present chloramphenicol resistance rate of
249 60% is markedly different from the 38.1% reported from Nigeria by Onwubiko et al
250 [40].

251 Drug resistance could be associated with earlier exposure of these drugs to isolates,
252 which may have enhanced the development of resistance. The most effective
253 chemotherapeutic agent observed against MRSA in this study was linezolid. The
254 97.61% sensitivity observed is similar to that reported by Rajaduraipandi et al.[31] in

255 2006; however, it is somewhat lower than that reported by Hasani et al [41]. In 2013
256 in Iran. The MDR character of the MRSA isolates (Table 2) could be a result of the
257 use of inappropriate antimicrobials because of the widespread availability of
258 antimicrobials without a prescription or those prescribed by non-skilled practitioners.
259 The consequences of using these antimicrobials can be inadequate therapy and
260 further drug resistance.

261

262 CONCLUSION

263 MRSA comprised 64% of the *S. aureus* isolates, which represents a high
264 prevalence, and treatment of infections caused by *S. aureus* with β -lactam antibiotics
265 and other drug groups can be undependable. The cefoxitin disc diffusion test was
266 revealed to be an accurate phenotype-based method for the detection MRSA in a
267 laboratory where PCR is not available. The most significant principal finding was the
268 MDR nature of the *S. aureus* isolates, except for linezolid, which can be considered
269 as the drug of choice for treatment of MRSA infections. Furthermore, the incidence
270 of MRSA infection can be reduced by monitoring the antibiotic susceptibility of MRSA
271 isolates, identification of MRSA infections within the hospital, and increasing
272 awareness of MRSA in the general public. Further studies will be required to confirm
273 the present results.

274

275 CONFLICT OF INTEREST

276 The authors declare that they have no conflict of interest.

277

278 AUTHOR'S CONTRIBUTIONS

279

280 Jeza Muhamad Abdul Aziz

281 Group 1 – Substantial contributions to conception and design, Acquisition of data,
282 Analysis and interpretation of data.

283 Group 2 - Drafting the article, revising it critically for important intellectual content.

284 Group 3 -Final approval of the version to be published

285

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289 interpretation of data.
290 Group 2- Revising it critically for important intellectual content
291 Group 3- Final approval of the version to be published
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443 **TABLES**

444

445 Table: 1: Occurrence isolates *S. aureus* from clinical samples

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Clinical samples	Total. No isolated samples	S. aureus	MSSA	MRSA
High Vaginal swap	22	15(68.1%)	3(20%)	12(80%)
Wound swabs	45	16(35.5%)	5(31.25)	11(68.7%)
Nasopharyngeal swab	33	14(42.4%)	8(57.1%)	6(42.8%)
Total % Occurrence	100	45(45%)	16 (20%)	29(64.4%)

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466 Table 2: Antibiotic susceptibility tests of Staphylococcus aureus from clinical swab
 467 samples

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S.NO	Antibiotics	Con µg	MRSA n=29	
			R	S
1.	Ampicillin	10	29(100%)	0.00
2.	Amoxicillin	10	29(100%)	0.00
3.	Ciprofloxacin	10	21(72.4%)	8(27.5%)
4.	Cotrimoxazole	25	19(65.5%)	10(34.4%)
5.	Chloramphenico l	30	13(44.8%)	16(55.1 %)
6.	Gentamicin	10	16(55.1%)	13 (44.8%)
7.	Kanamycin	30	26(89.6%)	3(10.3%)
8.	Linezolid	30	1(3.4%)	28(96.5%)

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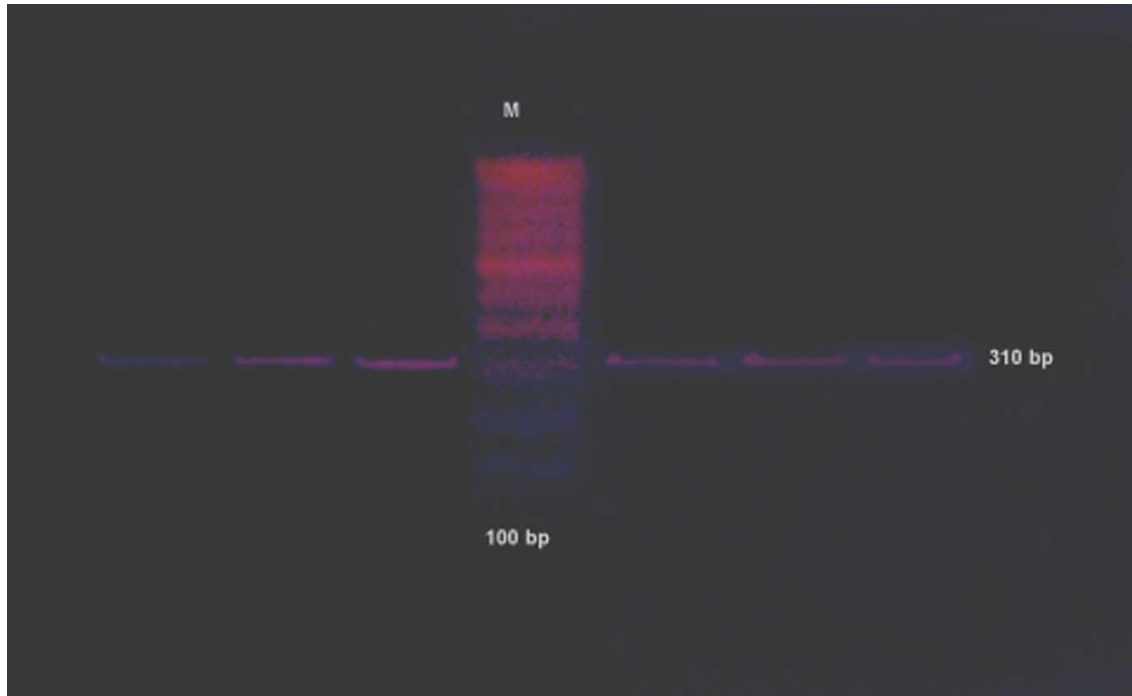
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484 **FIGURE LEGEND**

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486 Figure 1: PCR results for some of the tested isolated samples, positive bands at 310
487 bp *mecA* gene. M 100bp molecular-weight markers

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492 bp *mecA* gene. M 100bp molecular-weight markers