INTRODUCTION

Staphylococcus aureus, particularly methicillin-resistant S. aureus (MRSA), is of global concern as it can cause serious infections in both hospitals and the community [1-3]. Increasing resistance to antibiotics among staphylococcal isolates limits the choices of antibiotics available to treat infections caused by these bacteria [4]. For Gram-positive bacteria, against Methicillin-resistant Staphylococcus aureus (MRSA), ciprofloxacin (CIP) combined with vancomycin or an amino glycoside was indifferent in inhibition; however, decreased killing occurred with vancomycin combined with CIP or pefloxacin. Against Staphylococcus spp., combination with rifampin was antagonistic with CIP or pefloxacin but not enoxacin. Killing of Staphylococcus aureus was blunted when pefloxacin was combined with rifampin, but pefloxacin suppressed emergence of rifampin-resistant organisms. For Staphylococcus aureus, combination of enoxacin with oxacillin, clindamycin, or vancomycin was indifferent. For Enterococcus faecalis CIP, CIP combined with ampicillin, penicillin, or gentamicin was also indifferent, as usually was combinations of ofloxacin (OF) and amoxicillin, vancomycin, or netilmicin [5].

Fluoroquinolones are broad-spectrum antibiotics widely used in the treatment of bacterial infections such as Gram-positive cocci; however, resistance to these antibiotics has significantly increased worldwide [5,6]. Three different mechanisms of fluoroquinolones resistance have been described in staphylococci. The first is the mutation in the grlA and grlB genes that encode the subunits of DNA topoisomerase IV, the second is the mutation in the gyrA and gyrB genes that encode the subunits of DNA gyrase, and the third is an active efflux pump mediated by mutations in the norA gene [5-9]. In most cases, mutations occur in the highly conserved quinolone resistance-determining regions (QRDRs) of the gyrA and gyrB genes [10].

It is imperative to figure out whether these mutations are responsible for affecting the drug affinity and susceptibility profile of common pathogens. In the present venture, efforts were made to analyze gyrA mutations in Staphylococcus aureus that could be responsible for increased quinolone resistant mechanisms among clinical pathogens.

MATERIALS AND METHODS

This is a cross-sectional study conducted in the Department of Microbiology, Saveetha Medical College and Hospitals, Thandalam, Chennai. Ethical clearance was obtained on November 26, 2018 (number SMC/IEC/2018/11/256).

Continuous sampling method was used in the study. Samples received in the Clinical Microbiology Laboratory during the period of 6 months (December 2018 to May 2019) were included in the study. A total of 3919 samples (wound swab, pus, blood, urine, and body fluids) were received from the inpatient and outpatient department.

SAMPLE PROCESSING

Sample inoculation

The samples were inoculated on nutrient agar, blood agar, and mannitol salt agar (MSA) plates by quadrant streaking methods. The plates were then incubated at 37°C for 18–24 h and observed for the growth.
Cultural characteristics were identified by biochemical tests.

**ANTIBIOTIC SUSCEPTIBILITY TEST (ABST) BY KIRBY–BAUER DISK DIFFUSION METHOD [11]**

The Kirby–Bauer (disk diffusion) method was used to test sensitivity and resistance of antibiotics. The disks which were used included in Table 1.

Inoculums were prepared in sterile saline solution from grown culture of nutrient agar 0.5 McFarland turbidity value which was obtained for each bacterial inoculums and by sterile cotton swab was incubated on Mueller-Hinton agar, and then, antibiotic disks were placed on plates. The plates were incubated at 37°C for 18–24 h. After incubation time, inhibition zone diameters were measured and the results were interpreted according to CLSI standard.

**DETERMINATION OF CIP AND OF MINIMUM INHIBITORY CONCENTRATION (MIC) BY AGAR DILUTION METHOD [12]**

MIC of CIP and OF was determined by agar dilution method. Antimicrobial substances such as CIP and OF were used in powder form.

**DNA Extraction and Identifying gyrA Gene**

The template DNA was prepared and extracted for polymerase chain reaction (PCR) amplification through using Gram-positive bacteria. DNA extraction and extracted DNAs stored at ~20°C until needed. Polymerase chain reaction was also carried out for detecting fire (885 bp), with specific primer sequences.

**PRIMERS**

PCR primers were designed for each target gene and obtained from Eurofins Genomics, Bengaluru. Primers were designed for gyrA gene, which codes for CIP and OF resistance.

**Preparation of primers**

1. Take 2X Master Mix, forward and reverse primer (reaction mix) — 23 µl
2. Template DNA – 2 µl (forward – 1 µl and reverse – 1 µl)
3. Molecular grade water – up to 25 µl (if needed)

**Primers used in this PCR**

| Target       | Sequence                      | Amplicon |
|--------------|-------------------------------|----------|
| gyrA – Forward | 5’-GGCACCCGTTGATAAAACTGAG-‘3 | 850 bp   |
| gyrA – Reverse | 5’-ATACTTACGGCATAACCGTAG-‘3 | 850 bp   |

**Sample preparation for PCR reaction mix [13]**

A master mix (Taq TM PCR Master Mix) was used to facilitate the PCR reaction preparation and it has the advantage of including loading buffer, allowing for direct loading on electrophoresis gel after PCR amplification. Each PCR mix contained 23 of 2× PCR master mix (1 µl of forward primer and 1 µl of reverse primer), 2 µl of extracted DNA and PCR grade water. The mixture was centrifuged for few seconds and loaded in DNA Eppendorf Thermocycler.

**Template**

As template for the PCR, 2 µl of the above prepared DNA was used in a 25 µl PCR reaction.

**PCR protocol**

1. Initial denaturation – 94°C for 10 min
2. Denaturation – 94°C for 30 s, 30 cycles
3. Annealing – 53.1°C for 30 s, 30 cycles
4. Extension – 72°C for 60 s, 30 cycles
5. Final extension cycle at 72°C for 10 min

**Agarose gel electrophoresis**

1. Run 10 µl of the PCR products (you do not need to mix loading buffer for the electrophoresis in case you use the Taq Master Mix).
2. Run in parallel with a 100 bp Ladder molecular weight marker on 1.5% agarose gel in TBE 1 x. Run for 1 h at 100 V.
3. Stain the gel in ethidium bromide circa 20–30 min.
4. Destain briefly in Milli-Q water.

Take a picture in the transilluminator under UV light. Observe the bands and interpret the results.

**Statistical analysis**

Logistic regression was used to determine the effects of different prophylactic regimens on the probability of infection and to compare the levels of statistical significance of differences between regimens.

**RESULTS**

Out of 3919 samples received, 309 (7.88%) were respiratory samples, 809 (20.64%) were exudates samples, 492 (12.55%) were blood, and 2309 (58.91%) were urine samples.

Out of 91 samples from which S. aureus was isolated, majority of the samples 37 (40.65%) were from the age group 41–60 years. The least number of samples 5 (5.49%) were from the age group of 0–20 years. The highest prevalence was seen in the age group of 41–60 years.

The total numbers of *Staphylococcus aureus* isolated from various clinical samples were 91 based on morphology, cultural characteristics, and biochemical tests.

**Antibiotic susceptibility test by Kirby–Bauer disk diffusion method**

The resistance and sensitive pattern of *S. aureus* isolates is depicted in Figs. 1 and 2.

Out of 91 isolates, 77 (84.61%) were resistant to CIP and 47 (51.65%) were resistant to OF.

Out of 91 isolates, 56 (61.54%) were sensitive to cefoxitin which is MSSA (methicillin-sensitive *S. aureus*) and 35 (38.46%) were resistant to cefoxitin which is MRSA (Methicillin-resistant *S. aureus*). It shows that MSSA was predominant than MRSA in this study. Among the 91 isolates, only 6 (6.59%) were sensitive to all the antibiotics.

**Determinance of minimum inhibitory concentration of CIP and OF by agar dilution method**

The minimum inhibitory concentration of CIP and OF was done by agar dilution method of all the 91 isolates.

On treating with different dilutions (128 µg/ml, 64 µg/ml, 32 µg/ml, 16 µg/ml, 8 µg/ml, 4 µg/ml, 2 µg/ml, 1 µg/ml, 0.5 µg/ml, and 0.25 µg/ml) of CIP and OF drugs, *S. aureus* showed different inhibitory patterns. The minimum inhibitory concentration values are given in Tables 2 and 3.

The minimum inhibitory concentration by which maximum numbers of organisms were inhibited was found to be 64 µg/ml for both CIP and OF.

| S. No. | Antibiotics     | Units |
|-------|-----------------|-------|
| 1.    | Ampicillin (AMP) | 10mcg |
| 2.    | Cefazolin (CZ)  | 30mcg |
| 3.    | Cefotaxine (CTX)| 30mcg |
| 4.    | Cefotin (CX)    | 30mcg |
| 5.    | Ciprofloxacin (CIP) | 5 mcg |
| 6.    | Clindamycin (C) | 2 mcg |
| 7.    | Cotrimoxazole (CDT) | 25 mcg |
| 8.    | Erythromycin (E) | 15 mcg |
| 9.    | Gentamicin (GEN) | 10 mcg |
| 10.   | Lincomycin (LZ) | 30 mcg |
| 11.   | Ofloxacin (O)   | 5 mcg |
| 12.   | Penicillin (P)  | 10 units |
| 13.   | Tetracycline (TE) | 30 mcg |
| 14.   | Vancomycin (VA) | 5 mcg |
Confirmation with agar dilution test showed that 57 samples were resistant to CIP, 38 samples were resistant to OF, and 29 samples were resistant to both CIP and OF.

**MOLECULAR DETECTION OF GYRA GENE**

In this study, for genotypic detection of gyrA gene, five resistance isolates and two sensitive isolates were selected as follows: One from 128 µg/ml for CIP, 1 from 128 µg/ml for OF, 1 from 64 µg/ml for CIP, 1 from 64 µg/ml for OF, 1 from both CIP and OF (64 µg/ml), and 2 from both CIP and OF (1 µg/ml and 0.5 µg/ml). All the five resistant isolates were found to be positive for the presence of fluoroquinolones resistance gene (gyrA gene) and the two sensitive isolates were found to be negative (Fig. 3).

**Statistical analysis**

For S. aureus strains, vancomycin was more effective than CIP or OF (p<0.001 for all comparisons), except in the case of gyrA producing strains, which exhibited similar ID50s with CIP and vancomycin. In general, the quinolones were more active in vivo than vancomycin despite the administration of a second dose of the latter agent. The ID50s of 7 gyrA strains with CIP and OF were significantly greater than those with vancomycin. The quinolones were comparably effective among all the strains.

CIP was more effective than OF against strain S2 and S3 (p<0.0001), whereas OF was more active than CIP against strain S1 and S4 (p<0.02).

Screen for emergence of quinolone resistance during prophylaxis. To determine if and to what degree the emergence of quinolone resistance contributes to the failure of CIP and OF to prevent infection. These strains were spread on agar plates containing twice the MICs of CIP or OF for the strains. No growth on quinolone-containing media was detected.

**DISCUSSION**

In a study, Suneel Bhooshan et al., they observed 12.69% inducible clindamycin resistance in plus isolates, 10% in blood and 9.25% in isolated from the catheter. The observation of drug resistance in MRSA is leading toward the use of the last resorts of antibiotics such as vancomycin by clinicians, which can be avoided if alternate antibiotic (erythromycin and clindamycin) which has good efficacy and tissue penetration is used in the treatment [14].

In agreement with many other studies, the results showed that all the fluoroquinolone-resistant isolates had a single mutation at codon 80 in the gyrA gene and a mutation at codons 84 in the gyrA gene [13,15,16]. Among the 69 tested isolates, 23 had an additional mutation at codon 106 and 1 had a mutation at codon 90 in the gyrA gene. The mutation at codon 106 has also been reported in a few studies, but its effect on resistance was reported to be unknown [17]. According to our literature review, the point mutation at codon 90 (Tyr to Ser) in the gyrA gene has not been reported previously and further studies are needed to determine its effect on fluoroquinolone resistance. In the current study, all the fluoroquinolone-resistant isolates had a mutation at codon 86 in the gyrA gene; this point mutation is a silent mutation and has already been reported by others [18,19]. Our results are consistent with those of others who found that the same mutations in the QRDRs were detected in different PFGE types, and different combinations of mutations were also found in the isolates of the same PFGE type [11,19,20].

Fluoroquinolone resistance has posed a serious challenge to the Iranian medical community. This study aimed to characterize the phenotypic and genotypic resistance to fluoroquinolone among S. aureus isolates in Saveetha Medical College, Chennai. In our study, 38.46% of the MRSA and 61.54% of the MSSA isolates were found. Our study results similar from that of Emaneini where they have used 152 Staphylococcus aureus isolates. Among them, MRSA were found to be less than MSSA [21].
Antibiotic resistance due to widespread use of antibiotics is a major concern. Fluoroquinolone use in particular is associated with a high rate of bacterial antibiotic resistance. Several studies have demonstrated an association between increased systemic fluoroquinolone use and resistance in *S. aureus*. For example, the study of Miller et al. MRSA isolated from anybody site increased with the use of systemic fluoroquinolones in a study of French hospitals, and systemic fluoroquinolone use has been associated with higher colony counts of nasal MRSA. Various risk factors have been found to be associated with antibiotic resistance in monocular bacterial isolates. In the present study, *Staphylococcus aureus* isolates, 77 samples were resistant to CIP and 44 samples were resistant to OF. In Blumberg et al., resistance to CIP was 100% [12,22].

The aim of Touaiia et al. study was to investigate the methicillin resistance gene and some virulence factors in MRSA isolates by PCR. The obtained results showed that MRSA isolates exhibited low resistance to chloramphenicol and trimethoprim/sulfamethoxazole. This supports the potential utility of chloramphenicol and trimethoprim/sulfamethoxazole as empiric treatment agents for MRSA in Algeria.

This finding highlights the importance of modified empiric therapy and infection control policies in Saveetha Hospitals in Chennai. The results of this study show a fluoroquinolone resistance rate of 81% among *S. aureus* isolates, which was higher than 13–20.7% reported by similar investigations [12,22]. Many studies have shown that 1 or 2 point mutations in the QRDRs region of both *gyrA* and *gyrB* genes are the main mechanisms of fluoroquinolone resistance, in particular CIP resistance, among *S. aureus* isolates [23,24].

The present study was that bacterial target of the quinolones is desosylriburacil (DNA) gyrase, an essential bacterial enzyme. Likewise studies by (Tanaka et al.) DNA gyrase has been most extensively studied in *Escherichia coli*, but DNA gyrase has also been purified from *Micrococcus luteus*, *Bacillus subtilis*, *Haemophilus influenzae*, *Citrobacter freundii*, and *Pseudomonas aeruginosa*, *Staphylococcus aureus*. Except as otherwise indicated, the information discussed here refer to studies of *Staphylococcus aureus*.

Additional limitations include that as dosing, local immunity, and patient compliance are not taken into account. However, in vitro studies are considered the standard in determining antibiotic resistances. With the introduction of broad-spectrum antibiotics such as the fluoroquinolones, community is more comfortable empirically treating smaller uers without laboratory support [25,26].

Amplification of partial sequences of the QRDRs in *gyrA* and *gyrB* genes in the same 12 MRSA isolates was done in an attempt to assess the association of mutations in *gyrA* and *gyrB* genes with fluoroquinolone resistance. Similar mutation at the same position in *GyrA* (S84L) was previously reported by Schmitz et al. 2007. In addition, three other mutations: E88K, G106D and S112R the same silent mutation at 186; and another silent mutation at F110 were recorded by the same study. In Wang et al. study, mutations in similar or neighboring positions were also recorded in other studies: S84L, S84A, S85P and E88K in *GyrA*, and D437N, R458Q in *GyrB*. Mutations conferring quinolone resistance in the *gyrA* gene of *E. coli* were also confined to that region [24].

In this study, out of the 91 isolates tested, five resistant samples and two sensitive samples were selected as follows: One from 128 µg/ml for CIP, 1 from 64 µg/ml for OF, 1 from 64 µg/ml for OF, 1 from both CIP and OF (64 µg/ml), and 2 sensitive samples from both CIP and OF (1 µg/ml and 0.5 µg/ml) and subjected for DNA extraction and amplification. The samples were subjected to genotypic detection of *gyrA* gene (fluoroquinolone resistant). All the five resistant samples were found to be positive for the presence of fluoroquinolone resistance gene (*gyrA* gene) and the two sensitive samples were found to be negative.

**CONCLUSION**

The result from this study will be useful to update the antibiotic policy in our hospital setup and controlling the irrational use of antibiotics among health care workers. The information obtained will provide a baseline data that can be used to design further research for the prevention of drug resistance caused by *Staphylococcus aureus*.

**FUNDING**

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**CONFLICTS OF INTEREST**

None declared.

**ETHICAL APPROVAL**

The study was approved by the Institutional Ethics Committee.

**AUTHORS’ CONTRIBUTIONS**

Shelina Nameirakpam made substantial contributions to conception, acquisition of data, took part in drafting the article, or revising it critically for important intellectual content, S. S. M. Uma Mageswari made statistical analysis and final approval of the version to be published, and agreed to be accountable for all aspects of the work.

**REFERENCES**

1. Brumfit W, Hamilton-Miller JM. The worldwide problem of methicillin-resistant *Staphylococcus aureus*. Drugs Exp Clin Res 1990;16:205-14.
2. Emaneini M, Taherikalani M, Esfampour MA, Sedaghat H, Aligholi M, Jabalameli F, et al. Phenotypic and genotypic evaluation of aminoglycoside resistance in clinical isolates of *staphylococci* in Tehran, Iran. Microb Drug Resist 2009;15:129-32.
3. Nicholas BD, Bhargave G, Hatipoglu A, Heffelfinger R, Rosen M, Pribitkin EA. Preoperative prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) colonization in patients undergoing intranasal surgery. Med Sci Monit 2010;16:CR365-8.
4. Chambers HF. Methicillin resistance in *staphylococci*: Molecular
and biochemical basis and clinical implications. Clin Microbiol Rev 1997;10:781-91.
5. Hooper DC. Fluoroquinolone resistance among gram-positive cocci. Antimicrob Agents Chemother 1996;40:1079-85.
6. Schnitz J, Higgins PG, Mayer S, Fluit AC, Dalhoff A. Activity of quinolones against gram-positive cocci: Mechanisms of drug action and bacterial resistance. Eur J Clin Microbiol Infect Dis 2002;21:647-59.
7. Horii T, Suzuki Y, Takeshtaa A, Maekawa M. Molecular characterization of 8-methoxyfluoroquinolone resistance in a clinical isolate of methicillin-resistant Staphylococcus aureus. Chemotherapy 2007;53:104-9.
8. Tanaka M, Wang T, Onodera Y, Uchida Y, Sato K. Mechanism of quinolone resistance in Staphylococcus aureus. J Infect Chemother 2000;6:131-9.
9. Tanaka M, Onodera Y, Uchida Y, Sato K. Quinolone resistance mutations in the GrlB protein of Staphylococcus aureus. Antimicrob Agents Chemother 1998;42:3044-6.
10. Iihara H, Suzuki T, Kawanura Y, Ohkusu K, Inoue Y, Zhang W, et al. Emerging multiple mutations and high-level fluoroquinolone resistance in methicillin-resistant Staphylococcus aureus isolated from ocular infections. Diagn Microbiol Infect Dis 2006;56:297-303.
11. Noguchi N, Okihara T, Namiki Y, Kumaki Y, Yamanaka Y, Koyama M, et al. Susceptibility and resistance genes to fluoroquinolones in methicillin-resistant Staphylococcus aureus isolated in 2002. Int J Antimicrob Agents 2005;25:374-79.
12. Blumberg HM, Rimland D, Carroll DJ, Terry P, Wachsmuth IK. Rapid development of ciprofloxacin resistance in methicillin-susceptible and-resistant Staphylococcus aureus. J Infect Dis 1991;163:1279-85.
13. Mahon CR, Lehman DC, Manuelsin G. Text Book of Diagnostic Microbiology. 3rd ed. Philadelphia, PA, USA: Saunders; 2007.
14. Bhooshan S, Prasad J, Dutta A, Ke V, Mukhopadhyay C. Reduced susceptibility of mrsa to vancomycin. Int J Pharm Pharm Sci 2017;10:59-61.
15. Fatholahzadeh B, Emameini M, Gilbert G, Udo E, Aligholi M, Modarressi MH, et al. Staphylococcal cassette chromosome mec (SCCmec) analysis and antimicrobial susceptibility patterns of methicillin-resistant Staphylococcus aureus (MRSA) isolates in Tehran, Iran. Microb Drug Resist 2008;14:217-20.
16. Reisner SB, Woods GL, Thomson RP. Specimen collection. In: Murray PR, Baron EJ, Pfaffer MA, editors. Manual of Clinical Microbiology. 7th ed. Washington, DC: American Society for Microbiology; 1999. p. 64-76.
17. Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing. Twenty-first Informational Supplement. United States: Clinical and Laboratory Standards Institute; 2011. p. 31.
18. Fatholahzadeh B, Emameini M, Aligholi M, Gilbert G, Taherikalani M, Jonaidi N, et al. Molecular characterization of methicillin-resistant Staphylococcus aureus clones from a teaching hospital in Tehran. Jpn J Infect Dis 2009;62:309-11.
19. Horii T, Suzuki Y, Monji A, Morita M, Muramatsu H, Kondo Y, et al. Detection of mutations in quinolone resistance-determining regions in levofloxacin-and methicillin-resistant Staphylococcus aureus: Effects of the mutations on fluoroquinolone MICs. Diagn Microbiol Infect Dis 2003;46:139-45.
20. Schnitz JF, Jones ME, Hofmann B, Hansen B, Scheuring S, Lückefahr M, et al. Characterization of grlA, grlB, gyrA, and gyrB mutations in 116 unrelated isolates of Staphylococcus aureus and effects of mutations on ciprofloxacin MIC. Antimicrob Agents Chemother 1998;42:1249-52.
21. Emameini M, Aligholi M, Hashemi FB, Jabalameli F, Shahsavani S, Dabiri H, et al. Isolation of vancomycin resistant Staphylococcus aureus in a teaching hospital in Tehran. J Hosp Infect 2007;66:92-3.
22. Marangon FB, Miller D, Maulllem MS, Romano AC, Alfonso EC. Ciprofloxacin and levofloxacin resistance among methicillin-sensitive Staphylococcus aureus isolates from keratitis and conjunctivitis. Am J Ophthalmol 2004;137:453-8.
23. Coskun-Ari FF, Bosphelmez-Tinaz G. grlA and gyrA mutations and antimicrobial susceptibility in clinical isolates of ciprofloxacin-resistant methicillin-resistant Staphylococcus aureus. Eur J Med Res 2008;13:366-70.
24. Takahata M, Yonezawa M, Kurose S, Futakuchi N, Matsubara N, Watanabe Y, et al. Mutations in the gyrA and grlA genes of quinolone-resistant clinical isolates of methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother 1996;38:543-6.
25. Yoon EJ, Lee CY, Shim MJ, Min YH, Kwon AR, Lee J, et al. Extended spectrum of quinolone resistance, even to a potential latter third-generation agent, as a result of a minimum of two GrlA and two GyrA alterations in quinolone-resistant methicillin-resistant Staphylococcus aureus. J Antimicrob Chemother 1996;38:543-6.
26. Wang T, Tanaka M, Sato K. Detection of grlA and gyrA mutations in 344 Staphylococcus aureus strains. Antimicrob Agents Chemother 1998;42:236-40.
27. Touaitia R, Bektache S, Boutefhouechet N, Djahoudi A, Bachtarzi M. Molecular characterization of methicillin-resistant Staphylococcus aureus isolated from clinical cases in east Algeria. Asian J Pharm Clin Res 2017;10:59-61.