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Research Article

PHENOTYPIC AND GENOTYPIC CHARACTERIZATION OF FLUOROQUINOLONE DRUG RESISTANCE AMONG *STAPHYLOCOCCUS AUREUS* IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Objective: The objective of the study was to determine whether recent use of topical fluoroquinolones is a risk factor for *in vitro* fluoroquinolone resistance in *Staphylococcus aureus* ocular isolates. Fluoroquinolone antibiotics such as ciprofloxacin (CIP) are useful drugs against infections caused by *Staphylococcus aureus* and mutations in deoxyribonucleic acid (DNA) gyrase which control bacterial DNA topology, can be one of the reasons of occurrence resistance to this class of antibiotics. Therefore, finding new mutations and study of the quinolone interaction with mutated GyrA can provide important issues for explanation resistance.

Methods: Bacterial identification was confirmed by appropriate morphological, cultural, and biochemical tests. The antibiotic susceptibility pattern was determined for all isolates. The possible involvement of efflux pumps in mediating fluoroquinolone resistance as well as changes in the quinolone resistance-determining region (QRDR) of *gyrA* gene was investigated.

Results: Differences in methicillin resistance among staphylococci were observed based on patient age, with higher rates observed in older patients ($p<0.0001$). Out of 91 isolates, 77 (84.61%) were resistant to CIP and 47 (51.65%) were resistant to ofloxacin (OF). 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. By polymerase chain reaction (PCR) testing, *gyrA* genes in resistance strains were amplified. All the five resistant isolates were found to be positive for the presence of *a fluoroquinolones resistance gene* (*gyrA* gene) and the two sensitive isolates were found to be negative. Resistance among CIP and OF in *isolated* harboring *a mutation Gyr*A was of statistical significance among S. aureus ($p<0.001$).

Conclusion: The result of this study will be useful to update the antibiotic policy in our hospital set up 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 prevention of drug resistance caused by *Staphylococcus aureus.*

Keywords: Fluoroquinolones, *Staphylococcus aureus, gyrA* gene, Ciprofloxacin, Ofloxacin and polymerase chain reaction.

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INTRODUCTION

Staphylococcus aureus, particularly methicillin-resistant *S. aureus* (MRSA), is of great 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 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 *grlA* and *gyrA* 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 gyrase mutations in *Staphylococcus aureus* that could be responsible for increased quinolone resistive 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 ul
2. Template DNA – 2 ul (forward – 1 ul and reverse – 1 ul)
3. Molecular grade water – up to 25 ul (if needed)

Primers used in this PCR

Target	Sequence	Amplicon
gyrA – Forward	5'- GCCACCGTTGTATAAACTGAC- '3	850 bp
gyrA – Reverse	5'- ATACCTACCGCGATACCTGATG- '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.

Table 1: List of antibiotics used in the study

S. No.	Antibiotics	Units
1.	Ampicillin (AMP)	10mcg
2.	Cefazolin (CZ)	30 mcg
3.	Cefotaxime (CTX)	30 mcg
4.	Cefoxitin (CX)	30 mcg
5.	Ciprofloxacin (CIP)	5 mcg
6.	Clindamycin (CD)	2 mcg
7.	Cotrimoxazole (COT)	25 mcg
8.	Erythromycin (E)	15 mcg
9.	Gentamicin (GEN)	10 mcg
10.	Linezolid (LZ)	30 mcg
11.	Ofloxacin (OF)	5 mcg
12.	Penicillin (P)	10 units
13.	Tetracycline (TE)	30 mcg
14.	Vancomycin (VA)	5 mcg

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° 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×. 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.

Determination 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.

Table 2: MIC of ciprofloxacin showing resistant to different concentrations

Values (µg/ml)	128	64	32	16	8	4	2	1	0.5	0.25
No. of isolates	12	24	6	5	7	3	3	17	6	8

Table 3: MIC of ofloxacin showing resistant to different concentrations

Values (µg/ml)	128	64	32	16	8	4	2	1	0.5	0.25
No. of isolates	3	11	10	6	4	4	2	25	17	9

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 *grlA* 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

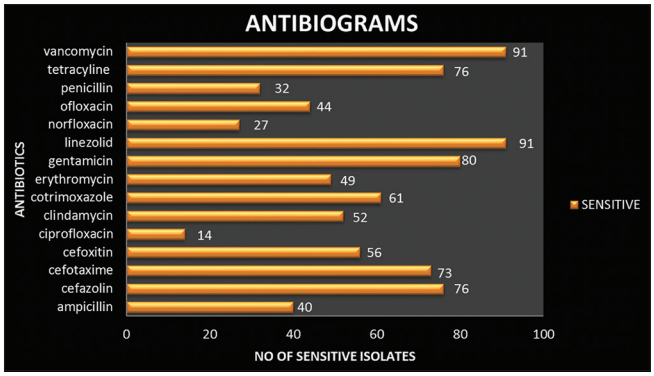


Fig. 1: Sensitive pattern of *Staphylococcus aureus* isolates

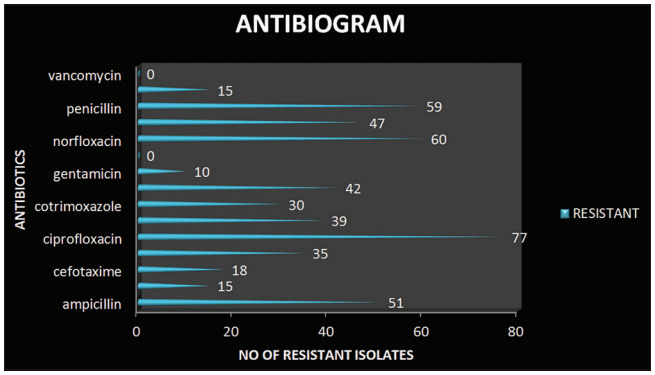
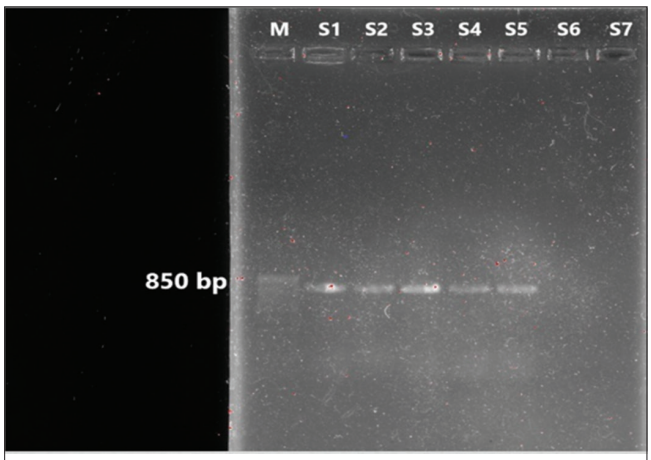


Fig. 2: Resistance pattern of *Staphylococcus aureus* isolates



- Lane M- DNA ladder
- Lane 1, 2, 3, 4 and 5 -Isolates positive for gyrA gene (850 bp)
- Lane 6 and 7 - Isolates positive for gyrA gene (850 bp)

Fig. 3: Genotypic detection of gyrase a gene sequence

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 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 Touaitia *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 *grlA* and *gyrA* 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 deoxyribonucleic acid (DNA) gyrase, an essential bacterial enzyme. Likewise studies by (Tanaka *et al*.,) DNA gyrase has been most extensively studied in *Escherichia coli*, but DNA gyrases have 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 ulcers 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 I86 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 and R458Q in GyrB. Mutations conferring quinolone resistance in the *gyrA* gene of *E. coli* were also confined to that region [26].

The aim of this present study was identification of amino acid mutations in GyrA protein encoding subunit A of DNA gyrase of *Staphylococcus aureus* which is isolated from different clinical infections and studying the influence of identified mutations on the structure of *GyrA* gene for interaction with CIP and OF. *S. aureus* mutations occur first in the *par*C (topoisomerase IV) gene, leading to moderate levels of resistance to fluoroquinolones (MIC: 8 µg/ml).

Another mechanism involved in quinolone resistance in *S. aureus* is overexpression of *nor*A gene. This gene encodes a multidrug efflux protein (NorA) capable of transporting fluoroquinolones outside the bacteria. Studies have shown that a mutant of *S. aureus* with a knockout in the *nor*A gene – coding for the MDR pump – has a substantially increased sensitivity to a large number of antimicrobials, including therapeutically significant compounds [20]. As inhibitors of the NorA efflux pump, reserpine, omeprazole, and lansoprazole can improve fluoroquinolone activity against strains expressing different levels of NorA.

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 I86; 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 inGyrA, 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 CIP, 1 from 128 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

No funding sources.

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.

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