

Original Research Article

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Mutations within the Quinolone Resistance Determining Region in Fluoroquinolone-Resistant *Staphylococcus epidermidis* Recovered from Different Ocular Isolates

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ABSTRACT

Staphylococcus epidermidis (*S. epidermidis*) is a common pathogen in ocular infection. Mutations contribute to drug resistance. We intended to identify mutations in genes within the quinolone resistance determining region (QRDR) of fluoroquinolone-resistant *S. epidermidis* ocular isolates and to study their phenotypic and genotypic correlation. A total of 50 phenotypically fluoroquinolones-resistant *S. epidermidis* isolates were studied. Fluoroquinolones susceptibility was evaluated by Kirby- Bauer disk diffusion method. Polymerase chain reaction (PCR) was optimized and applied followed by DNA sequencing to detect mutations in *gyrA*, *gyrB*, *parC* and *parE* in the QRDR region among the fluoroquinolone-resistant *S. epidermidis* isolates recovered from ocular specimens. The majority of the samples (74%) were from conjunctival swabs ($n = 37$). *gyrA*, *gyrB*, *parC*, and *parE* genes were detected in 47 samples (94%). *gyrA* gene ($n = 47$) was the most common, followed by *parE* ($n = 35$), *gyrB* ($n = 30$) and *parC* ($n = 28$). In 25 isolates, all four mutated genes were present. In 25(50%) *S. epidermidis* isolates mutations were observed in all four genes of QRDR region of *S. epidermidis* genome. This is the first study in a tertiary eye care hospital in India to characterise ocular *S. epidermidis* for fluoroquinolone resistance which showed mutations were predominant in *gyrA* gene in the QRDR region compared to 3 other genes.

Keywords

Staphylococcus epidermidis (*S. epidermidis*),
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Introduction

Staphylococcus epidermidis (*S. epidermidis*) is a most common cause of keratitis and endophthalmitis (O'Brien *et al.*, 1995; Graves *et al.*, 2001).

Fluoroquinolones are the drugs of choice based on their good safety profile, excellent penetration into aqueous and vitreous humor, long duration of tear concentration, and broad

spectrum antimicrobial activity (Neu, 1991; Leibowitz, 1991). However, continued use in the population has contributed to emergence of drug resistance (Chalita *et al.*, 2004; Goldstein, 1999). The incidence of resistance has been steadily increasing. Resistance mechanisms include mutations of DNA gyrase and topoisomerase, decreased outer membrane permeability, or the development of changes in the mechanism of efflux pumps.

The primary targets are the two essential enzymes, DNA gyrase and topoisomerase IV (Dubin *et al.*, 1999; Li *et al.*, 1998). In *S. epidermidis*, DNA gyrase is composed of the GyrA and GyrB subunits encoded by the *gyrA* and *gyrB* genes, respectively.

Topoisomerase IV is composed of ParC and ParE subunits encoded by *parC* and *parE* genes, respectively. Mutated *gyrA*, *gyrB*, *parC* and *parE* genes within the quinolone resistance determining region (QRDR) are known to be responsible for clinically evident resistance of bacteria to fluoroquinolones.

Although there are numerous studies which have elucidated this phenomenon in case of *Staphylococcus aureus*, only a few studies have done the same with respect to *Staphylococcus epidermidis*, the bacterium of interest in this study.

Materials and Methods

The study was carried out using the *S. epidermidis* strains isolated from ocular specimens in the L&T Microbiology Research centre (SNSC) Chennai from December 2017 to July 2018. *S. epidermidis* isolates were obtained from various ocular samples like conjunctival swab, corneal scraping, lacrimal pus, bondage contact lens (BCL) & intraocular specimens. *S. epidermidis* was identified using standard microbiological procedures.

The Kirby-Bauer Disk Diffusion method (KBBD) was carried out for antimicrobial susceptibility testing as per CLSI guidelines 2014 for ciprofloxacin, moxifloxacin, gatifloxacin, norfloxacin and gatifloxacin.

S. epidermidis isolates were also classified as methicillin-susceptible or methicillin-resistant based on oxacillin susceptibility, using clinical and laboratory standard institute-defined break points. The fluoroquinolone resistance group

was defined as *S. epidermidis* showing resistance to any one of following tested fluoroquinolones: ciprofloxacin, moxifloxacin, norfloxacin, ofloxacin and gatifloxacin

A total of 50 fluoroquinolone-resistant *Staphylococcus epidermidis* isolates from various ocular specimens (37 from conjunctival swabs, 6 from corneal scrapings, 5 from canalicular pus, 2 from bandage contact lens) were included in the study.

Optimization of PCR targeting the genes of QRDR region

DNA extraction method

The boiling method was used to extract DNA from the bacteria. (Ali A Dashti *et al.*, 2009). Two to three morphologically identical colonies were picked up by just touching the colonies with a sterile loop from a pure culture of *S. epidermidis* and suspended in 50 µl of sterile water and heated at 100°C. for 15 minutes. After centrifugation in a micro centrifuge (6, 000 × g for 3 min), the supernatant containing the DNA, was stored at -20°C for further use.

Sensitivity and specificity and optimization of PCR

Primers were designed for detection of *gyrA*, *gyrB*, *parC* and *parE* gene targeting the QRDR region. PCR was optimised using these primers. Sensitivity and specificity were carried out using the primers mentioned below. PCR was found to be sensitive to detect DNA concentration of 120 pico gram for *gyrA*, *gyrB* and *parC* gene and 120 femto gram for *parE* gene.

Details of Primers used for detection of *gyrA*, *gyr B*, *parC* and *parE* gene targeting the QRDR region by PCR with the amplicon size in (Table 1).

DNA amplification and sequencing of QRDR

The PCR conditions for *Staphylococcus epidermidis* were as follows: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 60 s, followed by an elongation step at 72°C for 5 min. The PCR products of *gyrA* gene (284 bp), *gyrB* gene (251 bp), *parC* (197 bp) and *parE* (324 bp) were visualized by agarose gel electrophoresis, using ethidium bromide incorporated in the agarose gel.

PCR based DNA sequencing

PCR products were purified using ExoSAP according to the manufacturer's instructions (Fermentas LIFE SCIENCES). PCR-amplified product was sequenced by the dye terminator method (AB applied biosystems) in both the forward and reverse directions. Sample sequences were compared with a reference sequence and mutations were detected. The strain *S. epidermidis* ATCC 35984 (RP62A) was used as a reference. Sequences were edited using the software SeqMan (Lasergene Software package) and then aligned against the reference *S. epidermidis* RP62A sequence from GenBank using the "blastx program" with automatically adjusted parameters.

Results and Discussion

In this study, out of the 50 fluoroquinolone-resistant *Staphylococcus epidermidis* were included, 37 were isolated from conjunctival swab (74%), followed by 6 from corneal scraping, 5 from canalicular pus and 2 from Bandage contact lens (BCL). Thirty isolates were Methicillin resistant and 20 were Methicillin sensitive (Table 2).

gyrA, *gyrB*, *parC* and *parE* genes in the QRDR region was detected in 47 isolates (94%). Mutations in *gyrA* gene ($n = 47$) was

present in all the resistant isolates, followed by *parE* ($n = 35$), *gyrB* ($n = 30$) and *parC* ($n = 28$) mutations. In 25 isolates, all four genes were present. In this study, 30 (60%) of fluoroquinolone resistant strains were MRSE which also is a useful information (Fig. 1–12).

To determine the contribution of mutation in QRDR which attributes FQ resistance, sequencing of *gyrA*, *gyrB*, *parC* and *parE* patterns were done. When the DNA sequence of the *gyrA*, *gyrB*, *parC* and *parE* patterns were compared with the sequence of *S. epidermidis* RP62A, it revealed nucleotide differences at many positions.

The genes that were studied (*gyrA*, *gyrB*, *parC* and *parE*), when mutated give rise to resistance in isolates of *S. epidermidis*. The present study included fluoroquinolone resistant isolates of *S. epidermidis* recovered from the ocular samples.

Of the 50 resistant isolates, it was inferred that 94% of them were due to mutated genes (any one or more of the above) while the remainder were purportedly due to mechanisms like decreased outer membrane permeability, or the development of efflux pumps, as have been mentioned previously (Iihara *et al.*, 2006; Noguchi *et al.*, 2005).

The primary targets of fluoroquinolones are two essential enzymes of bacterial cells, DNA gyrase and topoisomerase IV.

In most bacterial species the mutations in the genes that lead to fluoroquinolone resistance are limited to a few point mutations at restricted positions of the genes called QRDR.

The present study revealed that approximately 97% of *S. epidermidis* isolates in the human conjunctival flora have mutation(s) in the QRDR area of *gyrA*, *gyrB*, *parC* and *parE* genes (Table 3 and Fig. 13–19).

Table.1 Primers for *Staphylococcus epidermidis*

Target gene	Primer sequence (5` to 3`)	Product size (bp)
<i>gyrA</i>	ATGCGTGAATCATTCTTAGACTATGC GAGCCAAAGTTACCTTGACC	284
<i>gyrB</i>	CAGCATTAGACGTTTCAAG CCAATACCCGTACCAAATGC	251
<i>parC</i>	TCGCAATGTATTCAAGTGGG ATCGTTATCGATACTACCATT	197
<i>parE</i>	AAGCTCAACAAGCACGCGAGGCTG TTAAAGTCAGTACCAACACCAGCAC	324

Table.2 Clinical specimens showing isolation rates from different clinical samples

Types of samples	Isolates of <i>Staphylococcus epidermidis</i>	Methicillin resistant isolates (n = 30)
Conjunctival swabs	37	24
Corneal scrapings	6	3
Canalicular pus specimens	5	2
Bandage contact lens (BCL)	2	1
Total	50	30

Table.3 Sensitivity, specificity and detection of *gyrA*, *gyrB*, *parC*, *parE* of *S.epidermidis*

PCR targeting QRDR region	Sensitivity	Specificity
<i>gyrA</i>	120 pico gram	All four sets of primers were specific to amplify only <i>S. epidermidis</i> DNA.
<i>gyrB</i>	120 pico gram	
<i>parC</i>	120 pico gram	
<i>parE</i>	120 femto gram	

Fig.1 Agarose gel electrophoretogram showing sensitivity of the *gyrA* primer (*S. epidermidis*)



Detection of *gyrA* gene (*S. epidermidis*) (284 bp) by Polymerase Chain Reaction. Schematic representation of agarose gel (1%) showing the (284 bp) amplified products by conventional polymerase chain reaction.

NC: Negative control

Lane 1: Neat DNA

Lanes 2-11: 10 fold serial dilutions of *Staphylococcus epidermidis* DNA bp band found till 10⁴ (120 pico gram)

Mwt: Molecular weight marker (100 bp ladder)

Fig.2 Agarose gel electrophoretogram showing sensitivity of the *gyrB* primer (*S. epidermidis*)



Detection of *gyrB* gene (*S. epidermidis*) (251 bp) by Polymerase Chain Reaction. Schematic representation of agarose gel (1%) showing the (251 bp) amplified products by conventional polymerase chain reaction.

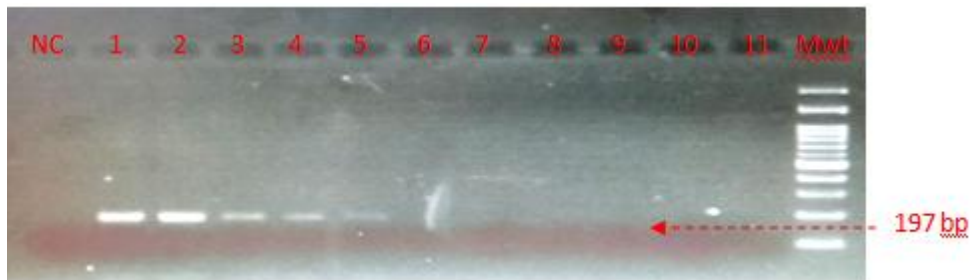
NC: Negative control

Lane 1: Neat DNA

Lanes 2-11: 10 fold serial dilutions of *Staphylococcus epidermidis* DNA bp band found till 10^4 (120 pico gram)

Mwt: Molecular weight marker (100 bp ladder)

Fig.3 Agarose gel electrophoretogram showing sensitivity of the *parC* primer (*S. epidermidis*)



Detection of *parC* gene (*S. epidermidis*) (197 bp) by Polymerase Chain Reaction. Schematic representation of agarose gel (1%) showing the (197 bp) amplified products by conventional polymerase chain reaction.

NC: Negative control

Lane 1: Neat DNA

Lanes 2-11: 10 fold serial dilutions of *Staphylococcus epidermidis* DNA bp band found till 10^4 (120 pico gram)

Mwt: Molecular weight marker (100 bp ladder)

Fig.4 Agarose gel electrophoretogram showing sensitivity of the *parE* primer (*S. epidermidis*)



Detection of *parE* gene (*S. epidermidis*) (324 bp) by Polymerase Chain Reaction. Schematic representation of agarose gel (1%) showing the (324bp) amplified products by conventional polymerase chain reaction.

NC: Negative control

Lane 1: Neat DNA

Lanes 2-11: 10 fold serial dilutions of *Staphylococcus epidermidis* DNA bp band found till 10^7 (120 femto gram)

Mwt: Molecular weight marker (100 bp ladder)

Fig.5 Agarose gel electrophoretogram showing specificity of the *gyrA* PCR primers (*S.epidermidis*)



NC: Negative control, Lane 1: *S. aureus* ATCC 25923, Lane 2: *Bacillus subtilis* lab isolate, Lane 3: *Escherichia coli* ATCC 25922, Lane 4: *P. aeruginosa* ATCC27853, Lane 5: *Streptococcus viridans* lab isolate, Lane 6: *Streptococcus pneumoniae* lab isolate, Lane 7: *Enterococcus faecalis* lab isolate, Lane 8: *Streptococcus pyogenes* ATCC 12384, Lane 9: *Nocardia spp.* lab isolate, Lane 10: Human DNA, PC: Positive Control DNA, Mwt: 100 bp molecular weight marker

Fig.6 Agarose gel electrophoretogram showing specificity of the *gyrB* PCR primer (*S. epidermidis*)



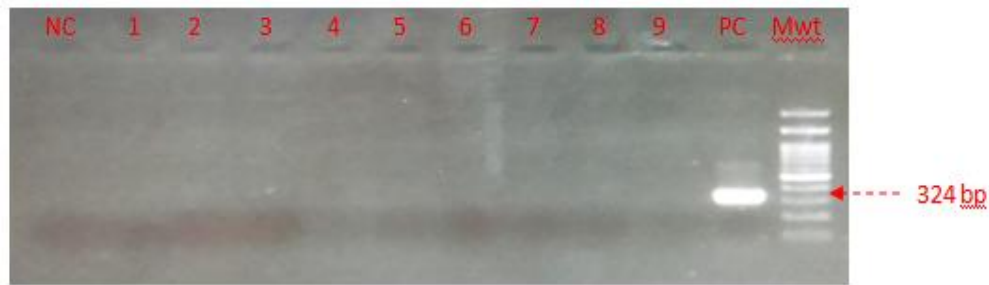
NC: Negative control, Lane1: *S. aureus* ATCC 25923, Lane 2: *Bacillus subtilis* lab isolate, Lane 3: *Escherichia coli* ATCC, Lane 4: *P. aeruginosa* ATCC, Lane 5: *Streptococcus viridans* lab isolate, Lane 6: *Streptococcus pneumoniae* lab isolate, Lane 7: *Enterococcus faecalis* lab isolate, Lane 8: *Streptococcus pyogenes* ATCC 12384, Lane: 9: *Nocardia spp* lab isolate, Lane 10: Human DNA, PC: Positive Control DNA, Mwt: 100 bp molecular weight marker

Fig.7 Agarose gel electrophoretogram showing specificity of the *parC* primer (*S. epidermidis*)



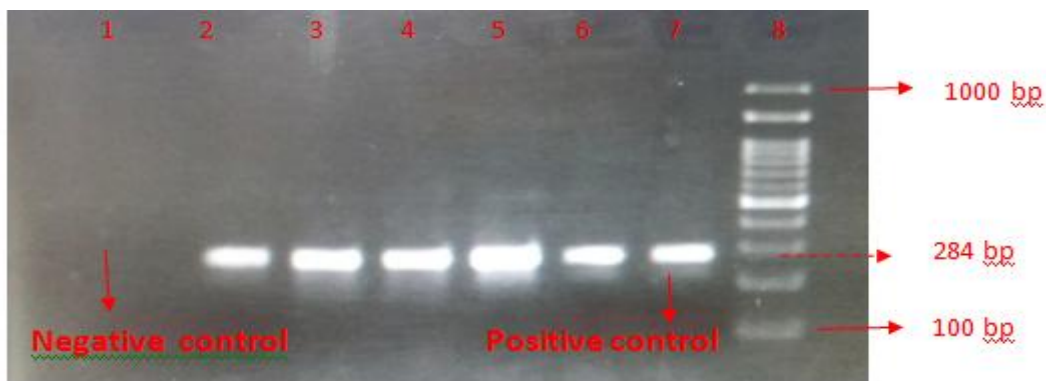
NC: Negative control, Lane 1: *S. aureus* ATCC 25923, Lane 2: *Bacillus subtilis* lab isolate, Lane 3: *Escherichia coli* ATCC 25922, Lane 4: *P. aeruginosa* ATCC 27853, Lane 5: *Streptococcus viridans* lab isolate, Lane 6: *Streptococcus pneumoniae* lab isolate, Lane 7: *Enterococcus faecalis* lab isolate, Lane 8: *Streptococcus pyogenes* ATCC 12384, Lane 9: *Nocardia spp.* lab isolate, Lane 10: Human DNA, PC: Positive Control DNA, Mwt: 100 bp molecular weight marker

Fig.8 Agarose gel electrophoretogram showing specificity of the *parE* primer (*S. epidermidis*)



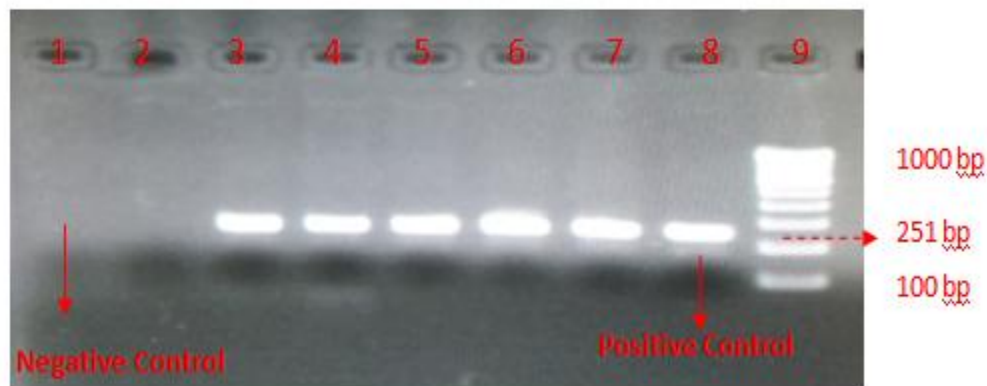
NC: Negative control, Lane 1: *S. aureus* ATCC 25923, Lane 2: *Bacillus subtilis* lab isolate, Lane 3: *Escherichia coli* ATCC 25922, Lane 4: *Pseudomonas aeruginosa* ATCC 27853, Lane 5: *Streptococcus viridans* lab isolate, Lane 6: *Streptococcus pneumoniae* lab isolate, Lane 7: *Enterococcus faecalis* lab isolate, Lane 8: *Streptococcus pyogenes* ATCC 12384, Lane 9: *Nocardia spp.* lab isolate, Mwt: 100 bp molecular weight marker

Fig.9 Detection of *gyrA* gene (*S. epidermidis*) (284 bp)



PCR amplification of the QRDRs of the *gyrA* gene in *S. epidermidis* isolates. Lane 1: Negative Control, Lanes 2- 6: PCR products of the corresponding genes; Lane 7: Positive Control, Lane 8: 100 bp plus DNA Ladder

Fig.10 Detection of *gyrB* gene (*S. epidermidis*) (251 bp)



PCR amplification of the QRDRs of the *gyrB* gene in *S. epidermidis* isolates. Lane 1: Negative Control, Lanes 2: Negative sample, Lanes 3- 7: Positive PCR products of the corresponding genes; Lanes8: Positive Control, Lanes 9:100 bp Plus DNA Ladder

Fig.14 Sequence alignment of the two types of *gyrA* Reverse sequence with the sequence of *Staphylococcus epidermidis* RP62A, complete genome Sequence ID: CP000029.1 (Length 2616530)

```
Query 8      GAAAACT-AAGTCTTGGG-CATTCTGTACCATTGCTTCATAA-TTG-ATAATCACCATGA 63
             ||  ||||  |||||||||  |||||  |||||||||||||||||  |||  |  |||||||||
Sbjct 2609478 GATAACTAAAGTCTTGGGCATTCT-TACCATTGCTTCATAAATTGAAGAATCACCATGA 2609536
             -
Query 64     GGGTGATATTACCCATGACATCCCGACTATACGTGCAGATTCTTATAAGGTTTATCG 123
             |||||||||  |||||||||  |||||||||  |||||||||  |||||||||  |||||||||
Sbjct 2609537 GGGTGATATTACCCATGACATCCCGACTATACGTGCAGATTCTTATAAGGTTTATCG 2609596
```

Fig.15 Sequence alignment of the two types of *gyrB* Forward sequence with the sequence of *Staphylococcus epidermidis* RP62A, complete genome ID: CP000029.1 (Length 2616530)

```
Query 4      TGGCGAAATTGGTTCTAGT-AG-ACCCTGTAG-AAGTGAAATTTCTTGGTAGAAGGTGA 60
             |||  |  ||  |||||||  ||  |||||  |  |||||||||  |||||||||  |||||||||
Sbjct 2610494 TGGC-AGATT-GTTCTAGTAAGAACCCTGAAGAAGTGAAATTTCTTGGTAGAAGGTGA 261043
```

Fig.16 Sequence alignment of the *gyrB* Reverse sequence with the sequence of *Staphylococcus epidermidis* RP62A, complete genome Sequence ID: CP000029.1 (Length 2616530)

```
Query 7      AATTTCTTTATTGTT-AGATACGATCT-AACGTGCTTTTCCACATTCAAATTTTACC 63
             |||||  |||||||  |||||||||  |||||||||  |||||||||  |||||||||
Sbjct 2610313 AATTTCTTTATTGTTAAGATACGATCTAACGTGCTTTTCCACATTCAAATTTTACC 2610372
```

Fig.17 Sequence alignment of the *par C* Forward sequence with the sequence of *Staphylococcus epidermidis* RP62A, complete genome Sequence ID: CP000029.1 (Length 2616530)

```
Query 16     AAGTGCGAAACTGTCGGTGATGTAATAGGTCAATATCATCCTCATGGAGACTATTTCAGT 75
             |||||||||  |||||||||  |||||||||  |||||||||  |||||||||  |||||||||
Sbjct 939231  AAGTGCGAAACTGTCGGTGATGTAATAGGTCAATATCATCCTCATGGAGACTTTCAGT 939290
```

Fig.18 Sequence alignment of the *parC* Reverse sequence with the sequence of *Staphylococcus epidermidis* RP62A, complete genome Sequence ID: CP000029.1 (Length 2616530)

```
Query 33     CTTAAGCGCCCAATAACATCATATACTGAATAGTCTCCATGTATGATGATATTGACCTAT 92
             |||||||||  |  |||  |||||||||  |||||||||  |||||||||  |  |||||||||
Sbjct 939314  CTTAAGCGCACCATAGCATCATATACTGAAGAGTCTCCATG-AGGATGATATTGACCTAT 939256
```


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