

## RESEARCH ARTICLE



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## Investigation for the Ciprofloxacin Resistance Genes *Gyr A* and *Par C* in *E. Coli* Isolates from Urinary Tract Infected Patients

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### Abstract

**Background and Objective:** *Gyr A* and *Par C* genes, are known to cause resistance especially in Alterations in proteins of fluoroquinolones. Here, we investigated human pathogens *Escherichia coli* causing Urinary Tract Infection [UTI] to explore the possible link between the abundance of mutations, and the exposure to fluoroquinolones. In this study, we investigated the occurrence of *Gyr A* and *Par C* gene producers among Quinolones resistant [QR] *Escherichia coli* isolated. **Methods:** 148 Urine samples were collected from a patients with UT infections. Phenotypically, Ciprofloxacin resistance was screened by micro broth dilution method. Multiplex PCR was carried out to determine the mutations in *Gyr A* and *Par C* genes. We have determined partial sequences of the *Gyr A* and *Par C* genes of *E. coli* including the regions analogous to the quinolone resistance-determining region of the *E. coli Gyr A* gene. **Results:** Out of 148 urine samples, 100 *E. coli* were isolated and identified. We analysed 20 quinolone-resistant strains for alterations in *Gyr A* and *Par C*. Of these, 11 *Gyr A*-positive isolates were identified using the *Gyr A* specific primers and were clearly Ciprofloxacin resistant. The other 9 Ciprofloxacin-resistant isolates were found to have *Par C* genes using specific primers. We observed an unexpectedly high prevalence of *Gyr A* than *Par C* in patients attending a tertiary care hospital by PCR with an estimation of 9.0% (95% confidence interval). This study demonstrated that the number of mutations in QRs of *Gyr A* and/or *Par C* was significantly associated with the MICs of quinolones ( $P < 0.01$ ). **Conclusion:** The *Gyr A* and *Par C* genes were detected predominantly in *E. coli*. The data emerging out of this study helps in understanding the dynamics of this infection and provide inputs for antibiotic policy in the treatment of urinary tract infections.

**Keywords:** Ciprofloxacin; resistance; *Escherichia coli*; Polymerase chain reaction; microdilution method

## 1 Introduction

*Escherichia coli* is an important pathogen causing septicemia, wound, and urinary tract infections. Fluoroquinolones (FQs) are synthetic compound derivatives of quinolones that are currently one of the main classes of agent used for treatment of many types of bacterial infection, including *E. coli* infection<sup>(1)</sup>. FQs form complex with bacterial DNA *Gyr Ase* and *topoisomerase IV*, two crucial enzymes used during DNA- replication process, thereby inhibiting bacterial growth<sup>(2)</sup>. Ciprofloxacin is among the most frequently prescribed FQs, which was introduced into clinical use more than 30 years ago. It has been widely used to treat infections caused by bacteria due to its effective inhibitory activity against Gram-positive and Gram-negative bacteria, especially the *Enterobacteriaceae*<sup>(3)</sup>.

A plasmid-mediated Ciprofloxacin resistance gene, *Gyr A*, harbored by *E. coli* isolated from animals and hospital inpatients, was first reported in China<sup>(4)</sup>. Then it became popular all over the world, demonstrating a horizontal transfer mechanism for Ciprofloxacin resistance<sup>(5)</sup>. Additional novel plasmid-encoded Ciprofloxacin resistance genes were identified as well: *Par C*, identified in *E. coli* isolates and sharing 76.7% of nucleotide identity with *Gyr A*; identified in porcine *E. coli* isolates and sharing 45.0% of nucleotide sequence identity with *Gyr A*.<sup>(6)</sup>

These genes (mention them) encode a phosphoethanolamine transferase family protein that modifies the lipid A component of LPS and confers a low level of Ciprofloxacin resistance (MIC=4-8mg/L)<sup>(7)</sup>.

Presently, nucleotide- sequencing analysis is a common method for the detection of mutations of *Gyr A* and *Par C* in the QRDRs. However, conventional sequencing is time- intensive and expensive. Various alternative methods to replace sequencing have been proposed, including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)<sup>(8)</sup>, multiplex real-time (RT) based detection<sup>(9)</sup>, and single-stranded conformation polymorphism (SSCP).

The present study is to check the prevalence, demonstrate the virulence factors and study the antibiotic susceptibility pattern of *E. coli* in our clinical settings. Studies on the prevalence of drug resistant *E. coli* have been done in the hospital. The data emerging out of this study helps in understanding the dynamics of this infection and provide inputs for antibiotic policy in the treatment of such infections.

## 2 Materials and Methods

### Methodology and research design

This is a prospective study conducted in the department of microbiology, Saveetha Medical College and Hospital, Thandalam, Chennai.

### Sample size and sampling techniques

Continuous sampling method was used in the study, urine samples received in the clinical microbiology laboratory during the period of three months (July 2019 – October 2019) were included in the study. *E. coli* isolates were collected from urine specimens of hospitalized patients with suspected UTI, who had not yet received antibiotics, during the study period. To avoid testing multiple isolates from a single patient, *E. coli* was isolated in only one urinary culture from each patient. Urine specimens were collected by clean-catch midstream or from catheter in catheterized patients. In this interpretation informed consent is obtained by generally accessible information.

### Sample processing

#### Microscopy

Smears were prepared by placing a loopful of the sample on a clear glass slide and gram staining was done for microscopic examination.

#### Culture

All the samples were inoculated onto Blood agar and MacConkey agar and the plates were incubated at 37°C. Biochemical Identification of *E. coli* was done with IMVIC tests.

#### Virulence factor testing — bio film [tissue culture plate assay]<sup>(10)</sup>

Isolates from fresh agar plates were inoculated in Trypticase Soy Broth and incubated for 24 hours at 37°C, then diluted with fresh Trypticase Soya Broth in 1 in 100 dilution. Individual wells of sterile, polystyrene, 96 well & #8209; flat bottom tissue culture plate (TCP) wells filled with 0.2 mL aliquots of the diluted cultures and only broth served as control to check sterility

and nonspecific binding of media. The TCP was incubated for 18–24 h at 37°C. After incubation content of each well was gently removed by tapping the plates. Then wells were washed four times with 0.2 ml of PBS (pH 7.2) to remove free & #8209; floating “planktonic” bacteria. Wells were stained with crystal violet (0.1%). Excess stain was rinsed off by washing with deionized water, and the plate was kept for drying. If bio film is formed by organisms, then wells are uniformly stained with crystal violet. Optical density (OD) of stained adherent bacteria was determined with a micro ELISA auto reader at a wavelength of 570 nm (OD 570 nm). Experiment was repeated thrice, and the data then were averaged, and standard deviation was calculated. The mean OD value obtained from media control was deducted from all the test OD values.

### Antibiotic Sensitivity Testing

Antimicrobial susceptibility testing by disk diffusion was performed as a part of the routine testing and interpretation was done according to the Clinical and Laboratory Standard Institute (CLSI) guidelines<sup>(11)</sup>. Isolates were tested for susceptibility to Ampicillin (10µg), Gentamicin (10µg), Cefuroxime (30µg), Cefotaxime (30µg), Norfloxacin (5µg), Amikacin (30µg), Nitrofurantoin (30µg), Ceftazidime/Clavulanic acid (30µg), Ceftazidime (30µg), Co-trimoxazole (1.25/23.75mcg), Polymyxin B (300µg), Ciprofloxacin (10µg) by Kirby-Bauer disk diffusion method.

### Minimum inhibitory concentration — broth dilution method (mic)

For Ciprofloxacin susceptibility testing, the isolates (n=105) were subjected to the broth microdilution (BMD) method, with susceptible *E. coli* ATCC 259226. The antibiotic pure substance, Ciprofloxacin sulphate powder, was obtained from Sigma-Aldrich.

16µg/mL, 8 µg/mL, 4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, 0.25 µg/mL dilutions of Ciprofloxacin were prepared.

### Determination of minimum bactericidal concentration (mbc)

The minimum bactericidal concentration (MBC) is the amount of agent that will prevent growth after subculture of the organism to antibiotic free medium.

An aliquot from each wells of microtitre plate was inoculated and streaked on to nutrient agar plate. The plates were incubated at 37°C for 24 hours and the minimum concentration at which the bactericidal activity occurred were determined.

### PCR (Polymerase Chain Reaction):

PureFast® Bacterial DNA mini spin purification kit (Kit contains Lysozyme, Lysozyme digestion buffer, Proteinase-K, Binding buffer, Wash Buffer-1, Wash Buffer-2, Spin columns with collection tube and elution buffer. HELINI 2X ReDdye PCR Master Mix, Agarose gel electrophoresis consumables and *Gyr A* and *Par C* Primers are from HELINI Biomolecules, Chennai, India.

Primers used for PCR assay

Target gene	Primer	Sequence 5'-3'	Product size [bp]
Gyr A	gyr A F	5'-TACACCGGTCAACATTGAGG-3'	647
Par C	par C F	5'AAACCTGTTTCAGCGCCGCATT-3'	395

### PCR Procedure:<sup>(12)</sup>

- Reactions set up as follows;

COMPONENTS	QUANTITY
HELINI RedDye PCR Mater mix	10µl
HELINIRedDye to use-Primer Mix	5µl
Purified Bacterial DNA	10µl
Total volume	25µl

- Mixed gently and spin down briefly.
- Place into PCR machine and program it as follows.

**Initial Denaturation:** 95°C for 5 min.

**Denaturation:** 94°C for 30sec

**Annealing :** 58°C for 30sec

**Extension :** 72°C for 30sec

**Final extension:** 72°C for 5 min

**LOADING:**

1. Prepared 2% agarose gel [2gm of agarose in 100ml of 1X TAE buffer].
2. Run electrophoresis at 50V till the dye reaches three fourth distances and observe the bands in UV Transilluminator.

### Agarose Gel Electrophoresis

Prepared 2% agarose. (2gm agarose in 100ml of 1X TAE buffer and melted using micro oven). When the agarose gel temperature was around 60°C, added 5 $\mu$ l of Ethidium bromide. Poured warm agarose solution slowly into the gel platform. Kept the gel set undisturbed till the agarose was solidifies. Poured IXTAE buffer into submarine gel tank. Carefully placed the gel platform into tank. Maintained the tank buffer level 0.5cm above than the gel. PCR Samples are loaded after mixed with gel loading dye along with 10 $\mu$ l HELINI 100bp DNA ladder.[100bp, 200bp, 300bp, 400bp, 500bp, 600bp,700bp, 800bp, 900b, 1000bp and 1500bp]. Run electrophoresis at 50V till the dye reaches three fourth distance of the gel. Gel viewed in UV Transilluminator and observed the bands pattern.

### Data Analysis

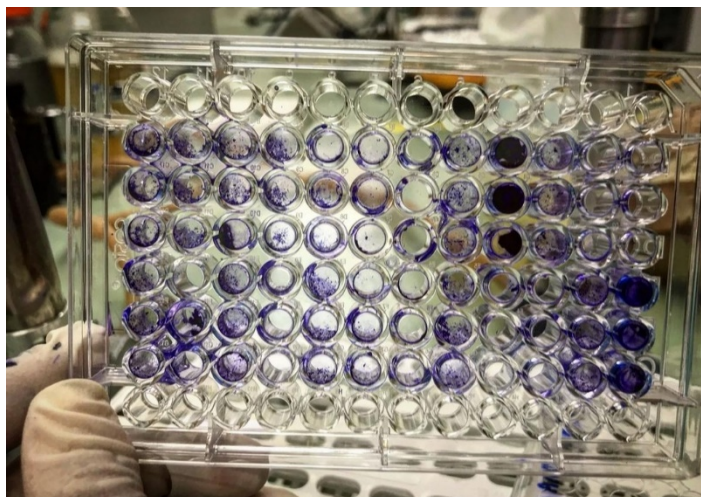
We calculated the frequency of identification of Gyr A and par C genes and their antibiotic resistance pattern for quinolones mutated genes positive bacteria. Pivot table function of Microsoft Excel 2016 was used to calculate the descriptive analysis (as a percentage), and the prevalence of Gyr A and par C harboring strains among the total strains including 95% confidence intervals (CIs), the total number of resistant isolates (number of resistant isolates/total number of positive isolates from same species) to each individual antimicrobial drug.

## 3 Results

The present study was conducted at Clinical Microbiology Laboratory of Saveetha Medical College and Hospital during the period of Nov 2019 to Feb 2020. Ethical clearance was obtained.

Out of 148 urine samples, 100 samples were *E. coli*, which was statistically significant\* ( $p=0.005$ ).

Virulence factors like bio film activity by TCP method detected 49(49.0%) as biofilm producers and 51 (51%) as non biofilm producers. [Figure 1 ]



**Fig 1.** Biofilm activity

The sensitivity and resistance pattern of *E. coli* against various antibiotics (Amikacin (30 $\mu$ g), Ampicillin (10 $\mu$ g), Cefotaxime (30 $\mu$ g), Cefepime (30 $\mu$ g), Cefoperazone sulbactam (30 $\mu$ g), Ciprofloxacin (30 $\mu$ g), Cotrimoxazole (1.25/23.75mcg),

Ciprofloxacin (10 $\mu$ g), Ertapenem (10 $\mu$ g), Gentamicin (10 $\mu$ g), Imipenem (10 $\mu$ g), Meropenem (10 $\mu$ g), Nitrofurantoin (30 $\mu$ g), Norfloxacin (10 $\mu$ g), Ofloxacin (30 $\mu$ g), Piperacillin tazobactam (30 $\mu$ g) and Polymyxin B (300 $\mu$ g) were analysed by Kirby Bauer disc diffusion method. The ABST pattern is depicted in *E. coli* is shown in Figure 2.

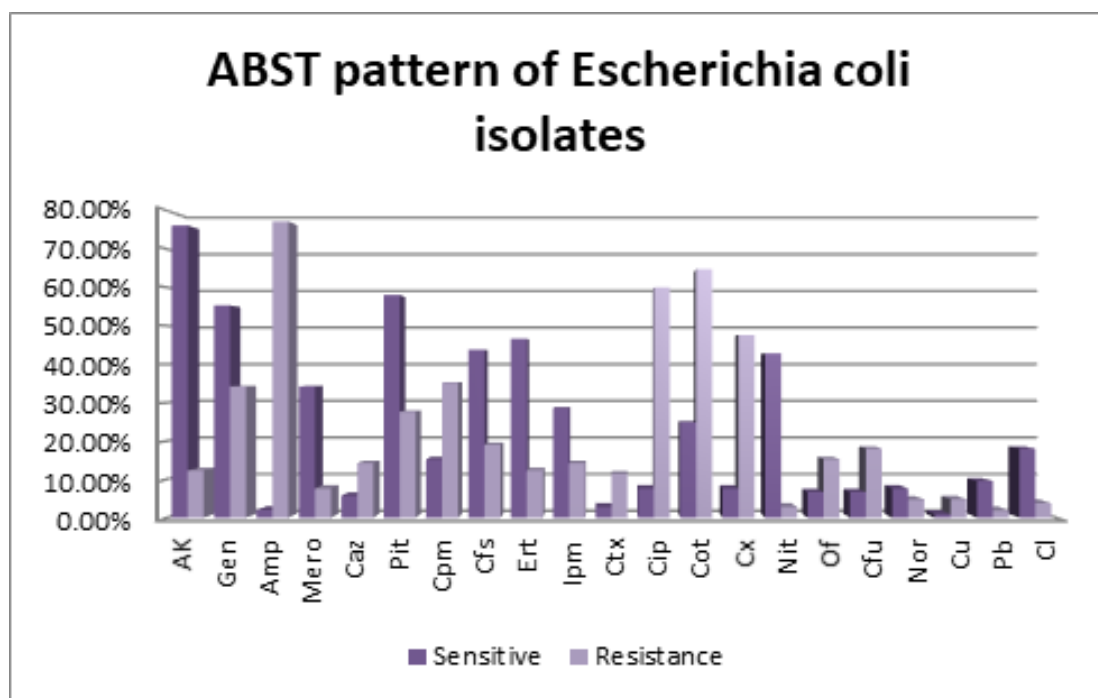


Fig 2. Antibiotic Susceptibility Pattern of Escherichia coli

The higher rate of resistance were found to be for the following drugs Amikacin (78%), Piperacillin tazobactam (69%), Gentamicin (66%).

### MIC and MBC for Ciprofloxacin

On treating with different serial dilutions 16 $\mu$ g/mL, 8 $\mu$ g/mL, 4 $\mu$ g/mL, 2 $\mu$ g/mL, 1 $\mu$ g/mL, 0.5 $\mu$ g/mL and 0.25 $\mu$ g/mL of Ciprofloxacin. *Escherichia coli* showed different inhibitory and bactericidal patterns.

From the 100 *E. coli* strains 20 (3.80%) were found to be resistant to the Ciprofloxacin by broth dilution method, according to the CLSI guidelines.

Out of 100 *E. coli*, 4 isolates were in the MIC range of 0.25 $\mu$ g, 5 isolates were in the MIC range of 0.5 $\mu$ g, 3 isolates were in the range of 1 $\mu$ g, 3 isolates were in the MIC range of 2 $\mu$ g, 4 isolates were in the MIC range of 4 $\mu$ g, 1 isolates were in the MIC range of 8 $\mu$ g, 0 isolates were in the MIC range of 16 $\mu$ g.

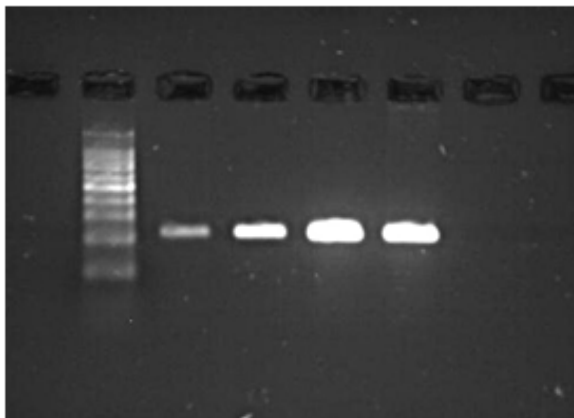
Out of 100 *E. coli*, 6 isolates were in the MBC range of 0.25 $\mu$ g, 1 isolates were in the MBC range of 0.5 $\mu$ g, 3 isolates were in the MBC range of 1 $\mu$ g, 4 isolates were in the MBC range of 2 $\mu$ g, 2 isolates were in the MBC range of 4 $\mu$ g, 1 isolates were in the MBC range of 8 $\mu$ g, 3 isolates were in the MBC range of 16 $\mu$ g.

### Molecular detection of *Gyr A* and *Par C* gene:

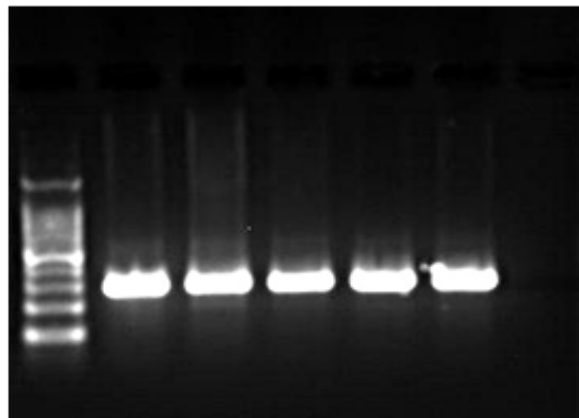
From 100 clinical *E. coli* isolates, we identified 20 isolates that exceeded the Ciprofloxacin resistance breakpoint (>2 mg/mL) using the agar dilution method. [Figure 3 a,b] Full gene sequencing confirmed that all these 11 strains encoded *Gyr A*. The other 9 Ciprofloxacin-resistant isolates were found to have *Par C* genes using specific primers.

The MICs value and GenBank accession numbers of the *E. coli* strains have been mentioned in Table 3. The choice of *E. coli* in this study was to attribute its prevalence as a urinary tract infection.

### 3a: *Gyr A* gene



### 3b: *Par C* gene



**Fig 3.** 3a and 3b Molecular detection

**Table 3.** Accession numbers and quinolone susceptibility of *E. coli* isolates.

Strain	Accession number	MIC
M38	KP276747	>2 mg/mL
M45	KP276762	>2 mg/mL
M22	KP276740	>2 mg/mL
M32	KP276750	>2 mg/mL
M46	KP276734	>2 mg/mL
M52	KP276721	>2 mg/mL
M38	KP276735	>2 mg/mL
M12	KP276753	>2 mg/mL
M53	KP276748	>2 mg/mL
M79	KP276756	>2 mg/mL
M71	KP276733	>2 mg/mL
M13	KP276720	>2 mg/mL
M5	KP276727	>2 mg/mL
M83	KP276725	>2 mg/mL
M102	KP276731	>2 mg/mL
M123	KP276719	>2 mg/mL
M145	KP276728	>2 mg/mL
M98	KP276739	>2 mg/mL
M100	KP276711	>2 mg/mL
M93	KP276722	>2 mg/mL

## Statistical analysis:

Overall, the *Gyr A* prevalence determined by both culture and PCR methods was estimated to be 9.0% (95% confidence interval of 5.7%–13.7%, Wilson score interval). This was statistically significant \* ( $p=0.005$ ).

## 4 Discussion

In the present study, during a period of six months from Nov 2019 to Feb 2020, 100 samples received to Clinical Microbiology Laboratory of Saveetha Medical College and Hospital were included.

In this study, 100 *Escherichia coli* strains were collected. (100%) were from urine. A study done by N Prim et al, Barcelona, Spain<sup>(13)</sup> closely related to our study, in that study they isolated 76 *Escherichia coli* between January 2012 to March 2012 66 (90.85%) were from urine, 10 (9.5%) were from wound swab.

Biofilm producing bacteria are responsible for many recalcitrant infections and are difficult to eradicate. Biofilm production in *E. coli* promotes colonization and lead to increased UTI. Such infections may be difficult to treat as they exhibit multiple drug resistance. Ponnusamy et al. showed 69% isolates as biofilm producers by TM and TCP methods. Congo red method showed 59.4% strains to be biofilm producer.<sup>(14)</sup> Significant production of biofilm was seen in 67.5% isolates of *E. coli* in a study conducted by Sharma et al. by TCP method. In our study 49 were bio film producers and 51 were non bio film producers by tissue culture assay.<sup>(15)</sup>

Antibiotic susceptibility testing was done by the following antibiotics- Amikacin, Ampicillin, Cefazolin, Cefoxitin, Cefotaxime, Cefepime, Cefoperazone sulbactam, Ciprofloxacin, Cotrimoxazole, Ciprofloxacin, Ertapenem, Gentamicin, Imipenem, Meropenem, Nitrofurantoin, Norfloxacin, Ofloxacin, Piperacillin tazobactam and Polymyxin B. The percentage of susceptibility were towards Amikacin 78%. The percentage of susceptibility Gentamicin was 54%. The percentage of susceptibility for Ertapenem 47%.

The highest Resistance was noted for Ampicillin 78%. The resistance to Cotrimoxazole was 64% and resistance to Ciprofloxacin 60%. In the study of Kareem et al.,<sup>(16)</sup> The antimicrobial agents tested included meropenem, imipenem, tigecycline, Ciprofloxacin, aztreonam, amikacin, levofloxacin, cefoperazone-sulbactam, cefotaxime, cefepime, and trimethoprim/sulfamethoxazole. The highest susceptibility were found to be towards the Cefoperazone-sulbactam 48% and the highest rate of resistance were found to be towards Amikacin 82% which is least similar to our study.

When we talk about Ciprofloxacin resistance exclusively in our study *Escherichia coli* isolates were 1% resistance towards Ciprofloxacin by antibiotic susceptibility testing. The MIC showed 1% of the strains were resistance to Ciprofloxacin.

Out of 100 *Escherichia coli* isolates, four (4%) were resistance in MIC to Ciprofloxacin by Broth dilution method.

Among 100 isolates four (3.8%) isolates which were showing resistance to Ciprofloxacin by MIC method were sent for molecular detection for *Gyr A* and *Par C* gene all 4 isolates were positive for both the genes. This study correlates Kareem et al.,<sup>(16)</sup> the *Gyr A* gene was detected by PCR.

## 5 Conclusion

To conclude, since Disk diffusion and Vitek interpretation for Ciprofloxacin is not recommended, Minimum inhibitory concentration is the only method by which the susceptibility of Ciprofloxacin can be reported. Incidence of Ciprofloxacin resistance might be higher among MDR isolates for which Ciprofloxacin might be used in treatment. Hence, judicious use of this drug will help in preserving this drug usage in infections with multi drug resistant strains. Misuse and overuse of antibiotics can be prevented by constant monitoring of the antibiotic susceptibility testing for the bacterial isolates in the hospital and by framing antibiotic policy and initiating antibiotic stewardship program.

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