Detection of Quinolone resistance in *Staphylococcus Aureus* isolates in a tertiary care centre

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DOI:10.53555/ecb/2022.11.03.23

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Background: *Staphylococcus aureus* is a gram-positive coccus frequently found in the nose, respiratory tract, and on the skin. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. Quinolone resistance among *Staphylococcus aureus* emerged quickly, more prominently among the methicillin-resistant strains. Fluoroquinolones cannot be used as anti-staphylococcal agents effectively and its efficacy was dramatically reduced. **Aim:** The study is aimed to examine the mechanisms of fluoroquinolone resistance and to rule out the epidemiologic factors which contribute to the prevalence of antibiotic resistance in clinical settings. **Methodology:** Biochemical tests and antibiotic sensitivity procedure were done among the isolates obtained from various clinical samples. In our study a representative population of fifty isolates of resistant *Staphylococcus aureus* by both disk diffusion and agar dilution were subjected to real time PCR assay for detection of topoisomerase iv and gyrase. **Result & Discussion:** The incidence of MRSA in our study was found to be 74%. High prevalence of MRSA (64%) was observed from pyogenic infections followed by blood stream infections.

Keywords: Quinolone, *Staphylococcus Aureus*, topoisomerase, gyrase

Introduction

Staphylococcus aureus is a gram-positive coccus frequently found in the nose, respiratory tract, and on the skin[1]. Infections are produced by virulent strains that induce potent protein toxins, and expressing cell-surface proteins that bind and inactivate antibodies. *Staphylococcus aureus* can cause a range of illnesses, from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome, and abscesses, to life-threatening diseases of respiratory tract, CNS and bones toxic shock syndrome, bacteremia, and sepsis[2]. It is still one of the five most common causes of hospital-acquired infections and is often the cause of postsurgical wound infections.[3]

The mortality of *Staphylococcus aureus* bacteremia remains approximately 20–40% despite the availability of effective antimicrobials.[4] *Staphylococcus aureus* is now the leading overall cause of nosocomial infections and as more patients are treated outside the hospital setting, is an increasing concern in community[5].

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterium responsible for several difficult-to-treat infections in humans. MRSA is any strain of *Staphylococcus aureus* that has developed, through horizontal gene transfer and natural selection, multi- resistance to beta -lactam antibiotics and Quinolones[6]. The emergence of antibiotic-resistant strains of *Staphylococcus aureus* such as MRSA is a worldwide problem in clinical medicine[7].

Nosocomial infections are produced mostly by MRSA in patients with poor immune systems, open wounds and devices which are invasive. This situation is common in hospitals, prisons, and nursing homes than the general public. The healthcare-associated MRSA is designated as HA-MRSA and community-associated MRSA is defined as CA-MRSA and LA-MRSA (livestock-associated) reflect this distinction[8].

Quinolone resistance among *Staphylococcus aureus* emerged quickly, more prominently among the methicillin-resistant strains. Fluoroquinolones can not be used as antistaphylococcal agents effectively and its efficacy was dramatically reduced[9]. The rates of quinolone resistance between MSSA and MRSA differ significantly and reason for this is uncertain. One contributing factor is likely antibiotic selective pressure, especially in the hospital setting, resulting in the selection and spread of the more antibiotic-resistant MRSA strains.[10] These resident, resistant strains then become the reservoir for future infections.

The alarming rise in antibiotic resistance among pathogenic bacteria is a persistent issue in antibiotic therapy in health care and community settings[11].

The fluoroquinolone class of antimicrobial agents has broad acceptance in hospitalized and community patients, and usage appears to be increasing. Although some members of the class (temafloxacin, grepafloxacin, gatifloxacin and trovafloxacin) have been withdrawn or restricted because of adverse events. New members of fluoroquinolones continue to be developed and approved for treating patients with respiratory tract infections, the single most common group of infections. It can be used once or twice a day, oral dosing and this easy way increases its use. As we approach the halfway point of the second decade of fluoroquinolone use, resistance has already emerged in some species of bacteria and some clinical settings.[12]

This study has been undertaken to examine the mechanisms of fluoroquinolone resistance and discuss epidemiologic factors that may have contributed to the prevalence of antibiotic resistance in clinical settings.

Aim and objectives:

To detect phenotypic and genotypic resistance among *Staphylococcus aure by i*solation and characterisation *Staphylococcus aureus* from clinical isolates using disk diffusion and agar dilution sensitivity methods for detection of quinolone resistance.

Materials and method

Isolates were obtained from various clinical samples and the sample size was calculated using the formula $N = 4pq/d^2$ where n is required sample size p is expected prevalence q is 100-p and d is degree of prevalence. Convenience sampling technique was used and this is a cross sectional prospective study. Institutional Human ethical clearance was obtained.

Sample collection:

All clinical samples which were sent to diagnostic microbiology laboratory were included in the study. Around 120 clinical isolates of *Staphylococcus aureus* from various clinical samples such as blood, wound swab, pus, sputum, broncho - alveolar lavage, tracheal aspirate, which grew beta haemolytic golden yellow colonies on 5 % sheep blood agar were processed.

All processing work was done in bio safety cabinet class II type A2 under strict aseptic precautions. Clinical samples were streaked on plates with 7% sheep blood agar and Mac Conkey agar. All culture plates were incubated at 35 to 37^0 C up to 24 – 48 hours. Cultures were examined after 48 hours of inoculation to detect growth. Blood agar plates were observed for the growth of beta haemolytic golden yellow colonies. The Mac Conkey agar was examined for small lactose fermenting colonies without any contamination.

Bio chemical tests:

Catalase test: The presence of catalase enzyme was detected in the test isolate using hydrogen – peroxide. **Tube coagulase test:** This test was done to differentiate between S*taphylococcus aureus* and coagulase negative *Staphylococcus* CONS. The staphylocoagulase produced by the *Staphylococcus aureus* reacts with coagulase reacting factor which is a thrombin like molecule. They both combine to indirectly convert fibrinogen to fibrin and gives positive result.

Antibiotic sensitivity procedure:

Kirby Bauer disc diffusion:

The peptone water was inoculated with the test organism and incubated for four hours at 37^0C. The turbidity was matched with 0.5 Macfarlands. A sterile swab was soaked in the inoculum and squeezed against the wall of the test tube and was spread over the Muller – Hinton agar plate. Antibiotic discs like Vancomycin (30 µg

), linezolid (30 µg), Amoxy-clav (30 µg), Clinadamycin (2 µg), Erythromycin (15 µg), Penicillin (6 µg), Ciprofloxacin (5 µg), Levofloxacin (5 µg), Ofloxacin (5 µg) were placed on the MHA plate and tested for sensitivity pattern. A cefoxitin 10 µg disc was placed over the MHA plate and incubated at 37 ^{0}C for 24 hours. Next day zone size was measured. A zone size of > 22mm was interpreted as Methicillin sensitive *Staphylococcus aureus (*MSSA*)* and less than 22 mm was considered as Methicillin resistant *Staphylococcus aureus* (MRSA)

The zone diameter of fluroquinolones like Ciprofloxacin, Ofloxacin. Levofloxacin with appropriate disc strength was interpreted as follows Fluoroquinolones Staphylococcus species.

Antimicrobial agent	Disk content	SENSITIVE	INTERMEDIATE	RESISTANT
Ciprofloxacin	5µg	≥21	16-20	≤15
Levofloxacin	5 µg	≥19	16-18	≤15
Ofloxacin	5 µg	≥18	15-17	≤14

CLSI guidelines for Antimicrobial susceptibility testing – 2016

Testing of MIC by Agar Dilution Method.

MIC was done by agar dilution as per the (CLSI-2013) guide lines. The materials used for the agar dilution were Mueller Hinton agar, Pure pharmaceutical products of antimicrobial powder, petriplates, pipettes, antimicrobial solvents, disposable tips, sterilized test tubes etc. The antimicrobial agents used for testing were Ciprofloxacin, Levofloxacin, and ofloxacin for the species *Staphylococcus aureus*.

SCHEMATIC DRUG DILUTION

Drug and step no.	**Concentration (µg/ml)**	**Source**	**Volume (Ml)**	**Diluents**	**Intermediate concentration**	**Final concentration 1ml Mixed with 20 ml Media (µg/ml)**
1	5120	Step 1	1ML	3 ML	1280	64
2	1280	Step 1	1ML	1ML	640	32
3	640	Step 2	1ML	1ML	320	16
4	320	Step 3	1ML	1ML	160	8
5	160	Step 4	1ML	1ML	80	4
6	80	Step 6	1ML	1ML	40	2
7	40	Step 7	1ML	1ML	20	1
8	20	Step 8	1ML	1ML	10	0.5

Preparation of inoculum:

The inoculums were prepared from fresh subculture of *Staphylococcus aureus* isolates by using a sterile inoculation loop by picking 4 to 5 similar colony in to the sterile saline.

The suspensions were matched to McFarland 0.5 turbidity standard (approximately 1.5×10^8 CFU/ml). The working dilutions were made further by diluting 25 times in to CAMHB (Cation-adjusted Mueller-Hinton broth) to obtain a final organism concentration of 3×10^5 to 5×10^5 CFU/ml in each.

Interpretation of MIC:

The results were interpreted by checking Growth on drug free medium, control plate showing satisfactory growth. The MIC was calculated by the inhibition of visible growth in least concentration of each isolate in particular concentrations were considered MIC of each isolate.

Antimicrobial agent	**MIC(µg/ml) SENSITIVE**	**MIC(µGg/ml) INTERMEDIATE**	**RESISTANT**
Ciprofloxacin	≤1	2	≥4
Levofloxacin	≤1	2	≥4
Ofloxacin	≤1	2	≥4

CLSI guidelines for Antimicrobial susceptibility testing – 2016

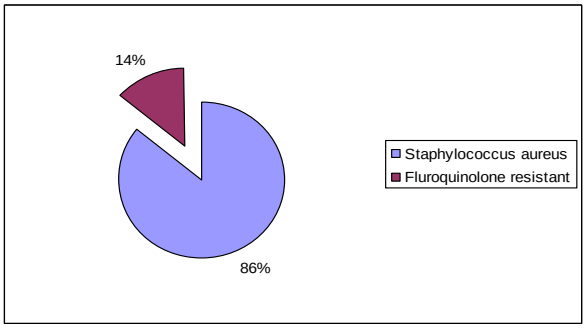
Genotypic Method for detection of Fluroquinolone resistance

The DNA purification procedure using the miniprep spin columns were done by adsorption of DNA to the membrane, removal of residual contaminants and elution of pure genomic DNA. The extracted DNA from the isolates were amplified by using Real time PCR

Target	**Primer**	**Sequence 5' to 3'**	**Expected Amplicon size(bp)**
grlA-	Forward primer Reverse	5'-TGC CAG ATG TTC GTG ATG-3' 5'CCT TGA ATA ATA CCA CCA GTT G-3'	5'nucleotide at position 2467) (5′ nucleotide at position 3040)
grlB	Forward primer Reverse primer	5'-TGT TGT GTC TGT TCG TAT TCC-3' 5'-GCA CCA TCA GTA TCA GCA TC-3'	(5′ nucleotide at position 1353) (5′ nucleotide at position 1910)
gyrA	Forward primer Reverse primer	5'-GAG TGT TAT CGT TGC TCG TG-3' 5'-GAC GGC TCT CTT TCA TTA CC-3'	(5′ nucleotide at position 2333) (5′ nucleotide at position 2725)
gyrB	Forward primer Reverse primer	5'-CCA CAA GTC GCA CGT ACA G-3' 5'-ATC CAC ATC GGC ATC AGT C-3'	(5′ nucleotide at position 1407) (5′ nucleotide at position 1817).

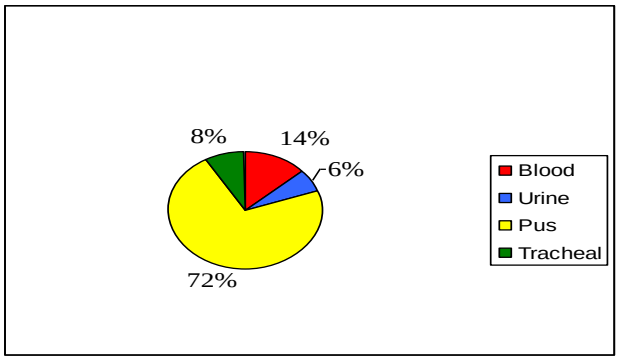
Results

A **t**otal of 24,024 samples of pus, blood, wound swab, tracheal aspirate and urine were processed in microbiology laboratory between April 2016 to July 2017. The incidence of *Staphylococcus aureus* from the samples were 3.5%. **Graph: 1** shows the distribution of *Staphylococcus aureus* that were resistance to fluroquinolones was about 120 among the total 859 isolates received from various samples. It was found to be 14.3%

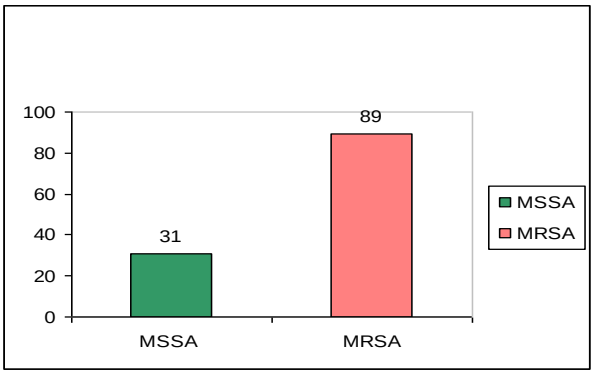


Graph: 1 Distribution of fluroquinolones resistance among total number of *Staphylococcus aureus*

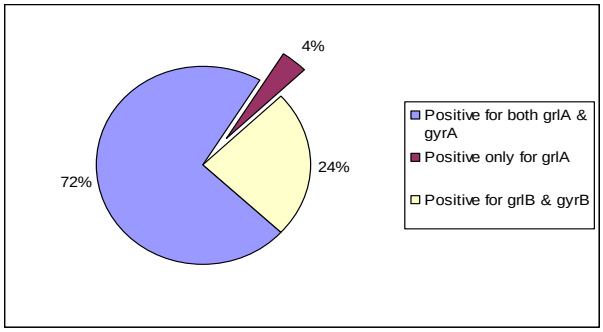
Graph: 2 depicts the distribution of *Staphylococcus aureus* from various clinical samples received which was most commonly isolated from pus sample 72%,followed by14% from blood 8% from tracheal aspirate and 6% from urine sample



Graph: 2 Distribution of *Staphylococcus aureus* from various clinical samples.



Graph : 3 shows the pattern of fluroquinolones resistance among the isolates of *Staphylococcus aureus*.



Graph : 4 Shows Distribution of Flouroquinolone resistant genes among test isolates

Gram's stain picture shows the gram positive cocci in clusters (figure 1). Growth of *Staphylococcus aureus* as beta haemolytic golden yellow colonies on blood agar (Figure 2). Growth on Mac Conkey agar showing small lactose fermenting colonies in (Figure 3)**.** Bio chemical tests to differentiate *Staphylococcus aureus.* e.g. Tube coagulase, Mannitol with both positive and negative controls are shown in (Figure 4 & 5)**.** Antibiotic sensitivity was performed by Disc diffusion and Agar dilution methods (Figure 6 & 7).

Figure 1: Gram's stain shows the gram positive cocci in clusters.

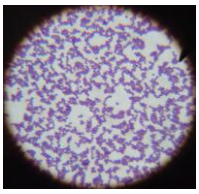
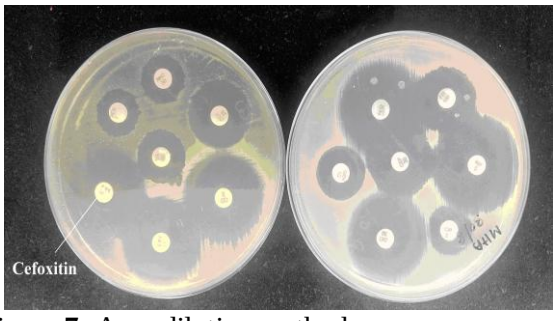


Figure 2: Beta haemolytic golden yellow colonies on blood agar.

Figure 3: Small lactose fermentors on MacConkey agar

Figure 4: Tube coagulase and mannitol fermentation

Figure 6: Antibiotic sensitivity by Disc diffusion



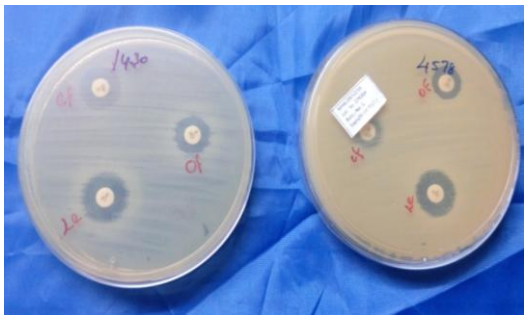


Figure **7:** Agar dilution method

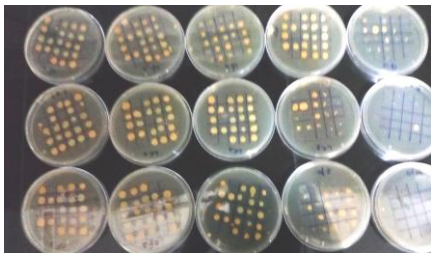


Table 1. Pattern of susceptibility of the isolates of *Staphylococcus aureus* for various antibiotics.

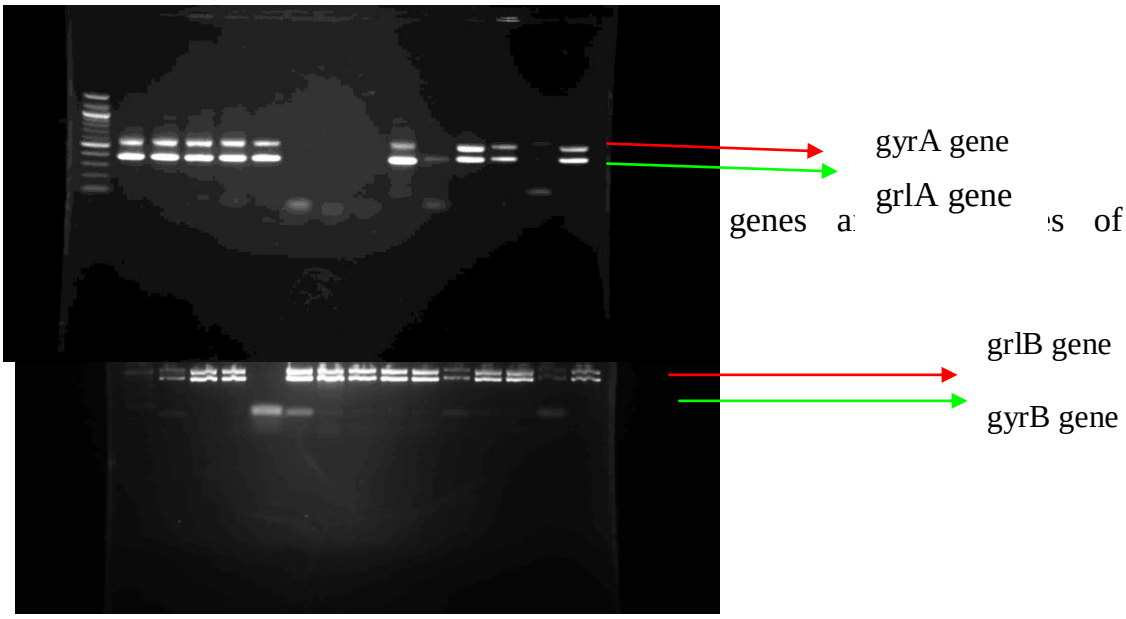
Antibiotic (µg)	**Sensitive (%)**	**Resistance (%)**
Vancomycin (30 µg)	100	0
Linezolid (30 µg)	100	0
Amoxy-clav (30 µg)	74	26
Clinadamycin (2 µg)	88	12
Erythromycin (15 µg)	69	31
Penicillin (6 µg)	13	87
Ciprofloxacin (5 µg)	0	100
Levofloxacin (5 µg)	0	100
Ofloxacin (5 µg)	0	100
Cefoxitin(10 µg)	37	73

Table 2: Minimum Inhibitory Concentration (MIC) for the Fluroquinolone resistant *Staphylococcus aureus.*

Antibiotic	**MIC 8 - 16µg**	**MIC 16 - 32 µg**	**MIC 32 - 64 µg**
Ciprofloxacin	52	38	30
Levofloxacin	16	45	39
Ofloxacin	18	43	49

The molecular characterisation for the gyrA gyrB grlA grlB was done by Real time PCR and amplification by gel electrophoresis is shown in figure 10. The expression of grlB and gyrB was found in 24% of isolates which is depicted in figure 11.

Figure 10: The molecular characterisation for the gyrA gyrB grlA grlB done by Real time PCR**.**



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Discussion

Staphylococcus aureus constitutes an important pathogen and is found to be colonised in approximately 30% of the population who are asymptomatic. Drug resistance has evolved due to increasing inadvertent use of high end antibiotics and the incidence of multidrug resistant bacteria is emerging rapidly.

The incidence of MRSA in our study was found to be 74%. This correlates well with other studies in and around world. MRSA isolates reported across various parts of the world range between 28 % to 87%.[13]

Literature surveys reveal that the high percentages of isolates are of MRSA are found to be resistant to fluroquinolones as compared to MSSA. In our study 74% of MRSA were resistant to fluroquinolones among which most predominant was 43.3% to ciprofloxacin (43.3%) followed by ofloxacin (37.5%) and levofloxacin (34.2%).

MRSA colonisation and infection in patients relates to the effect of fluroquinolones and cotrimoxazole. Among the isolates of MRSA tested , in one study from patients 74% resistant to ciprofloxacin and 68% were resistant to cotrimoxazole were noted. In our study, among clinical isolates of MSSA, 26% were resistant to ciprofloxacin and 32% were resistant to cotrimoxazole.

High prevalence of MRSA (64%) was observed from pyogenic infections followed by blood stream infections. In a study in New Delhi high prevalence of MRSA 35% in ward and 43% in ICU was observed from blood culture specimens[14].

Anti microbial susceptibility testing of isolates of *Staphylococcus aureus* showed maximum sensitivity to vancomycin (100%), linezolid (100 %)followed by clindamycin(88%) amoxyclav(74%). Highest resistance was noted for ciprofloxacin followed by ofloxacin.

First generation cephalosporins form the main stay of treatment for MSSA infections in patients intolerant to anti staphylococcal penicillins. If the patient has been empirically started on vancomycin and the culture report reveals MSSA de-escalation to beta lactams is the preferred mode of treatment. Glycopeptides-vancomycin and teicoplanin should be used only as a last resort and reserve drug.[15]

The minimum inhibitory concentration susceptibility profile to fluroquinolones was tested by agar dilution method. Among the 120 isolates of *Staphylococcus aureus* tested MIC value 32 to 64µg for ciprofloxacin, 16 to 32µg for Ofloxacin and 8 to 16µg for Levofloxacin. The range of MIC value for fluroquinolones was between 32 to 64µg for MRSA as against 8 to 16µg for MSSA.

Studies on ocular infections have reported ciprofloxacin resistance ranging from 3 to 11% as compared to Levofloxacin 25.5%. Increasing resistance among *Staphylococcus aureus* isolates (MSSA) for ciprofloxacin and levofloxacin poses a threat for management of infections as treatment choices are narrowed.[16]

In another study fluoroquinolones resistant rate of 41.8% was found among *Staphylococcus aureus* isolates which was comparatively higher than similar reports which showed resistance range between 13% and 20.7%[17, 18]

The mechanism of genotypic resistance to fluroquinolones in *Staphylococcus aureus* has been extensively studied. In majority of isolates single mutations in DNA topoisomerase IV and DNA gyrase have been reported. DNA topoisomerase IV is the primary target of fluroquinolones in S*taphylococcus aureus* with the first step to these agents being mutations in grlA.[19]

In our study a representative population of fifty isolates of resistant *Staphylococcus aureus* by both disk diffusion and agar dilution were subjected to real time PCR assay for detection of topoisomerase iv and gyrase. The results of our study showed

majority of the isolates (72%) of *Staphylococcus aureus* were detected to express grlA and gyrA. This correlates well with other studies which report similar results.[20]

There have been reports showing one or two point mutations in the QRDR regions of both grlA and gyrA genes to be the main mechanism of ciprofloxacin resistance among isolates of *Staphylococcus aureus*.[20] as shown by pulsefieldgel electrophoresis studies.

We noted high level ciprofloxacin resistance for 43.3% of the isolates tested (MIC 32-64 µg /ml) This level of high resistance is usually associated with grlA and gyrA mutations. This finding also correlates well with other studies.[19]

Though other molecular methods for detection of fluroquinolones exist eg: Restricted fragment length polymorphism (RFLP), High performance liquid chromatography (HPLC) and gene sequencing, they are cumbersome and labour intensive. The advantages of Real time PCR include high sensitivity and the turnaround time is far less compared to other methods. In laboratories which have facilities for molecular testing of Real time PCR could be used as a routine diagnostic tool.

Conclusion

Injudicious use of antibiotics has led to widespread emergence of resistant organisms. Newer compounds have been introduced in the market but inadvertent use of these drugs by clinicians have left us with resistant bugs which are very difficult to eradicate. The strict implementation of antibiotic policy tailor made to the individual hospital needs and prompt regular audits of compliance to the same are the only means of saving the populations from newer pathogens and multi drug resistant bugs.

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