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Original Research Article

A pilot study on comparative analysis of minimum inhibitory concentration and mutant prevention concentration of conjunctival bacterial isolates against fluoroquinolones

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ABSTRACT

Background: The aim of this study was to compare the Minimum Inhibitory Concentration (MIC) and Mutant Prevention Concentration (MPC) of fluoroquinolones, including ciprofloxacin, moxifloxacin, and gatifloxacin, against *Staphylococcus aureus* and coagulase-negative Staphylococci (CONS) isolated from conjunctival swabs.

Materials and Methods: 25 isolates of *Staphylococcus* spp., obtained from conjunctival swabs submitted to the Department of Microbiology, Vision Research Foundation, Sankara Nethralaya, were included in this study. The identification and confirmation of *Staphylococcus* spp. were performed using standard microbiological techniques. The MIC and MPC were determined using the agar dilution method, following protocols from previous studies. The MIC_{50}, MIC_{90}, MPC_{50}, and MPC_{90} values for the above three fluoroquinolones were calculated and analysed.

Results: Out of all 25 isolates, 20 were CONS and 5 were *Staphylococcus aureus*. In our study, gatifloxacin had least MIC and MPC values when compared to ciprofloxacin and moxifloxacin of gatifloxacin had lower MPC_{50} and MPC_{90} values in comparison to ciprofloxacin and moxifloxacin. Our study shows that Gatifloxacin had least MIC and MPC values when compared to ciprofloxacin and moxifloxacin. Besides, MPC of ciprofloxacin, moxifloxacin and gatifloxacin showed wider range of distribution than the MIC.

Conclusion: Gatifloxacin demonstrated effective inhibition of resistant mutant strains at lower concentrations compared to ciprofloxacin and moxifloxacin. Additionally, future studies with a larger number of isolates, incorporating pharmacokinetic and pharmacodynamic parameters, will provide essential information on therapeutic outcomes and resistance prevention.

1. Introduction

Ocular microflora plays a vital role in maintenance of ocular homeostasis by diverse mechanisms.[1] Eyelids and conjunctiva of the eye harbour a significant number of normal bacterial flora; those are CONS, *Staphylococcus aureus, Streptococcus viridians, Haemophilus aegyptius, Streptococcus pneumonia, Chlamydia trachomatis, Corynebacterium diphtheriae, Haemophilus influenza,*

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Chlamydophila pneumoniae, Propionibacterium acnes, Moraxella spp, and *Neisseria* sp.[2] They do not cause infection in normal conditions but in certain circumstances like surgery, ocular injection, and trauma serve as a source of ocular infection. Among all, *Staphylococcus aureus* and *Staphylococcus epidermidis* are frequently isolated from the conjunctival swabs in the case of bacterial conjunctivitis and as a part of the pre-operative procedure before undergoing ocular surgeries. It is mandatory to evaluate the significance and insignificance of normal bacterial flora present in the conjunctiva by collecting conjunctival swabs to reduce the number of post-operative infections.

Probably the most prevalent ocular infection, bacterial conjunctivitis is self-limiting and does not pose a threat to vision which is frequently treated by primary care physicians. On the other end of the severity scale, endophthalmitis is not a common but an important complication of ocular surgery, intravitreal injections, cataract surgery, and ocular trauma.[3,4] Fluoroquinolones is one of the broad-spectrum antibiotics commonly used to treat ocular infection caused by Gram-positive bacterial pathogens.[5,6] Unfortunately, fluoroquinolone resistance has emerged worldwide. Infections caused by these antimicrobial-resistant strains are difficult to treat resulting in increased morbidity.[7] The enhanced pharmacodynamic characteristics of the newer generation fluoroquinolones will optimize antibiotic concentration at infection sites, thereby decreasing the likelihood of bacterial resistance. Approaches to prevent or delay antibiotic resistance in ocular pathogens include careful antibiotic selection and employing sensitivity testing to ensure the prescription of the most effective antibiotic.

Minimal inhibitory concentration (MIC) is defined as the lowest concentration of antibacterial required to inhibit the visible growth of microorganisms and mutant prevention concentration (MPC) is defined as the antimicrobial drug concentration threshold that would require an organism simultaneously to possess two resistance mutations to grow in the presence of the drug. The MPC may also be defined as the drug concentration required to block the growth of the most resistant first-step resistant mutant(s) present in a heterogeneous bacterial population. Determination of MPC value of a particular antibiotic is imperative to prevent or prolong the resistance of the antimicrobials. The MPC concept can be used to decide on the dosing regimen with respect to the potential for the selection and enrichment of mutants. As antimicrobial resistance rises worldwide, it is a great concern to develop methods to limit its further spread.

It has been suggested that the Mutant Prevention Concentration (MPC) can be used to determine whether it is still appropriate to use monotherapy with minimal risk of resistance development or if there is a need to switch to combination therapy. Recent studies have emphasized the significance of MPC-based dosing strategies to improve the therapeutic outcome and limit the selection of resistant mutants.[8] MICs and MPCs have been reported for a variety of bacterium-drug combinations which along with the knowledge of pharmacokinetic/pharmacodynamics parameters, provide necessary information on therapeutic outcomes and resistance prevention.[7,9–11] In this context, the present study was aimed to determine the MIC and MPC values of the commonly used fluoroquinolones such as ciprofloxacin, moxifloxacin and gatifloxacin especially *Staphylococcus aureus* and CONS obtained from eye. This study will be developed further in the future by pharmacokinetic and pharmacodynamics principles.

2. Materials and Methods

25 bacterial isolates (*Staphylococcus aureus* and CONS) obtained from conjunctival swabs from normal healthy subjects for a period of three months (December 2018 to February 2019) in the Microbiology department, Vision Research Foundation, Sankara Nethralaya, Chennai, India were included in this study. No selection criteria were included for the isolates tested. Duplicate isolates from the similar patient were excluded. Genus and Species identification of the isolates was done by standard microbiological techniques. Antibiotics susceptibility testing was performed by Kirby Bauer disc diffusion method as per CLSI guidelines.[12]

Sources of antimicrobials and media were as follows: Ciprofloxacin, Moxifloxacin (Himedia), and Gatifloxacin (Allergan), Muller Hinton agar (Himedia) for MIC testing, Trypticase soy agar (Himedia) for MPC testing, Peptone water (Himedia).

2.1. Cefoxitin disc diffusion method

Staphylococcus aureus and CONS isolates were also screened for susceptibility to Methicillin by cefoxitin disc diffusion test as per CLSI guidelines.

2.2. Determination by agar dilution method

Mueller–Hinton agar plates with two-fold serial dilutions of antimicrobial agents, ranging from 0.015 to 128 μg/ml, were prepared. Inoculum was prepared by suspending overnight culture of the isolate into peptone water. The suspension was matched to 0.5 McFarland standard which was further diluted to achieve final inoculum of 1×10^4 CFU/ml. 5μl of the prepared inoculum was added to antibiotic dilution plates and incubated for 18–24 h at 35-37°C. For agar dilution, the MIC was noted as the lowest dilution showing no growth. For *Staphylococcus aureus* ATCC 25293 and CONS ATCC 12228 were used as controls. The controls were found to be satisfactory.[13]

2.3. MPC determination by agar dilution method

A lawn culture of test organisms was inoculated onto Trypticase Soy Agar plates and incubated overnight (18–24 hrs) at 35–37 ∘C to produce confluent growth. The next day, the contents of the plates were transferred into 2-3ml of peptone water/normal saline to make the suspension of inoculum containing $>10^{10}$ CFU/ml. Viable counts were conducted on the high-density bacterial cultures to confirm the presence of more than $10^{1\circ}$ CFU/ml.[11]

From the high-density suspension, 10μl was inoculated to trypticase soy agar plates containing two-fold concentration increments of antimicrobial agents as like MIC. Inoculated plates were incubated for 24 hours and examined for growth. The plates were then re-incubated for an additional 24 hours and re-examined.

Colonies growing on plates containing drug concentrations exceeding the susceptibility breakpoint by ≥3 doubling dilutions above the MIC were sub cultured onto tryptic soy agar plates with the same drug concentration as the original plate. Plates with a confluent film, making it difficult to identify individual colonies, were re-streaked onto fresh drug plates, re-incubated overnight, and examined for colonies the following morning. This process confirmed the presence or absence of the organism and determined the MPC value. The MPC was recorded as the lowest concentration that prevented the growth of any colonies (Figures 1 and 2).

3. Results

A total of 25 clinical isolates of *Staphylococcus aureus* and CONS from ocular conjunctival swabs were determined for both MIC and MPC values. Among 25 isolates, 20 were CONS and 5 were *Staphylococcus aureus*. Methicillin resistance of the 25 isolates were given in (Table 1).

Table 1: Distribution of methicillin susceptible and methicillin resistant among 25 isolates

S. No	Organism	Methicillin Susceptible (n=18)	Methicillin Resistant (n=7)
1.	*Staphylococcus aureus*	3(16.6%)	2(28.5%)
2.	Coagulase Negative *Staphylococci*	15(83.3%)	5(71.4%)
	Total	18(72%)	7(28%)

MIC values for ciprofloxacin, moxifloxacin and gatifloxacin on different dilutions starting from 0.015μg/ml to 128μg/ml were performed (Table 2). Out of 25 isolates, 10 isolates ranges from 0.25 - 1 μg/ml, 13 isolates were 2 μg/ml and 2 isolates were 4 and 8 μg/ml for ciprofloxacin. For moxifloxacin, MIC for 9 isolates were 0.03-0.5 μg/ml, 6 isolates were 1μg/ml and 10 isolates were 2μg/ml. MIC for gatifloxacin all 25 isolates ranges from 0.03-1 μg/ml.

Table 2: Comparison of MIC values of ciprofloxacin, moxifloxacin and gatifloxacin

MIC (μg/ml)	Ciprofloxacin	Moxifloxacin	Gatifloxacin
0.015	0	0	0
0.03	0	3	5
0.06	0	1	5
0.125	0	2	0
0.25	3	0	6
0.5	4	3	8
1	3	6	1
2	13	10	0
4	1	0	0
8	1	0	0
16	0	0	0
32	0	0	0
64	0	0	0
128	0	0	0
MIC50	2	1	0.25
MIC90 -	4	2	0.5

By MPC testing, 15 isolates showed between 2-8 μg/ml, 10 isolates were 16-128 μg/ml for ciprofloxacin (Figure 3). For moxifloxacin 17 isolates MPC values ranged between 2-8 μg/ml and 8 isolates MPC values were between 16-128 μg/ml (Figure 4). For Gatifloxacin, MPC values were between 0.125-4 μg/ml for all 25 isolates (Table 3).

Table 3: Comparison of MPC values of ciprofloxacin, moxifloxacin and gatifloxacin

MPC (μg/ml)	Ciprofloxacin	Moxifloxacin	Gatifloxacin
0.015	0	0	0
0.03	0	0	0
0.06	0	0	0
0.125	0	0	4
0.25	0	0	2
0.5	0	0	7
1	0	0	7
2	2	12	4
4	7	2	1
8	6	3	0
16	3	4	0
32	4	1	0
64	2	2	0
128	1	1	0
MPC50 -	8	2	0.5
MPC90 -	64	64	2

The MIC_{50} MIC_{90} values (Table 2) and MPC_{50} and MPC_{90} values (Table 3) were calculated. In our study, MIC and MPC values for gatifloxacin were the lowest among all 25 isolates when compared to ciprofloxacin and moxifloxacin. The MPC_{50} (0.5) and MPC_{90}[(2)] values for

gatifloxacin were also the lowest in comparison (Figure 5). Our findings indicate that gatifloxacin demonstrates the least resistance in terms of MIC and MPC values compared to ciprofloxacin and moxifloxacin. Additionally, the MPC values for ciprofloxacin, moxifloxacin, and gatifloxacin exhibited a broader range of distribution than the MIC values.

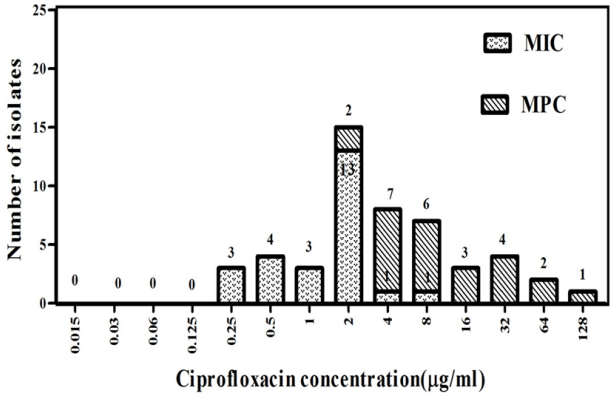

Figure 1: Comparison of MIC and MPC values of ciprofloxacin

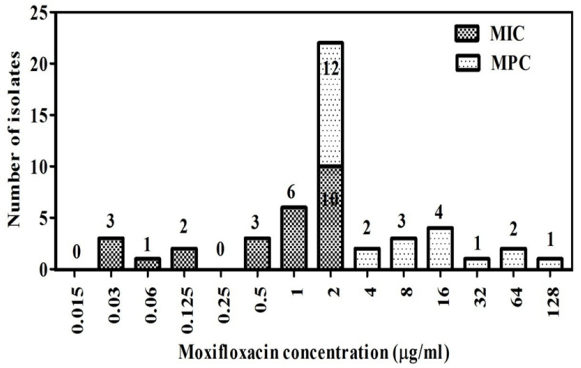

Figure 2: Comparison of MIC and MPC values of moxifloxacin

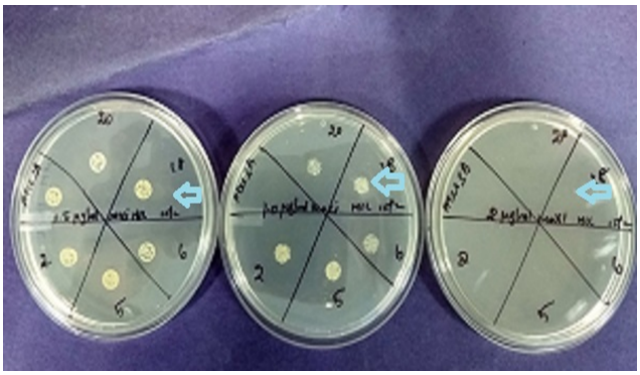

Figure 3: MIC for moxifloxacin

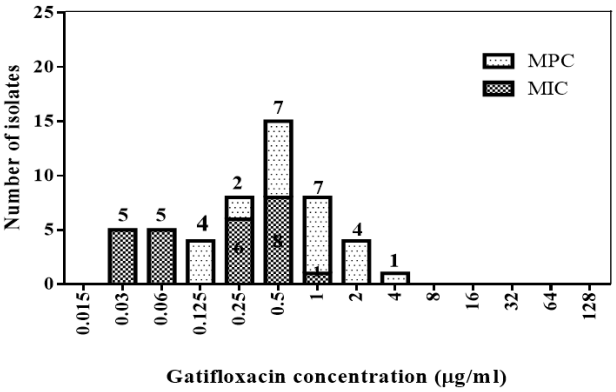

Figure 4: Comparison of MIC and MPC values of gatifloxacin

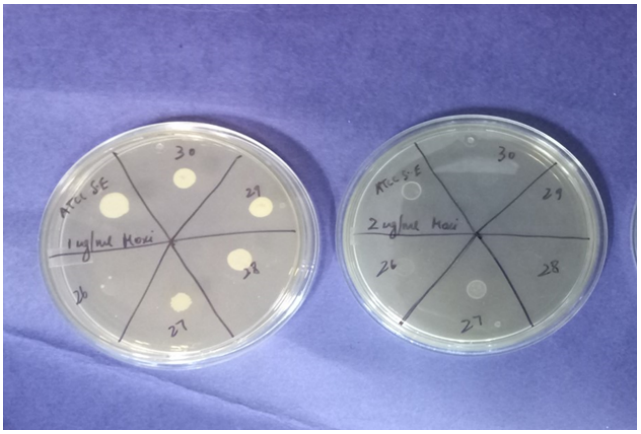

Figure 5: MPC for moxifloxacin

4. Discussion

Both culture-independent and culture-dependent methods reveal a variety of microorganisms present on the ocular surface in both healthy and diseased states.[14–17] The predominant pathogens include *Staphylococcus aureus*, Streptococcus pneumoniae, Coagulase-negative Staphylococci (CoNS), H. influenzae, P. aeruginosa, Candida albicans, Fusarium species, and Aspergillus species. *Staphylococcus aureus* is notably the most common bacterial pathogen among Gram-positive isolates, while P. aeruginosa is the predominant Gram-negative pathogen.[18] Despite achieving higher drug concentrations in ocular tissues with topical antibiotics, there is a growing number of reports indicating clinical failures and suboptimal outcomes with empirical treatment using each new generation of fluoroquinolones.[4,19] Fluoroquinolones inhibit DNA synthesis by targeting two essential topoisomerases in the bacterial cell.[20] Since the 1900s Fluoroquinolones were first used to treat ocular infections with topical preparations of ciprofloxacin, ofloxacin, and norfloxacin. For the past two decades, levofloxacin, gatifloxacin, and moxifloxacin

are preferred because of their higher activity against gram-positive organisms and some atypical mycobacteria, elevated drug delivery into the anterior segment of the eye, and lesser tendency for selecting resistant bacterial strains.[5]

The MPC measurement has been previously applied to clinical isolates of Streptococcus pneumoniae and Pseudomonas aeruginosa.[21,22] We aimed to investigate whether variations in MIC values among the compounds correspond to differences in mutant prevention concentrations (MPC), a novel metric used to assess antimicrobial potency by evaluating the risk of resistance development when exposed to a higher bacterial inoculum.Dosing based on MPC drug concentrations will not only decrease the total bacterial count but also inhibit the selective growth of any resistant subpopulations within the bacterial population.Hence in our study, we determined the MIC & MPC of three fluoroquinolones against both methicillin-susceptible and methicillin-resistant strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Recent studies have reported that newer fluoroquinolones (such as gatifloxacin, gemifloxacin, moxifloxacin) exhibit enhanced activity against Gram-positive pathogens compared to older agents like ciprofloxacin and levofloxacin, as evidenced by lower MIC values.[23,24] These observations have been consistently confirmed by numerous investigations. In our study, the lower MIC values were found in newer fluoroquinolones (gatifloxacin, moxifloxacin) compared to ciprofloxacin. Christine K. Hesje et al.[25] showed that potency of moxifloxacin was higher compared to Gatifloxacin and ciprofloxacin. Our study is mainly from isolates obtained from ocular diseases where fluoroquinolones are largely used as empirical as well as therapeutically.

In this current study, we reported that the MPC of both moxifloxacin and ciprofloxacin showed a wider range of distribution than the MIC. Our study reveals the need for both MIC and MPC breakpoints for deciding the optimum antibiotic concentration to prevent bacterial resistance in ocular infections. Extensive development of resistance suggests that consideration should also be given to restricting the selection of resistant mutants.

The limitation of our study is the small number of isolates because of the limited duration of the study and lack of pharmaco dynamic properties. To conclude, we have tested 25 isolates of methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis* from conjunctival swabs by MIC and MPC against ciprofloxacin and moxifloxacin. To be clinically useful, the MIC or MPC obtained in vitro at constant antibiotic concentrations cannot be used without consideration of the drug's pharmacokinetic properties.[26] Though it is an in vitro study, the subsequent addition of in vivo data with larger sample size and longer duration will help to clarify the in vitro observations.

5. Conflict of Interest

Nil.

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