MUTANT PREVENTION CONCENTRATION OF LEVOFLOXACIN ALONE AND IN COMBINATION WITH CEFTAZIDIME AGAINST LEVOFLOXACIN AND CEFTAZIDIME SENSITIVE AND RESISTANT ISOLATES OF *Pseudomonas aeruginosa*

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Abstract

The study includes 23 isolates of *Pseudomonas aeruginosa* isolated from wound infections .The Minimum Inhibitory Concentration (MIC) to each of Levofloxacin and Ceftazidime for these isolates were determined. The results showed 11(47.8%) isolates sensitive to both antibiotics, 5(21.7%) isolates resistant to each one of the antibiotics and 7(30.5%) isolates appeared resistance to one of them and sensitive to the other. Mutant Prevention Concentration (MPC) to both Levofloxacin and Ceftazidime alone and in combination were determined to the 5 sensitive and 5 resistant isolates to both antibiotics. Mutant Selection Window (MSW) was calculated according to the data of MPC and MIC to both levofloxacin and Ceftazidime alone and in combination to the same isolates which their MPC were determined (10 isolates). The decrease in the value of MSW by 1-2 times were noted when both antibiotics together (in combination) were used in comparison with its value when Levofloxacin was used alone (before combination) to the sensitive isolates (5 isolates), and this indicates a synergistic action whereas, no synergistic action appeared in the resistant isolates (5 isolates) according to the MSW values, this emphasizes that the combination between levofloxacin and ceftazidime against resistant isolates is useless.

التركيز المانع للطفرات (MPC) لليفوفلوكساسين لوحده مره,واخرى بعد دمجه مع السيفتازديم ضد عزلات الـ *Pseudomonas aeruginosa* الحساسه والمقاومه لكلا المضادين

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الخلاصة

شملت الدراسة 23 عزلة من بكتريا *pseudomonas aeruginosa* والمعزولة من اخماج الجروح.حدد التركيز المثبط الادنى (MIC) لكل من المضادين levofloxacin و ceftazidime لتلك العزلات. اظهرت النتائج وجود 11 (47.8%) عزلة حساسة لكلا المضادين و 5 (21.7%) عزلات مقاومة لكلا المضادين و7 (30.5%) عزلات حساسة لاحد المضادين ومقاومة للاخر.تم تحديد التركيز المانع للطفرات (Mutant prevention concentration) MPC) لكل من المضادين levofloxacin و ceftazidime كلا على حدة مرة واخرى بعد دمجهما وذلك لخمسة من العزلات الحساسة و خمسة من العزلات المقاومة لكلا المضادين. اعتمادا على قيمة الMPC والMIC تم حساب قيم الـ Mutation selection window

(MSW) لكل من المضادين levofloxacin وceftazidime كلا على حدة مرة واخرى بعد دمجهما معا للعزلات نفسها التي تم تحديد الMPC لها (10 عزلات). لوحظ انخفاض في قيمة الMSW عند دمج المضادين معا بمقدارضعف الى ضعفين بالمقارنة مع قيمتها للمضاد levofloxacin (قبل الدمج) للعزلات الحساسة (5عزلات) مما يشير الى وجود فعل تآزري عند دمج كلا المضادين على تلك العزلات،اما فيما يخص العزلات المقاومة (5 عزلات) فاظهرت قيم الMSW لها عند دمج كلا المضادين عدم وجود فعل تآزري بين المضادين على تلك العزلات مقارنة مع قيم الMSW للمضادين كلا على حدة مما يؤكد عدم امكانية استعمال الدمج بين كلا المضادين ضد العزلات المقاومة لهما.

Introduction

Pseudomonas aeruginosa is ubiquitous organism causing world-wide morbidity and mortality. This species readily develops resistance among human pathogens now occurs in almost every bacterial species for which antibiotic therapies exist (1).

Development of resistance to antimicrobial agents and the emergence of multi resistant pathogens have generated world wide concern in the medical community.Infctions caused by resistant bacteria are associated with higher rates of hospitalization, greater length of hospital stay and higher rates of illness and death (2).Fluoroquinolones , such as ciprofloxacin and levofloxacin, are routinely used to treat patients with *P. aeruginosa* infections .Floroquinolones resistance can be selected for upon exposure to the floroquinolones ,leading to a dramatic increase in MICs subsequent treatment failure (3,4).

A major goal of antimicrobial therapy is to achieve a sufficient drug exposure in relation to MIC, at the site of infection, for optimal efficacy .However, bacterial infection may contain subpopulation of mutant variants with reduce susceptibility to the antimicrobial agent. Thus, a therapy effective against the major part of the population might select for growth of the less subpopulation single-step mutant (5).

The mutant prevention concentration (MPC) is the concentration of drug that prevent the growth of the least susceptible single-step mutant presenting large bacterial population (5, 6). The antibiotic concentration range between MIC and MPC is the mutant selection window (MSW), whereas single-step mutant will be enriched. The MSW is bound by the MIC at its lower end and the organisms MPC at its upper end (6).

The addition of a second antibiotic to a floroquinolone treatment regimen has been shown to lower an organisms MPC (6) .In order to survive treatment with two antimicrobials, an organism has to develop spontaneous mutations causing resistance to both drugs, assuming that the two antimicrobials act via different mode of action and that the organism is initially susceptible to both agents (6,7).

Approach designed to reduce the rate at which antibiotic resistance developed is the use of combination therapy, whereby the additive or synergistic action of two or more drugs is exploited (8).

The aim of our work is to determine the MIC, MPC and the MSW of levofloxacin and ceftazidime each alone and in combination with each other for ceftazidime and levofloxacin sensitive and resistant clinical isolates of *P.aeruginosa*

Materials and methods

- **Bacterial isolates**

Twenty three clinical isolates of *P.aeruginosa* isolated from wound infections were collected from Al-Yarmok hospital (9 isolates) and Al-Wasiti hospital (14 isolates). These isolates diagnosed as *P.aeruginosa* according to Stolp and Starr. (9) and marked as M1 to m23.

- **Media and growth conditions**.

Muller Hinton broth and Muller Hinton agar (HiMedia –India) were used for bacterial growth. isolates were grown at 37°c and liquid culture were aerated by shaking.

- **Antibiotics**

The antibiotic used in this study were levofloxacin(Ortho-McNeil pharmaceutical. USA) and ceftazidime (LDP-laboratories, spain).

- **MIC determination**

The MIC were determined by broth dilution method using Muller Hinton broth and recorded as antibiotic concentration required to inhibit visible growth (10). All MIC determinations were conducted in duplicate on separate day.

• **MPC determination**

The isolates were grown overnight on Muller Hinton agar at 37°c in ambient air. The overnight growth was inoculated in to Muller Hinton broth and incubated for three hours at 37°c in ambient air in order to achieve inocula of ~10^{10} CFU/ml (6, 7).

The inocula were quantified through the serial dilution and plating of 0.1 ml samples on antibiotic-free medium

Simultaneously, *P.aeruginosa* mutants were selected by plating the inocula on Muller Hinton agar containing 1X, 2X, 4X, 8X, 16X or 32X of the levofloxacin MIC alone and in combination with ceftazidime (32µg/ml), the selected concentration of ceftazidime used in combination with levofloxacin reflects it's average 24 hours serum concentration in healthy adults and held static in all plates per combination experiment, regardless of the levofloxacin concentration (7, 11).

The inocula were also plated on Muller Hinton agar containing 1X, 2X, 4X, 8X, 16X or 32X the MIC of ceftazidime.

The antibiotic-containing plates were incubated in ambient air at 37°c for 48 hours, the antibiotic –free pates were incubated under the same conditions for 24 hours (7, 12). All MPCs determination were conducted in duplicate on separate day.

Results and Discussion

This study included 23 clinical isolates of *P.aeruginosa* isolated from wound infections these isolates were divided according to the susceptibility to both levofloxacin and ceftazidime determined by minimum inhibitory concentration (MIC) according to the national committee for clinical laboratory standard (10). the use of disk method was less reliable than the dilution test in predicting levofloxacin susceptibility results(13)

The results shows that 11 (47.8%) isolates were sensitive to both levofloxacin and ceftazidime, 5(21.7%) isolates were resistant to both antibiotics and 7(30.5%) isolates were sensitive to one of them and resistant to the second (Table-1).

The study concentrated on the isolates which were resistant to both antibiotics (levofloxacin and ceftazidime) and also to those which were sensitive to both, whereas the isolates that were resistant to one and sensitive to the second antibiotic were neglected. The study groups include 5 resistant isolates and 5 sensitive one's.

Mutant prevention concentration to levofloxacin alone, levofloxacin in combination with ceftazidime and to ceftazidime alone were determined to both resistant and sensitive isolates as shown in Table (2) and Table (3).

The MPC of levofloxacin to the five sensitive isolates were 1-5 folds more than it's MIC this emphasizes that levofloxacin at high doses prevent resistance in *P.aeruginosa* and consistent with the fact that this agent is concentration-dependent bacterial killer (12). Whereas the MPC of ceftazidime to the same isolates were 4-5 folds more than it's MIC and can't find the MPC to the M4 and M10 isolates, this indicates that ceftazidime was not able to prevent resistance when used alone and provide a caution regarding using ceftazidime alone instead of in combination for the treatment of infections caused by *P.aeruginosa* (7). MPC of levofloxacin in combination with ceftazidime in sensitive isolates were less than MPC of levofloxacin alone by 1-2 folds table (3).

What is novel about combination of MPC is the concept of using specific combination of antimicrobials not to simply increase bacterial killing but to actually maximize resistance prevention (7).

The MSW was calculated by dividing MPC/MIC to levofloxacin alone and in combination with ceftazidime and to ceftazidime alone for each resistant and sensitive isolates.

The MSW of levofloxacin in combination with ceftazidime in sensitive isolates were less than MSW of levofloxacin alone by 1-2 folds as shown in Table (3).

This means that the combination of levofloxacin and a second antimicrobial(with each antimicrobial possessing independent activity against *P.aeruginosa* and acting with a different mechanism of action) is more effective at preventing resistance selection in *P.aeruginosa* than are the two agents individually, these findings is compatible with the results of Zhanel *et al.,* (7).

concerning the resistant isolates (five) ,MPC of levofloxacin was 2-3 folds more than it's MIC except the isolate M21 in which the MIC equals to the MPC .Whereas, MPCs of ceftazidime in three isolates could not be measured, in isolate M2 MPC=MIC and in isolate M1 the MPC is more than it's MIC by 4 folds (Table-2).

Resistance to flouroquinolones (levofloxacin) happened as a result of reduced affinity of Topoisomerase II and /or Topoisomerase IV, while to ß-lactame (ceftazidime) happened as a

result of derepression of ß-lactamase AmpC which is either partial or total derepression(14). Mutant Prevention Concentration of levofloxacin in combination with ceftazidime resistant isolates were equal or more than it's value in levofloxacin alone (Table-2) and also MSW for levofloxacin in combination with ceftazidime were either equal or more than it's value in levofloxacin alone (Table-2).

These results in resistance isolates indicates that dosing above MPC during monotherapy with levofloxacin or ceftazidime would not be possible with approved dosing procedures and evaluations of drugs toxicity (15).In addition, the absence of a decrease MSW in the levofloxacin combination regimen support the hypothesis that the dual-drug therapy can be effective in preventing selection for resistance mutants, hence bacteria must be susceptible to both antimicrobials (7).

Table 1: Minimum inhibitory concentrations (MIC's) for 23 clinical *P.aeruginosa* isolates determined by broth dilution.

Sensitive isolates			Resistant isolates			Sensitive to one of them and resist to the second		
No. of isolates	MIC of ceftazidime µg/ml	MIC of levofloxacin µg/ml	No. of isolates	MIC of ceftazidime µg/ml	MIC of levofloxacin µg/ml	No. of isolates	MIC of ceftazidime µg/ml	MIC of levoflox-acin µg/ml
M3	4	2	M1	1024	32	M5	8	1
M4	4	1						
M7	1	0.5	M2	512	64	M6	8	0.5
M10	2	0.25						
M11	2	0.25	M13	32	16	M8	32	2
M12	4	0.25						
M14	4	0.25	M18	2048	16	M9	512	2
M17	2	0.5						
M19	1	0.25	M21	32	16	M15	1024	0.25
M20	4	1				M16	32	
M23	4	2				M22	8	8

Table 2: Mutant prevention concentration (MPC) and MPC/MIC (MSW) to levofloxacin alone and in combination with ceftazidime and to ceftazidime alone for five resistant (to levofloxacin and ceftazidime) P.aeruginosa isolates.

Resistant isolates						
Isolates No.	MPC µg/ml ceftazidime	MPC µg/ml levofloxacin	MPC µg/ml combination	MPC/MIC ceftazidime	MPC/MIC levofloxacin	MPC/MIC combination
M1	16384	256	256	16	8	8
M2	512	256	256	1	4	4
M13	>1024	64	128	>32	4	8
M18	>65536	64	512	>32	4	32
M21	>1024	16	16	>32	1	1

Table 3: Mutant prevention concentration (MPC) and MPC/MIC (MSW) to levofloxacin alone and in combination with ceftazidime and to ceftazidime alone for five sensitive (to levofloxacin and ceftazidime) *P.aeruginosa* isolates.

Sensitive isolates						
Isolates No.	MPC µg/ml ceftazidime	MPC µg/ml levofloxacin	MPC µg/ml combination	MPC/MIC ceftazidime	MPC/MIC levofloxacin	MPC/MIC combination
M3	64	4	2	16	2	1
M4	>128	8	2	>32	8	2
M7	16	4	2	16	8	4
M10	>64	8	2	>32	32	8
M11	64	2	1	32	8	4

Conclusion

- Mutaunt prevention concentration (MPC) potentiates successful therapy with fluoroquinolone antibiotics.
- Combination therapy might not only provide a greater likelihood of pathogen killing but also a greater likelihood of resistance prevention.
- In combination therapy, bacteria must be susceptible to both antimicrobials.

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