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Comparative Study of the Mutant Prevention Concentrations of Sulfamethoxazole-Trimethoprim Alone and in Combination with Levofloxacin against *Stenotrophomonas maltophilia*

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Abstract

Objectives: To determine the mutant prevention concentration (MPC) of sulfamethoxazole-trimethoprim (SXT) alone and in combination with levofloxacin (LVX) against *Stenotrophomonas maltophilia* (*S. maltophilia*) and to determine if the combination may decrease the emergence of resistant mutants. **Methods:** The MPC with 20 *S. maltophilia* strains which were both susceptible to SXT and LVX were determined by inhibiting visible growth among 10^{10} CFU on four agar plates after 72 hours incubation at 37˚C. **Results:** All except two strains (18/20) showed a mutant prevention concentration ≥ 152/8 μg/mL for SXT and the range of the mutant prevention concentration for the SXT in combination with LVX is 9.5/0.5~608/32 μg/mL, which demonstrates at least 2 fold reduction except one strain. There was a significant difference ($P < 0.01$) between SXT alone and in combination with LVX on the mutant prevention concentration and mutant prevention concentration/minimum inhibitory concentration values. **Conclusions:** The MPC/MIC values were narrowed for SXT by combining with LVX against the *S maltophilia*. The combination may decrease the enrichment of mutant bacterial populations. Much study is needed to verify whether the using of drug combinations may restrict or even block the selection of *S. maltophilia* mutants.

Keywords

Stenotrophomonas maltophilia, Mutant Prevention Concentration, Sulfamethoxazole-Trimethoprim, Levofloxacin

1. Introduction

S. maltophilia is an important non-fermentative gram-negative bacterium isolated from clinical infectious samples. Macrolide antibiotics, sulfamethoxazole-trimethoprim (SXT) and β-lactam antibiotics are the common therapeutic strategies used to against the *S maltophilia* [1] [2]. *S. maltophilia* demonstrates a broad resistance to multiple classes of antibiotics such as SXT, β-lactam antibiotics, fluoroquinolones, aminoglycosides, macrolides and so on. The intrinsic resistance determinants including encoding β-lactamases, antibiotic-modifying enzymes and multidrug resistance efflux pumps and the acquired drug-resistance by the horizontal transfer of antibiotic resistance through integrons, transposons, and plasmids. The antibiotic resistance of *S. maltophilia* is a growing problem that lacks effective solutions [3] [4]. One of the important contributions to increased resistance is the inadequate use of antibiotics [5]. New therapeutic strategies and antibiotic-drugs should be developed to prevent the outgrowth of resistant mutants.

The "mutant prevention concentration" (MPC) concept and the hypothesis of "mutant selection window" (MSW) may be a useful way to control the resistance by restricting the enrichment of mutants. The MPC, proposed by Drlica, is the drug concentration to prevent the emergence of the mutants. The MSW is a range of drug concentrations exists in which mutants can be enriched. The lower boundary of the range is the minimum inhibitory concentration (MIC) and the upper is the mutant prevention concentration (MPC) [6] [7] [8]. This concept and hypothesis have been used to study a variety of bacteria to achieve slowing the amplification of resistance mutants [9] [10] [11] [12]. Many studies have suggested that the combined treatments might be an effective regimen to restrict the emergence of drug-resistance mutants [13] [14].

The purpose of this study is to investigate the effect of MPC for SXT alone and in combination with levofloxacin (LVX) against *S. maltophilia*. In order to find a way to slow or even block the emergence of the SXT-resistant mutants and want to know whether combination therapy for *S. maltophilia* infections would be effective to slow or prevent the emergence of the SXT-resistance.

2. Materials and Methods

2.1. Bacterial Isolates and Drugs

In our study, susceptible isolates were selected for testing and the MPC/MIC was used to interpret the results. 20 nonduplicate *S. maltophilia* strains were part of clinical isolates collected from 34 hospitals in Anhui, China, during 2010 to 2014. Isolates identifications were carried out by a MicroScan WalkAway 96 System (Dade Behring, Deerfield, IL). *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 35218 and *E. coli* ATCC 25922 were stored in Anhui Center for Surveillance of Bacterial Resistance (Hefei, Anhui, China) as the quality control strains. The antibiotics in this study including: sulfamethoxazole-trimethoprim (SXT); levofloxacin (LVX). Two of the antibiotics were obtained

from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP).

2.2. MIC and MPC Determinations

The Minimum Inhibitory Concentration (MIC) for two antibiotics was determined by Mueller-Hinton agar (Oxoid Ltd., Cambridge, UK) doubling dilution method according to Clinical and Laboratory Standards Institute (CLSI 2013) [15]. MPCs were determined as follows: each strain was inoculated onto Mueller-Hinton agar (MHA) plates and grown overnight at 37˚C in ambient air. A single colony of the strains were swabbed into 20 ml of Mueller-Hinton broth (MHB) and were cultured at 37˚C for 24 h. Bacterial suspensions were centrifuged (3500 rpm for 15 min) and transferred into 200 ml of MHB for 6 h with shaking at 37˚C in order to achieve a concentration of 3×10^{10} CFU ml^{-1}. Suspensions of 100 μL (3×10^{9} CFU ml^{-1}) were plated onto MHA plates containing 1×, 2×, 3×, 4×, 5×, 6×, 7× MIC concentrations of SXT alone or in combination with LVX (2 μg/mL). Plates contained drugs were cultured at 37˚C in ambient air for 72 h. The resistant mutants were confirmed by regrowth on agar containing antibiotics at the concentration used to select the mutants. All experiments were performed separate days. The MPC were determined as the lowest antibiotic concentrations that inhibited visible bacterial growth among 10^{10} CFU.

The MPC/MIC was defined as a range of concentration that resistant mutant subpopulations are selected and amplified in. The lower values mean the better ability of antibiotic to prevent emergence of mutants [16] [17].

2.3. Statistical Analysis

Univariate assessment of characteristics was performed using Fisher's exact test or the Mantel-Haenszel test for categorical variables. Independence of data was assumed, $P < 0.05$ in the two-tailed test was used as the criterion of significance. Statistical analyses were performed using SPSS V11.0 software (SPSS Inc., 2000).

3. Results

Table 1 shows MICs, MPCs and MPC/MIC ratios for SXT alone and in combination with LVX against 20 stranins of *S. maltophilia* in vitro activities. The results shows that the MPCs for SXT alone and in combination with LVX against susceptible *S. maltophilia* strains were 38/2-1216/64 μg/mL and 38/2-608/32 μg/mL, respectively. The MPC/MIC ratio of SXT alone is 2 - 64. After the addition of LVX at a concentration (2 μg/mL), the MPC/MIC ratio decreased 2-to 16-fold except No. 40 Strain which have no change. There are 4 isolates demonstrate a remarkable decrease in the MPC/MIC ratio after combination with LVX. No. 20, No. 47 and No. 023 strains decreased 4-fold and strain 036 decreased 16-folds whose MPC/MIC ratio became 1 (Figure 1). Different MPC/MIC ratio of SXT and SXT plus LVX indicated that they have different abilities in preventing the growth of first-step mutants of *S. maltophilia*. Statistically significant differences exist between the values of MPC and MPC/MIC for SXT alone and in

Table 1. MICs, MPCs and MPC/MIC ratio for SXT alone and in combination with LVX in 20 strains of *S. maltophilia.*

Isolate No.	MIC (μg/mL)		MPC (μg/mL)		MPC/MIC	
	SXT	LVX	SXT	SXT + LVX	SXT	SXT + LVX
1	9.5/0.5	0.5	152/8	76/4	16	8
6	19/1	0.5	152/8	76/4	8	4
12	38/2	0.5	152/8	76/4	4	2
20	9.5/0.5	0.25	152/8	38/2	16	4
40	19/1	1	38/2	38/2	2	2
47	19/1	0.5	152/8	38/2	8	2
57	19/1	4	76/4	38/2	4	2
67	38/2	0.5	1216/64	608/32	32	16
80	19/1	1	152/8	76/4	8	4
83	19/1	0.5	152/8	76/4	8	4
023	4.75/0.25	0.25	152/8	38/2	32	8
031	9.5/0.5	0.25	304/16	152/8	32	16
036	9.5/0.5	0.25	152/8	9.5/0.5	16	1
1103	19/1	0.25	152/8	76/4	8	4
1113	19/1	0.5	1216/64	608/32	64	32
1122	19/1	0.25	304/16	152/8	16	8
1308	9.5/0.5	0.5	152/8	76/4	16	8
1313	9.5/0.5	0.5	608/32	304/16	64	32
1321	4.75/0.25	2	304/16	152/8	64	32
1326	4.75/0.25	0.5	304/16	152/8	64	32

MIC, minimum inhibitory concentration; MPC, mutant prevention concentration; MPC/MIC, defined as the ratio of the MPC obtained to the original MIC.

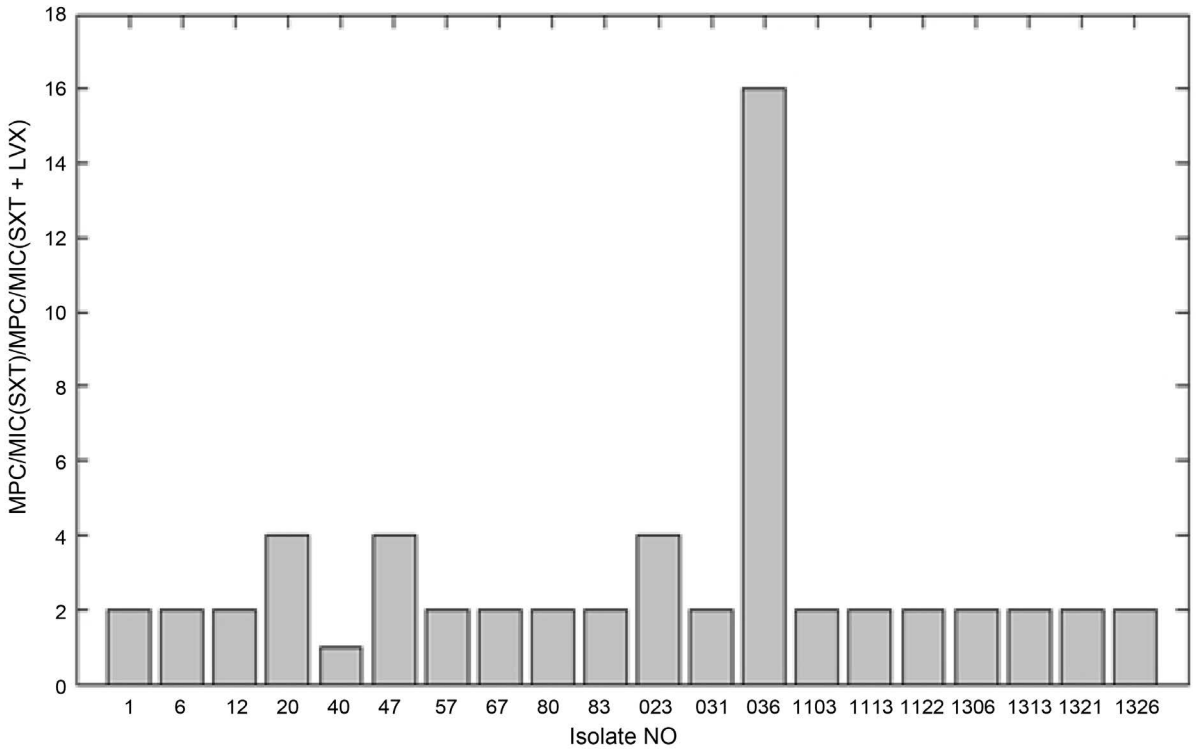

Figure 1. The SXT comparison with the SXT + LVX in MPC/MIC. After combination with LVX, most of the isolates demonstrate a 2flod reduction except No. 20, No. 47, and No. 023 a 4foldreduction, No. 036 a 16 fold reduction.

combination with LVX ($P < 0.01$).

Table 2 and Table 3 describe the effects of MIC of LVX and SXT on MPC and MPC/MIC, respectively. Combining the same MICs of SXT with various MICs of LVX result in a significant ($P < 0.005$) decrease only on MPC/MIC (Table 2). Different MIC in the sensitive range of SXT combining with the same MIC of LVX can make a statistical significant difference ($P \leq 0.01$) between SXT and SXT + LVX on MPC and MPC/MIC (Table 3).

4. Discussion

S. maltophilia is an important opportunistic pathogen that can be isolated from a variety of sources and lead to bacteremia, endocarditis and pneumonia under the condition of the patients with hypoimmunity [18]. As the recommended drug for the treatment of *S. maltophilia* infections, SXT has been applied in clinical for many years. Many resistance mechanisms and resistance genes of *S. maltophilia* have been widely studied. More attentions were paid to some new methods trying to impede this emergence. One of them is combination therapy,

Table 2. The effect of LVX for MIC on MPC.

Isolate No.	MIC (μg/mL)		MPC (μg/mL)		MPC/MIC	
	SXT	LVX	SXT	SXT + LVX	SXT	SXT + LVX
6	19/1	0.5	152/8	76/4	8	4
40	19/1	1	38/2	38/2	2	2
47	19/1	0.5	152/8	38/2	8	2
57	19/1	4	76/4	38/2	4	2
80	19/1	1	152/8	76/4	8	4
83	19/1	0.5	152/8	76/4	8	4
1103	19/1	0.25	152/8	76/4	8	4
1113	19/1	0.5	1216/64	608/32	64	32
1122	19/1	0.25	304/16	152/8	16	8

Table 3. The effect of SXT for MIC on MPC.

Isolate No.	MIC (μg/mL)		MPC (μg/mL)		MPC/MIC	
	SXT	LVX	SXT	SXT + LVX	SXT	SXT + LVX
1	9.5/0.5	0.5	152/8	76/4	16	8
6	19/1	0.5	152/8	76/4	8	4
12	38/2	0.5	152/8	76/4	4	2
47	19/1	0.5	152/8	38/2	8	2
67	38/2	0.5	1216/64	608/32	32	16
83	19/1	0.5	152/8	76/4	8	4
1113	19/1	0.5	1216/64	608/32	64	32
1308	9.5/0.5	0.5	152/8	76/4	16	8
1313	9.5/0.5	0.5	608/32	304/16	64	32
1326	4.75/0.25	0.5	304/16	152/8	64	32

the method that had been studied by our team completely [19]. Another is using MSW theory, defining a range of concentrations between MIC and MPC, to prevent the selective enrichment of mutant bacterial and restrict the development of antibiotic resistance. The MPC is the lowest concentration that can limit the emergence of MIC, minimum inhibitory concentration; MPC, mutant prevention concentration; MPC/MIC, defined as the ratio of the MPC obtained to the original MIC limit the emergence of resistance at the infectious site. Our study is focusing on the MPCs of the SXT in *S. maltophilia*.

Many studies including vitro studies and animal models have led to a better knowledge about the MPC and MSW which showed positive effects on decreasing the emergence of resistance bacteria [20] [21]. To our knowledge, this is the first report to demonstrate the usage of the theory on SMA. Initially, the concept of MPC and the MSW hypothesis were only applied to those bacteria and antibiotics that did not associate with horizontal gene transfer. However, Rafael and Maria-Isabel point out that resistance mechanisms involving horizontal gene transfer can be researched by MPC and MSW [22]. In their opinion the MPC concept also can be used in variety of antibiotics, including fluoroquinolones, glycopeptides, mcrolide, and tetracycline and so on.

According to MSW theory, antimicrobial treatment failure and emerging of drug-resistant strains maybe related to the clinical dose felled into the resistance mutations selected by the window. Which means only sensitive bacteria can be killed, whereas few spontaneous resistant mutants tend to be enriched selectively. When the concentration of the drug is higher than MPC, only can pathogens enduring two or more times of mutation be growing, so the possibility of the emergence of resistant mutants is pretty small [23].

Trimethoprim and Sulfamethoxazole are both well absorbed from the gastrointestinal tract. From the pharmacokinetic point of view, after the usual oral dose of 160 mg of Trimethoprim and a 1200 mg oral dose of Sulfamethoxazole, a Peakserum level of about 2 μg/ml and 60 μg/ml are reached in 4 hours, respectively [24]. And the mean half-lives of trimethoprim and sulfamethoxazole were about 10 hours. The peak serum level of SXT may can be considered as 60/2 μg/ml. In this study, the MPC of SXT was tested among 20 strains show high resistance to these agents. The MPC values of SXT against SMA most are ≥ 152/8 μg/ml (**Table 1**). It shows that the plasma concentrations (≤60/2 μg/ml) in bacterial infection site are far below the MPC value (≥152/8 μg/ml) of the drug, and just falls within the MSW, which facilitates the selective enrichment of drug-resistant mutants. The results suggest that the clinical routine doses of single-agent application SXT easily lead to increased cases of bacteria resistant to SXT, and a large dose of SXT may cause serious side effects. So narrowing the MSW through combination therapy is a good way. Zhanel *et al*. [25] and Cai *et al*. [11] showed that combination of LVX with colistin is more efficient than colistin alone at preventing selection of colistin resistance in *Pseudomonas aeruginosa*. Mohammed *et al*. exhibited LVX in combination with Ceftazidime can result in a synergistic action in sensitive isolates of *Pseudomonas aeruginosa* [26]. The

MPC values and MPC/MIC ratio of SMA have decreased (except for the isolate 40) after SXT combined with LVX The MPC and MPC/MIC of SXT in combination with LVX were less than the MPC and MPC/MIC of SXT alone by 1 - 16 folds (Table 1). The change of MPC and MPC/MIC ratio by statistical analysis was statistically significant ($P < 0.01$). The results suggest that LVX combined SXT have a better ability to prevent the production of SXT resistant mutants. Concerning the impact of the two drugs on the results of different MIC, select 10 bacteria having the same MIC of SXT, as (Table 2), combined with LVX having the same MIC. The MPC and MPC/MIC to SXT alone were less than MPC and MPC/MIC of SXT significant difference was found between the two MPC groups. Another 10 strains have the same MIC of LVX and different MIC of SXT. There is a significant difference between two MPC groups and two MPC/MIC groups as shown in Table 3. The reason of the difference between the two tables is unclear.

In this study, the hypothesis that the dual-drug therapy may prevent the production for resistance mutants can be supported by the SXT combination regimen against *S. maltophilia*. But a limited number of samples may result in biased results. More samples and the amount of experimental data are needed to validate the results.

5. Conclusion

The MPC/MIC values were narrowed for SXT by combining with LVX against the *S. maltophilia*. The combination may decrease the enrichment of mutant bacterial populations. Much study is needed to verify whether the using of drug combinations may restrict or even block the selection of *S. maltophilia* mutants.

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Competing Interests

No conflict of interest among the authors of this paper.

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