원 저

Mutant Prevention Concentration of Polymyxin B for the Clinical Isolates of *Pseudomonas aeruginosa*

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임상적으로 동정된 *Pseudomonas aeruginosa*에 대한 Polymyxin B의 Mutant Prevention Concentration

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Backgroud : Infection caused by multi-drug resistant (MDR) Gram-negative organisms such as *Pseudomonas* and *Acinetobacter* species is one of emerging important problems in modern hospitals. To treat multi-drug resistant non-fermenting Gram-negatives, polymyxins which were used in 1960s, but abandoned because of grave toxicities such as renal toxicity are reused. The objective of this study was to estimate the probability of resistance development of the clinical isolates of Pseudomonas aeruginosa to polymyxins.
Methods and Materials : Twenty-nine multidrug-resistant *P. aeruginosa* isolates were collected from Dankook University Hospital and Seoul National University Hospital in 2000 and tested for antimicrobial susceptibility test, minimal inhibitory concentration (MIC), mutant prevention concentration (MPC) and mutant frequency to ciprofloxacin and polymyxin B.
Results : The MIC_{50} and MIC_{90} of polymyxin B for the isolates were 2 and 2 μg/mL, and those of ciprofloxacin were 0.5 and 4 μg/mL, respectively. Thirteen of 29 isolates developed polymyxin B-resistant mutants but all 29 isolates, ciprofloxacin-resistant mutants. The MPC_{50} and MPC_{90} of polymyxin B were 32 and 64 μg/mL, and those values of ciprofloxacin were 4 and 64 μg/mL. Mutation frequencies of polymyxin B ranged from 2×10^{-9} to 2×10^{-7}, and those of ciprofloxacin from 4×10^{-10} to 5×10^{-7}.
Conclusions : Mutation frequencies of polymyxin B were similar to those of ciprofloxacin, suggesting appreciable development of resistant mutants with wide usage of polymyxins.

Key Words : *Pseudomonas aeruginosa*, Polymyxin B, Mutant prevention concentration, Mutant frequency

Introduction

Multi-drug resistant (MDR) Gram-negative infections are one of emerging important problems in modern hospitals. Non-fermenters such as *Pseudomonas* and *Acinetobacter* spp. are usually the most resistant organisms and readily acquire resistance (1-3). Considering the dearth of available antimicrobials to these resistant organisms, the appropriate use of antimicrobial is becoming increasingly important.

Mutant selection window is an antimicrobial concentration range, extending from the minimal concentration required to block the growth of wild-type bacteria up to that required to inhibit the growth of the least susceptible, single-step mutant. The upper boundary is called the mutant prevention concentration

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(MPC) (4). To prevent the development of resistant mutant, the serum concentration of antimicrobials higher than MPC is required theoretically, however, achievement of the serum concentration higher than MPC is practically difficult in most circumstances (4, 5).

The increasing prevalence of multi-drug resistant non-fermenting Gram-negatives has revived the interest in polymyxins. Polymyxins are bacteriostatic antibiotics which were used in 1960s, but abandoned because of grave toxicities such as renal toxicity (6). However, recent clinical data on the treatment results of MDR *P. aeruginosa* or *Acinetobacter* spp. showed that polymyxins can be an acceptable substitute for the serious infections by MDR Gram-negatives (1, 2, 7). Recently, *Acinetobacter* spp. resistant to polymyxins were identified in So Paulo Hospital in Brazil shortly after the use of polymyxins. The MICs of polymyxins for those isolates from blood ranged from 8 to 32 μg/mL, and all the 4 organisms disclosed distinct PFGE types (8).

The objective of this study was to estimate the probability of resistance development of the clinical isolates of *P. aeruginosa* to polymyxins.

Materials and Methods

Bacterial strains Twenty nine multidrug-resistant *Pseudomonas aeruginosa* isolates were collected from Dankook University Hospital and Seoul National University Hospital in 2000. *P. aeruginosa* PAO1 was used as a reference strain.

Antimicrobial susceptibility test Antibiotic susceptibility was determined by the disk diffusion test, according to National Committee for Clinical Laboratory Standards (NCCLS) (9). Antimicrobial disks tested included cefoxitin, cefotaxime, ceftazidime, cefepime, gentamicin, amikacin, and imipenem (Becton & Dickinson, NJ, U.S.). Minimal inhibitory concentrations (MICs) of polymyxin B and ciprofloxacin were determined by agar dilution method, according to National Committee for Clinical Laboratory Standards (NCCLS) (9, 10).

Mutant prevention concentration Mutant prevention concentrations and mutation rates of polymyxin B and ciprofloxacin were determined as described (4, 5, 11). Briefly, clinical isolates were grown overnight on Trypticase soy agar (TSA), and they were then inoculated in 100 mL of LB broth and grown at 37℃ for 18 hours. Broth cultures were centrifuged, and the pellet was resuspended in 10 mL of phosphate buffered saline (PBS) (100 μL of each strain contained $\geq 10^9$ cfu). One hundred μl of each strain were plated onto each plate containing 8, 16, 32, and 64 μg of polymyxin B (3 plates per each concentration) or 1, 2, 4, 8, and 16 μg of ciprofloxacin (3 plates per each concentration), and 32, 64, 128, and 256 μg of ciprofloxacin if necessary. Plates were incubated at 37℃ for 48 hours, and the number of colonies on each plate was counted. For ciprofloxacin, MPC value was recorded as the lowest concentration of ciprofloxacin to prevent the emergence of any mutants after 48 hours of incubation. On the other hand, however, polymyxin B showed strong tendency of inoculum effect, and colonies grew in clusters on the plate. Therefore, the method to determine MPC of polymyxin B was slightly modified as follows. Among the colonies which grew on the plates of the highest and the second highest concentrations of polymyxin B, 10 separate colonies were picked up. The colonies were cultured serially twice on the TSA without any antimicrobial (12), and the MIC of polymyxin B for each colony was determined by agar dilution test. The colonies whose MIC of polymyxin B was 4-fold higher than that for mother strain were considered as mutants. The MPC values of polymyxin B were determined as the lowest concentration of polymyxin B to prevent the emergence of any mutants after 48 hours of incubation. Mutation rate of ciprofloxacin was calculated as the mutant number divided by the number of inoculum of each strain. However, the approximate mutation rate of polymyxin B was obtained by the following equation; $R=P\times M/I$, where P represents the percentage of mutants among 10 colonies whose MICs of polymyxin B were tested previously, M, the total number of colony on the plate with maximum concentration of polymyxin B, and I, the number of inoculum of each strain.

Results

The MIC_{50} (median value of MIC) and MIC_{90} (90 percentile of MIC) of polymyxin B for the isolates were 2 and 2 μg/mL, and those of ciprofloxacin were 0.5 and 4 μg/mL, respectively (Table 1). We selected mutants

Table 1. MIC*, MPC†, Mutation Rate of Ciprofloxacin and Polymixin B and Susceptibilities of Other Antimicrobials for the Clinical Isolates of *P. aeruginosa*

No.	Ciprofloxacin				Polymyxin B				Susceptibility by disc diffusion							
	MIC (μg/mL)	MPC (μg/mL)	MPC/ MIC	Frequency	MIC (μg/mL)	MPC (μg/mL)	MPC/ MIC	Frequency	CTX	CAZ	FOX	FEP	NA	AN	GM	IPM
PAO1	0.5	4	8	3.E-7	2	NC										
2	0.25	2	8	4.E-10	2	NC			R	R	R	I	R	S	S	R
3	1	8	8	6.E-08	2	NC			R	R	R	I	R	S	S	R
4	0.13	2	16	6.E-08	2	NC			I	S	R	S	R	S	S	S
5	0.5	2	4	3.E-07	1	NC			R	S	R	S	R	S	S	I
6	1	16	16	2.E-07	1	NC			R	I	R	I	R	S	S	R
7	0.25	2	8	6.E-08	2	16		2. E-07	I	S	R	S	R	S	S	S
8	2	8	4	1.E-08	2	64	8	7. E-08	R	S	R	S	R	S	R	I
9	<0.03	1	>32	7.E-10	2	NC	32		I	S	R	S	R	R	I	S
10	2	16	8	2.E-08	2	64		2. E-09	R	S	R	S	R	S	R	R
11	0.5	4	8	4.E-07	2	32	32	9. E-09	I	S	R	S	R	S	S	R
12	1	8	8	5.E-07	2	NC	16		R	S	R	S	R	S	S	R
13	1	16	16	8.E-08	2	32		8. E-08	R	S	R	S	R	S	R	R
14	0.5	64	128	2.E-07	1	NC	16		R	S	R	S	R	S	R	I
15	>16	256	<16	6.E-08	2	64		2. E-08	R	S	R	S	R	S	R	R
16	0.5	8	16	5.E-08	1	NC	32		R	S	R	S	R	S	R	R
17	0.25	4	16	6.E-09	2	32		2. E-08	R	S	R	S	R	S	R	S
18	0.13	4	32	1.E-08	2	64	16	2. E-09	I	S	R	S	R	S	S	S
19	<0.03	1	>32	2.E-07	2	64	32	2. E-08	S	S	R	S	I	S	S	I
20	4	32	8	2.E-08	2	NC	32		R	I	R	S	R	S	S	I
21	<0.03	2	>64	2.E-07	1	NC			R	R	R	S	R	S	S	S
22	8	128	16	5.E-08	2	NC			R	S	R	S	R	R	R	S
23	0.25	2	8	9.E-09	2	NC			R	S	R	S	R	S	S	S
24	0.25	2	8	2.E-09	2	NC			R	S	R	S	R	S	S	R
25	0.25	2	8	3.E-07	2	16		4. E-08	I	S	R	S	R	S	S	S
26	0.13	4	16	4.E-08	2	>64	8	7. E-08	R	S	R	S	R	S	S	S
27	0.5	2	4	1.E-09	2	NC	>32		R	S	R	S	R	S	S	S
28	<0.03	2	>64	2.E-08	1	NC			R	R	R	S	R	S	S	S
29	1	8	8	1.E-07	2	32		1. E-07	I	S	R	S	R	S	S	R
30	0.13	8	64	2.E-08	1	32	16	4. E-09	R	R	R	R	R	S	S	S
median	0.5	4		5.E-08	2	32	32	2. E-08								
90%	2	64		3.E-07	2	64		2. E-07								

Abbreviations : MIC, minimum inhibitory concentration; MPC, mutant prevention concentration; CTX, cefotaxime; CAZ, ceftazidime; FOX, cefoxitin; FEP, cefepime; NA, nalidixic acid; AN, amikacin; GM, gentamicin; IPM, imipenem; NC, not-checkable

from 13 of 29 clinical isolates shown by their elevated MICs of polymyxin B. As for the remaining 16 isolates, colonies grew on polymyxin B containing agar plates, however, their MICs of polymyxin B were not elevated. The MPC_{50} (median value of MPC) and MPC_{90} (90 percentile of MPC) of polymyxin B were 32 and 64 μg/mL, and those values of ciprofloxacin were 4 and 64 μg/mL, respectively (Table 1). The MPC/MIC ratio of polymyxin B ranged from 8 to >32 (median 16), and the ratio of ciprofloxacin was similar (4 to >64). The MPC/MIC ratios of polymyxin B were similar to those values of ciprofloxacin for the susceptible strains (t test, P= 0.57). Mutation frequencies of polymyxin B ranged from 2×10-9 to 2×10-7, and those of ciprofloxacin from 4×10-10 to 5×10^{-7}. MICs of mutants selected by polymyxin B ranged from 4-fold to 16-fold higher values than those of mother strains.

MPC represents a novel in vitro measurement of fluoroquinolone potency, and is recently applied to pharmacokinetic parameters such as peak and sustainable serum drug concentration not only to achieve significant kill of bacteria but also to minimize resistant development in body. However, applicability of MPC to other class antimicrobial agents is not clearly defined, and relationship between the MPC and pharmacodynamic effects is not established (13). Furthermore inoculum effect is a limitation for achieving target inocula for some antimicrobials. Indeed, the MPC values and

mutation frequencies of polymyxin B could be approximately measured with modification in this study because of strong inoculum effect of polymyxin *B. Furthermore*, the pharmacokinetic parameters for polymyxins have not yet been clearly determined.

Previous reports showed that serum peak concentration of polymyxin B was 8 mg/L when 50 mg of polymyxin B were intramuscularly administered (7), and that peak value of colistimethate was 21.4 mg/L on intravenous administration of 63 mg of colistimate (14). Furthermore, the half lives of polymyxin B sulfate and colistimate were 6 hours and 3.4 hours, respectively. For the ciprofloxacin, maximum serum concentration reached 4.5 mg/L for usual dosing regimen as intravenous administration of 400 mg of ciprofloxacin per 12 hours. The half life of ciprofloxacin was measured 3 to 4.5 hours (15).

Although 2/3 of clinical isolates of *P. aeruginosa* susceptible to ciprofloxacin in this study had MPC values of 4 mg/L or below and other previous reports presented low MPC values of ciprofloxacin for most isolates of *P. aeruginosa* (13), it is well known that ciprofloxacin and other fluoroquinolones select resistant subpopulation readily through the activation of efflux pumps (15, 16). Polymyxin resistance of *P. aeruginosa* was known to be associated with modulation of PmrAB two-component system, and polymyxin resistance phenotypes of *P. aeruginosa* were associated with mutations in H-box motif of the PmrB sensor kinase (12).

Although the pharmacokinetic parameters of polymyxins are not clearly evaluated and resistance mechanisms of both drugs are different, considering that MPC values of polymyxin B were far higher than the peak serum concentration of polymyxin B and mutation frequencies were similar to those of other antimicrobials, polymyxin B resistant mutant strains are highly expected to develop in clinical isolates of *Pseudomonas aeruginosa*.

Abstract

배 경 : 최근 항생제에 다제내성을 갖는 Pseudomonas나 *Acinetobacter*와 같은 그람 음성균들의 빈도가 증가하고 있다. 이로 인해 신장독성 등으로 인해 사용이 제한되었던 polymyxin이 이런 균주의 감염 치료를 위해 다시 사용되고 있다. 이에 본 저자들은 임상적으로 검출되는 *Pseudomonas aeruginosa*의 polymyxin B에 대한 Mutant prevention concentration (MPC), 돌연변이체빈도(mutant frequency)를 측정하고자 하였다.

재료 및 방법 : 2000년도에 단국대학병원과 서울대학병원에서 검출된 29개의 *P. aeruginosa*에 대하여 항균제 감수성시험, 최저억제농도(MIC) 및 ciprofloxacin과 polymyxin B에 대한 MPC와 돌연변이체빈도를 구하였다.

결 과 : *P. aeruginosa*의 polymyxin B에 대한 MIC_{50}과 MIC_{90}은 각각 2 ug/mL, 2 ug/mL였고 ciprofloxacin은 0.5 ug/mL, 4 ug/mL이었다. 29개의 균주 중에서 13개가 내성을 보였고 polymyxin B에 대한 MPC_{50}과 MPC_{90}은 각각 32 ug/mL, 64 ug/mL였고, Ciprofloxacin에 대해서는 4 ug/mL, 64 ug/mL였다. 돌연변이체빈도는 polymyxin B에 대해서는 2×10^{-9}에서 2×10^{-7}였고, ciprofloxain에 대해서는 4×10^{-10} 에서 $5\times10^{-}$였다.

결 론 : 이 연구에서 polymyxin B와 ciprofloxacin에 대한 *P. aeruginosa*의 돌연변이체빈도는 비슷하였다. 따라서 이 균주에 대해 광범위한 polymycin B를 사용하는 것은 상당한 정도의 내성균주의 출현을 야기할 것이다.

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The experiments comply with the current laws of the country in which they were performed.

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