EFFECTS OF ANTIBIOTICS ON ADHESION AND INVASION OF *PROTEUS MIRABILIS*

F. Jabalameli[*1], F. Malekzadeh[2] and A. Mirsalehian[1]

1) Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran

2) Department of Biology, Faculty of Sciences, Tehran University, Tehran, Iran

Abstract- The ability of subminimum inhibitory concentrations (sub-MICs) of antibiotics to affect the virulence factors of bacteria may be an important criterion in selecting an antibiotic for therapy. *Proteus mirabilis* is one of the most frequently seen pathogens in urinary tract infections. The aim of this study was to analyze the effects of some antibiotics on two important virulence factors of *P. mirabilis*. In this study the effects of 1/2, 1/4 and 1/8 of the MIC of amoxicillin, gentamicin and nalidixic acid on adhesion and invasiveness of two clinical isolates of *P. mirabilis* were evaluated. Sub-MICs of ampicillin significantly reduced adhesion of *P. mirabilis* to uroepithelial cells and gentamicin exerted the same effect to lesser extent. Invasion of kidney epithelial cells by *P. mirabilis* in the presence of antibiotics was also evaluated. Ampicillin and nalidixic acid caused a reduction in the number of intracellular bacteria. Gentamicin showed the lowest inhibitory effect on cell invasion. The results indicate that the sub-MICs of antibiotics can affect virulence factors of *P. mirabilis*. The presence of sub-MIC effect of antibiotics may be an important factor in determining the dosing regimen for urinary tract infections.

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INTRODUCTION

After administration, the concentration of any antimicrobial agent will reduce to sub-inhibitory levels in the human body (1). Although these subminimum inhibitory concentrations (sub-MICs) do not kill bacteria, they are still capable of modifying their physicochemical characteristics and architecture of their outermost surface and interfering with some important bacterial cell functions and host-bacteria interactions (2). Some of these effects are results of changes in cell morphology (3), rate of growth (4), production of enzyme and toxin (5) and adhesive properties (2,6).

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*** Corresponding Author:**
F. Jabalameli, Department of Microbiology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 8043219,
Fax: +98 21 8955810
E-mail: Jabalr@hotmail.com

Proteus mirabilis is a common cause of urinary tract infection (UTI) in catheterized patients and those with urinary tract abnormalities and occurs with significant frequency in hospitals. It shows a predilection for the upper urinary tract where it can cause serious kidney damage, acute pyelonephritis, bladder or renal stones, fever and bacteriemia (7).

Several characteristics of this organism have been studied in relation to its virulence of urinary tract, including hydrolysis of urea by urease, cell invasiveness, cytotoxicity induced by hemolysins, cleavage of IgA and IgG by proteolytic enzyme and adherence to the uroepithelium mediated by fimbriae (8).

The purpose of the present study was to investigate the *in vitro* effects of different sub-MICs of three antibiotics, ampicillin, gentamicin and nalidixic acid, on adhesion and invasion of two uropathogenic isolates of *P. mirabilis,* taken from clinical isolates.

MATERIALS AND METHODS

Two *P. mirabilis* strains taken from clinical isolates (urinary infection) were used. The strains were identified as *P. mirabilis* employing standard microbiological methods. Following antibiotics were used: ampicillin trihydrate (Gist brocades, Netherlands), gentamicin sulphate (Meijiserka Kaish, Japan) and nalidixic acid (Merck). The MICs of ampicillin, gentamicin and nalidixic acid were assessed by macrodilution method (9). Selected sub-MICs were 1/2, 1/4 and 1/8 × MIC levels of each antibiotics.

Urine samples from the healthy female volunteers were obtained aseptically, centrifuged at 450 rpm for 10 min. The precipitate was twice washed with sterile phosphate buffer saline (PBS) (pH= 7), treated with 0.4 NHCl and again washed with PBS. Washed cells at a concentration of 4.5 × 105 uroepithelial cells (UECs)/ml, counted by haemacytometer, were used. Bacteria grown in Mueller Hinton Broth (MHB) amended with sub-MIC of antibiotic, were harvested by centrifugation (10000 rpm for 20 min), resuspended in PBS and adjusted to a concentration of 1.5 × 108 CFU/ml. Then, 0.5 ml of UECs were added to 0.5 ml of bacterial cell suspension and the mixture was incubated for 1 hr at 37°C, with shaking.

The cells were harvested by centrifugation at 10000 xg for 20 min, washed four times with PBS to remove non-adherent bacteria and placed in 1 ml fresh PBS. A drop of the suspension was placed on a slide, stained with acridine orange for 2 min and was observed using light microscopy (Zeiss, Germany) the number of bacteria attached to UECs were counted with ≥ 20 adhering bacteria per 50 cells. Bovine kidney (BK) and baby hamster kidney (BHK) cells were maintained in yeast extract lactalbumin earle's (YLE) medium and stocker medium (Hesarak Razi Institute); the cells were grown in multi-well trays for 24 hr, at 30° C, in a 5% CO_2 humid atmosphere. Wells containing confluent cell mono layers were washed twice with HBSS (Hanks balanced salt solution).

Bacterial cultures grown in MHB for 24 hr were diluted using incubation medium. The incubation medium comprised HBSS (80% v/v), HEPES (N-2 hydroxy-ethyl-piperazine-N'-2-ethane-sulfonate) buffer 2 mM (10% v/v) and MHB (10% v/v). One ml of cultures (MHB, MHB amended with 1/2, 1/4 and 1/8 × MIC) was added to each well containing BK or BHK cells. A 1/150 dilution of 24 h bacterial culture was used in experiments, 1.5 × 107 CFU/ml *P. mirabilis* strains 1 and 2. After incubation for 2 hr at 37°C, growth was determined by serial dilution supernatant fluid and plating on nutrient agar (Merck). The cells were harvested and twice washed with HBSS, treated with streptomycin (30 µg/ml tissue culture medium, 50 fold MIC) to kill extracellular bacteria. Trays were incubated for 3 hr at 37°C and wells were treated with blood lysing solution (NaH2P04 0.01 M, tween 20 1 % v/v, trypsin 0.025% w/v) pH= 8. Incubation for 30 min at 37°C was sufficient to lyse BK and BHK cells, as determined by inverted microscopy (Olympus L×70). Bacterial counts were obtained by plating dilution of lysed cell suspensions on nutrient agar. Each test was repeated 3 to 4 times for each concentration.

The Kruskal-Wallis was used to analyze differences between the results for the control and antibiotic-treated-cultures. $P < 0.05$ was considered to be statistically significant.

RESULTS

The minimum inhibitory concentrations of ampicillin, gentamicin and nalidixic acid for *P. mirabilis* 1 and 2 are given in table 1. The effect of sub-MIC on adhesion of *P. mirabilis* 1 and 2 to UECs is presented in tables 2 and 3. Ampicillin, gentamicin and nalidixic acid inhibited bacterial attachment to UECs. No significant difference was observed between strains. Ampicillin caused the greatest suppression of adherence, the number of bacteria adhering to the surface of UECs were reduced to 15.22% (at 1/2 × MIC) compared with those bacteria grown in absence of antibiotics.

Table 1. MICs of gentamicin, ampicillin and nalidixic acid against *P. mirabilis*-1 and *P. mirabilis*-2

Bacterial	**Antibiotics (µg/ml)**		
strain	Ampicillin	Gentamicin	Nalidixic acid
P. mirabilis-1	0.5	1	4
P. mirabilis-2	1	1	4

Table 2. Effects of sub-MICs of the antibiotics on adhesion of *P. mirabilis*-1 to uroepithelial cells

	Antibiotics		
Concentration	Ampicillin	Gentamicin	Nalidixic acid
0	6.2±2/848	7.75±3.622	5.925±2.39
1/8× MIC	3.95±3.08	6.075±3.354	4.35±2.832
	(P= 310^{-5})	(P= 0.01)	(P= $7.16*10^{-4}$)
1/4× MIC	2.675±2.28	5.55±2.801	3.875±3.575
	(P<10^{-7})	(P= $4.8*10^{-3}$)	(P= $1.4*10^{-5}$)
1/2× MIC	0.95±0.904	4.225±1.954	3.15±2.824
	(P <10^{-7})	(P= 10^{-6})	(P= $10^{-6)}$

Abbreviations: SD, standard deviation; MIC, minimum inhibitory concentrations.
*Data are given as mean number of bacteria/uroepithelial cell±SD.
†*P* values are compared with control.

BK and BHK cell invasion by *P. mirabilis* 1 and 2 at sub-MICs of ampicillin, gentamicin and nalidixic acid were similar for both strains.

According to table 4, the number of intracellular bacteria in the effect of ampicillin and nalidixic acid reduced significantly compared with those intracellular bacteria grown in the absence of antibiotics.

The inhibition of invasion was high at the sub-MICs of ampicillin and nalidixic acid, but gentamicin showed the least inhibition of invasion, that was not significant at the most concentrations.

The percent of intracellular bacteria for *P. mirabilis* 1 is shown in figure 1.

Table 3. Effects of Sub-MICs of the antibiotics on adhesion of *P. mirabilis*-2 to uroepithelial cells

	Antibiotics		
Concentration	Ampicillin	Gentamicin	Nalidixic acid
0	11.5911±3.64	10.725±3.434	11.55±3.63
1/8× MIC	7.25±2.629	9.125±2.747	8.4±4.367
	(P< 10^{-6})†	(P= 0.01)	(P= 3.6×10^{-6})
1/4× MIC	2.761±2.174	7.852±2.96	6.815±3.531
	(P< 10^{-6})	(P= 3.1×10^{-4})	(P< 10^{-6})
1/2× MIC	2.575±1.662)	6.35±2.875	5.35±1.77
	(P< 10^{-6})	(P< 10^{-6})	(P< 10^{-6})

Abbreviations: SD, Standard Deviation; MIC, minimum inhibitory concentrations.
*Data are given as Mean number of bacteria/uroepithelial cell±SD.
† *P* values are compared with control.

Table 4. Effects of sub-MICs of ampicillin on invasion of *P.mirabilis*-1 and *P.mirabilis*-2 to cells BK and BHK*

			Ampicillin		
Strain	Cell line	Control	1/2× MIC	1/4×MIC	1/8× MIC
PM-1	BHK	18.4±0.926	0.14±0.035	2.4±0.042	4.35±0.065
			(P= 0.005)†	(P= 0.001)	(P= 0.005)
PM-1	BK	17±0.341	0.9±0.08	2±0.21	6±0.741
			(P= 0.001)	(P= 0.01)	(P= 0.001)
PM-2	BHK	6.6±0.594	0.51±0.071	1±0.034	3±0.212
			(P= 0.001)	(P= 0.003$^{)}$	(P= 0.000)
PM-2	BK	5±0.486	0.44±0.028	1±0.143	2.32±0.342
			(P< 10^{-7})	(P< 10^{-7})	(P< 0.001)

Abbreviations: BK, bovine kidney; BHK, baby hamster kidney; PM-1, *P.mirabilis*-1; PM-2, *P.mirabilis*-2
*Data are given as number of intracellular bacteria× 10^4±standard deviation.
†*P* values are compared with control

Table 5. Effects of sub-MICs of gentamicin on invasion of *P.mirabilis*-1 and *P.mirabilis*-2 to cells BK and BHK*

			Gentamicin		
Strain	Cell line	Control	1/2× MIC	1/4× MIC	1/8× MIC
PM-1	BHK	18.4±0.926	13.45±0.326	15.2±0.941	16.7±0.235
			(P= 0.01)†	(P= 0.01)	(NS)
PM-1	BK	17±0.341	13±0.821	15.68±0.94	15.98±0.89
			(P= 0.01)	(P= 0.01)	(NS)
PM-2	BHK	6.6±0.594	5.7±0.341	5.98±0.256	6.16±0.371
			(NS)	(NS)	(NS)
PM-2	BK	5±0.486	4.78±0.531	4.8±0.621	4.66±0.075
			(NS)	(NS)	(NS)

Abbreviations: BK, bovine kidney; BHK, baby hamster kidney; NS, not significant; PM-1, *P.mirabilis*-1; PM-2, *P.mirabilis*-2.
*Data are given as number of intracellular bacteria×10^4±standard deviation.
†*P* value are compared with control.

Table 6. Effects of sub-MICs of nalidixic acid on invasion of *P.mirabilis*-1 and *P.mirabilis*-2 to cells BK and BHK*

			Nalidixic acid		
Strain	Cell line	Control	1/2×MIC	1/4× MIC	1/8× MIC
PM-1	BHK	18.4±0.926	2±0.013	3.2±0.065	5.6±0.014
			(P= 0.005)†	(P=0.001)	(P= 0.001)
PM-1	BK	17±0.341	2±0.025	6±0.545	11±0.253
			(P= 0.005)	(P= 0.005)	(P=0.01)
PM-2	BHK	6.6±0.594	1.2±0.034	2.35±0.352	3.3±0.384
			(P= 0.001)	(P=0.001)	(P= 0.01)
PM-2	BK	5±0.486	1.4±0.043	2.3±0.211	3.95±0.062
			(P= 0.001)	(P= 0.001)	(P=0.005)

Abbreviations: BK, bovine kidney; BHK, baby hamster kidney.
*Data are given as number of intracellular bacteria×10^4±standard deviation.
†*P* value are compared with control.

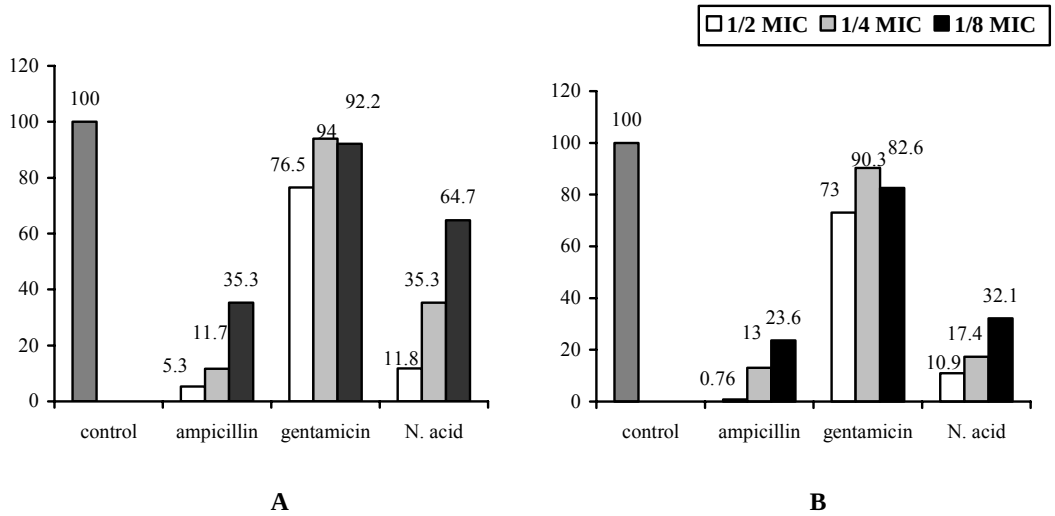

Fig. 1. Percent of penetration of *P. mirabilis*-1 to BK (A) and BHK (B) cells.

DISCUSSION

It has long been known that antibiotics may kill bacteria or inhibit their growth, but the effects of sub-MIC of antimicrobial agents on bacteria has attracted attention because knowledge of the effects on bacteria exposed to sub-MIC concentrations is considered to be useful for optimizing therapy.

Alteration of the morphology and physiology of bacteria at sub-MIC of antibiotics has been demonstrated by many investigators (1). Sub-MICs of antimicrobial agents examined in this study was found to affect adherence properties as described by other investigators. Ampicillin, gentamicin and nalidixic acid at sub-MICs inhibited attachment of *P. mirabilis* to UECs. As expected, the percentage of inhibition was dose-related, increasing according to the amount of the sublethal concentration for each drug. Tullio *et al.* reported that sub-inhibitory concentrations of netilmicin, ceftriaxone, cefotaxime, aztreonam and piperacillin resulted in decreased adhesive properties for proteus strains (10). Another report has demonstrated that amikacin significantly reduced adhesion of proteus strains to human uroepithelial cells and gentamicin exerts the same effect to a lesser extent (11). Sub-MICs of mupirocin dose-depently suppressed flagellin expression and flagella formation of *P. mirabilis*, (12).

Several theories have been exposed to account for the effect of antimicrobials at sub-inhibitory concentrations on bacterial adherence to epithelial cells. The agents may induce partial loss of fimbriae from the surfaces of bacteria which have been exposed to them. Ampicillin, a β-lactam, inactivates enzymes involved in peptidoglycan synthesis, thereby altering the surface of bacteria. Gentamicin, an aminoglycoside, reduces affinity of *P. mirbilis* for adhesion by blocking protein synthesis of fimbriae and nalidixic acid may affect the expression of fimbriae gene (13).

An increasing interest has been expressed in cell invasiveness as an important step in pathogenesis in UTI caused by different bacteria. The persistence of infection may be a result of ability of *P. mirabilis* to invade human epithelial cells *in vivo* and *in vitro*, thereby evading the humoral system and destroying tissue. Peerbooms *et al.* described invasion of mammalian cells, both *in vivo* and *vitro*, by *P. mirabilis* and reported that invasion efficiency depends on hemolytic activity of proteus strains (14).

Investigators have observed that inhibition of bacterial protein, RNA or DNA synthesis with bacteriostatic antibiotics reduces invasion drastically (15). We observed influence of sub-MICs of antibiotics on this virulence factor for two strains of *P. mirabilis* using BK and BHK cells. Inhibitory effect of ampicillin and nalidixic acid at sub-MIC was significant. Ampicillin showed highest inhibition at 1/2× MIC, with significant reduction in the number

of intracellular bacteria. The effect of gentamicin was the least. Antibiotics of different structure and known inhibitory activity show extensive stimulation or inhibition of a large number of promoters when target bacteria are exposed to sub-inhibitory concentrations of the drugs. In fact factors or processes necessary for efficient invasion by *P. mirabilis* may be affected by inhibition of protein, RNA or DNA synthesis. However, *in vivo* studies in humans and with animal models are needed to better delineate the role of sub inhibitory concentrations of antibiotics in UTI.

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