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Original scientific paper

In vitro Effect of Subinhibitory Concentrations of Ceftazidime and Meropenem on the Serum Sensitivity of *Pseudomonas aeruginosa* Strains

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A B S T R A C T

The aim of this study was to determine the effect of subminimal inhibitory concentrations (subMICs) of ceftazidime, meropenem and gentamicin on the in vitro serum sensitivity of Pseudomonas aeruginosa *strains isolated from a variety of isolation sites at two medical wards and an intensive care unit in a government university hospital in Croatia. A total of 20 serum-resistant* P. aeruginosa *strains isolated from different clinical specimens were selected. Bacteria were exposed to 1/2, 1/4, 1/8, 1/16, and 1/32 x MIC of each antibiotic tested. Sensitivity of* P. aeruginosa *strains to bactericidal activity of normal human serum before and after bacterial exposure to subMICs was determined. Significant difference in serum sensitivity of the strains was observed after the bacteria were exposed to subMICs of ceftazidime and meropenem ($p<0.01$), while the exposure to subMICs of gentamicin did not affect significantly the resistance of tested strains to the serum bactericidal activity. Comparing the number of serum-resistant strains before and after exposure to subMICs of antibiotics, statistically significant differences were determined ($p<0.01$) after exposure of the strains to 1/2, 1/4, 1/8 and 1/16 x MIC of meropenem, and after exposure to 1/2, 1/4 and 1/8 x MIC of ceftazidime. SubMICs of ceftazidime and meropenem affected not only the resistance to serum bactericidal activity of bacteria, but also their morphology. The alterations in bacterial morphology caused by subMICs of ceftazidime and meropenem could be connected with consecutive bacterial serum sensitivity.*

***Key words**:* Pseudomonas aeruginosa*, antibiotics, subminimal inhibitory concentrations, serum bactericidal activity*

Intoduction

Hospitalized patients are particularly susceptible to *Pseudomonas aeruginosa* infections and this organism has emerged as an important nosocomial pathogen during the past three decades[1]. This is partly due to the increased number of patients particularly prone to such infections: immunocompromised patients, patients with malignancy, cystic fibrosis, burns or traumatic wounds. Infections with *P. aeruginosa* is associated with severe morbidity, and despite antibiotics, lethality to this bacterium is one of the highest among the nosocomial pathogens[1]. *P. aeruginosa* is responsible for approximately 200 000 nosocomial infections that occur annually[2].

Antibiotics are often present at the site of infection at subinhibitory concentrations, because in human treated with intermittent dosage schedules of antibiotics, subinhibitory levels will always follow suprainhibitory concentrations. Values such as minimal inhibitory concentrations (MICs), and serum levels of antibiotics do not necessarily reflect the situation at the cellular level and, moreover, antibiotics are often present in subinhibitory concentrations at sites of infections[3]. Exposure of bacteria to subminimal inhibitiory concentrations (subMICs) of diverse antibiotics, although not able to kill bacteria, can modify their biological characteristics, may cause al-

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teration in bacterial morphology[4], proliferation[5], also can modulate bacterial gene expression[6], and may interfere with some essential bacterial functions such as motility or adhesiveness to different surfaces[7,8], etc., thus influencing bacterial virulence. Also, antibiotics act on susceptible bacteria by modifying their susceptibility to serum antibacterial activity and their interactions with cellular host defense components[9]. The increasing number of immunocompromised patients with infections that are difficult to treat and the attempt to reduce the problems of nosocomial infections has stimulated the research for a better understanding of the interactions between antibiotics and host defense mechanisms, and the selection of agents with immunostimulatory properties, which can contribute to recovery of the infection[10].

The aim of this research was to determine which of the studied antibiotics has the best characteristic in order to be capable to affect the serum resistance of *P. aeruginosa* strains isolated from a variety of isolation sites.

Materials and Methods

Bacterial strains: A total of 20 serum-resistant *P. aeruginosa* strains isolated from different specimens (urine, bronchial secretion, endotracheal and wound aspirates) from patients hospitalized at the Osijek University Hospital, Osijek, Croatia were selected. Bacteria were characterized as *P. aeruginosa* by standard biochemical procedures and stored in deep-agar tubes at +4 °C (1.5% nutrient agar, Difco Lab., Detroit, MI, USA) and subcultured by passaging on Trypticase soy agar (TSA, Difco Lab., Detroit, MI, USA) before use.

Antibiotics and determination of MICs: The MICs of ceftazidime (Pliva, Zagreb, Croatia), meropenem (Astra Zeneca, Zagreb, Croatia) and gentamicin (Pliva, Zagreb, Croatia) were determined for each investigated strain by standard microdulution method[11], by using serial twofold dilution of each individual antibiotic in Mueller-Hinton broth medium (Becton Dickinson and Co., Cockeysville MD, USA) in 96-well microtiter plates (Falcon 3077, Becton Dickinson Labware, New Jersey, USA). MICs were determined in duplicate and *P. aeruginosa* strain ATCC 27853 was included as control. The MICs were defined as the lowest concentrations of antibiotics that inhibited the visible growth of bacteria after incubation at 37 °C for 18–24 h.

Exposure of bacteria to subMICs of antibiotics: Bacteria were exposed to 1/2, 1/4, 1/8, 1/16, and 1/32 of MIC of each antibiotic tested as described previously by Sandberg et al.[12]. In short, a part of one bacterial colony taken from a Mueller-Hinton agar plate was inoculated in 3 mL of Mueller-Hinton broth previously distributed in tubes (approximately 10^6 bacteria/mL). The tubes were incubated at 37 °C for two hours, at which point the bacteria were in the logarithmic growth phase. After growth of the bacteria in tubes for two hours, each antibiotic being tested was added to test tubes in proportion 1:1 to a final concentrations of 1/2, 1/4, 1/8, 1/16, and 1/32 × MIC, and Mueller-Hinton broth free of antibiotics was added to the control cultures. Incubation was then continued for a further four hours. After the total of six hours of incubation at 37 °C, the bacteria were harvested by centrifugation (1200 g, 10 min.), and resuspended in phosphate-buffered saline (PBS; pH 7.1, 300 mOsmol/L) to a concentration of 1.5×10^8 cells per mL in PBS.

*Serum and serum sensitivity assay***:** Blood was obtained by venipuncture from three healthy volunteers and was allowed to clot at room temperature for 30 min. After centrifugation at 1000 g for 15 min at 4 °C, serum was removed and pooled. A portion of the pooled serum was decomplemented by heating at 56 °C for 30 min and used as test controls. Bacterial susceptibility to serum killing was measured by assessing regrowth after incubation in normal human serum according to Schiller and Hatch method[13]. Briefly, after adjustment of each bacterial suspension to a concentration equivalent to the concentration of 10^3 bacteria/mL, the mixture of 0.1 mL of bacterial suspension and 0.1 mL of pooled fresh human serum were incubated at 37 °C for two hours. Finally, the percent bacterial survival was determined by plating 0.1 mL samples onto Mueller-Hinton agar plates and the number of bacteria per milliliter was determined after incubation at 37 °C for 18–24 hours and compared to control which contained mixture of bacterial suspension and decomplemented serum. The bacterial strain was determined as serum-sensitive if there were more than 90 percent killed bacteria regarded to serum bactericidal activity. Opposite, if there were less or equal to 90 percent killed bacteria, bacterial strain was determined as serum-resistant (SR).

Morphological changes of the bacteria: Morphological changes of bacteria after exposure to subMICs of antibiotics were determined by phase-contrast microscopy (Olympus CX41 microscope, Olympus Optical Co., Hamburg, Germany). Any differences from the normal morphological characteristics (shape, damage, filamentation) were recorded and the overall frequency of their occurrence in 100 randomly observed bacteria was determined. Untreated control organisms appeared as normal rods approximately 2.5 μm long and 0.6–1 μm wide. Organisms of up to 15 μm were classified as short filaments and those longer than 15 μm as long filaments.

Statistical analysis: Proportions were compared by the χ^2-test and by Fisher's exact test when the number in any of the 2×2 table was 5. P values <0.01 were considered to be statistically significant.

Results

Serum sensitivity of the 20 *P. aeruginosa* strains with or without exposure to subMICs of ceftazidime, meropenem and gentamicin is shown in Figures 1, 2, and 3, respectively. Ceftazidime and meropenem increased the serum sensitivity of *P. aeruginosa* strains in a dose-dependent manner, as is shown in Figures 1 and 2. On the other hand, such effect was not seen after exposure of strains to subMICs of gentamicin (Figure 3).

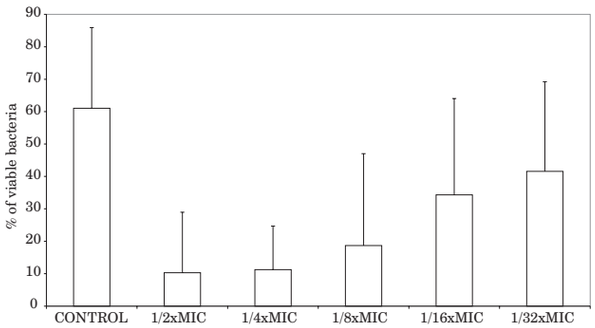

Fig. 1. Effect of subMICs of ceftazidime on serum sensitivity of the strains. MIC – minimal inhibitory concentrations.

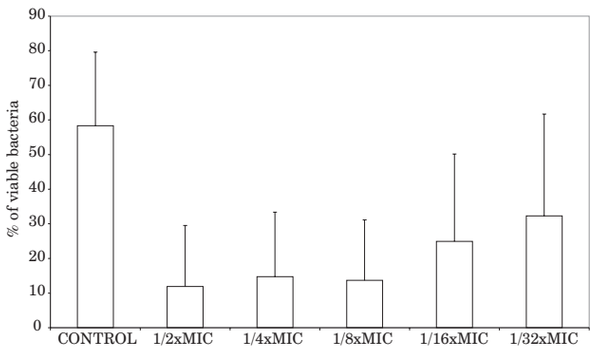

Fig. 2. Effect of subMICs of meropenem on serum sensitivity of the strains. MIC – minimal inhibitory concentration.

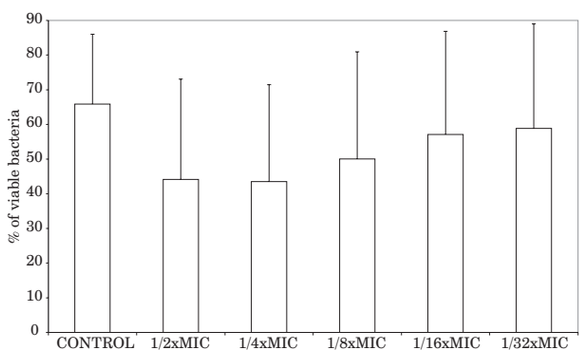

Fig. 3. Effect of subMICs of gentamicin on serum sensitivity of the strains. MIC – minimal inhibitory concentration.

Significant difference in serum sensitivity of the strains was observed after the bacteria were exposed to subMICs of ceftazidime (χ^2=42.046, DF=5, p<0.01) and meropenem (χ^2=28.687, DF=5, p<0.01), while the exposure to subMICs of gentamicin did not significantly affect the resistance of investigated strains to the serum bactericidal activity. Comparing the number of serum-resistant strains before and after exposure to subMICs of antibiotics, statistically significant differences were determined (p<0.01) after exposure of the strains to 1/2, 1/4, 1/8 and 1/16 × MIC of meropenem, and after exposure to 1/2, 1/4 and 1/8 × MIC of ceftazidime (Table 1).

As was shown by phase-contrast microscopy, subMICs of antibiotics altered not only susceptibility of bacteria to serum killing but also their morphology. Morphological changes were most prominent after bacterial exposure to 1/2, 1/4, and 1/8 × MIC of ceftazidime and meropenem. Higher concentrations of those antibiotics caused formation of very long filaments, which measured up to 30 μm. After incubation with ceftazidime at higher concentrations virtually all of the *P. aeruginosa* cells altered to a filamentous morphology. Moreover, higher concentrations of meropenem beside filamentous forms of bacterial cells, caused also the formation of irregular swellings of such filaments and formation of spheroplast-like structures, as are shown in Figure 4. The length of filaments decreased as the concentration of antibiotics was being reduced and at concentrations of 1/16 and 1/32 × MIC only short filaments were observed. SubMICs of gentamicin did not affect the bacterial morphology.

Discussion

Serum bactericidal activity has been regarded as one of the most important host defense mechanisms against bacterial infections[14]. It is known that invasive bacterial pathogens are commonly very resistant to serum bactericidal activity[15–18], and it appears that serum resistance is an important virulence factor of invasive bacteria. Although, precise mechanisms of bacterial resistance to a serum bactericidal activity is not known yet, it is known

TABLE 1
EFFECT OF SUBINHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON SERUM SENSITIVITY OF 20 SERUM RESISTANT STRAINS

Antibiotics		No. of SR[a] Strains	p-value
Ceftazidime	1/2×MIC	4	7.709×10^{-8}
	1/4×MIC	7	6.442×10^{-6}
	1/8×MIC	7	6.442×10^{-6}
	1/16×MIC	14	0.0101
	1/32×MIC	17	0.1154
Meropenem	1/2×MIC	5	3.854×10^{-7}
	1/4×MIC	7	6.442×10^{-6}
	1/8×MIC	9	7.265×10^{-5}
	1/16×MIC	12	1.638×10^{-3}
	1/32×MIC	13	0.0415
Gentamicin	1/2×MIC	17	0.1154
	1/4×MIC	18	0.2436
	1/8×MIC	18	0.2436
	1/16×MIC	18	0.2436
	1/32×MIC	19	0.5000

[a] Number of serum-resistant (SR) strains after expossure of 20 SR strains to subminimal inhibitory concentrations (subMICs) of antibiotics

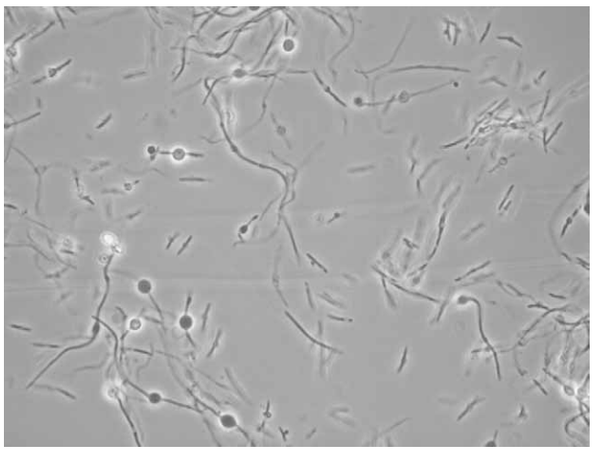

Fig. 4. Effect of 1/2× MIC of meropenem on the bacterial morphology (phase-contrast microscopy, magnification × 1000). MIC – minimal inhibitory concentration.

that such mechanisms include alterations of bacterial surface, which interfere with activation, or function of complement[19–21]. As expected, most strains of *P. aeruginosa* isolated from blood, wounds, urine, or burns are resistant to the bactericidal activity of normal human serum[13], and Schiller and Joiner suggested that serum resistance of *P. aeruginosa* does not represent a failure to activate complement, but instead reflects a failure of the assembled terminal complement complex C5b-9 to insert into the outer membrane of the strain[22]. The results of the present study indicated that the alterations in *P. aeruginosa* morphology caused by subMICs of ceftazidime and meropenem were associated with the consecutive bacterial sensitivity to serum bactericidal activity of diluted human sera. Altered cell surface characteristics of ceftazidime- and meropenem-treated bacteria may increase the bactericidal action of antibody and complement. Further studies are necessary to investigate the effects of subMICs of ceftazidime and meropenem on the molecular changes of *P. aeruginosa* cell wall components, such as expression of fimbriae, the structure and amount of outer membrane proteins and lipopolysaccharides, or other factors responsible for enhanced serum sensitivity.

Filament formation following the treatment of *P. aeruginosa* and other gram-negative bacilli with subMICs of various β-lactam antibiotics has been previously described[23–25]. This probably represents the preferential binding of those antibiotics to penicillin binding protein 3, which is required for the synthesis of peptidoglycan for cross walls, but not for side walls. Those studies indicated that the filaments produced at subinhibitory concentrations of antibiotics were devoid of fimbriae and ability to adhere to epithelial cells[24,25].

Other authors also reported that subMICs of antibiotics interfered with the pathogenic potential of *P. aeruginosa*. Fonesca et al. observed that the change in bacterial morphology and hydrophobicity caused by subinhibitory concentrations of piperacillin-tazobactam was associated with a significant decrease in the adherence ability, motility and biofilm formation of *P. aeruginosa* strains[23]. Garske et al. showed that subinhibitory concentrations of ceftazidime and tobramycin reduced the quorum sensing signals of *P. aeruginosa*[26]. On the contrary, recent study showed that subinhibitory concentrations of aminoglycoside antibiotics induced biofilm formation in *P. aeruginosa* and *Escherichia coli*[27].

Conclusion

Significant difference in serum sensitivity of the bacteria was observed after the serum-resistant *P. aeruginosa* strains were exposed to subMICs of ceftazidime and meropenem, while the exposure to subMICs of gentamicin did not affect significantly the resistance of tested strains to the serum bactericidal activity. The bacterial sensitivity to serum bactericidal activity after exposure of strains to subMICs of ceftazidime and meropenem correlated with the alterations in bacterial cell morphology.

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UTJECAJ SUBINHIBICIJSKIH KONCENTRACIJA CEFTAZIDIMA I MEROPENEMA NA *IN VITRO* OSJETLJIVOST NA BAKTERICIDNU AKTIVNOST SERUMA SOJEVA *PSEUDOMONAS AERUGINOSA*

S A Ž E T A K

Svrha istraživanja bila je odrediti djelovanje subminimalnih inhibicijskih koncentracija (subMIK) ceftazidima, meropenema i gentamicina na *in vitro* osjetljivost na baktericidnu aktivnost seruma sojeva *Pseudomonas aeruginosa* izoliranih iz različitih materijala u dva odjela te u jedinici intenzivnog liječenja jedne hrvatske kliničke bolnice. Odabrano je 20 serum-rezistentnih sojeva *P. aeruginosa* izoliranih iz različitih kliničkih uzoraka. Bakterije su izlagane koncentraciji od 1/2, 1/4, 1/8, 1/16 i 1/32 MIK-a svakog od istraživanih antibiotika. Određena je osjetljivost sojeva *P. aeruginosa* na baktericidnu aktivnost ljudskog seruma prije i nakon izlaganje bakterija subMIK-ovima. Opažena je značajna razlika u serumskoj osjetljivosti sojeva nakon što su bakterije izložene subMIK-ovima ceftazidima i meropenema, dok izlaganje subMIK-ovima gentamicina nije značajno utjecalo na otpornost istraživanih sojeva na baktericidnu aktivnost seruma. Uspoređujući broj serum-rezistentnih sojeva prije i nakon izlaganja subinhibicijskim koncentracijama antibiotika, uočena je statistički značajna razlika ($p<0,01$) nakon izlaganja sojeva koncentracijama od 1/2, 1/4, 1/8 i 1/16 MIK-a meropenema, te 1/2, 1/4 i 1/8 MIK-a ceftazidima. Subinhibicijske koncentracije ceftazidima i meropenema nisu samo utjecale na rezistenciju bakterija na baktericidnu aktivnost seruma, već i na njihovu morfologiju. Promjene bakterijske morfologije izazvane subMIK-ovima ceftazidima i meropenema mogle bi biti povezane s posljedičnom bakterijskom osjetljivošću na baktericidnu aktivnost seruma.