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Effect of different sub MIC concentrations of penicillin, vancomycin and ceftazidime on morphology and some biochemical properties of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolates

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ABSTRACT

Background and Objectives: Sub inhibitory concentrations (sub-MICs) of antibiotics, although unable to kill bacteria, can modify their physiologic and biochemical integrity and may to some extent interfere with some bacterial functions.

Materials and Methods: In this study the effect of penicillin, vancomycin, and ceftazidime were evaluated on two pathogenic and commonly encountered bacterial species in clinical practice. The test bacterial strains included *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 2783. In this study, coagulase and DNase activity, mannitol fermentation and morphologic change of *S. aureus* and oxidase activity, pigment production and morphologic change of *P. aeruginosa* were evaluated.

Results: Except for some changes in the morphology being apparent as enlarged and undivided cocci in 1/2 to 1/8 sub MIC of all above-mentioned antibiotics, *S. aureus* showed no change in any of its properties, like coagulase and DNase production and mannitol fermentation. In *P.aeuginosa,* except for some morphologic changes, i.e. elongation and filamentation, in 1/2 to 1/8 dilution of ceftazidime, no further changes were observed.

Conclusion: Exposure of bacteria to sub MICs of antibiotic can produce some detectable morphologic changes without any alteration in other biochemical properties.

Keywords: *Pseudomonas aeruginosa, Staphylococcus aureus,* Sub- MIC, Ceftazidime, Penicillin, Vancomycin.

INTRODUCTION

A critical facet of clinical microbiology is the determination of the antimicrobial susceptibility of an isolated microorganism. The *in vitro* sensitivity of an isolate to various antimicrobial agents is of great importance as a guide to therapy (1). However, while these *in vitro* analyses are relatively simple in both principle and performance, the *in vivo* response of both host and parasite to an antimicrobial agent is the sum of a complex interrelation (2). In addition to the bacterial strains susceptibility to a given antibiotic, other factor like age, immune status of patient, existing disorder, route of antibiotic administration, etc., can grossly affect the efficacy of that antibiotic (3). Due to the same reason, a given antibiotic dose may not reach its desirable level (minimal inhibitory concentration, MIC or minimal bactericidal concentration, MBC) at the affected tissue site, and may come down to a sub-inhibitory concentration (sub MIC). That is why the scientists have open a new research field by studying the effect of sub MICs of antibiotics on bacteria, in the aim to see whether these antibiotic levels could determine any modification able to reduce the expression of some bacterial virulence factors.

There is accumulating evidence that sub-MICs of antibiotics, though unable to kill bacteria, can modify their physico-chemical characteristics and the architecture of their outermost surface and may interfere with some bacterial functions (4). Recent studies show that changes may occur with quite variable mechanisms, namely, change in genetic integrity and its expression (5), alteration of growth

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and toxin production (6), inhibition of bacterial adhesion (4), increase susceptibility to phagocytosis (7) and change in morphology of the bacteria (8).

We began the present study by revisiting the reported interference of sub MICs of penicillin, vancomycin and ceftazidime on mannitol fermentation, coagulase and DNase activity and morphology of *Staphylococcus aureus,* and sub MICs of ceftazidime on pigment production, oxidase activity and morphology of *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

This study was conducted at the Diagnostic Laboratory of Valiasr Hospital, Zanjan Medical University, Iran. In this study, the effect of sub MICs of penicillin, vancomycin and ceftazidime on the mannitol fermentation, coagulase and DNase activity and morphology of *S. aureus* and the effect of ceftazidime on pigment production, oxidase activity and morphology of *P. aeruginosa* were evaluated.

Bacterial strains and culture media. The type strains including *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were received from the Iranian National Reference Laboratory. Culture media used comprised: Mueller Hinton broth and agar, DNase agar, mannitol salt agar, nutrient agar, trypticase soy agar (TSA) and trypticase soy broth (TSB).

Determination of MIC and sub MIC. The MICs were determined using the micro dilution broth method as instructed by Clinical and Laboratory Standards Institute (CLSI) (9). The obtained MIC values of penicillin, vancomycin, and ceftazidime for *S. aureus* were 0.25 µg/ml, 1 µg/ml, 8 µg/ml respectively and 2 µg/ml of ceftazidime for *P. aeruginosa*. From the different sub MIC dilutions of penicillin (1/2 to 1/128), ceftazidime (1/2 to1/128) and vancomycin (1/2 to 1/128) and ceftazidime (1/8 to 1/128), *S. aureus* was sub-cultured on Mueller Hinton, DNase and Mannitol salt agar. *P. aeruginosa* was similarly sub-cultured from ceftazidime (1/8 to 1/128) on Mueller Hinton agar. At the same time, control samples were cultured on antibiotic free media. Except for the controls, all solid media contained the same type and amount of antibiotic as in the primary sub MIC tube dilution. Finally, all plates were incubated at 37°C for 24 hours.

Morphologic evaluation and biochemical tests *of S. aureus*. Smears of *S. aureus* were prepared from visible colonies of test and control samples, and after staining, the slides were microscopically observed for any possible morphological change. Coagulase test was performed by adding a 24 hours old colony of test and control *S. aureus* into separate tubes containing 0.5 ml rabbit plasma with EDTA (Difco Laboratories and BBL Microbiology Systems) and incubating at 37°C for 24 hours. The tubes were observed with direct optic evaluation at half hour intervals for the first three hours and at one hour intervals for the remaining 21 hours (10). The test *S. aureus* strain was cultured on DNase media containing different sub MIC of antibiotics and control sample on antibiotic free media with subsequent incubation at 37 °C for 24 hours. Finally, the cultured plates were treated with normal HCl to trace the presence of any possible DNase positive colony. The test and control *S. aureus* cultures were plated on Mannitol salt agar and incubated at 37 °C for 24 hours.

Morphologic evaluation and biochemical tests *of P. aeruginosa.* Smears of *P. aeruginosa* were prepared from visible colonies of test and control samples and after staining, the slides were microscopically observed for any possible morphological change. The colonies were directly observed for presence or absence of pigment production. With the help of a glass Pasteur pipette, a small colony sample was rubbed on moistened filter paper containing 1% oxidase reagent (tetramethyl-p-phenylenediamine dehydrochloride) for detecting oxidase positive colonies (10).

RESULTS

Morphologic and biochemical test evaluations of *S. aureus*. Following microscopic evaluation of smears prepared from visible colonies of test and control *S. aureus* cultures, it was revealed that, as compared to the control sample, the bacteria in sub inhibitory concentrations of 1/2 to1/8 of penicillin, vancomycin and ceftazidime express some degree of morphologic change being apparent as enlarged and undivided Gram positive cocci (Table 1).

Since morphologic changes are less prominent in the higher dilutions, it might be possible to detect them with the help of electron microscopy. The coagulase test was positive within one hour of incubation,

similarly as the control sample, which means that these antibiotics had no effect on coagulase activity *of S. aureus*. The DNase test became positive in test as well as control samples, which means that they had no effect on DNase activity of *S. aureus*. Both the test and control *S. aureus* cultures developed colonies with a yellow color zone, because of induced acidic pH, which is indicative of a positive Mannitol fermentation test and the inability of antibiotics to inhibit this chemical property.

Morphologic and biochemical test evaluations of *P. aerugenusa.* Following the microscopic evaluation of smears prepared from visible colonies of *P. aeruginosa*, it was noticed that, as compared to the control sample, the bacteria in sub inhibitory concentrations of 1/2 and 1/4 of ceftazidime showed some morphologic changes, such as enlargement and filamentation (Table 2). The test and control *P. aeruginosa* cultures showed positive oxidase test, which means that this antibiotic had no effect on oxidase production. The pigment production by *P. aeruginosa* was unmodified for the test and control cultures in different sub MIC dilutions of ceftazidime, which means that this antibiotic had no effect on pigment production.

Table 1. Effect of Penicillin, Ceftazidime, Vancomycin sub MICs on morphology of *S. aureus*

sub - MIC concentrations	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC	1/32 MIC	1/64 MIC	1/128 MIC	control
final dilution of antibiotic in tube ug/ml	P-0.125 C-4 V-0.5	P-0.0625 C-2 V-0.25	P-0.032 C-1 V-0.125	P-0.015 C-0.5 V-0.062	P-0.0078 C-0.25 V-0.0312	P-0.00039 C-0.125 V-0.015	P-0.0019 C-0.0625 V-0.0078	0
morphologic change	P+ C + V +	P+ C + V +	P+ C+ V +	P- C- V -	P- C- V -	P- C- V -	P- C- V -	_

Table 2. Effect of Ceftazidime sub MICs on morphology of *P.aeruginosa*

Tube no.	1	2	3	4	5	6	7	8
sub - MIC concentrations	1/2 MIC	1/4 MIC	1/8 MIC	1/16 MIC	1/32 MIC	1/64 MIC	1/128 MIC	control
final dilution of antibiotic in tube ug/ml	1	0.5	0.25	012.5	0.062	0.031	0.155	0
morphologic change	+	+	-	-	-	-	-	-

DISCUSSION

We have revised an earlier view that the antibiotic at sub MIC level affect morphologic and biochemical property of bacteria. It rapidly became apparent, however, that *S. aureus* exposed to different sub MIC concentration of penicillin, vancomycin and ceftazidime showed no change in any of its properties, such as coagulase and DNase production and manitol fermentation. Morphologically it revealed some change in the form of enlarged and undivided cocci in 1/2 to 1/8 sub MIC of all above mentioned antibiotic dilutions. In the case of *P. aeruginosa*, except for some morphologic change, such as elongation and filamentation in 1/2 to 1/8 sub MIC dilution of ceftazidime, no change was observed in pigment and oxidase production.

In recent years, many researches have focused on studies regarding effect of sub MICs of various antibiotics on different morphologic and enzymatic properties of clinically important pathogenic bacteria (11). In 2003 Tsang *et al*. found that a sub inhibitory concentration erythromycin can cause some obvious morphologic, change in *P. aeruginosa* such as an increase in size, which were attested by SEM (Scanning Electron Microscopy) (12). In 2004, Fonseca *et al*. found that a 1/2 sub MIC dilution of piperacillin and tazobactam can interfere with bacterial virulence factors like adhesiveness, cell-surface hydrophobic property, motility, biofilm formation and sensitivity to oxidative stress (13). In 2004, Wozniak *et al.* showed that a sub MIC of clarithromycin could inhibit the motility of *P. aeruginosa* (14). In 1989, Grimwood *et al.* found that sub MICs of macrolides could inhibit the exoenzym production by *P. aeruginosa* (15). In 1998, Sheryok *et al*. exposed *P. aeruginosa*to sub MICs of ciprofloxacin, tobramycin and ceftazidime and observed that production of certain virulence factors like exoenzyme A, elastase, protease and phospholipase C had been inhibited (16). In 2004, Schultz found that sub MICs of macrolides could inhibit the biofilm formation in *P. aeruginosa*, as a causative agent of cystic fibrosis (17). In 2002, Gemmell *et al.* noticed a morphological change in *S. aureus*, exposed to 1/2 to 1/8 subMICs of linezolid (7). In 2004 Fonseca *et al*. studied the effect of piperacillin and tazobactam sub MICs on morphology of *P. aeruginosa*, and showed some enlargement and filamentation in 1/2 dilution (13).

Many reports have revealed that sub MICs of different antibiotics mostly cause increase in size of the tested bacteria, but in a study of Nosanchuk *et al.*, 1999, sub MICs of fluconazole and amphotericin-B had a reverse effect on *Cryptococcus neoformans* in which a decrease in size of the fungi is observed (18).

Similarly to our observations; sub MICs of various antibiotics have shown an impact on morphologic changes of different bacteria.

Nevertheless, in addition to the morphologic, modifications, some biochemical changes have also been reported. The effect of sub MICs of antibiotics on biochemical properties of bacteria is a variable phenomenon and changes depend on type of antibiotic and bacterial species. In 1986, Chopra et al. showed that sub MICs of lincomycin could reduce the production of DNsae and coagulase, whereas those of oxytetracycline inhibit coagulase production (19). In 2004, Godarzi et al. studied the different sub MICs of Chamomile alcoholic extract on DNase, catalase and hemolysin production of aureus and reported that in ¼ sub MIC, the DNase production was reduced by 45% (20). In 2000, Kavamura et al. reported a reduction in pigment production of P. aeruginosa exposed to sub MICs of macrolides (21). In 2002, Gemmell et al. showed a decrease in DNase and coagulase production of S. aureus exposed to sub MICs of linezolid. (15). In 1993, Tillioston et al. showed an increase in hemolysin production by S. aureus, exposed to sub MICs of methicillin, with no effect on coagulase production (22). Regarding DNase and coagulase activity, our study does not match with previously published findings. Following a thorough literature review and internet search, no article or paper was available regarding oxidase activity and pigment production in P. aeruginosa, and mannitol fermentation in S. aureus. As a result, no comparative comment can be presented on this issue. Now it should be determined to what extent a morphological change can affect the virulence factor pattern of a bacterial species. The reason for detecting no biochemical change in our study could be related to lack of more sensitive and more sophisticated instruments, able to detect qualitative and/or quantitative changes at

lowest concentrations. It is hoped that in future more comprehensive studies could be performed and more reliable findings be reported.

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