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Study of Antibiotic Resistance by Efflux in Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract: Twenty three multidrug resistant (MDR) strains were selected from 104 clinical isolates of *P. aeruginosa* and screened for resistance to ceftazidim, ceftriaxone, ciprofloxacin, ofloxacin and ethidium bromide by determining MICs. The MICs of EtBr and antibiotics were also measured in presence of proton conductor, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). The presence of proton gradient-dependent efflux mechanism was assessed using ethidium bromide accumulation assays. Drug accumulation studies for these antibiotics were performed to determine the drug specificity of efflux. PCR was used to identify the *mexAB-oprM* gene as a major factor in MDR intrinsic resistance of clinical isolates of *P. aeruginosa.* In absence of CCCP, the MICs of these antimicrobial agents were ≥ 4 μg L^{-1}. CCCP reduced the MICs of them at least in 1 dilution. Ethidium bromide accumulation assays confirmed the presence of proton gradient-dependent efflux mechanism in clinical isolates of *P. aeruginosa* and results of accumulation assays of drugs demonstrate that, active efflux in this bacterium are due to broadly-specific multidrug efflux system(s). PCR products demonstrate the presence of *mexAB-oprM* operon in 4 strains from 23 clinical isolates. These results confirmed the presence of proton gradient-dependent efflux mechanism in all of the clinical isolates of *P. aeruginosa* and demonstrate that, efflux pumps in this bacterium are broadly-specific multidrug efflux systems. In this study we show that MexAB-OprM multidrug efflux system was expressed in only 17% of clinical isolates of *P. aeruginosa*. These results confirmed the presence of other multidrug efflux pumps in clinical isolates of *P. aeruginosa*.

Key words: Accumulation, efflux pumps, *Pseudomonas aeruginosa*

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen exhibiting innate resistance to multiple antimicrobial agents. This intrinsic multidrug resistance is caused by synergy between a low-permeability outer membrane and expression of a number of broadly-specific multidrug efflux (Mex) systems, including MexAB-OprM and MexXY-OprM. In addition to this intrinsic resistance, these and three additional systems, MexCD-OprJ, MexEF-OprN and MexJK-OprM promote acquired multidrug resistance as a consequence of hyper-expression of the efflux genes by mutational events (Schweizer, 2003). MexAB-OprM is the major, constitutively expressed, multidrug efflux pump and the first discovered member of Resistance-Nodulation-Division (RND) family exporter in *P. aeruginosa*, is known to pump out a broad rang of structurally diverse antimicrobials including β-lactams, quinolones, tetracycline, chloramphenicol, novobiocin, macrolides and dyes. Although expressed constitutively in wild-type cells, where it contributes to intrinsic drug resistance, the operon is also hyper expressed in *nalB* multidrug-resistant mutants producing elevated levels of resistance to substrate antibiotics (Sirkumar *et al*., 1999). We evaluate here the contributions of active efflux in intrinsic multidrug resistance in clinical isolates of *P. aeruginosa* with emphasis on α-lactams, quinolones and ethidium bromide.

MATERIALS AND METHODS

Bacterial strain: Twenty three MDR strains were selected from 104 clinical isolates of *P. aeruginosa* from different clinical sources, including blood, urine, sputum, burns and bedsore. PAO1 was used as the wild-type strain. PAO1-M is a mutant strain derived from PAO1. *Pseudomonas aeruginos*a ATCC 27853 and *Staphylococus aureus* ATCC 25923 were used as controls strains for antibiotic susceptibility testing.

Antimicrobial susceptibility assay: Susceptibility of *P. aeruginosa* strains to ciprofloxacin, ofloxacin, ceftazidime, ceftriaxone, (Exir, Iran) and EtBr (Merck, Germany) measured in presence and absence of carbonyl

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cyanide m-chlorophenyl hydrazone (CCCP), using micro dilution method. CCCP (Sigma, USA) was added to a concentration of 100 μM. Results were reported as MIC, the concentration of antibiotic in presence and absence of CCCP that inhibited visible growth determined by absence of turbidity in the broth after 18 h of incubation at 37°C. This method used for first time in this laboratory. Previously, Piddock and Johnson (2002) used agar dilution method whereas micro dilution was the method of our choice.

Accumulation of ethidium bromide: Accumulation of ethidium bromide (EtBr) by all twenty three clinical isolates, the mutant PAO1-M and the wild types train was measured using a previously described method. Briefly, cells were grown overnight in nutrient broth, harvested and resuspended to an A_{600} = 0.2 in 50 mM sodium phosphate buffer, pH 7.0. EtBr was added to a final concentration of 2 μg mL^{-1}. Fluorescence of the samples was measured every 30 see for a period of 10 min at excitation and emission wave length of 530 and 600 nm, respectively, using a Shimadzu RF 5000 spectrofluorometer (Shimadzu Scientific Instruments, Inc., Columbia, Md.). CCCP was added to a final concentration of 100 μM. cells equilibrated with 50 mM sodium phosphate buffer without EtBr were used as blanks (Neyfakh, 1991).

Drug accumulation: Accumulation of ciprofloxacin, ofloxacin, ceftazidime and ceftriaxone was measured for clinical isolates showing an MIC of ≥4 mg L^{-1} of each of these antibiotics, following a method previously described by Mortimer and Piddock (1991). The fluorescence of antibiotics was measured as follows: ciprofloxacin at excitation and emission wavelengths of 279 and 447 nm; ofloxacin at 292 and 496 nm, respectively. Fluorescence of ceftazidime and ceftriaxone was measured by using innate excitation and emission wavelengths. Carbonyl cyanide *m*-chlorophenylhy drazone (CCCP) was added to a final concentration of 100 μM after 5 min. The concentrations of antibiotics were calculated using a standard curve for the respective antibiotic (concentration ranging from 100 to 1000 ng) in 0.1 M glycine hydrochloride pH 3.0. The results were expressed as nanograms of antibiotic incorporated per milligram (dry weight) of bacteria.

PCR: Two primers, Forward (5'-GATTGTCGATCCCGTTCATC-3') and reverse (5'-CATTGATAGGCCCATTTTCG-3') were used to amplify a 208 bp region from the *mexAB-oprM* operon. Reaction mixture (100 μL), including 2.5 U *Taq* DNA polymerase (CinnaGene Inc., Iran), 0.5 μM each primer, 0.2 mM each dNTP, 1 m M $MgCl_2$, 10 ng genomic DNA and 1X PCR buffer (CinnaGene Inc., Iran) was heated for 2 min at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, before completion of the reaction for 7 min at 72°C.

Selection of *P. aeruginosa* multidrug resistance (MDR) mutant: Wild-type *P. aeruginosa*, strain PAO1, was grown on Luria-Bertani (LB) agar plates and consecutively subcultured after overnight incubations on plates supplemented with increasing concentrations of ciprofloxacin. One colony capable of growing at 21 μg mL^{-1} ciprofloxacin was selected and named PAO1-M. This strain was subjected to the studies described above for clinical isolates (Kumar and Worobee, 2002).

RESULTS

Antimicrobial susceptibility assay: Wild-type strain of PAO1 was slightly more susceptible to all agents when compared with clinical strains. In clinical isolates of *P. aeruginosa* and efflux mutant PAO1M, MICs of these agents were ≥4 μg L^{-1} P reduced the MICs of these agents by ≥1-fold. In the presence of CCCP, the MICs of ciprofloxacin and ceftriaxone were lower by only 1 dilution. MICs of ofloxacin reduced in 2 dilutions and the MICs of ceftazidime were lowered by 3 dilutions, also CCCP reduced the MIC of EtBr in 4 dilutions. Consequently the MICs in presence of CCCP were lower than the MICs in absence of this PMF inhibitor (Fig. 1).

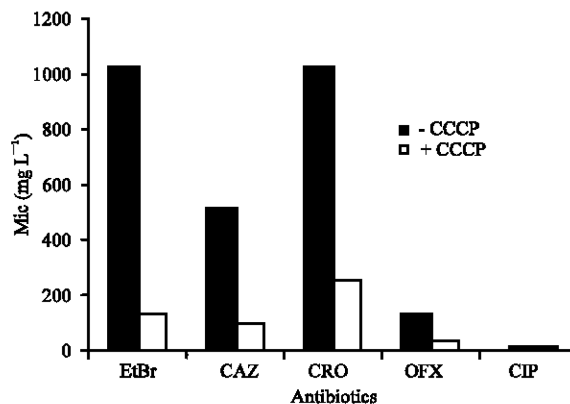

Fig. 1: MIC of antimicrobial agents in presence and absence of CCCP. Ethidium Bromide (EtBr), ceftazidime (CAZ), ceftriaxone (CRO), ofloxacin (OFX) and ciprofloxacin (CIP)

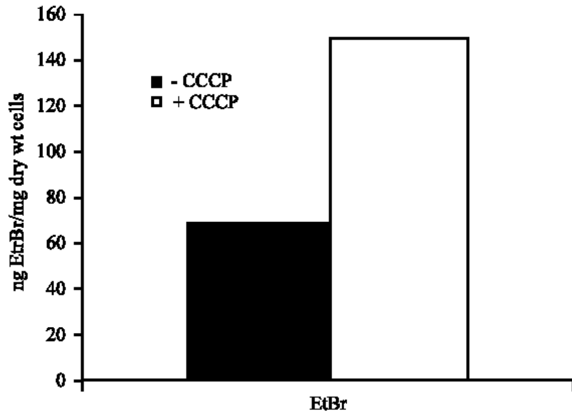

Fig. 2: Accumulation assay of ethidium bromide (EtBr) by clinical isolates of *P. aeruginosa* in absence and presence of CCCP

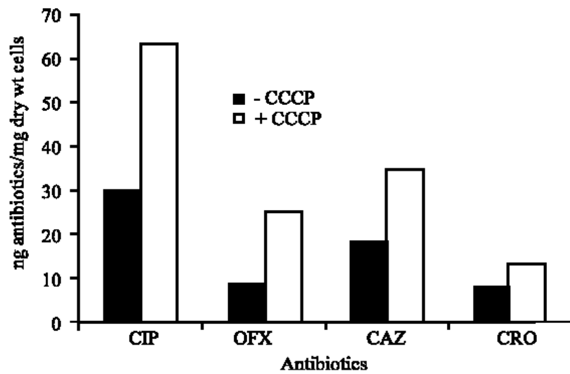

Fig. 3: Drug accumulation assays by clinical isolates of *P. aeruginosa* in presence and absence of CCCP. ceftazidime (CAZ), ceftriaxone (CRO), ofloxacin (OFX), ciprofloxacin (CIP)

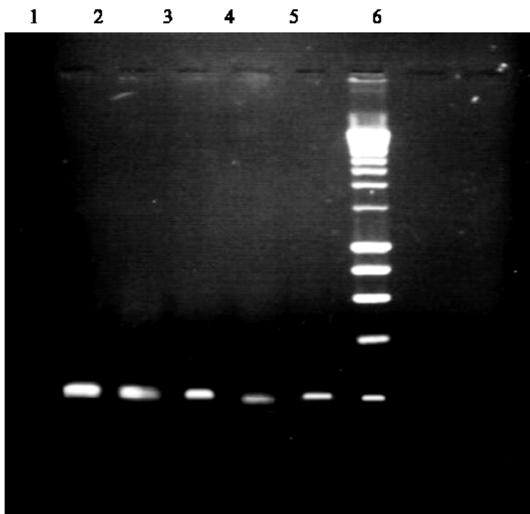

Fig. 4: Detection of *mexAB-oprM* by PCR. PAO1 M (Lane 1), 4 clinical isolates of *P. aeruginosa* (Lane 2-5), Hyper ladder 200-10000 bp (BIOLINE)

Ethidium bromide and antibiotics accumulation: All of the clinical strains and efflux mutant PAO1M were tested for their ability to antibiotics and EtBr accumulation. Results of accumulation in absence of CCCP were: EtBr; 40-140 ng mg^{-1} (dry weight) of cells, ceftazidime and ceftriaxone; 8-20 ng mg^{-1} (dry weight) of cells, ofloxacin; 3-16 ng mg^{-1} (dry weight) of cells, and ciprofloxacin; 28-32 ng mg^{-1} (dry weight) of cells. These results in presence of CCCP reached: EtBr; 68 -185 ng mg^{-1} (dry weight) of cells (Fig. 2), ceftazidime and ceftriaxone; 13-39 ng mg^{-1} (dry weight) of cells, ofloxacin; 22-32 ng mg^{-1} (dry weight) of cells and ciprofloxacin; 50 to 79 ng mg^{-1} (dry weight) of cells, (Fig. 3).

PCR: The amplicon obtained by PCR demonstrates the presence of *mexAB-oprM* operon in mutant strain, PAO1M and 4 strains from 23 clinical isolates that were studied (Fig. 4).

DISCUSSION

Active efflux is a mechanism of resistance to xenobiotics (antibiotics, biocides, dyes, detergents, metabolic inhibitors and organic solvent). In this mechanism there are membrane-bound xenobiotics transport proteins in gram negative and gram positive bacteria that expelled xenobiotics from bacteria cell. These transporters require the energy for xenobiotics efflux. In *P. aeruginosa* the most of efflux pumps energized by Proton Motive Force (PMF). In this pathogen efflux is a antiport of H+ and xenobiotics In this study we used CCCP for inhibition of proton gradient-dependent efflux pumps. Because, CCCP is a PMF inhibitor that disrupts the proton gradient across the membrane and thus inhibits proton gradient-dependent pumps (Mortimer and Piddock, 1991). In this study we examined ciprofloxacin, ofloxacin, ceftazidime, ceftriaxone and EtBr susceptibility of our clinical isolates. Consequently, in order to show that the high resistance to EtBr and these drugs are due to active efflux, we also measured the MICs in presence of CCCP. Reduction in the level of MICs in presence of CCCP shows the presence of proton gradient-efflux pump(s) in our isolates. CCCP reduced the MICs at least 2 fold which indicates the presence of active efflux in our strains. These results confirmed the presence of proton gradient dependent efflux mechanism in clinical isolates of *P. aeruginosa*. Ethidium bromide accumulation assays in presence of CCCP increased 2-2.5 fold. This difference in the level of accumulation of EtBr in presence and absence of CCCP showed active efflux in these strains. Accumulation assays of drugs in presence of CCCP increased 1.5 to 2 fold. PCR demonstrates the presence of

the major system contributing to intrinsic multidrug resistance, MexAB-OprM in 17% MDR clinical isolates of *P. aeruginosa*. This study indicates the presence the other MDR efflux pumps, in addition to MexAB-OprM in multidrug resistance in clinical isolates of *P. aeruginosa*.

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