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ResearchArticle

FUNCTIONAL STUDY OF EFFLUX PUMP INHIBITORS IN CLINICAL ISOLATES OF MULTIDRUG RESISTANT *KLEBSIELLA PNEUMONIAE*

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ABSTRACT

Objective: Recent studies showed that multidrug resistant (MDR) efflux mechanism is most common. The prevalence of active drug efflux pump was investigated in clinical isolates of *Klebsiella pneumoniae* (*K. pneumoniae)* exhibiting a multidrug-resistant phenotype in tertiary care hospital. Materials and Methods: The effects of efflux pumps inhibitors (EPI's) Reserpine 25 µg/ml and MC207-110 (Phe-Arg-beta-naphthylamide (PaβN), Sigma) 25 µg/ml, was studied on MDR clinical isolates of *K. pneumoniae*. The minimal inhibitory concentration(MIC) was performed for all the collected Clinical Isolates during the period of two years forfluoroquinolone in presence and in the absence of inhibitors as per the CLSI broth dilution method. Results: 10% of the isolates have shown reduction in MIC in the presence of PaβN and Reserpine.
Conclusions: The study indicates the necessity for the detection of overexpressing efflux pumps at diagnostic level at least in tertiary care hospitals.

Keywords: Efflux Pump, *K. pneumoniae,* Reserpine, PaβN, fluoroquinolone.

INTRODUCTION

Antibiotic efflux in bacteria was first reported in the late 1970s for tetracyclines, although drug efflux, mediated by P-glycoprotein, was originally discovered in mammalian cancer cells even earlier. Since then, efflux-mediated resistance to a wide range of antibacterial agents has been reported in a variety of bacterial species, and a number of efflux determinants have been identified [1]. Efflux-mediated tetracycline resistance was first characterized in *E. coli* and attributed to the plasmid-encoded Tet protein [2]. If the drug is pumped out faster than it can diffuse in, intra-bacterial concentrations of the antibiotic are kept low and ineffectual. Therefore, the bacterial protein synthesis proceeds at largely unimpeded rates. These pumps are variants of membrane pumps that all bacteria possess in order to move lipophilic and amphipathic molecules in and out of the cell. This acts as a protective mechanism for the microorganism and prevents it from being killed by its own chemical weapons [3]. Disruption of genes for multidrug efflux pumps resulted in a great decrease in the resistance against multiple antimicrobial agents in *E. coli* and *P. aeruginosa*. These results support the idea that multidrug efflux pumps are very important for the bacterial escape from the toxicity of many antimicrobial agents [4].

Multidrug RND (Resistance nodulation division) transporters are most frequently found in gram-negative bacteria, and are known to export a variety of clinically-relevant antimicrobial agents [5, 6]. RND protein situated within the inner membrane and function in complex with two other proteins, an outer membrane channel and a periplasmic adaptor protein, to form a tripartite efflux pump spanning both the inner and outer membrane. These multi-protein complexes transport a wide variety of substrates including antibiotics, dyes, detergents and host derived molecules from the periplasm to the extra-cellular space [7].

Overexpression of RND systems in clinical isolates is also associated with MDR [8]. Enhanced efflux is also implicated in rapidly emerging clinical resistance to new antimicrobials such as tigecycline, for which overexpression of AcrAB was detected in *E. coli* [9]. Efflux by RND pumps is driven by the proton motive force [10, 11, 12]. The efflux systems involved in *K. pneumoniae* resistance phenotype is AcrAB multidrug efflux system that is encoded by the *acrRAB*operon [13].

AcrB connects with TolC, an outer membrane protein that belongs to a family of envelope proteins found in all gram-negative bacteria and that is essential for the expulsion of a plethora of compounds [14]. However, little is known about the specific and direct role of the AcrAB multidrug efflux pump in the resistance of *K. pneumoniae.* The *K. pneumoniae* strains showed significant decrease in MICs of quinolones, chloramphenicol, and/or tetracycline was obtained with an efflux pump inhibitor PAβN [15].

By the Inhibition of activity of efflux pumps will thus have clear benefits for therapy since this will increase the susceptibility of gram negative bacilli, thus increasing the therapeutic efficacy of antibiotics used for treating such infections by these pathogens [16].

In the present study multidrug resistance (MDR) clinical isolates of *K. pneumoniae*were collected from the cultures of urine, catheter tip, and sputum etc., Isolates were tested for species conformation, sensitivity, and MIC was performed as per the guidelines of CLSI broth dilution method [17] for ciprofloxacin in presence and in absence of efflux pump inhibitor (Reserpine, and PAβN).

METHODS

An antimicrobial susceptibility test was done by Kirby Bauer disk diffusion method [18]. Isolates resistant to at least three classes of antimicrobial agents were considered as multidrug resistant [19]. Totally 20 strains were isolated. All isolates were tested for reduction in MIC of ciprofloxacin, in presences and in absence of efflux pump inhibitors 25µg/ml (Reserpine and PAβN) in triplicate [13, 20, 21, 22, 23, 24, 25].

RESULTS

Kirby Bauer disk diffusion

The antibiogram results of *K. pneumoniae* showed that, all the 20 isolates were resistant to gentamicin, amikacin, ciprofloxacin, nalidixic acid, cefotoxime, piperacillin/ tazobactum, 19 isolates were resistant to tetracycline, 18 isolates were resistant to cefaperazone/ sulbactum, 16 isolates were resistant to co-trimoxazole, 9 isolates were resistant to nitrofurantoin, and 3 isolates were resistant to meropenem (Table:1.1).

Table: 1.1. Antibiotic resistance pattern of 20 *K. pneumoniae* isolates.

Antibiotics	Resistant (%)	
	No.	%
Ct	16	80
GM	20	100
AK	20	100
NA	20	100
CI	20	100
MR	03	5
CS	18	90
PT	20	100
T	19	95
CX	20	100
Nf *	09	90

* % based on total number of urinary isolates.

Ct: Co-trimoxazole, Gm: Gentamicin, Ak: Amikacin, Na: Nalidixic Acid, Ci: Ciprofoxacin, Mr: Meropenem, Cs: Cefaperazone/ Sulbactum, Pt: Piperacilin/ Tazobactum, T: Tetracycline, Cx: Cefotoxime, Nf: Nitrofurantoin, R: Resistance, S: Sensitive.

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration results of 20 multidrug resistance clinical isolates of *K. pneumoniae* for ciprofloxacin in presence and in absence of efflux pump inhibitors PAβN and reserpine were tested. In the presence of PAβN following changes in MIC for ciprofloxacin was noted: 2 (10%) isolate have showed reduction in MIC, isolate (L13) shown 4 fold reduction in the presence of PAβN and reserpine. Isolate (L14) has shown 2 fold reduction in MIC in the presence of PAβN and reserpine. Remaining 18 isolates have shown no change in MIC in the presence of both the inhibitors.

DISCUSSION

Epidemiological evidence supports the concern regarding the emergence of gram-negative bacteria and their role in serious healthcare associated infections. Recent reports reveal an increase in the incidence of nosocomial infections caused by gram-negative bacteria, such as *E. coli, Klebsiella* spp, *Acinetobacter,* and *P. aeruginosa*spp, which are branded as the "Bad Bugs, No Drugs" [26]. Effective antibiotic transport has now been observed for many classes of drug efflux pumps. Most drug efflux pumps confer a multidrug resistance phenotype, corresponding to the large variety of substrates they may recognize, including several classes of antibiotics as well as non-antibiotic drugs [27]. More recent studies have revealed that the AcrAB-TolC system is actually inducible by certain fatty acids and bile salts, consequently leading to the export of multiple detergents, dyes and antimicrobial agents [28].

The MIC analysis in the current study has shown that in the presence of PAβN has shown reduction in 2 clinical isolates of *K. pneumoniae.* These results clearly indicate the presence of over expressing Resistance Nodulation cell-Division (RND) efflux pumps [29, 30]. Reduction in MIC in presence of reserpine was observed in two clinical isolates of *K. pneumoniae* L13 and L14.The reduction of MIC in presence of reserpine reveals that the monocomponent efflux pump might be in operation. Reserpine is an effective inhibitor of monocomponent efflux pump system like SMR, MFS, MATE, ABC family commonly found in gram positive bacteria and in few gram negative bacteria in which the mechanism is plasmid mediated [31, 32, 33, 34,35].

One of the efflux systems commonly involved in resistant *K. pneumoniae* phenotype is the AcrAB multidrug efflux system that is homologous to the AcrAB described in *E. coli.* Schneider*et al.,*[36] found that increased AcrAB efflux pump expression in resistant *K. pneumoniae* strains was caused by mutations in the *acrR* repressor of *acrAB*, where mutations in the repressor led to a marked increase in resistance to quinolones due to overexpression of AcrAB [36, 37].In the present study the two isolates of *K. pneumoniae* L13 and L14 showed reduction in MIC in the presence of inhibitors reserpine and PAβN which may due to the inhibition of overexpressing efflux pump. In other isolates which has not shown reduction in MIC to ciprofloxacin in presence of efflux pump inhibitors may be due to mechanisms other than efflux mechanism like target modification[1, 38].

CONCLUSION

Efflux pump inhibitors (EPIs) are promising therapeutic agents, as they should restore the activity of standard antibiotics. The efflux pump inhibitor-antibiotic combination is expected to increase the intracellular concentration of antibiotics that are expelled by efflux pumps, decrease the intrinsic bacterial resistance to antibiotics, reverse the acquired resistance associated with efflux pumps overexpression, and reduce the frequency of the emergence of resistant mutant strains. The EPIs hold promise for the development of a new class of antibiotic-potentiating agents that may extend the clinical utility of both existing and future antibiotics. However, the available data support the contention that the development of EPIs for use in combination with antibiotics.

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