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Study on the Role of Efflux Pump in Drug Resistance of *Pseudomonas aeruginosa*

R. Chavan*, A. De and S. Baveja

Department of Microbiology, L.T.M. Medical College, Mumbai, India
**Corresponding author*

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A B S T R A C T

Four different antibiotic efflux systems have been described in *P. aeruginosa*. Use of efflux pump inhibitors (EPIs) improve and potentiate the activity of exported antibiotics. The present study was undertaken to study the role of efflux pumps in drug resistance of *Pseudomonas aeruginosa.* Total 126 *P. aeruginosa* isolates from different samples were identified. Antibiotic susceptibility patterns of all the isolates were performed by MIC method. The five antibiotics studied were Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem and Meropenem. Efflux pump mechanisms were studied by phenylalanine argynyl β-naphthylamide (PAβN), a synthetic EPI and by curcumin, a natural extract. By MIC, susceptibility to imipenem was maximum (59.52%). Resistance to meropenem, gentamicin and ceftazidime was > 53%. Resistance to ciprofloxacin was highest (71.43%). After addition of PAβN to all five antibiotics, susceptibility to imipenem, meropenem, ceftazidime and ciprofloxacin increased but susceptibility to gentamicin remained same. Total 22 resistant strains of *P. aeruginosa* became susceptible after addition of PAβN. Using concentration of Curcumin 50 µg/ml, total seven resistant strains became susceptible with four antibiotics, except with ceftazidime. MIC lowered significantly with addition of PAβN in all antibiotics, except gentamicin, as compared to curcumin. Overall resistance due to efflux pump in multidrug resistant *P. aeruginosa* was encountered in 14.89% patients. The present study indicates the presence of efflux pump mediated drug resistance among clinical isolates of *P. aeruginosa* against one or more antimicrobials employed. This study indicates both PAβN and curcumin as potential EPIs, with PAβN being a better one.

Introduction

The 20th century was considered the antimicrobial era, whereas the 21st century may well represent the post-antimicrobial era. The reason for this dramatic change, should it come to pass, is the development of bacterial resistance to antimicrobial agents. Multidrug resistant (MDR) and pan drug resistant (PDR) bacteria are on the rise, leaving clinicians with few or no therapeutic options[1]. An understanding of these important microbial resistance mechanisms will help the clinicians to identify circumstances in which resistance may be a problem, as well as evaluating the potential usefulness of an alternate antimicrobial agent against resistant microbes.

Pseudomonas aeruginosa is frequently implicated in healthcare associated infections (HAIs). Efflux pumps are transport proteins involved in the extrusion of toxic substrate,

including all classes of clinically relevant antibiotics from within cells, into the external environment. In Gram negative organisms, efflux pump was first identified in *Escherichia coli.* Four different antibiotic efflux systems have been described in *P. aeruginosa* – MexAB-oprM, MexXY-oprM, MexCD-oprJ and MexEF-oprN. All classes of antibiotics except the polymyxins are susceptible to extrusion by one or more of these efflux systems. MexAB-oprM is responsible for extrusion of β-lactams, quinolones and a range of disinfectants. MexXY-oprM extrudes aminoglycosides. The third efflux operon MexEF-oprN confers resistance to quinolones, chloramphenicol and trimethoprim.[2]

Use of efflux pump inhibitors improve and potentiate the activity of exported antibiotics. One known efflux pump inhibitor (EPI) in *Pseudomonas aeruginosa* is phenylalanine argynyl β-naphthylamide (PAβN). It is apeptidomimetic compound, also called MC-207,110. It is capable of significantly reducing fluoroquinolone resistance in *P. aeruginosa*.[2]

Curcumin is a natural extract derived from rhizomes of the plant *Curcuma longa* (Zingiberaceae) grown in regions of India and South East Asia. Extensive research in this field has shown its activity as an anti-inflammatory, antiviral, antioxidant and anticancer agent. It was observed that curcumin potentiated the effect of various antibiotics against *Staphylococcus aureus* and reduced the virulence in animal pathogenicity models. Effort is also being made to explore a natural analogue of the above drug curcumin, which may be biocompatible and safe for human consumption.

Therefore, this study was undertaken to detect minimum inhibiting concentration (MIC) of selected antibiotics; to detect reduction in MIC by the use of an efflux pump inhibitor phenylalanine argynyl β-naphthylamide (PAβN) and to explore the possible efflux pump activity of a natural compound curcumin derived from curry spice turmeric (*Curcuma longa*) which may act as an efflux pump inhibitor.

Materials and Methods

A total of 126 laboratory confirmed non duplicate isolates of *Pseudomonas aeruginosa* from all clinical samples received in this tertiary care hospital in Mumbai were included in this study for the period of one year, i.e. from 1st January to 31st December 2014. All new born infants, geriatric patients and HIV positive patients were excluded. All isolates were identified by standard biochemical reactions[3]. Antibiotic susceptibility testing was done on Mueller Hinton Agar (MHA) plate by Minimum Inhibitory Concentration (MIC) by agar dilution method by using five antibiotics – Ceftazidime, Ciprofloxacin, Gentamicin, Imipenem and Meropenem. Results were recorded as per Clinical Laboratory Standards Institute (CLSI) 2014 guidelines (M07-A9)[4].

Minimum inhibitory concentration (MIC) method[3]

Minimum inhibitory concentration (MIC) by agar dilution method was performed by using the same five antibiotics. Organisms were inoculated in 4ml Tryptic soy broth (Hi–Media, Mumbai) and incubated overnight at 35^0C and growth in broth was observed the next day.

The antibiotic was incorporated in MHA (Becton Dickinson and Co., Sparks, MD 21152, USA) plates by addition of appropriate concentrations of antibiotics in 20 ml of molten MHA in a flask. Serial two-fold drug dilutions of respective antibacterial agents

were made in dilution tubes. Each antibiotic was weighed and dissolved in 2 ml of sterile triple distilled water in separate vials to make stock solutions of 4 mg/ml. Then serial dilutions of each antibiotic solution ranging from 32 µg/ml to 0.25 µg/ml were made. *Pseudomonas aeruginosa* ATCC 27853 was used as positive control. Plates were incubated at 35^0C for 16-20 hours. MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibited the growth. MIC was recorded as growth inhibition in antibiotic containing plate, when compared with plate without any antibiotic.

For ceftazidime, susceptible range was taken as ≤ 8µg/ml; Resistance range as ≥ 32 µg/ml; Intermediate susceptible as 16 µg/ml. For ciprofloxacin, susceptible range was taken as ≤ 1µg/ml; Resistance range as ≥ 4 µg/ml; Intermediate susceptible as 2 µg/ml. For gentamicin, susceptible range was taken as ≤ 4µg/ml; Resistance range as ≥ 16 µg/ml; Intermediate susceptible as 8 µg/ml. For imipenem, susceptible range was taken as ≤ 2µg/ml; Resistance range as ≥ 8 µg/ml; Intermediate susceptible as 4 µg/ml. For meropenem, susceptible range was taken as ≤ 2µg/ml; Resistance range as ≥ 8 µg/ml; Intermediate susceptible as 4 µg/ml. In this study, all intermediate susceptible strains were considered as susceptible.

Comparative MIC reduction after addition of Phenylalanine argynyl β-naphthylamide (PABN)[5]

Phenylalanine argynyl β-naphthylamide (PAβN), a broad spectrum efflux pump inhibitor, (Sigma Aldrich, USA) was dissolved in distilled water to prepare fixed concentrations of 50µg/mL. Stock solution of 10 mg/ml was prepared. Appropriate dilutions of each antimicrobial solution ranging from 32 µg/ml to 0.25 µg/ml were made and added to corresponding amount of molten MHA along with PAβN separately. Uninoculated MHA plates and MHA with only PAβN without antibiotics were included as growth controls in each experiment. The proportion of MIC reduction with PAβN was determined comparing the MIC of respective antimicrobials when tested alone.

Comparative MIC reduction study after addition of curcumin to each antibiotic solution[5]

Curcumin (Hi-Media RM1449-54) was dissolved in Dimethyl Sulphoxide (DMSO) (Sigma Chemical, USA) and stock concentration of 10 mg/ml was stored at -20^0C. Final test concentrations were 20 µg/ml, 30 µg/ml and 50 µg/ml for each sample. Appropriate dilution of each antimicrobial solution ranging from 32 µg/ml to 0.25 µg/ml are made and added to corresponding amount of molten MHA along with Curcumin in above concentrations separately. Uninoculated MHA plates and MHA with curcumin only without antibiotics were included as growth controls in each experiment. Proportion of MIC reduction with Curcumin was determined by comparing the MIC of antimicrobials when tested alone.

Results and Discussion

Majority of the isolates were from pus/wound swabs samples (53.17%), followed by urine samples (19.84%), as shown in Pie Diagram 1. By antibiotic susceptibility testing by MIC, most isolates were susceptible to Imipenem (59.52%), followed by Meropenem (46.82%) and Gentamicin (46.03%). Resistance to ciprofloxacin was highest (71.43%) (Bar Diagram2). Bar Diagram 3 shows MIC by agar dilution method with PaβN. Susceptibility to imipenem, meropenem, ceftazidime and ciprofloxacin increased after addition of PAβN, but susceptibility to gentamicin remained same (Table 1). Total

five isolates became susceptible to ceftazidime resistant strains and six to ciprofloxac in resistant strains after addition of PAβN. There was no change in total number of susceptible strains even after addition of PAβN to gentamicin. Total seven resistant strains became susceptible after addition of PAβN to Imipenem and four resistant strains became susceptible after addition of PAβN to Meropenem (Table 1). MIC lowered significantly with addition of PAβN in all antibiotics, except gentamicin in a total of 54 (42.86%) strains (Table 2). Significant MIC reduction was highest in ciprofloxacin (42.86%).

By using 20 µg/ml and 30 µg/ml concentrations of curcumin, no change was observed (Table 3). Using concentration of curcumin 50 µg/ml, only one strain became susceptible to imipenem, two strains became susceptible to ceftazidime and meropenem, and three strains became susceptible to gentamicin, which were previously resistant to it (Table 4). Curcumin resulted in decrease in MIC significantly, which was seen maximum with meropenem, where a total of nine strains showed decrease in MIC after addition of curcumin. This was followed by imipenem, where six strains showed significant decrease in MIC. Table 5 shows the no. of strains in which MIC was lowered significantly after addition of PAβN and curcumin.

Bacterial infectious diseases have been an important cause of morbidity and mortality in the history of mankind. The ability of bacteria to overcome antibacterial agents is increasing gradually.

Pseudomonas aeruginosa is a versatile human pathogen, particularly seen in critically ill or immunocompromised patients.[6] They are responsible for morbidity, mortality and healthcare costs both in hospitals and in the community.[7] This 'superbug' is responsible for hospital associated infections (HAIs). Infections due to *Pseudomonas aeruginosa* is quite difficult to treat due to its rapidly developing inherent resistance mechanism against many antimicrobials and disinfectants.[8] The difficulty in treatment of pseudomonas infections is due to its multidrug resistance (MDR) and exhibits resistance to most antimicrobial agents due to the expression of different mechanisms, overcoming their effects.

Pseudomonas aeruginosa is known for phenomenon of bacterial resistance, as practically all types of resistance mechanisms are seen. It exhibits resistance by virtue of expression of AmpC β-lactamase and efflux pumps, combined with a low permeability of the outer membrane. Its remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including β-lactams, aminoglycosides and fluoroquinolones.

Efflux pumps in *P. aeruginosa* belong to Resistance Nodulation Division (RND) family. MexA–MexB–OprM, MexC–MexD–OprJ, MexE–MexF–OprN and MexX–MexY–OprM are common efflux pumps known in *P.aeruginosa*.[2,9]

This study was undertaken to know the role of efflux pump in drug resistance of *Pseudomonas aeruginosa*. For this, a known efflux pump inhibitor Phenylalanine argynyl *β*-naphthylamide (PAβN), a broad-spectrum synthetic and toxic efflux pump inhibitor was used. Also, curcumin, a natural compound (a herbal extract) was also investigated for its possible role as an efflux pump inhibitor.

The present study of one year duration was undertaken to investigate the contribution of efflux pumps in drug resistance of

P. aeruginosa prevailing amongst hospital isolates from patients admitted in different wards of a tertiary care hospital in Mumbai. In this study, 126 *P. aeruginosa* strains were included, which were isolated from wound swabs/pus, endotracheal aspirates, sputum, urine, blood, etc.

Antibiotic susceptibility pattern of *P. aeruginosa* isolates by Minimum Inhibitory Concentration (MIC) by agar dilution method

Minimum Inhibitory Concentration (MIC) study was done for all 126 *P. aeruginosa* isolates by using all the above five antibiotics by agar dilution method and susceptible, intermediate susceptible and resistant ranges taken were according to CLSI 2014 guidelines. All intermediate susceptible strains were considered as susceptible in this study. Maximum resistance was seen in Ciprofloxacin (71.43%), followed by Ceftazidime and Gentamycin (55.56% and 53.97%) respectively. Resistance to Imipenem and Meropenem was (40.48% and 53.17% respectively (Bar Diagram2).

In a study by Negi *et al.,* resistance to ciprofloxacin was 57.64%, followed by gentamicin 51.17%, whereas resistance to ceftazidime and meropenem was 41.76% and 29.41% respectively by MIC.[5] In a study by Shashikala *et al.,* resistance to imipenem and meropenem was 10.9% each, resistance to both ciprofloxacin and gentamicin was 97% among imipenem resistant *P. aeruginosa* by MIC.[10] In another study by Ballal *et al., P. aeruginosa* isolates showed 0% resistance to imipenem, resistance to ciprofloxacin and gentamicin was 60% and 54% respectively, followed by ceftazidime (44%)[11].

In a study by Carmeli *et al.,* from USA, 7% strains were resistant to ceftazidime, 13% were resistant to imipenem and 21% were resistant to ciprofloxacin by MIC[12], which is lower than the present study. In a study by Khan *et al.,* from Pakistan, resistance pattern by MIC, for gentamycin was 67.8%, which is much higher as compared to the present study and for ceftazidime, meropenem and imipenem it was 30.2%, 28% and 26.7% respectively[13].

A study from Jakarta found resistance to both ceftazidime and gentamicin to be 68% and ciprofloxacin 44%, by MIC, whereas the carbapenems showed good activity – only 20% and 25% resistance to imipenem and meropenem respectively[14].

A surveillance study was conducted by Obritsch *et al.,* over a 10-year period from 1993 to 2002 in patients admitted in ICUs in District of Columbia. They observed increasing resistance to all antipseudomonal agents, with the greatest increase involving ciprofloxacin (15 to 32%), imipenem (15 to 23%) and ceftazidime (15 to 19%)[15].

This indicates that significant increase in resistance of *P. aeruginosa* isolates over period of time, which may compromise the ability to choose efficacious empirical regimens for treatment of this formidable pathogen in critically ill patients.

MIC of *P. aeruginosa* with selected antibiotics after addition of PAβN

When MIC was performed after adding PAβN, it was found that five (7.14%) strains which were previously resistant to ceftazidime, became susceptible to it (Table 1), indicating the mechanism of resistance in these strains to be due to efflux pumps. A study from Canada by Lamers *et al.,* showed that PAβN reduced the minimal inhibitory concentrations (MICs) of several β-lactam antibiotics against *P.aeruginosa*[16].

Table 1. Susceptibility pattern of *P. aeruginosa* by MIC with and without PAβN (n=126)

Antibiotic	Total no. of susceptible strains No. (%)	Total no. of strains susceptible after addition of PaβN No. (%)	Total no. of strains showing resistance due to efflux pump No. (%)
Ceftazidime	56 (44.44%)	61 (48.41%)	05 (7.14%)
Ciprofloxacin	36 (28.57%)	42(33.33%)	06 (6.67%)
Gentamicin	58 (46.03%)	58 (46.03%)	00 (0.00%)
Imipenem	75 (59.53%)	82 (65.08%)	07 (13.73%)
Meropenem	59 (46.83%)	63 (50%)	04 (5.97%)

Table 2. Strains in which MIC was lowered significantly after addition of PAßN and Curcumin

Antibiotic	PAβN	Curcumin
Ceftazidime (n=70)	27 (21.43%)	04 (3.17%)
Ciprofloxacin (n= 90)	54 (42.86%)	02 (1.59%)
Gentamicin (n=68)	04 (3.17%)	03 (2.38%)
Imipenem (n= 51)	15 (11.90%)	06 (4.76%)
Meropenem (n=67)	13 (10.31%)	09 (7.14%)

Table 3. MIC of *Pseudomonas aeruginosa* by agar dilution method using different concentrations of Curcumin (n=126)

Antibiotic	Curcumin 20 µg/ml		Curcumin 30 µg/ml		Curcumin 50 µg/ml	
	S	R	S	R	S	R
Ceftazidime	54	72	54	72	56	70
Ciprofloxacin	38	88	38	88	38	88
Gentamicin	57	69	57	69	60	66
Imipenem	75	51	75	51	76	50
Meropenem	61	65	61	65	61	65

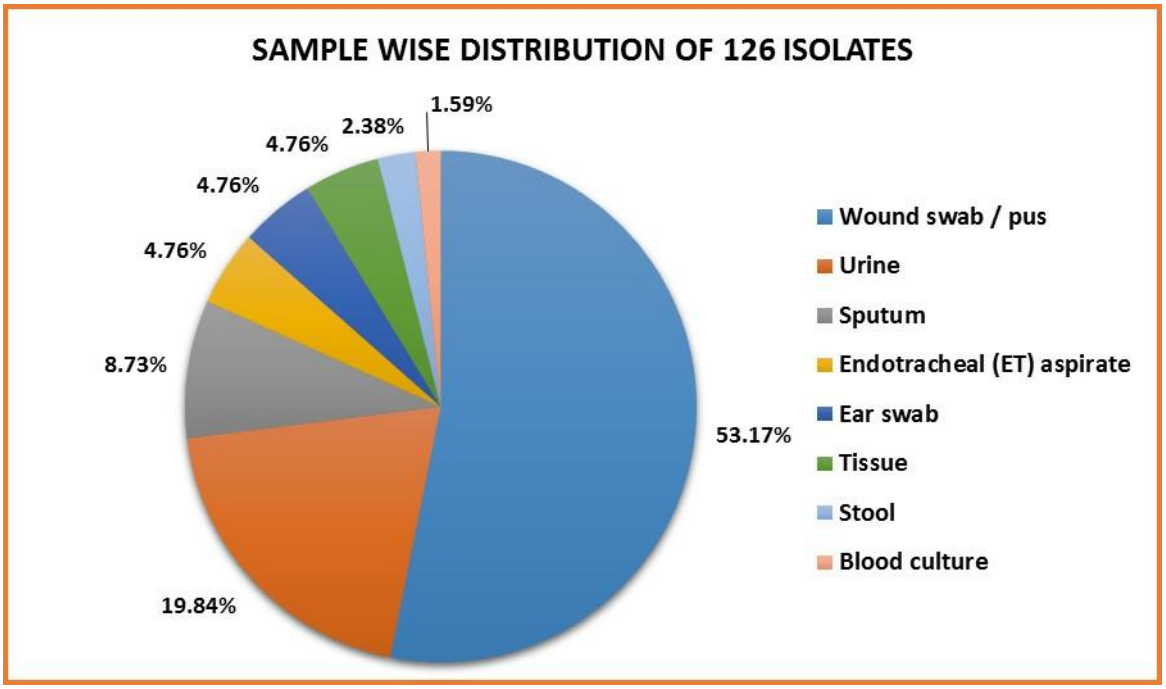
Table 4. Comparison between sensitivity of *P. aeruginosa* strains with and without addition of 50 µg/ml Curcumin

Antibiotic	Total no. of susceptible strains No. (%)	Total no. of susceptible strains after addition of 50 µg/ml Curcumin No. (%)
Ceftazidime	56 (44.44%)	56 (44.44%)
Ciprofloxacin	36 (28.57%)	38 (30.16%)
Gentamycin	58 (46.03%)	60 (47.62%)
Imipenem	75 (59.52%)	76 (60.32)
Meropenem	59 (46.83%)	61 (48.41%)

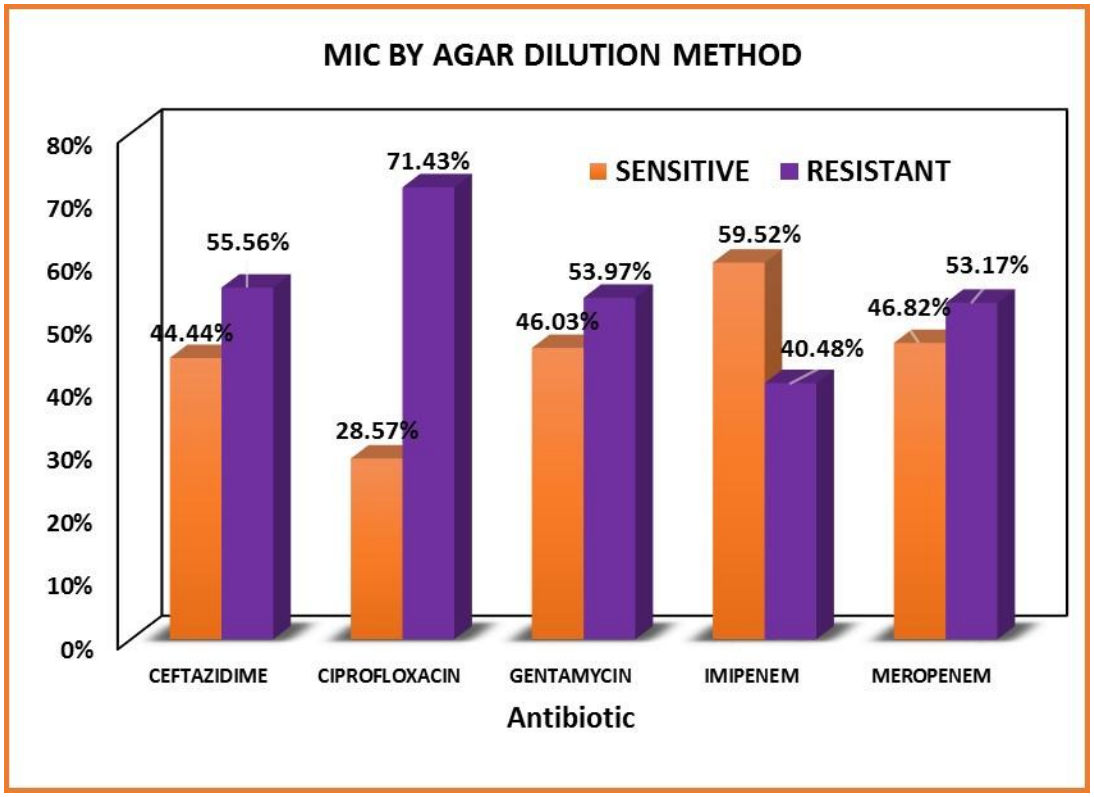
Table 5. Comparison of total number of *P. aeruginosa* strains which became susceptible after addition of PAβN and curcumin

Antibiotic	Susceptible strains after addition of PAβN	Susceptible strains after addition of Curcumin
Ceftazidime (n=70)	05 (7.14%)	00 (0.00%)
Ciprofloxacin (n= 90)	06 (6.67%)	02 (2.22%)
Gentamicin (n=68)	00 (0.00%)	02 (2.94%)
Imipenem (n= 51)	07 (13.73%)	01 (1.96%)
Meropenem (n=67)	04 (5.97%)	02 (2.99 %)

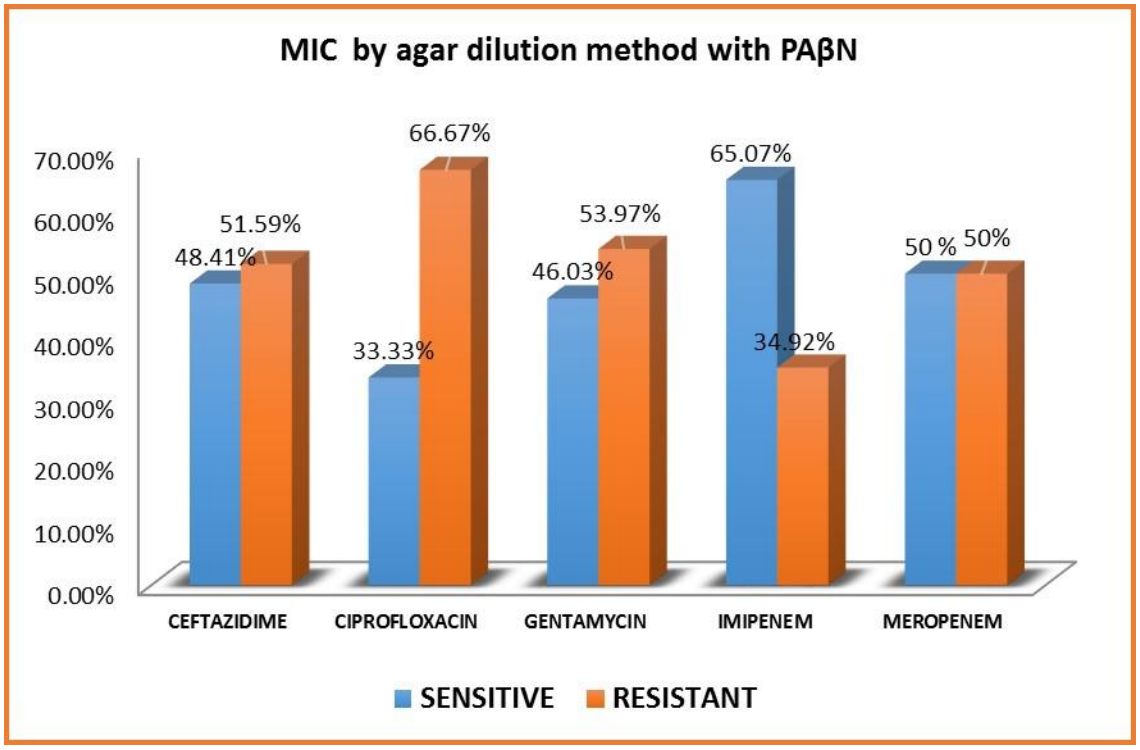
Pie diagram 1



Bar diagram 1



Bar Diagram 2



In the present study, MIC for ceftazidime lowered significantly in 27 (21.43%) strains (Table 2). In a study by Negi *et al.,* from North India, 21.12% strains showed resistance to ceftazidime due to efflux pumps[5].

A total of six (6.67%) strains which were previously resistant to ciprofloxacin, became susceptible after addition of PAβN (Table 1). This is close to the findings of an Indian study, where they found 9.18% strains resistant due to efflux pumps.[5] Another study from Belgium, showed decrease in MIC of a fluroquinolone (norfloxacin), where 57.14% strains became susceptible[17].

In the present study, MIC significantly lowered after addition of PAβN in a total of 54 (42.86%) strains (Table 2). In a study from Egypt, PAβN resulted in significant reduction in the MIC of ciprofloxacin in 71.4% isolates[18].A significant reduction was also seen in MIC to levofloxacin in a study by Lomavskya *et al.,*[19].

In case of gentamicin, there was no reduction in resistant strains after addition of PAβN (Table 1), which is in contrast to the study by Negi *et al.,*[5], who showed 14.9% strains showed resistance to gentamicin due to efflux pumps. In a study by Mesaros *et al.,* two out of seven strains became susceptible to gentamicin after addition of PAβN[17]. However, in the present study, in four (3.17%) strains, MIC for gentamicin lowered significantly after addition of PAβN (Table 2).

A total of seven (13.73%) strains which were previously resistant, became sensitive to imipenem after addition of PAβN (Table 1). In 15 (11.90%) strains, MIC lowered significantly after addition of PAβN (Table 2). In a similar study at Belgium, only one strain of imipenem became susceptible and three strains showed decrease in MIC[17].

A total of four (5.97%) strains which were previously resistant became sensitive to meropenem after addition of PAβN (Table 1), indicating the resistance due to efflux pumps. In the present study, in 13 (10.31%) strains, MIC for meropenem lowered significantly after addition of PAβN (Table 2).

However, in another study from India[5], 26% strains showed resistance due to efflux pump mechanism, which is much higher as compared to the present study.

MIC of *P. aeruginosa* with selected antibiotics after addition of curcumin

In the present study, curcumin, which is a natural compound, i.e. an herbal extract, was also studied for its possible role as an efflux pump inhibitor. When it was used in concentration of 20 µg/ml and 30 µg/ml, no significant change in total number of susceptible strains were observed. Change was observed at concentration of 50 µg/ml only (Table 3).

Two previously resistant strains became susceptible after addition of curcumin to ciprofloxacin, gentamicin and meropenem individually. When curcumin was added to imipenem, only one previously resistant strain became susceptible to it. No change in total susceptible strains was observed after addition of curcumin to ceftazidime (Table 4).

This finding is similar to the study by Negi *et al.,*[5] who found that two strains which were resistant previously became susceptible to ciprofloxacin and gentamicin each, after addition of curcumin.

After adding curcumin to meropenem, five resistant strains became susceptible. However, in their study six resistant strains became susceptible to ceftazidime, which is in contrast to the present study.

Comparison of PAβN and curcumin

When we compared the efflux pump inhibiting activity of PAβN and curcumin, it was found that 22 strains became susceptible which were previously resistant after addition of PAβN. After addition of curcumin, only seven strains which were previously resistant became susceptible (Table 5). Therefore, in the present study, it was observed that PAβN is a better efflux pump inhibitor as compared to curcumin but the former has toxic properties whereas the latter is non-toxic herbal product, safe for human consumption.

Amongst 47 multi drug resistant strains, eleven (23.4%) strains became susceptible after addition of PAβN but only five (10.63%) strains became susceptible after addition of curcumin. In the study by Negi *et al.*,[5] 30% MDR isolates of *P. aeruginosa* showed decrease in MIC to susceptible level after addition of curcumin, which is much higher as compared to the present study.

In this study, overall resistance due to efflux pump in multidrug resistant *P. aeruginosa* was demonstrated in 14.89% (7/47) patients. Henrichfreise *et al.,* from Germany demonstrated efflux pumps in 40.9% amongst multidrug resistant *P. aeruginosa*, which is much higher than the present study[20].

Although synthetic efflux pump inhibitor (EPI) PAβN is better than natural extract curcumin in inhibiting the resistant strains but keeping in mind their toxic potential and harmful effects, search for a natural homologue other than curcumin, which can mimic their action can never be ignored.

It might be acting either as drug analogue, hence becoming a substrate for efflux channels or it may inhibit the expression of different genes responsible for efflux pump mechanism.

The present study indicates the presence of efflux pump mediated drug resistance among clinical isolates of *P. aeruginosa* against one or more antimicrobials employed. Overall resistance due to efflux pump was seen in 14.89% MDR strains of *P. aeruginosa*. This study indicates both PAβN and curcumin as potential EPIs, with PAβN being a better one but further confirmation is required by observing the molecular mechanisms.

Considering the seriousness of prevailing and emerging drug resistance in *Pseudomonas aeruginosa*, extensive research is needed for finding out some suitable alternatives, e.g. a synthetic EPI with less toxic effects, or a better natural extract like curcumin.

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