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The Antipsychotic Drug Thioridazine as A Highly Promising Agent in the Treatment of Acute Infections by *Pseudomonas Aeruginosa*

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Abstract

Pseudomonas aeruginosa poses a serious threat to clinicians since almost all isolates are resistant simultaneously to multiple antibiotics at very high levels. Elimination of such resistances by a known pharmacological compound would be advantageous to control infections caused by this bacterium. Resistance levels of various antibiotics and antipsychotic drug thioridazine (Tz) were determined in *P. aeruginosa* strains, elimination of drug resistance plasmids in such bacteria were performed with Tz. Agarose gel electrophoresis was performed in both wild type and their cured clones. A simple process called cart wheel method was employed to find out presence of efflux pumps in all strains. Then both varieties were tested to determine their capacity to hemolyse sheep RBC. The results revealed that all the strains were multiply resistant to most antibiotics and Tz at high levels; however, piperacillin, carbencillin, amikacin and ciprofloxacin showed lower resistance levels. Agarose gel electrophoresis showed large plasmids in wild types which were totally absent in cured clones. No difference in fluorescence was noted among wild type pseudomonads and their cured varieties. There was a distinct difference in hemolytic character in the two varieties. Thus, Tz can successfully eliminate drug resistance plasmids in hemolytic character is plasmid mediated while action of efflux is not. Therefore, this study shows that this particular antipsychotic drug Tz poses a fairly good chance of being administered along with antibiotics to patients suffering from multi-drug resistant pseudomonas infections where Tz would hopefully support the action of antibiotics by simultaneously eliminating drug resistant plasmids.

Keywords: *Pseudomonas Aeruginosa; Multi-Drug Resistance; Thioridazine; Plasmid-Curing; Efflux Pump*

Introduction

Pseudomonads include a group of non-fermenting Gram negative motile aerobic rods, being capable of producing water soluble pigments. The most important member *Pseudomonas aeruginosa* is highly invasive and toxigenic, can produce infections in patients with abnormal host defenses and is a common nosocomial pathogen. In hospitals *P. aeruginosa* invades patients suffering from burns, wounds, scars and also those in the immuno-compromised state where infections occur in any external site or internal organs. The pre-eminence of this bacterium as a powerful pathogen is due its easy adaptability, its innate resistance to almost all antibiotics and disinfectants, its armory of putative virulence factors that promote hemolysis of red blood cells [1]. The acute virulent property of the bacterium is not only due to the plasmid borne characters, potent exotoxins, cytotoxic substances or pyocyanin [2], but also due to presence of efflux pumps in the cells [3,4]. These pumps can prevent antibiotics from entering into the bacterial cells resulting in drug resistance [5]. Although polymyxin had been used for many years to treat patients suffering from infections due to this bacterium such an antibiotic had to be discon-

tinued for its severe toxicity. As a newer antibiotic is discovered *P. aeruginosa* develops resistance to the new agent in a very short period of time. Hence treatment with combination therapy was initiated [6]. It has been suggested that there are multiple virulence factors that promote drug resistances in *P. aeruginosa*. Elimination of resistances by known pharmacological compounds would be advantageous in successful therapeutic control of infections by *P. aeruginosa*. There have been a large number of investigations on the discovery and application of newer antibiotics alone and in combination, but the multiple virulence factors present in *P. aeruginosa* continuously try and develop ways and means to overcome their actual application.

Medicinal compounds that are used in non-infectious pathology but have antimicrobial property are termed as "non-antibiotics" [7]. One of most potent non-antibiotics are phenothiazines which occupy the largest of five main classes of antipsychotic drugs. Phenothiazines have been found to be the most potent source of antimicrobials [8-15] whose potentiation may be further advanced by synergism with antibiotics [16-18]. Earlier studies have shown that the drug thioridazine (Tz) is highly active against many Gram positive and Gram negative bacteria [19] and even against *M. tuberculosis* [20] and is able to cure drug resistance plasmids in such organisms [19,21].

Multidrug resistant (MDR) clinically isolated bacteria have been found to be related to over expression of efflux systems [22,23]. Although efflux systems are present in both Gram positive and Gram negative bacteria, efflux mediated resistance in the latter is more complex due to the molecular architecture of the cell envelope [24]. Since evaluation of efflux system by conventional techniques requiring specialized instrumentation which may not be available in all laboratories a simple instrument-free method with the help of agar plates containing increasing amounts of ethidium bromide (EB) has been developed [5]. This protocol is now termed as cartwheel method. Identification of over expressed efflux pump systems contributing to MDR phenotype can be made quite easily [25]. Here EB acts as the pump substrate that helps in verification of the presence of overexpressed efflux pump system with respect to the efflux activity of the reference control strain. The amount of EB which is effectively extrude by the cells and a high EB concentration can be retained. Thus, the bacterial mass fluorescence when this is exposed to ultraviolet (UV) light [25]. Pseudomonads are known for their hemolytic character [26], though less prominent than their property of pyocyanin production. The present study has been designed to find out the capacity of Tz to eliminate the drug resistance factors of the highly virulent and multiply drug resistant clinical isolates of *P. aeruginosa*. Simultaneously attempts have been made to determine if elimination of drug resistant plasmids can influence the action of efflux pumps and hemolytic property of pseudomonads.

Materials and Methods

Bacteria

A total of 14 strains of *P. aeruginosa* were taken for this study; of these 13 were collected from patients who were suffering from various types of acute infections, and were admitted in different hospitals for their illnesses in Kolkata, India. The remaining one was the standard strain ATCC 27853T that was obtained from the American Type Culture Collection.

Media

Liquid media were nutrient broth (NB, Oxoid) and Mueller Hinton broth (MHB, Oxoid); solid media were nutrient agar (NA, Oxoid) and Mueller Hinton agar (MHA, Oxoid).

Drugs

The antibiotics tetracycline (Tc), chloramphenicol (Cm), azithromycin (Az), streptomycin (Sm), amikacin (Ak), penicillin (Pc), carbenicillin (Cb), piperacillin (Pp), imipenem (Im), ciprofloxacin (Cp), norfloxacin (Nf), ceftazidime (Cd), ceftriaxone (Ct), cefoperazone (Cz) and Tz were purchased from Sigma Chemicals (USA).

Determination of minimum inhibitory concentration (MIC) of antibiotics and Tz

Each antibiotic and Tz was added to molten NA and MHA at the concentrations (µg/ml) of 10, 50, 100, 200, 300, 400, 500, 1000, 2000, 3000, 4000 and 5000 and poured in sterile Petri dishes. Overnight grown broth cultures of *P. aeruginosa* were spot inoculated on dried

drug plates and incubated at 370C. Appearance of growth was recorded after 24h and again after 48h and 72h. The lowest concentration that inhibited the growth of a bacterium was taken as its MIC and the concentration prior to this level that produced growth was considered as the resistance level.

Plasmid curing test

This was performed by taking 50% MIC of Tz with respect to a particular bacterium and inoculating the same in NB with approximately 500 cells. After 18 h a loopful of growth was plated out on NA containing the same amount of Tz to produce numerous isolated colonies. At least 100 colonies of a culture were tested for single or multiple loss of antibiotic resistances at 50% MIC level of a particular antibiotic [27,28]. Plates were incubated overnight to detect presence or absence of growth suggestive of possibility of elimination of plasmid responsible for a particular antibiotic.

Extraction of plasmid DNA and their demonstration by gel diffusion

Colonies in control plate corresponding to no growth in antibiotic plates were inoculated to fresh NB along with original control bacterial strain. The broths were incubated for 24h at 37^0C and plasmids were extracted following the method of Birnboim & Doly [29] and Birnboim [30]. The DNA samples of Tz treated and their respective untreated wild type controls were mixed with loading dye and 20 µl of each sample was loaded in agarose gel trough containing agarose gel. This was allowed to run for 45 - 60 min, stained with ethidium bromide solution, destained with distilled water and placed under BIO-RAD gel docking system for detection of presence or absence of plasmid bands [31,32].

Experiments to detect efflux pumps in P. aeruginosa following cartwheel method with the help of ethidium bromide (EB)

For this purpose, cured clones as well as their respective wild types were tested on the same plate simultaneously. Twenty ml of NA supplemented with varying concentrations of EB ranging from 0 to 5 mg/l were dispersed to individual plates and allowed to cool. Concentration of EB used was dependent on empirical demonstration of range expected for a given reference and multi-drug resistance (MDR) clinical isolates. Both the cured clones and their respective wild types were grown in NB overnight. Sterile swabs were dipped individually in the culture and each swab was initially pressed to drain out excess fluid and then streaked onto agar plates from the centre to the margins, care being taken not to deviate from the line [25]. Identification of the strain was made on the side of the bottom plate corresponding to the end of swabbed line. The arrangement of the swabbed lines was as follows: reference stain at 12' o clock position. The MDR clinical strains and their respective cured clones were at 1, 2, 3 etc o'clock positions (Figure 3). The EB-agar plates were incubated for 16 - 18h at 37^0C. Each EB plate was examined under a transilluminator. The minimal concentration of EB that produced fluorescence of the bacterial swabbed mass was recorded for the reference and MDR clinical strains. The higher the concentration of EB required for the presentation of fluorescence the greater the presumptive efflux pump activity of the organism [25].

Hemolytic assay

This was performed as described by Blocker., *et al.* [26]. Sheep RBC was washed three times in Phosphate Buffer Saline (PBS) at pH 7.4 (150 mM NaCl), and resuspended in RPMI 1640 medium (Sigma) at $5x10^8$ RBC ml^{-1} at 4^0C. Bacteria were grown in LB to an OD_{600} of 1.0 - 1.5, centrifuged and resuspended in RPMI 1640 at $5x10^8$ RBC ml^{-1}. Hemolysis assays were started by mixing 100 µl of RBC and 100 µl of bacterial suspension in round bottomed, 96 well plates, which were then centrifuged at 1500g for 10 min and incubated at 37^0C for 1 h. The release of hemoglobin was measured at 540 nm, after centrifugation, in 100 µl of cell supernatants. The percentage (%) of total lysis was calculated as where B (baseline) was negative control, corresponding to RBC incubated with 100 µl of RPMI 1640, and T was a positive control, corresponding to total lysis obtained by incubating the cells with 0.1% sodium dodecyl sulfate (SDS). X was the OD value of the analysed sample.

When indicated, RBC suspension was resuspended in 60 mM sterile solution of osmoprotectants, made up in RPMI 1640, given a final concentration of molecules to 30 mM. All experiments were performed at least three times.

Results

MIC of different antibiotics and Tz in the test bacteria

The inhibitory spectra of Tz and various antibiotics against 14 strains of *P. aeruginosa* are being present in Table 1. All the strains including ATCC 27853T were highly resistant to the age-old antibiotics Tc, Cm, Sm and Pc and also Tz. The bacteria were almost equally resistant to Az and Im. The organisms were comparatively less resistant to Ak, Pp, Nf, Cb and Ct. However, the antibiotics that exhibited least sensitivity were Cp and Cz. Among the test bacteria ATCC 27853T, C15, C17 and BVC1 revealed maximum sensitivity, although the MIC level was never below 100 µg/ml (Table 1).

P. aerugnosa	**Minimum inhibitory concentration (MIC) µg/mL**														
	Tc	**Cm**	**Az**	**Sm**	**Ak**	**Pc**	**Cb**	**Pp**	**Im**	**Cp**	**Cd**	**Ct**	**Cz**	**Nf**	**Tz**
ATCC 27853^T	2000	2000	1000	2000	200	2000	200	100	500	100	500	500	100	100	2000
APC 1	2000	2000	2000	>3000	500	>3000	500	500	2000	100	500	500	100	2000	2000
PS7	2000	2000	2000	2000	500	>3000	500	500	2000	100	500	500	200	2000	2000
C15	2000	2000	1000	2000	500	>3000	500	200	1000	100	500	500	100	200	2000
C17	2000	2000	1000	2000	500	>3000	500	200	1000	100	500	500	100	200	2000
Kr 8/89	2000	2000	2000	2000	500	>3000	500	500	2000	100	500	500	100	200	2000
Kr 12/3	2000	2000	2000	2000	500	>3000	500	500	2000	100	500	500	100	200	2000
732	2000	2000	3000	>3000	1000	>3000	1000	500	2000	100	1000	1000	100	2000	2000
AMRI 100	>3000	>3000	3000	>3000	2000	>3000	1000	2000	3000	1000	2000	2000	2000	2000	>3000
BVC 1	2000	2000	1000	2000	500	>3000	200	100	2000	100	500	1000	1000	1000	2000
BVC 2	2000	2000	1000	>3000	500	>3000	1000	500	2000	100	500	1000	1000	1000	2000
BVC 3	>3000	>3000	3000	>3000	2000	>3000	2000	2000	3000	1000	2000	2000	2000	2000	>3000
BVC 4	2000	2000	1000	2000	500	>3000	1000	500	2000	100	1000	1000	1000	1000	2000
BVC 5	2000	>3000	3000	>3000	2000	>3000	2000	2000	3000	100	2000	2000	2000	2000	>3000

Table 1*: Antibiotic resistance pattern of Ps. Aeruginosa.*

Tc: Tetracycline; Cm: Chloramphenicol; Az: Azithromycin; Sm: Streptomycin; Ak: Amikacin; Pc: Penicillin; Cb: Carbenicillin; Pp: Piperacillin, Im: Imipramine; Cp: Ciprofloxacin; Nf: Norfloxacin; Cd: Ceftazidime; Ct: Ceftriaxone; Cz: Cefoperazone, Tz: Thioridazine

Effect of Tz on elimination of antibiotic resistances

Out of 14 test bacteria 6 highly drug resistant pseudomonads including ATCC 27853T were subjected to plasmid curing test. These were APC1, AMRI 100, BVC 3, BVC 4 and BVC 5. One hundred colonies of all the strains grew on NA and 4 to 8 colonies failed to grow on all the test antibiotics (Figure 1). Such colonies were selected and picked up from the NA plates and tested further to determine their levels of resistance in different antibiotics. It was found that the levels of drug resistance came down to 10 to 25 µg/ml with respect to most of the test antibiotics (Figure 1). Such colonies were tested for elimination of plasmids.

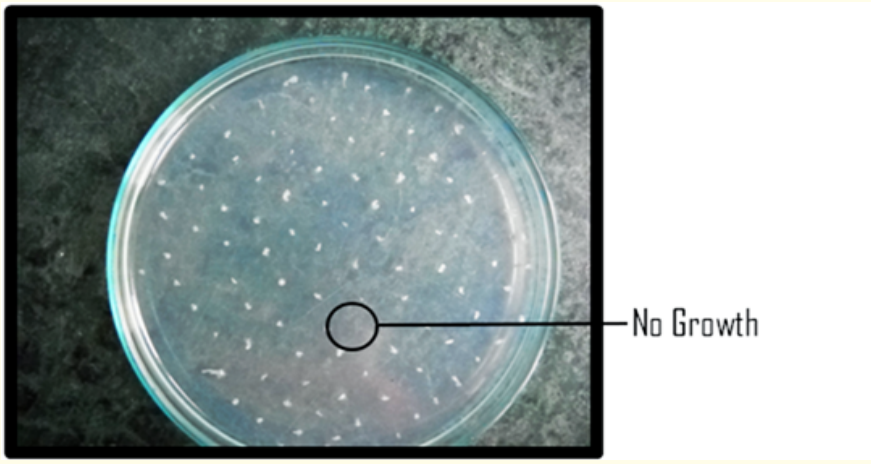

Figure 1: *Growth of isolated colonies on NA in the curing test; the absence of growth of a colony is marked here by a circle.*

Detection of plasmids by agarose gel electrophoresis

P. aeruginosa BVC 2 was found to possess two large plasmids while its cured clones were found to have lost both the plasmids. The test was performed with plasmids from two organisms *E. coli* K12 V517 and *Shigella flexneri* YSH 6000 as controls (Figure 2). Almost identical data were obtained with other strains of Pseudomonas and their cured clones.

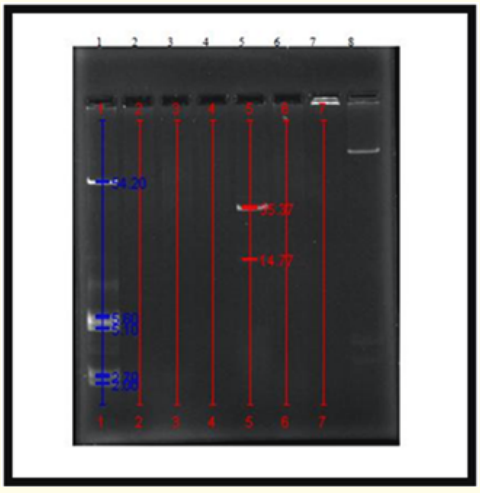

Figure 2: *Agarose gel electrophoresis: lane 1: E. coli k12 (v517) marker, lane 5: wild type P. aeruginosa bvc2, lanes 2, 3, 4, 6 & 7: Tz -treated BVC 2 with plasmid missing. lane 8: standard Shigella flexneri (YSH6000 plasmid).*

Effect of EB for detection of efflux pump in *P. aeruginosa*

When culture swabbed agar containing EB were viewed under suitable ultraviolet radiation at 354 nm long wavelengths out of the total of 14 strains tested only ATCC 27853T showed intense fluorescence right from the initial concentration of 0.5 µg/L (Figure 3). With increase in concentration of EB the intensity of fluorescence produced by the strains APC 1, 732, BVC 2, BVC 4, BVC 5, Kr/12/3 and C17

increased gradually from 1.0 mg/ml concentration. Strains BVC 3, Kr 8/89, C 15 exhibited fluorescence from concentrations of 2 mg/L increasing further with increase in concentration of EB. *P. aeruginosa* strains AMRI 100, BVC 1, *P. aeruginosa* 7T showed faint fluorescence at the highest concentration of EB 5 mg/L (Table 2).

Figure: 3A

Figure: 3B

Figure 3: *Fluorescence of known efflux pump containing strain S. aureus ATCC 25923T (control) at 12 O'clock position. Wild types and their respective cured clones showing same pattern of fluorescence in increasing concentrations of EB. Faint fluorescence at 1mg/ml of EB (Figure 3A) very distinct fluorescence at 5mg/L of EB (Figure 3B). No difference of fluorescence seen in a wild type strain and its cured clone.*

P. aeruginosa	**Fluorescence shown by P. aeruginosa strains in EB plates at 365nm**						
	Type	**Control**	**0.5 mg/L**	**1 mg/L**	**2.5 mg/L**	**3 mg/L**	**5 mg/L**
ATCC 27853^T	Wild type	-	+	+	+	++	++
	Cured type	-	+	+	+	++	++
C15	Wild type	-	-	-	-	-	-
	Cured type	-	-	-	-	-	-
BVC 2	Wild type	-	+	+	++	+++	+++
	Cured type	-	+	+	++	++	++
BVC 3	Wild type	-	-	-	-	+	+
	Cured type	-	-	-	-	+	+
BVC 4	Wild type	-	-	-	-	+	+
	Cured type	-	-	-	-	+	+
BVC 5	Wild type	-	-	-	-	+	+
	Cured type	-	-	-	-	+	+

Table 2: *Intensity of fluorescence exhibited by wild type strains of P. aeruginosa and their cured clones.*
-, no fluorescence; +/-, very faint fluorescence; +, ++, +++ denote increasing intensities of fluorescence

Presence of hemolytic activity in *P. aeruginosa*

Percentage (%) of hemolysis was calculated after OD value was measured for the sample (X), positive control (T) and negative control (B) for each *P. aeruginosa* strain tested. The formula used for calculation was: [(X-B) / (T-B)] x 100.

Although hemolytic property was evaluated in all the 14 bacteria; complete data of only 6 strains are presented in Table 3 (Figure 4).

P. aeruginosa	Values of hemolysis depicted from the formula			
	Negative control	Positive control	Sample	% of hemolysis
27853^T	3.42	4.018	3.588	33.74
AMRI 100	3.542	4.04	4.024	96.78
C15	3.548	4.058	4.032	94.9
APC 1	3.534	4.028	3.958	85.3
732	3.55	4.05	4.014	92.8
BVC 1	3.6	4.088	3.716	23.3
BVC 2	3.544	4.048	3.978	86.11
BVC 3	3.58	4.09	4.05	92.16
BVC 4	3.53	4.022	3.968	89.02
BVC 5	3.544	4.044	3.779	47

Table 3: *Determination of hemolytic activity in ten strains of P. aeruginosa.*

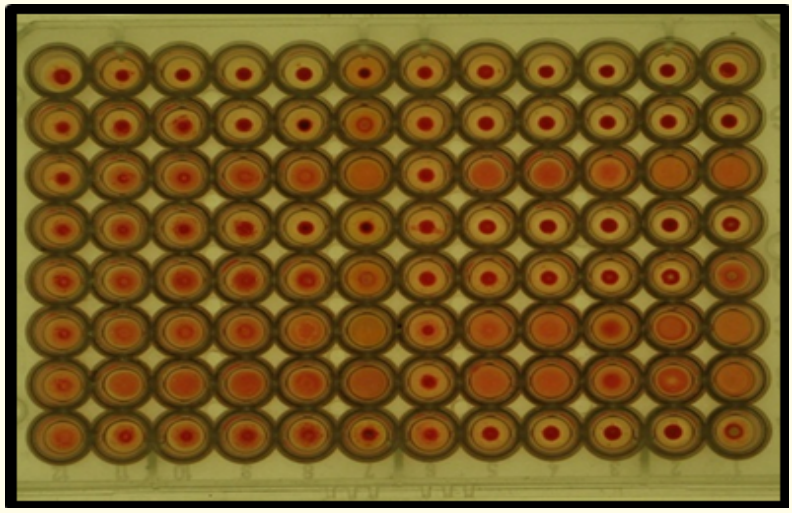

Figure 4: *Distinct hemolysis by virulent cultures and its absence in cured clones.*

Discussion

P. aeruginosa is a highly prevalent opportunistic pathogen. However, one of the most worrisome characteristics is its low antibiotic susceptibility which is attributable to the combined action of multidrug resistance plasmids, the efflux pumps with chromosomally encoded antibiotic resistance genes like MexAB and MexXY and the low permeability of bacterial cell envelope [1]. Additionally, hypermutation in this bacterium supports the selection of mutation – driven antibiotic resistance leading to chronic infections, whereas clustering of several different antibiotic resistance genes in integrons favours the concerted acquisition of antibiotic resistance determinants.

The emergence of multiple drug resistant Gram negative bacteria, particularly *P. aeruginosa* is currently a matter of great concern. *P. aeruginosa* can become multidrug resistant very swiftly due to its intrinsic resistant mechanisms. This bug is particularly responsible for

hospital-acquired infections of bloodstream, urinary tract, pulmonary and device related ailments and can also be isolated from immunocompromised patients in the intensive care unit of a hospital. One of the most common causes of resistance in *P. aeruginosa* is the presence of extended spectrum β- lactamases (ESBL) plasmids. PER-1 β- lactamase was the first ESBL identified in *P. aeruginosa* in France [33]. Subsequently the same plasmid was detected in other European countries, Asian countries, Greece and Iran [34]. Other drug resistant plasmids like VEB-1, SHV-2a, SHV-12, TEM-4, TEM-21 were subsequently reported in *P. aeruginosa* [35]. All these confer resistances to most of the β- lactam antibiotics. Carbapenems resistance in *P. aeruginosa* often related to carbapenamases, which are metallo-β- lactamases of the IMP, VIM, SPM and GIM types. Although IMP-1 was first reported from members of Enterobacteriaceae, it was subsequently distributed globally suggesting horizontal transmission of all these plasmids among unrelated Gram negative organisms including *P. aeruginosa* [36-38]. In this way, it may be clearly evident that the antibiotic resistances observed in the test strains of *P. aeruginosa* in the present study were primarily plasmid borne.

Elaborate scientific research on detection of antimicrobial action in various pharmacological groups revealed that the tricyclic phenothiazines have a vast potential as antimicrobics [7,13,20,39]. Such phenothiazines act not only on Gram positive and Gram negative organisms, but also on *Mycobacterium tuberculosis* [40-43]. In 1999 Radhakrishnan., *et al*. [19] made an elaborate study on the antibacterial activity of Tz and observed that Tz was bactericidal for Gram positive bacteria and bacteriostatic against Gram negative organisms. Moreover, Tz could efficiently eliminate antibiotic resistances singly and jointly in *E. coli* and shigellae [19]. In the present study, it was observed that Tz could ably eliminate several antibiotic resistances simultaneously in MDR *P. aeruginosa* strains rendering them sensitive to antibiotics at much lower levels.

Special care was taken while performing the cartwheel method to determine the presence of efflux pumps in *P. aeruginosa*. All the solutions were prepared freshly on the day of the experiment. Since the recommended diameter of the central circle is 1.0 cm it was always ensured that no contamination between swabbed materials could occur. Illumination was best observed by keeping the UV light source below the test plate. It is assumed that the strains which did not have significant efflux-pump activity allowed EB present in the medium to enter through the porin channels crossing the lipopolysaccharide and outer membrane proteins. With increase in the concentration of EB the intensity of fluorescence increased due to its easy accessibility inside bacterial cells. The strains that failed to show fluorescence and/or fluorescence at higher concentrations of EB possibly had effective that prevented EB to penetrate efflux pump activity the cell wall and thus accounting for drug resistance. The strain ATCC 27853T and its cured clone produced fluorescence right from the lowest amount of EB proving thereby the absence of efflux pump in this particular strain. However, most of the highly multiply drug resistant pseudomonads demonstrated fluorescence either at very high levels of EB or none at all, which proved presence of definitely active efflux pumps. The cured clones of these bacteria behaved almost identically signifying thereby the absence of efflux pumps in these clones as well. Therefore, action of efflux pumps is certainly not connected with presence or absence of drug resistant plasmids. The results corresponded to the fact that the strains that did not fluorescence even at the highest amount of the test drug had the maximum efflux pump activity while the pseudomonads whose fluorescence gradually increased with increase in the amount of EB had lesser efflux pump activity. This indicated that Tz had no effect on efflux pump of *P. aeruginosa.*

It may be assumed from the data in Table 3 that most of the pathogens with nosocomial background had moderate to very high haemolytic activity, the highest being 96.78%. This haemolytic activity may well be assumed as one of the virulence factors and responsible for the multi-drug resistances of this bacterium. Moreover, Tz treated cured clones had much lesser hemolytic activity or none at all. Therefore, such observations convincingly prove that haemolytic character is plasmid borne and that Tz can efficiently eliminate this property in *P. aeruginosa*. This bacterium is intrinsically resistant to almost all class of antibiotics and chemotherapeutics and even various categories of detergents. Increasing rates of bacterial resistance among non-fermenting bacteria are threatening the effectiveness of antibiotics that are used as the last resort therapeutic options. Acquisition of resistant traits to these molecules in *P. aeruginosa* has become extremely frequent leading to multidrug and pandrug resistances. In the last decade, most of the drug resistance patterns identified in Enterobacteriaceae are found prevalent in *P. aeruginosa*. Such multidrug resistant *P. aeruginosa* is intrinsically resistant to almost

all classes of antibiotics, therefore pseudomonads can cause an outbreak in hospitals. Such a bacterium has complex mechanisms of drug resistances, including reduced membrane permeability due to decreased outer membrane protein (D2 porin), over expression of efflux pumps, mutation of the quinolone target (DNA gyrase), production of aminoglycoside modification enzymes and production of metallo-beta-lactamases [44]. Thus, simultaneous application of Tz with one or two antibiotics of choice would not only act as an additional antibacterial drug but also would help to eliminate the drug resistant plasmids from the infectious bacterial cells [45]. Therefore, this study opens up an avenue for patients suffering from MDR *P. aeruginosa* infections who may be administrated Tz at standard human doses along with the antibiotics of choice.

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