IJBPAS, September, 2015, 4(9): 5967-5979

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

EFFLUX AS AN ARISING CAUSE OF DRUG RESISTANCE IN PUNJAB (INDIA)

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ABSTRACT

MDR strains of bacteria which are a serious public health problem may arise due to efflux pump over expression. Detection of efflux pumps in bacteria requires specialized instruments. In present study simple instrument free agar cartwheel method was used to detect efflux pumps in bacteria with brief modification. Forty clinical isolates of *K. pneumoniae* and 100 clinical isolates of *P. aeruginosa* were collected and processed. Then MDR strains were processed to detect efflux pump containing strains. Further cartwheel assay was performed. Knockout strains of these two bacteria were also included in the study along with standard strains. MIC determination method was used to confirm the observations of agar cartwheel assay. In Cartwheel assay, a total of 8 strains of *K. pneumoniae* were analyzed for the presence of efflux pumps, active efflux pump was detected in 6 isolates while it was not observed in 2 negative control strains, similar results were observed for *P. aeruginosa*. Bacterial isolates containing efflux pumps also showed high MIC value for the antibiotics used. But in the presence of an EPI, a considerable decrease in the MIC of antibiotics was observed among isolates containing efflux pumps, while there was no decrease in MIC of antibiotics for strains without efflux pumps. Present study revealed that cartwheel assay to detect efflux pumps is a reliable, fast and sensitive technique, which may reduce time and efforts to detect efflux pumps.

Keywords: MDR, Bacteria, MIC, efflux pumps, antibiotics, EPI

INTRODUCTION

Antibiotic resistance is a phenomenon in which antibiotic used to treat a particular bacteria becomes useless due to resistance mechanism evolved by it **[1]**. Increased drug resistance in bacteria has dramatically reduced the possibilities of treating infectious diseases **[2]**. In the late 1940s, after less than a decade of penicillin discovery, resistant strains of bacteria had been reported from different parts of the world **[3]**. There are two mechanisms involved in the origin of multidrug resistance are: accumulation of multiple genes, each encoding for resistance to a single drug and increased expression of genes that code for multidrug efflux pumps **[4]**. Efflux pumps are one of the major causes of multidrug resistance in bacteria which effluxes out the drugs accumulated. Therefore, inhibition of the activity of these pumps with efflux pumps inhibitors (EPIs) appears to be a promising approach for restoring the activity of drugs which are the substrates of these pumps **[5]**.

There are five super families of efflux pumps found in microorganisms, these are, ATP –binding cassette super family (ABC), major facilitator super family (MFS), resistance – nodulation cell division super family (RND), small multidrug resistance family (SMR), multi- antimicrobial extrusion protein family (MATE) **[6]**. Resistance nodulation division (RND) transporters are most frequently found in gram-negative bacteria and are known to export a variety of antimicrobial agents **[7-8]**. They transport a wide variety of substrates including antibiotics, detergents, dyes and host derived molecules from the periplasm to the extra-cellular space **[9]**.

The conventional methods for detection of efflux pump are costly affairs and time consuming, also the use of radioactive substances is dangerous and biohazardous. Hence there is a need to develop a fast and cost effective method for detecting efflux pumps in efflux mediated multidrug resistant (MDR) bacteria **[10-11]**. The Ethidium bromide (Et Br) agar cartwheel assay is a newly discovered simple, instrument free, safe and cost effective method utilized for the demonstration of efflux pump activity in bacteria **[12]**. The present study was aimed to detect efflux pumps in Gram negative bacteria (*K. pneumoniae* and *P. aeruginosa*) in Punjab with the help of EtBr agar Cartwheel assay.

MATERIALS AND METHODS

The Gram negative bacteria *K. pneumoniae* and *P. aeruginosa* were used to detect efflux pumps by using EtBr agar Cartwheel assay.

***K. pneumoniae* strains:** A total of 40 clinical isolates of *K. pneumoniae* were collected from Gian Sagar Medical College and Hospital, Rajpura, Distt. Patiala, Punjab (India) & PGI Chandigarh. One wild type and two AcrAB efflux pump knockouts strains were obtained from Dr. Enrique Llobet, CIBERES, Bunyola, Spain. Two AcrAB efflux pump over expressing strains were obtained from Annarita Mazzariol, *Dipartimento di Patologia, Sezione di Microbiologia, Strada Le Grazie*, 8, 37134 Verona, Italy. One standard strain MTCC 109 was obtained from IMTECH, Chandigarh as control as shown in Table 1&2.

***P. aeruginosa* strains:** A total of 100 clinical isolates of *P. aeruginosa* were collected from Gian Sagar Medical College and Hospital, Rajpura, Distt. Patiala, Punjab (India) & PGI Chandigarh. A wild type and three efflux pump knockout strains were obtained from Dr. Thio Kohler, Dept. of Microbiology and Molecular Medicine Geneve, University of Geneva, Switzerland. One standard strain was obtained from IMTECH, Chandigarh as a control as shown in table 1&2.

In-vitro Culture of Bacterial strains: All clinical isolates were cultured on nutrient agar then isolated strains of *P. aeruginosa* and *K. pneumoniae* were further cultured on selective media Cetrimide media and Hicrome *Klebsiella* selective agar base respectively. *K. pneumoniae* showed purple magenta coloured colonies on Hicrome *Klebsiella* selective agar base and *P. aeruginosa* showed pigmented greenish colonies on Cetrimide media.

Antibiotic susceptibility assay was performed as described by Bauer et al., (1966) **[13]**. The MDR strains on *K. pneumoniae* and *P. aeruginosa* were further cultured with Modified EtBr Cartwheel method to detect efflux pumps (Fig1).

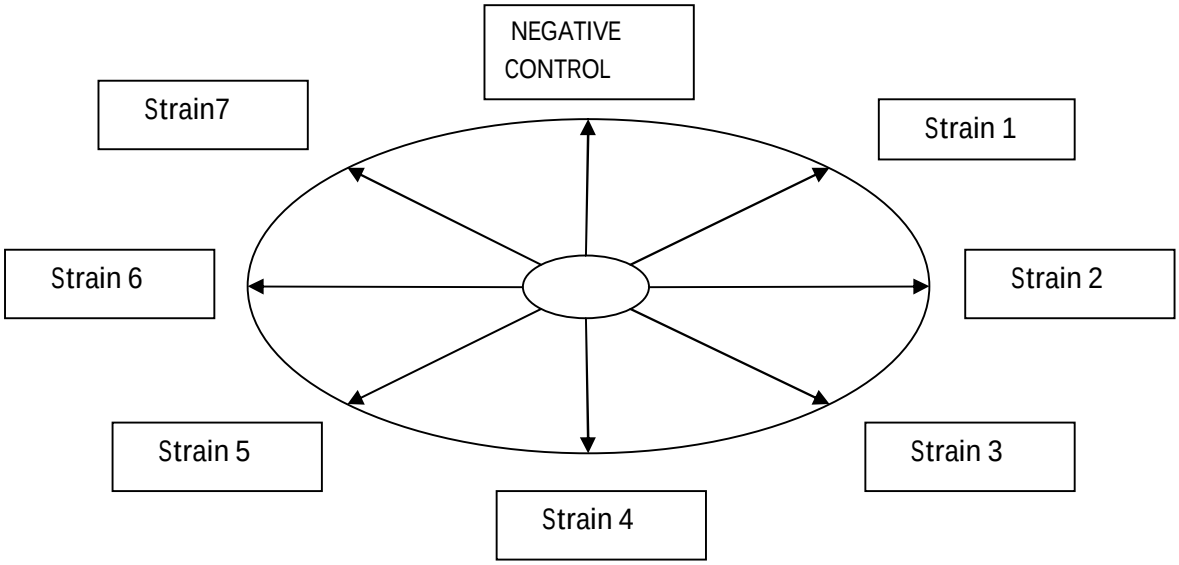

Fig 1: Schematic representation of EtBr agar cartwheel assay

EtBr Agar Cartwheel assay:

The EtBr- agar cartwheel method was used to assess the presence of efflux activity with brief modifications **[12, 14]**. In brief all the strains of bacteria were grown in nutrient broth instead of tryptic soya broth and incubated at 37^0 C for 24 hrs. Then culture was inoculated by swabbing on Tryptic soy agar (TSA) containing Ethidium Bromide. Three different concentrations of EtBr were used i.e., 2µg/mL, 1µg/mL and 0.5µg/mL, these concentrations were lower than the MIC of EtBr. Plates were incubated for 16 hrs at 37^0C and then observed under UV light. Effect of temperature was also recorded by incubation at 37^0C and 4^0C for 16 hrs. The fluorescence at each temperature was compared to that evident after the first incubation (at 37^0C).

MIC Determination:

The MIC was determined in all strains used for modified EtBr agar cartwheel assay to confirm the presence of efflux pumps. Pure cultures were diluted in nutrient broth to a concentration between $1x10^5$ and $1x10^6$cfu/mL. A stock dilution of the antibiotics i.e., ciprofloxacin, tetracycline & chloramphenicol were made at approximately 100x the level of the expected MIC for *K. pneumoniae* & *P. aeruginosa*. Further 1:1 dilutions of antibiotics were made with nutrient broth in 96 well microtiter plates. Then the bacterial culture was inoculated from lower concentration to higher concentration of antibiotic in equal

amount. Positive and negative control was also included. Then microtiter plates were incubated at 37^0C for 24 hrs and then plates were observed in ELISA plate reader (BioTek ELx800) at 490 nm. MIC was recorded as the lowest concentration where no growth is visually observed. Then MIC of antibiotics was again observed in the presence of an EPI (CCCP). CCCP (Carbonyl cyanide m-chlorophenyl hydrazone) is a standard EPI that blocks the efflux pumps and helps antibiotic accumulation inside the bacteria which decreased MIC value.

Results: Antibiotic susceptibility of all the strains of bacteria was recorded and it was found that 12.5% (5/40) clinical isolates of *K. pneumoniae* were multidrug resistant while 87.5% were found sensitive. Out of 100 isolates of *P. aeruginosa* 6% (6/100) isolates were found multidrug resistant while 96% were sensitive to various drugs.

Out of 5 MDR clinical isolates of *K. pneumoniae* 2 isolates were selected for EtBr Agar Cartwheel assay because these two strains showed phenotypic features of AcrAB efflux pump containing *K. pneumoniae*. In case of *P. aeruginosa* out of 6 MDR isolates 3 were selected for EtBr Agar Cartwheel assay because these three strains showed phenotypic features of mexAB-OprM efflux pump containing *P. aeruginosa*.

When EtBr Agar Cartwheel assay was performed for *K. pneumoniae* with 2 clinical isolates, 1 wild type, 4 knockout / overexpressing and 1 standard strain. Then fluorescence was observed in two strains only while 6 strains did not show fluorescence due to the presence of efflux pumps. In case of *P. aeruginosa*, EtBr Agar Cartwheel assay was performed for 3 clinical isolates, 1 wild type, 3 knockout/ overexpressing and 1 standard strain and fluorescence was observed only in 2 strains while 6 strains did not show fluorescence due to the presence of active efflux pumps in them. The absence of fluorescence determines the presence of active efflux pumps in MDR strains while the bacteria which showed pink fluorescence did not contain active efflux pumps (Fig 2& 3 and table 3). At 4^0C more fluorescence was observed in comparison to 37^0C at similar concentrations of EtBr. The effect of temperature suggested that low temperature decreases the membrane energy due to which efficacy of efflux transporters is decreased and hence less efflux occurs at low temperature.

The results of EtBr- agar cartwheel method were further explored by the determination of MIC values for selected antibiotics, known to be efflux pump substrates viz., Tetracycline, chloramphenicol & ciprofloxacin. The antibiotic sensitive strains of *K. pneumoniae* and *P. aeruginosa* showed MIC of antibiotics which was very low (table 4 &5), while antibiotic resistant strains showed very high MIC value which confirmed the presence of efflux pumps. When MIC of antibiotics was observed in the presence of an EPI (CCCP) then there was a significant decrease in the MIC of antibiotics for efflux pump containing strains but there was no decrease found in the MIC of antibiotics for strains without efflux pumps (table 4&5). Hence results of MIC assay were found similar to EtBr assay.

When MICs of antibiotics were compared for different strains of *K. pneumoniae* then it was found high for resistant strains while less for sensitive strains. In case of *P. aeruginosa*, MIC of antibiotics were found higher for one knockout strain than wild type while for second knockout strain it was found less in case of tetracycline & chloramphenicol but higher in case of ciprofloxacin.

Table 1: Description of different strains of bacteria used in the study:

S.No.	Name of the bacteria	Total clinical isolates	MDR Strains	Efflux pump containing clinical strains	Wild type strains	Knockout strains /Over expressing strains	Standard strain
1.	*K. pneumoniae*	40	5	2	1	4	1
2.	*P. aeruginosa*	100	6	3	1	3	1

Table 2: Strains selected for EtBr agar cartwheel assay:

S. No.	Name of bacteria	Strains chosen for cartwheel assay	No. of strains
1.	*Klebsiella pneumoniae*	Clinical	2
		Wild type	1
		Knockouts/ Over expressing strains	4
		Standard strains	1
2.	*Pseudomonas aeruginosa*	Clinical	3
		Wild type	1
		Knockouts/ Over expressing strains	3
		Standard strains	1

Table 3: EtBr agar cartwheel assay showing the presence of efflux pump among MDR clinical isolates, and standard controls (n=8)

S. No.	Name of strains	Strains with efflux pump
1.	*K. pneumoniae* MDR Clinical Isolate, Resistant strain (KC4)	Efflux pump detected
2.	*K. pneumoniae* MDR Clinical Isolate, Resistant strain (KC18)	Efflux pump detected
3.	*K. pneumoniae* AcrAB efflux pump repressor gene knockout strain, Resistant strain (1740)	Efflux pump detected
4.	*K. pneumoniae* AcrAB Wild type strain, Resistant strain (52145)	Efflux pump detected
5.	*K. pneumoniae* AcrAB efflux pump regulator gene knockout strains, Sensitive strain (1739)	Efflux pump not detected
6.	*K. pneumoniae* AcrAB efflux pump overexpressing strain, Resistant strain (KLPN86)	Efflux pump detected
7.	*K. pneumoniae* AcrAB efflux pump overexpressing strain, Resistant strain (KLPN105)	Efflux pump detected
8.	*K. pneumoniae* Standard strain, Sensitive strain (MTCC109)	Efflux pump not detected
9.	*Pseudomonas aeruginosa* MexAB-oprM efflux pump overexpressing strain (TETR)-Resistant strain	Efflux pump detected
10.	*Pseudomonas aeruginosa* Wild type strain of (PA01) MexAB-oprM efflux pump-Resistant strain	Efflux pump detected
11.	*Pseudomonas aeruginosa* MexAB-oprM efflux pump overexpressing strain (PT629)-Resistant strain	Efflux pump detected
12.	*Pseudomonas aeruginosa* MDR Clinical Isolate (Ps3)-Resistant strain	Efflux pump detected
13.	*Pseudomonas aeruginosa* MDR Clinical Isolate (PsT10)-Resistant strain	Efflux pump detected
14.	*Pseudomonas aeruginosa* MDR Clinical Isolate (Ps11)-Resistant strain	Efflux pump detected
15.	*Pseudomonas aeruginosa* MexAB-oprM efflux pump knockout strain (TETR-T)-Sensitive strain	Efflux pump not detected
16.	*Pseudomonas aeruginosa* (MTCC-471) (Standard)-Sensitive strain	Efflux pump not detected

Table 4: MIC determination in MDR and standard *K. pneumoniae* strains for ciprofloxacin, Tetracycline and Chloramphenicol (µg/mL) to confirm the presence of active efflux pump

S. no.	Sensitive/Resistance (S/R)	Name of the strain	MIC for Ciprofloxacin		MIC for Tetracycline		MIC for Chloramphenicol	
			Without CCCP	With CCCP	Without CCCP	With CCCP	Without CCCP	With CCCP
1.	R	*K. pneumoniae* MDR Clinical Isolate, Resistant strain (KC4)	0.5	0.125	2	1	2	1
2.	R	*K. pneumoniae* MDR Clinical Isolate, Resistant strain (KC18)	2	1	4	2	2	1

3.	**R**	***K. pneumoniae* AcrAB Wild type strain, Resistant strain (52145)**	**0.06**	**0.003**	**2**	**1**	**2**	**1**
4.	**S***	***K. pneumoniae* AcrAB efflux pump regulator gene knockout strains, Sensitive strain (1739)**	**0.06***	**0.06***	**0.5 ***	**0.5 ***	**1***	**1***
5.	**R**	***K. pneumoniae* AcrAB efflux pump repressor gene knockout strain, Resistant strain (1740)**	**0.125**	**0.5**	**4**	**2**	**4**	**2**
6.	**R**	***K. pneumoniae* AcrAB efflux pump overexpressing strain, Resistant strain (KLPN86)**	**64**	**8**	**2**	**1**	**2**	**1**
7.	**R**	***K. pneumoniae* AcrAB efflux pump overexpressing strain, Resistant strain (KLPN105)**	**4**	**2**	**4**	**2**	**4**	**2**
8.	**S***	***K. pneumoniae* Standard strain, Sensitive strain (MTCC109)**	**0.003***	**0.003***	**0.003***	**0.003***	**1***	**1***

Table 5: MIC determination in MDR and standard *P. aeruginosa* strains for ciprofloxacin, Tetracycline and Chloramphenicol (µg/mL) to confirm the presence of active efflux pump

S. No.	Name of the strain with their Sensitive/Resistance (S*/R)	MIC for Ciprofloxacin		MIC for Tetracycline		MIC for Chloramphenicol	
		Without CCCP	With CCCP	Without CCCP	With CCCP	Without CCCP	With CCCP
1.	**R1-Knockout TETR-T (S*)**	**0.5***	**0.003***	**0.06***	**0.003***	**0.125***	**0.06***
2.	**R2-Over expressing strain TETR (R)**	**0.5**	**0.125**	**64**	**8**	**128**	**64**
3.	**R3-Wild type strain PAO1(R)**	**2**	**1**	**4**	**2**	**8**	**4**
4.	**R4-Over expressing strain PT629 (R)**	**0.06**	**0.003**	**16**	**8**	**64**	**16**
5.	**Standard strain MTCC-471(S*)**	**0.125***	**0.06***	**0.06***	**0.003***	**0.5***	**0.125***
6.	**Ps3-Clinical strain (R)**	**1**	**0.5**	**16**	**8**	**64**	**16**
7.	**PsT10-Clinical strain (R)**	**2**	**1**	**64**	**8**	**128**	**64**
8.	**Ps11-Clinical strain (R)**	**1**	**0.5**	**16**	**8**	**8**	**4**

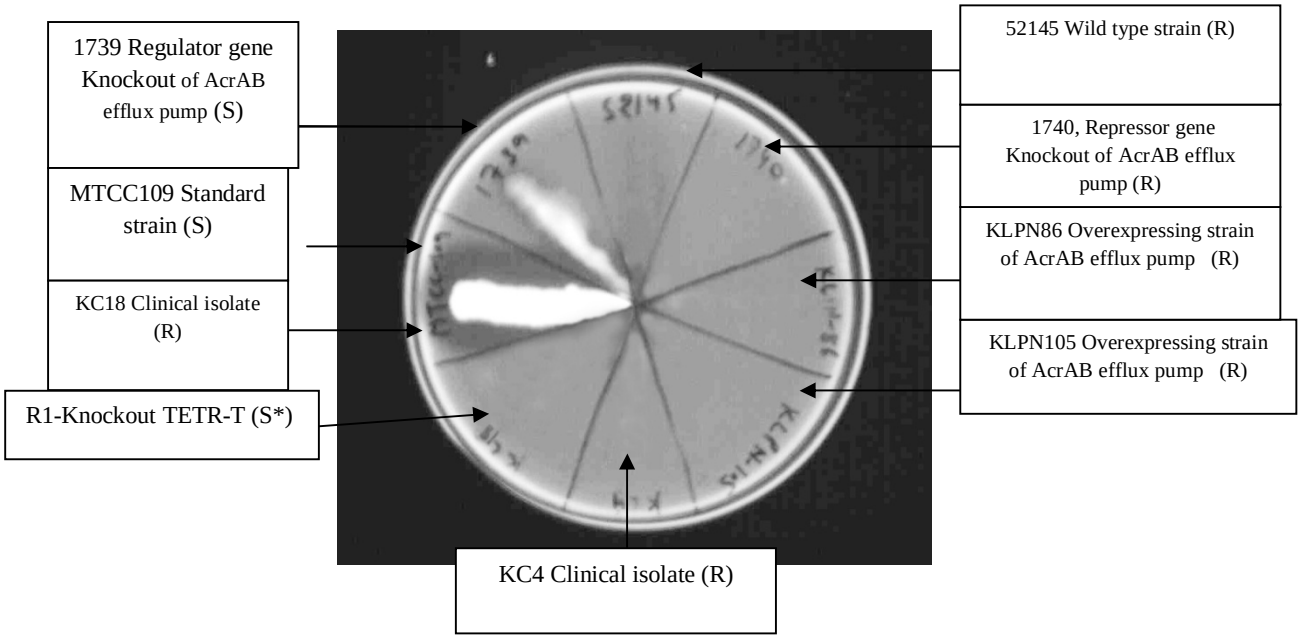

Fig 2: Fluorescence in two strains of *K. pneumonia*

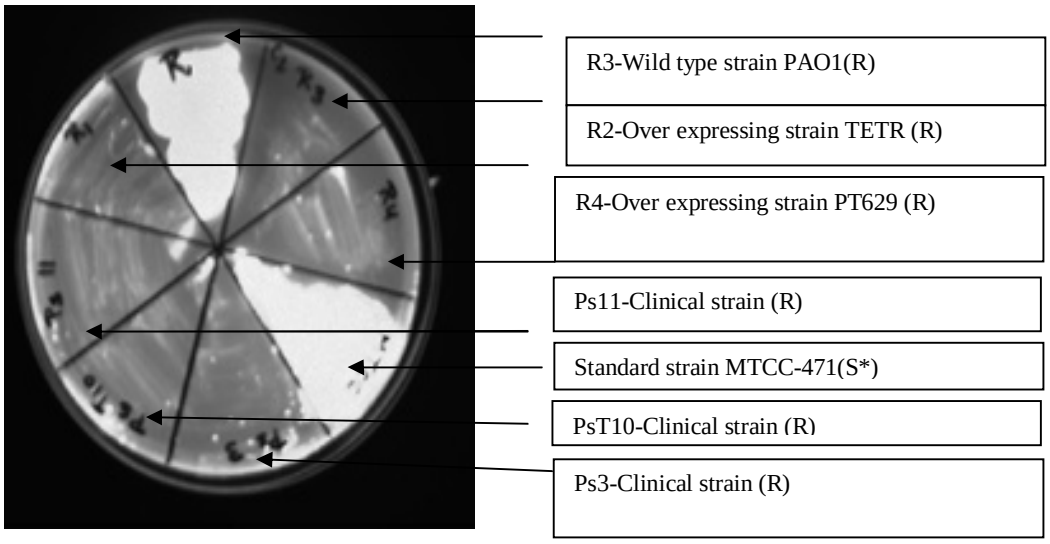

Fig 3: Fluorescence in two strains of *P. aeruginosa*

DISCUSSION

EtBr is an intercalating agent commonly used as a fluorescent tag in molecular biology. It forms fluorescent complexes by intercalation of DNA, which is visible under ultraviolet light **[15]**. Ethidium bromide used as a substrate to detect efflux pumps in drug resistant bacteria and is not allowed to accumulate inside the bacterial cell **[16]**. In the present study, Ethidium bromide cartwheel assay was used to detect active AcrAB efflux pump in multidrug resistant strains of *K. pneumoniae* and also to detect active MexAB-oprM efflux pump in *P. aeruginosa*. In the present study the assay was performed for all isolates, MDR, sensitive as well as for control strains.

In the present study, EtBr fluorescence was not observed among selected antibiotics resistant clinical isolates of *K. pneumoniae*, in AcrAB efflux pump overexpressed control strains of *K. pneumoniae* and also in wild type strains of AcrAB efflux pump of *K. pneumoniae*. Contrarily all the sensitive isolates including sensitive controls isolates (efflux pump knockout strain and in MTCC 109) have shown EtBr fluorescence. The absence of EtBr fluorescence among MDR isolates and control resistant isolates suggested that all strains contains AcrAB efflux pump which effluxed out EtBr from bacterial cell.

The effect of temperature on efflux activity of bacteria has also been recorded and the maximum fluorescence was observed on 4^0C in comparison to 37^0C at similar concentrations of EtBr. According to Martins et al., (2013) **[12]**, the Gram negative bacteria *Acinetobacter* has shown significant increase in the fluorescence after the incubation at 4^0C while less fluorescence was observed at 37^0C. The observations of the present study suggested that the drug efflux mechanism is an energy mediated mechanism and direct proportionate to the temperature when the temperature decreased the energy of membrane also decreased and the other factor may be responsible for less efflux is low metabolic rate at low temperature, which is not able to produce sufficient energy, hence the available energy is not sufficient to efflux out the accumulated compounds. Therefore, more fluorescence occurs at low temperature. In addition to this, the isolates having potentially less active efflux systems showed higher fluorescence and isolates having more active efflux systems

showed less fluorescence (Martins *et al*., 2011) **[11]**.

In the present study, the MIC value of selected antibiotics was found very high (for active efflux pump containing strains) which are known as efflux pump substrates viz., Tetracycline, Chloramphenicol and Ciprofloxacin. While for the sensitive control strains of *K. pneumoniae* very low MIC value was observed. Further the assay was performed with an Efflux pump inhibitor (EPI) CCCP. A significant decrease was observed in MIC values of antibiotics up to 0.003 µg/mL. While the MIC for control sensitive strains (1 MTCC 109 and 1 regulator gene knockout strain) without efflux pump was not changed and remain constant even in the presence of an EPI (CCCP). Similarly, Kishk et al., (2014) **[17]**, has reported the significant decrease in the MIC of Clorhexidine in the presence of efflux pump inhibitor CCCP.

All the active efflux pump containing strains of *P. aeruginosa* have shown very high MIC values ranging upto 128 µg/mL confirm the presence of MexAB-oprM efflux pump. While the sensitive control strains have shown very low MIC value. When the assay was performed with an Efflux pump inhibitor (EPI) CCCP, a significant decrease was observed in MIC of antibiotics for all resistant strains and these values were ranging upto 0.003 µg/mL. While the MIC of control sensitive strains without efflux pump was not changed and remain constant even in the presence of an EPI (CCCP). Similarly, Abdi-Ali et al., (2006) **[18]** has demonstrated the significance decrease in MIC of resistant antibiotics used with EPI (CCCP).

The observations of the present study again suggested that the drug resistance in MDR clinical isolates is mediated by an active efflux pump which effluxed out the accumulated drug.

CONCLUSION

EtBr agar cartwheel assay is a trustworthy method and can be used for the detection of efflux pumps in bacteria in laboratories and on the basis of present study it can be concluded that efflux pump over expression is an arising cause of drug resistance in Punjab therefore proper measures should be used to tackle this problem.

REFERENCES

[1] Gothi D, Joshi JM, Resistant TB: Newer drugs and community approach, Recent Pat Anti-Infect Drug Discov, 6, 2011, 27-37.

[2] WHO, Shaping the future, World Health Report, 2003.

[3] Cohen ML, Epidemiology of drug resistance: Implications for a post-antimicrobial era, Science, 257, 1992, 1050–1055.

[4] Nikaido H, Multidrug Resistance in Bacteria, Ann Rev Biochem, 78, 2009, 119-146.

[5] Bardwaj AK, Moanty P, Bacterial Efflux Pumps Involved in Multidrug Resistance and their Inhibitors: Rejuvinating the Antimicrobial Chemotherapy, Recent Patents on Anti-Infective Drug Discovery, 7, 2012, 73-89.

[6] Ren Q, Paulsen IT, Comparative analyses of fundamental differences in membrane transport capabilities in prokaryotes and eukaryotes. PLoS Comput Biol, 2005, 3: 27.

[7] Elkins CA, Nikado H, Substrate specificity of the RND type multidrug efflux pumps AcrB and AcrD of *Escherichia coli* is determined predominantly by two large periplasmic loops, J Bacteriol, 184, 2002, 6490-6498.

[8] Mao W, Warren MS, Black DS, Satou T, Murata T, Nishino T, Geton N, Lomovskaya O, On the mechanism of substrate specificity by resistance pumps: the large periplasmic loops of MexD from *P. aeruginosa* are involved in substrate recognition. Mol Microbiol, 46, 2002, 889-901.

[9] Jessica MAB, Laura JVP Structure, function and inhibition of RND efflux pumps in Gram-negative bacteria: an update. Current opinion in Microbiology, 12, 2009, 512-519.

[10] DeMarco CE, Cushing LA, Frempong-Manso E, Seo SM, Jaravaza TAA, Diixa Patel, Christos Kosmidis, Susan MS, Glenn WK, Ethidium Bromide MIC Screening for Enhanced Efflux Pump Gene Expression or Efflux Activity in *Staphylococcus aureus*, Antimicrob. Agents Chemother, 54(12), 2010, 5070-5073.

[11] Martins M, Viveiros M, Couto I, Costa SS, Pacheco T, Fanning S, Pagès JM, Amaral L, Identification of efflux pump-mediated multidrug-resistant bacteria by the Ethidium Bromide-Agar Cartwheel Method, In Vivo, 25, 2011, 171–178.

[12] Martins M, McCuskin MP, Viveiros M, Couto I, Fannings S, Pages JM *et al.*, Simple Method for Assessment of MDR Bacteria for Over –Expressed Efflux Pumps, Open Microbiol J, 7: (1-M6), 2013, 72-82.

[13] Bauer AW, Kirby WMM, Sherris JC, Turck M Antibiotic susceptibility testing by a standardized single disk

method, Am. J. Clin. Pathol, 36, 1966, 493-496.

[14] Costa SS, Junqueira E, Palma C, Viveiros M, Cristino JM, Amaral L, Couto I Resistance to Antimicrobials Mediated by Efflux Pumps in *Stapylococcus aureus*, Antibiotics, 2, 2013, 83-99.

[15] Genium's handbook of Safety, health and environmental data for common hazardous substances, 1999, 456-478.

[16] Patel D, Kosmidis C, Seo SM, Kaatz GW: Ethidium bromide MIC screening for enhanced efflux pump gene expression or efflux activity in *Staphylococcus aureus***,** *Antimicrob Agents Chemother*, 54, 2010, 5070-5073.

[17] Kishk R, Mandour M, Hessam W, Nemr N, Efflux pump genes and chlorhexidine resistance: Clue for *Klebsiella pneumoniae* infections in intensive care units, Egypt. Afr J Microbiol Res, 8(21), 2014, 2162-2167.

[18] Abdi-Ali A, Mohammadi-Mehr M, Agha Alaei Y, Bactericidal activity of various antibiotics against biofilm-producing *Pseudomonas aeruginosa*. Int J Antimicrob Agents, 27, 2006, 196–200.