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Research Article

EVALUATION OF EFFLUX PUMP ACTIVITY IN ESBL-PRODUCING *ESCHERICHIA COLI* ISOLATES RECOVERED FROM ENVIRONMENTAL SOURCES OF MIRZAPUR, UTTAR PRADESH

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ABSTRACT

The overexpression of efflux pumps is one of the mechanisms of acquisition of multidrug resistant (MDR) traits in bacteria. In the current study, a total of 24 extended spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolates recovered from various environmental sources of Mirzapur, Uttar Pradesh were evaluated for the presence of efflux pump activity by ethidium-bromide (Et-Br) agar cartwheel method (EBACM) followed by the efflux pump inhibition assay (EPIA) against different antibiotics in the presence and absence of phenylalanine-arginine β-naphthylamide (PAβN), a broad-spectrum efflux pump inhibitor (EPI). The difference in the zone of inhibition (ZOI) of ≥5 mm around the antibiotic discs in the presence and absence of PAβN was considered as a significant change. All the isolates exhibited an efflux activity up to an Et-Br concentration of 1.5µg/mL. A total of 19 (79.16%) isolates revealed an MDR phenotype. An absolute resistance (100%) towards ampicillin and ceftriaxone was exhibited by all the isolates, followed by azithromycin (54.16%), ciprofloxacin (33.33%), kanamycin (25%), and doxycycline (16.66%). In the presence of PAβN, a significant increase in the ZOI against azithromycin (100%) and doxycycline (76.19%) was observed. However, there was a reduction in ZOI against ceftriaxone, ampicillin/sulbactam, and ciprofloxacin in the presene of PAβN in 70.83%, 87.5%, and 12.5% of the isolates, respectively. The exhibition of a high degree of MDR phenotype and presence of active efflux pumps in environmental ESBL-producing *E. coli* isolates is a matter of serious concern from public health point of view.

Keywords: MDR, ESBL, *Escherichia coli*, Efflux pump, Et-Br agar cartwheel method, PAβN

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The indiscriminate antimicrobial usage in clinical medicine and food animal production along with the presence of various environmental factors, host factors, and pathogen's intrinsic or acquired characteristics act as drivers in the emergence and dissemination antimicrobial resistance (AMR) (Manoj *et al.*, 2023). The efflux-pump mediated AMR is an intrinsic mechanism possessed by the bacteria to extrude metabolic waste, toxic substances and antimicrobial agents (Blanco *et al.*, 2016). The AcrAB-TolC is an important resistance nodulation division (RND) family efflux pump which plays a major role in expression of multi-drug resistant (MDR) phenotype in *Escherichia coli* because of their broad specificity (Weston *et al.*, 2018). A simple and cost-effective phenotypic method to demonstrate the efflux pump activity in bacteria is ethidium-bromide (Et-Br) agar cartwheel method (EBACM). The absence of fluorescence under UV light by bacterial mass in the agar plates supplemented with Et-Br indicates an overexpression of efflux pumps (Martins *et al.*, 2011).

Certain molecules known as the efflux pump inhibitors (EPIs) can be used in combination with antibiotics (known as adjuvant therapy) to enhance their activity against the strains which overexpress efflux pumps (Sharma *et al.*, 2019). Among the EPIs, phenylalanyl-arginine-β-naphthylamide (PAβN), a peptidomimetic EPI was the first inhibitor of RND family efflux pumps, specifically the AcrB (Huang *et al.*, 2022). In addition to its role as a potentiator of fluoroquinolones, macrolides and chloramphenicol, the phenylalanine-arginine β-naphthylamide (PAβN) itself acts as a substrate for RND efflux pumps as well, thereby competitively inhibiting the substrate binding and efflux (Viveiros *et al.*, 2008). Although, the efflux pump inhibition assay (EPIA) in MDR *Salmonella* Typhimurium has been studied (Anbazhagan *et al.*, 2019), the literatures are scarce with regard to the extended spectrum β-lactamase (ESBL)-producing *E. coli*. Therefore, the current study aims to detect the efflux pump activity in ESBL-producing *E. coli* isolates recovered from environmental sources of Mirzapur district, Uttar Pradesh, India by EBACM as well as subjecting these isolates to EPIA using PAβN against different antibiotics.

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MATERIALS AND METHODS

Determination of efflux pump activity by Ethidium-Bromide (Et-Br) Agar Cartwheel Test (EBACT)

Atotal of 24 previously characterized ESBL-producing *E. coli* isolates recovered from various environmental sources in and around Mirzapur, Uttar Pradesh (data not shown) were included in this study. The efflux pump activity of the ESBL-producing *E. coli* isolates was determined by the ethidium-bromide (Et-Br) agar cartwheel test (EBACT) as described previously (Martins *et al.*, 2011). In brief, sterile Mueller Hinton Agar (MHA) plates with varying concentration (i.e., 0, 0.5, 1, 1.5 and 2 µg/mL) of Et-Br were prepared. The plates were divided into six sections forming a cartwheel pattern. The overnight-grown culture of ESBL-producing *E. coli* isolates (inoculum adjusted to 0.5 McFarland standard) were streaked on those plates starting from the centre to the periphery of the plate. The plates were wrapped in aluminium foil and incubated overnight at 37º C and then observed under UV light in a gel documentation system (Biorad, USA). The minimum concentration of Et-Br that exhibited fluorescence in the streaking line (bacterial mass) was recorded. The *E. coli* ATCC 25922 was used as a control strain.

Antibiotic sensitivity test

The antibiotic sensitivity test of the isolates against 7 antibiotics (Himedia, India) representing different classes namely, ampicillin (penicillin), kanamycin (aminoglycoside), azithromycin (macrolide), ciprofloxacin (fluoroquinolone), ceftriaxone (cephalosporins), ampicillin/sulbactam (penicillin with a β-lactamase inhibitor) and doxycycline (tetracycline) was performed as per the Kirby-Bauer disc diffusion method in sterile MHA plates. In brief, the plates were swabbed with the standard inoculum (adjusted to 0.5 McFarland standard) of the test isolates. The above-mentioned antibiotic discs were placed at a distance of 30 mm centre-to-centre and the plates were incubated overnight at 37º C. The zone of inhibition (ZOI) around the discs were measured with a ruler against a bright background and the results were interpreted according to the CLSI guidelines (CLSI, 2018).

Efflux pump inhibition assay against different antibiotics

The efflux pump inhibition assay was performed in the presence and absence of a broad-spectrum efflux pump inhibitor i.e., phenylalanine-arginine β-naphthylamide (PAβN) (Sigma Aldrich, USA). In brief, the sterile MHA plates supplemented with PAβN (at a concentration of 25 µg/mL) and that without PAβN were prepared (Anbazhagan *et al.*, 2019). The plates were swabbed with the standard inoculum (adjusted to 0.5 McFarland standard) of the test isolates. The antibiotic discs mentioned earlier were placed at a distance of 30 mm centre-to-centre and the plates were incubated overnight at 37º C. A difference in the ZOI of ≥5 mm in the presence (P) and absence (A) of PAβN around the antibiotic discs was considered significant for efflux pump inhibition against that antibiotic.

RESULTS AND DISCUSSION

Determination of Efflux pump activity in the ESBL-producing *E. coli* isolates by Et-Br agar cartwheel test (EBACT)

In this study, all the ESBL-producing *E. coli* isolates (100%) exhibited an efflux activity (absence of fluorescence) at an Et-Br concentration of 1.5 µg/mL indicating the presence and expression of active efflux pumps in those isolates. Fluorescence was exhibited by the isolates at an Et-Br concentration ≥ 2 µg/mL (Fig. 1). Ethidium bromide is considered as a common substrate of efflux pump in *Enterobacteriaceae* (Schumacher *et al.*, 2007) and has been used to assess the intrinsic efflux activity in *E. coli* (Amaral *et al.*, 2014). The efflux pumps are generally involved in the extrusion of harmful chemicals (like Et-Br) and toxic metabolites from the bacterial cells (Anbazhagan *et al.*, 2019).

Antibiogram of the isolates

A total of 19 (79.16%) isolates revealed a multi-drug resistant (MDR) phenotype, i.e., these were resistant to three or more than three classes of antibiotics used in the study. An absolute resistance (100%) towards ampicillin and ceftriaxone was exhibited by all the isolates. This was in concordance to the study of Yadav *et al.* (2021) in which 78 % of the ESBL *E. coli* and *Klebsiella* isolates recovered from milk and faecal samples of buffaloes collected in Ayodhya and Sultanpur district of Uttar Pradesh were observed to be MDR. Resistance towards azithromycin was observed in 54.16% of isolates followed by ciprofloxacin (33.33%), kanamycin (25%) and doxycycline (16.66%). All the non-MDR isolates (n=5) were sensitive to azithromycin and doxycycline (Fig. 2). The presence of functional efflux pumps as observed in EBACT may be involved in the emergence of MDR traits in these isolates. It has been reported that, the emergence of MDR phenotypes is linked to the overexpression of efflux pumps (Huang *et al.*, 2022). The efflux of different antibiotics was evaluated in the presence and absence of PAβN, a broad-spectrum EPI.

Efflux pump inhibition assay (EPIA)

In the presence of PAβN, the difference in the ZOI of ≥5 mm against azithromycin was evident in all the isolates (100%), whereas, that against doxycylcine was observed in 19 (76.19%) isolates (Table 1). This suggests an over expression of efflux pumps in acquisition of resistance towards these antibiotics. It also indicates that azithromycin and doxycycline are the substrates for the efflux pumps

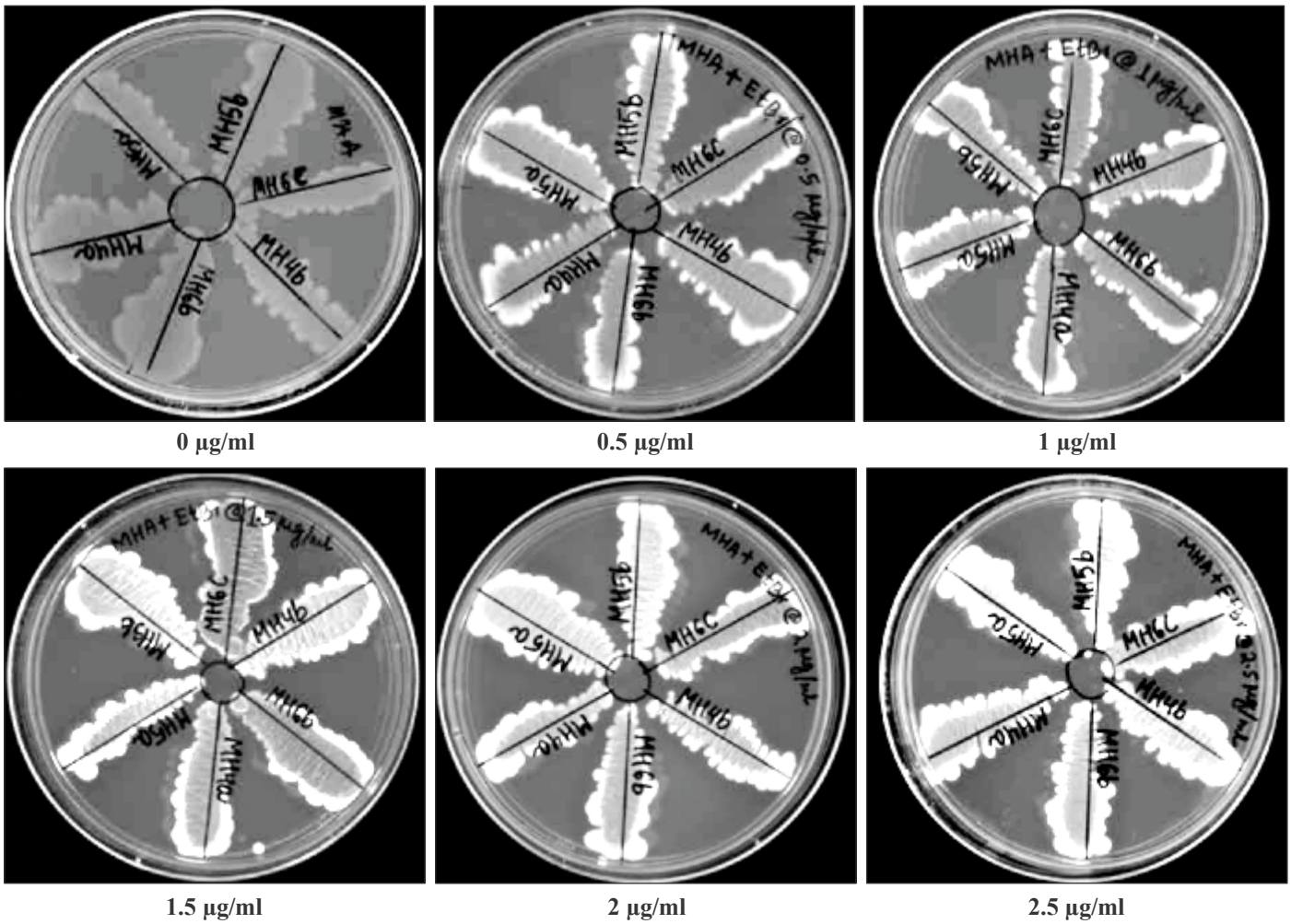

Fig. 1. Determination of efflux pump activity in ESBL-producing *E. coli* isolates at various concentrations of Et-Br by the ethidium-bromide (Et-Br) agar cartwheel method

present in these isolates. Also, it was well documented that, PAβN reverts the bacterial resistance towards tetracyclines (e.g., doxycycline) and macrolides (e.g. azithromycin) in comparison to other EPIs (Kern *et al.*, 2006). Moreover, in all the azithromycin and doxycycline-resistant isolates under this study, the resistant status changed from resistant to sensitive in the presence of PAβN against these two antibiotics.

The contribution of PAβN-inhibited efflux pumps towards ciprofloxacin (a quinolone) resistance was observed in 16 isolates in our study (Table 1). This was in agreement with a study in which, a 4-32-fold decrease in the MIC of nalidixic acid (a quinolone) in the presence of PAβN among ESBL-producing *E. coli* isolates recovered from rivers and lakes in Switzerland was observed (Zurfluh *et al.*, 2014). In another study, a ≥4-fold decrease in the MIC of ciprofloxacin in 7 of the 17 K. *pneumoniae* isolates were documented (Vera-Leiva *et al.*, 2018). Additional mechanisms like mutation in DNA gyrase, plasmid-mediated quinolone resistance or decreased uptake of quinolones due to loss of membrane bound porins may contribute towards

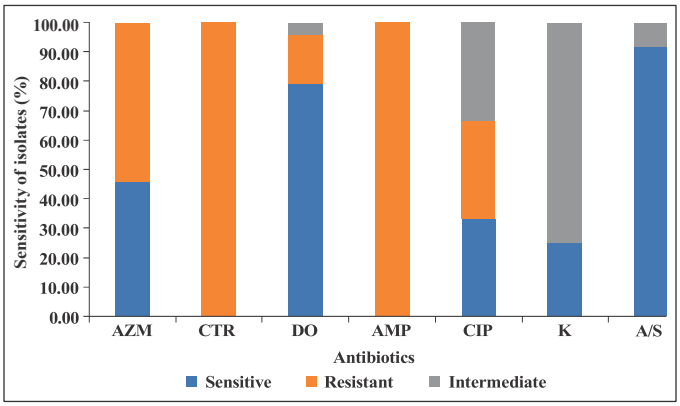

Fig. 2. Antibiogram of ESBL-producing *E. coli* isolates recovered from environmental sources of Mirzapur, Uttar Pradesh (Abbreviations: AZM: Azithromycin, CTR: Ceftriaxone, DO: Doxycycline, AMP: Ampicillin, CIP: Ciprofloxacin, K: Kanamycin, A/S: Ampicillin/Sulbactam)

ciprofloxacin resistance (Bush *et al.*, 2020).

However, there was a reduction in ZOI against ceftriaxone, ampicillin/sulbactam and ciprofloxacin in presene of PAβN in 17 (70.83%), 21 (87.5%) and 3 (12.5%) isolates, respectively (Table 1). It can be assumed that,

Table 1. Evaluation of efflux pump inhibition assay in the ESBL-producing *E. coli* isolates in the presence (P) and absence (A) of PAβN against different antibiotics

Isolate id	Differences (P-A) in ZOI (in mm) in the presence (P) and absence (A) of PAβN						
	AZM	CTR	DO	AMP	CIP	K	A/S
MH1a	6	-4	5	0	9	0	-1
MH3b	8	0	7	0	1	2	-7
MH4a	8	-1	3	0	0	0	-3
MH4b	8	0	4	0	1	2	1
MH4c	7	-3	2	0	2	0	-4
MH5a	6	-2	5	0	3	0	-12
MH5b	7	-4	1	0	2	0	-4
MH5c	7	-3	3	0	1	2	-6
MH6a	9	0	5	0	-8	3	-7
MH6b	7	0	6	0	0	1	0
MH6c	8	-2	5	0	1	1	-3
MH7a	9	-2	5	0	3	2	-5
MH7b	5	0	6	0	2	0	0
MH7c	7	-2	5	0	2	1	-4
MW2c	7	-3	5	0	0	2	-8
MW3b	7	-4	5	0	0	1	-8
MW4a	5	-1	4	0	2	0	-5
MC1b	8	-3	7	0	4	2	-6
MC2c	9	-3	5	0	2	1	-6
MC3b	6	0	5	0	0	0	-6
MC4a	5	-2	5	0	-11	1	-8
MC4b	8	-1	5	0	3	1	-7
MC5c	8	0	5	0	-7	1	-5
MC6a	6	-2	5	0	4	3	-5
E. coli ATCC 25922	8	-1	5	0	1	1	0

Abbreviations: AZM: Azithromycin, CTR: Ceftriaxone, DO: Doxycycline, AMP: Ampicillin, CIP: Ciprofloxacin, K: Kanamycin, A/S: Ampicillin/Sulbactam

along with the production of ESBLs by these isolates, an increased efflux of ceftriaxone in presence PAβN might have resulted in the decrease of ZOI against that antibiotic. This is in agreement with a study where, a higher efflux activity of cephalosporins in the presence of PAβN in *E. coli* was reported which might be attributed to different binding sites of cephalosporins in the large binding pocket of the RND efflux pump (Kinana *et al.*, 2013). In a study, the β-lactamase inhibitors like clavulanate and sulbactam were found to be the substrates for the MexAB-OprM multi-drug efflux system in *Pseudomonas aeruginosa* (Li *et al.*, 1998; Nakae *et al.*, 2000). In another study, it was observed that, the PAβN treatment reduced the intracellular level of AmpC β-lactamase in *P. aeruginosa*. Even at a sublethal concentration, PAβN increased the permeabilization of outer cell membrane, thereby decreasing the intracellular accumulation of AmpC β-lactamases (Lamers *et al.*, 2013). Further, PAβN is also considered as a competitive inhibitor of RND efflux pumps (Viveiros *et al.*, 2008) and in low concentration, it inhibits AcrAB and AcrEF efflux system (Misra *et al.*, 2015). All these factors may explain why there was a reduction in the ZOI of ampicillin/sulbactam in presence of PAβN in these ESBL-producing *E. coli* isolates.

In this study, no contribution of PAβN-inhibited efflux pump activity towards ampicillin resistance was observed, as there was no change in the ZOI in the presence of PAβN in any of the studied isolates. Although, efflux activity by EBACT is complimentary to the EPIA, it may not be true that same efflux activity will be expected for Et-Br and other antibiotics (Anbazhagan *et al.*, 2019). It has been reported that, PAβN doesn't affect the efflux of all antibiotics, suggesting a different binding site of antibiotics to the efflux pumps (Schneiders *et al.*, 2003) or a different mechanism of resistance towards that antibiotic.

The efflux pumps are one of the mechanisms, but not always the only mechanism of antimicrobial resistance in bacteria. The EPIs usually show promising results against the efflux pumps when used alongside an antibiotic. Also, it requires a higher concentration of EPI for the competitive inhibition of substrate binding site with that of the antibiotics. Some antibiotics may have a different substrate binding site other than that of EPIs in the efflux pump. All these factors greatly narrow the spectrum of an EPI, thereby making it specific for particular substrates. However, it is extremely difficult to find an EPI that inhibits multiple antibiotics at different binding sites of an efflux pump (Misra *et al.*, 2015).

CONCLUSION

The current study demonstrates a simple and effective way for detecting efflux pump activity in ESBL-producing *E. coli* isolates by the EBACM which revealed the presence of active efflux pumps in all the isolates. The EPIA revealed a significant efflux activity by the isolates for azithromycin and doxycycline compared to other classes of antibiotics. However, the efflux activity for different antibiotics varied in the presence of PAβN, which suggests the presence of other potential resistance mechanisms or different binding sites of the antibiotics and PAβN in the efflux pumps for these isolates. Nevertheless, the presence of active efflux pumps and exhibition of a high degree of MDR phenotype along with the ESBL production among these environmental isolates is a matter of concern from public health point of view. It can be concluded that, the overexpression of efflux pumps has a significant role in the emergence of MDR ESBL-producing *E. coli* in the environmental milieu.

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