




# Identification of *AcrAB-ToIC* Efflux Pump Genes and Detection of Mutation in Efflux Repressor *AcrR* from Omeprazole Responsive Multidrug-Resistant *Escherichia coli* Isolates Causing Urinary Tract Infections

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**ABSTRACT:** Antimicrobial resistance poses a threat in the treatment of infectious diseases in Bangladesh as well as in the world. Multidrug-resistant (MDR) Enterobacteriaceae, the most common cause of one such infectious disease, urinary tract infection (UTI), has contributed to the escalating problem of selecting empiric antibiotics against UTIs. The aim of this study was to investigate the presence of the efflux pump in MDR *Escherichia coli* isolates from UTI in the North-East region of Bangladesh, to isolate and characterize the *AcrAB-ToIC* efflux pump genes of these locally isolated strains and to do mutation analysis of the efflux pump repressor *AcrR* gene to understand the *AcrAB-ToIC* efflux pump mechanism. In the presence of omeprazole, an efflux pump inhibitor, every MDR *E. coli* isolate showed increased susceptibility to at least 1 of the 7 antibiotics investigated, indicating that efflux pump might be involved in their antibiotic resistance. Omeprazole decreased the minimum inhibitory concentration of every antibiotics being investigated by 2- to 8-fold. DNA and the deduced amino acid sequences of the polymerase chain reaction (PCR) products analyzed by bioinformatics tools revealed that the chromosomal *AcrAB-ToIC* and *AcrR* genes were present in all MDR and antibiotic-susceptible *E. coli* isolates. However, the deduced amino acid sequences of the amplification refractory mutation system (ARMS) PCR product of the *AcrR* gene revealed that the substitution of arginine to cysteine at position 45 of *AcrR* was observed only in the MDR *E. coli* whose antibiotic susceptibility increased in the presence of omeprazole. Data reported herein support the notion that the increased antibiotic susceptibility of the MDR *E. coli* isolates in the presence of omeprazole might be due to efflux pump(s) inhibition and the *AcrAB-ToIC* efflux pump might be a contributor to antibiotic resistance when the mutation of arginine to cysteine occurs at position 45 of *AcrR*.

**KEYWORDS:** Multidrug resistant, MIC, UTI, efflux, *AcrAB-ToIC*, mutation, *AcrR*

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## Introduction

Multidrug resistance of pathogenic microorganisms, for example, bacteria, is a global noteworthy concerning issue. Pathogens resistant to 3 or more antibiotics are called multidrug resistant (MDR).<sup>1,2</sup> Infections by MDR pathogens are difficult to treat and have been associated with worse disease outcomes, and hence, impose a major threat.<sup>3,4</sup> Recent studies suggest that antibiotic-resistant bacteria can be more virulent than their antimicrobial-susceptible counterparts.<sup>5</sup> The antimicrobial resistance (AMR) in bacteria is emerging globally unprecedentedly and the resistant pattern differs based on geographical locations.<sup>6</sup> There are a plethora of means by which humans have inadvertently accelerated the evolution of bacterial resistance. The over prescription of antibiotics by doctors for symptoms that in many cases may not be caused, unnecessary use of antimicrobials in agriculture and aquaculture and their dissemination in the environment are contributing to AMR worldwide.

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Recently, global warming and climate change are also supposed to contribute to AMR.<sup>7,8</sup> Furthermore, in developing countries, such as Bangladesh, self-medication with antibiotics due to their easy availability in the pharmacy, selling pill-by-pill, misuse and abuse of antibiotics, and not fulfilling the prescribed antibiotic regimens once the patient feels better are the reasons of increasing AMR.<sup>2,9-11</sup> Emerging AMR in developing countries is not necessarily regionally confined, as today's globalized world allows for resistant bacteria as well as people to travel around the world.<sup>12</sup> Therefore, molecular elucidation of multidrug resistance in pathogen of different geographical regions is required due to variance in antibiotic intake, agricultural use, and climate change in different areas of the world.

Susceptible bacteria can acquire resistance to an antimicrobial agent through mutation, degradation, or changing the structure of antibiotic, efflux pump, and conjugation and/or transformation of the plasmid.<sup>2,13,14</sup> Efflux pump proteins, found in both Gram-positive and Gram-negative bacteria as well as in eukaryotic microorganisms,<sup>15</sup> are transporter proteins



involved in the extrusion of toxic substrates from within cells into the external environment. The impact of efflux mechanisms on AMR is large, and antibiotics can act as inducers and repressors of the expression of some efflux pump proteins.<sup>16</sup> AcrAB-TolC, one of the efflux pumps, constitutively expressed in *Escherichia coli*, is composed of the outer membrane protein TolC, the inner membrane transporter AcrB, and the periplasmic adaptor protein AcrA.<sup>14,17</sup> Overexpression of the AcrAB-TolC efflux pump is an intrinsic mechanism of multidrug resistance in Gram-negative bacteria.<sup>14,17,18</sup> It can be due to the mutation in *AcrR* gene which is the repressor of the *AcrAB* operon system.<sup>19</sup> Substrate profiles of the *E. coli* housekeeping efflux system AcrAB-TolC include chloramphenicol, fluoroquinolone, tetracycline, novobiocin, fusidic acid, nalidixic acid and  $\beta$ -lactam antibiotics, dyes, detergents, and most lipophilic antibiotics.<sup>16,20</sup> The expression of AcrAB is regulated by the repressor AcrR, encoded by *AcrR*.<sup>19</sup> It has been shown that a C133T transversion resulting in arginine to cysteine substitution at codon 45 of *AcrR* is responsible for overexpression of the AcrAB efflux system and results in AMR.<sup>21</sup> Therefore, the antibiotic therapy can be made effective if (1) efflux pumps are inhibited, (2) the expression of efflux pumps is downregulated, or (3) the antibiotics are structurally redesigned, so that they are no longer suitable efflux substrates.<sup>22</sup> One of the rational approaches toward confronting efflux of clinically relevant antibiotics is to discover or design potent efflux pump inhibitors. A number of known compounds have been reported as efflux pump inhibitors such as verapamil, biricodar, moxifloxacin, and omeprazole.<sup>14,19,23,24</sup> Although several reports show the inhibitory effects of omeprazole on bacterial efflux pumps,<sup>23,25,26</sup> very few studies have been done to show its inhibitory effects on efflux pumps of *E. coli*.

Urinary tract infection (UTI) is recognized as the second most common infection in community practice.<sup>27</sup> Globally, about 150 million people are diagnosed with UTI each year, costing the global economy in excess of US\$6 billion.<sup>28</sup> Recently, it is reported that about 60% of UTIs are caused by *E. coli* and ~90% of these *E. coli* isolates are MDR in the North-East region of Bangladesh.<sup>2</sup> In 2000, only 7.1% MDR *E. coli* were reported in patients of the United States,<sup>29</sup> whereas in 2011, 77% MDR *E. coli* were reported in UTI patients in Iran,<sup>30</sup> indicating rapid and threatening emergence of antibiotic resistance in *E. coli* causing UTI. Although about 90% of the *E. coli* isolates causing UTI are MDR,<sup>2</sup> the molecular reasons of multidrug resistance in these MDR *E. coli* are not yet elucidated. In this study, the efflux pump system has been assayed using omeprazole and ethidium bromide (EtBr). Because the sequence of *AcrAB-TolC* and mutation of *AcrR* in the *E. coli* isolates may differ due to the reasons aforementioned and contribute to AMR,<sup>2,6-11</sup> the *AcrA*, *AcrB*, *TolC*, and *AcrR* genes have been isolated and sequenced. The present report shows that the omeprazole increases the susceptibility of MDR *E. coli* isolates against almost all the antibiotics being investigated, *AcrAB-TolC* are constitutive genes, and there is a correlation between

antibiotic susceptibility and the mutation in *AcrR* in MDR *E. coli* isolates causing UTI in the North-East region of Bangladesh.

## Materials and Methods

### *E. coli* isolates and their maintenance

The MDR *E. coli* isolates were obtained from urine samples of UTI patients in North-East region of Bangladesh.<sup>2</sup> The pure culture of *E. coli* isolates was routinely subcultured on nutrient agar (NA; 0.5% peptone, 0.15% yeast extract, 0.15% beef extract, 0.5% NaCl, 1.5% agar; pH 7.0) and preserved at  $-20^{\circ}\text{C}$  in nutrient broth (NB; 0.5% peptone, 0.15% yeast extract, 0.15% beef extract, 0.5% NaCl; pH 7.0) with 15% glycerol.

### Antibiogram and efflux assay of antibiotics with proton pump inhibitor

The presence of efflux pumps in antibiotic-resistant *E. coli* isolates was screened by the increase in antibiotic susceptibility in the presence of omeprazole, an  $\text{H}^{+}/\text{K}^{+}$  proton pump inhibitor.<sup>14,23,25,26,31</sup> The antibiotic disks of amoxicillin (AMX), azithromycin (AZM), ciprofloxacin (CIP), cefixime (CFM), ceftriaxone (CTR), gentamicin (GEN), and sulfamethoxazole (SXT) were added to the surface of culture on Mueller Hinton Agar (MHA; beef extract 0.2%, acid hydrolysate of casein 1.75%, starch 0.15%, Agar 1.7%; pH 7.3) of each plate supplemented with or without 100  $\mu\text{g}/\text{mL}$  omeprazole. The zone of inhibition produced by the antibiotic disks was compared with the standard clear zone diameters of these disks against Enterobacteriaceae stated in the guidelines of Clinical and Laboratory Standards Institute (CLSI).<sup>32</sup>

The minimum inhibitory concentration (MIC) of each of the antibiotics aforementioned was determined in the presence or absence of 100  $\mu\text{g}/\text{mL}$  omeprazole by the broth dilution method as described previously.<sup>33</sup>

### EtBr efflux assay of *E. coli* isolates

Efflux of EtBr was tested by the EtBr cartwheel procedure.<sup>34</sup> Bacterial isolates were cultured overnight with NB (0.5% peptone, 0.15% yeast extract, 0.15% beef extract, 0.5% NaCl; pH 7.0) in a shaker and the cell concentration was adjusted to 0.5 of a McFarland standard<sup>35</sup> in the following day. The NA plates were divided by radial lines, forming a cartwheel pattern. The bacterial cultures were then swabbed (using sterilized cotton swab) on NA plates containing 1.5  $\mu\text{g}/\text{mL}$  EtBr in the presence or absence of 100  $\mu\text{g}/\text{mL}$  omeprazole, and incubated at  $37^{\circ}\text{C}$  for 16 h. The culture on the NA plates was examined under a UV transilluminator.

### Isolation and sequencing of *AcrA*, *AcrB*, and *TolC* genes

Chromosomal DNA was extracted from overnight culture of *E. coli* isolates in NB with Favorgen genomic DNA purification kit in accordance with the manufacturer's instruction (Favorgen

**Table 1.** Primers used for the detection of *AcrAB-TolC* efflux pump genes and ARMS PCR for *AcrR*.

PRIMER	SEQUENCE (5'→3')	REFERENCE
<i>AcrB</i> -FP	GAAAGGCCAACAGCTTAAC	36
<i>AcrB</i> -RP	GAGCTGGAGTCAGGATCAAC	
<i>TolC</i> -FP	TGCTCCCCATTCTTATCGGC	
<i>TolC</i> -RP	GCTCTTGCTTGGCGTTGTAC	
<i>AcrA</i> -FP	CAATTTGAAATCGGACACTCG	This study
<i>AcrA</i> -RP	GGCATGTCTTAACGGCTCCT	
<i>AcrR</i> FP	CGACCGCCAGAGGCGTAAACCC	
Inner FP	CGCTGGGCGAGATTGCAAAGCAGCTGGCGTTTCGT	
<i>AcrR</i> RP	ACGAAGCGTGGGGCACAGGAGA	

Abbreviation: ARMS PCR, amplification refractory mutation system polymerase chain reaction.

Biotech. Corp., Taiwan). *AcrA*, *AcrB*, and *TolC* genes were amplified by polymerase chain reaction (PCR) using gene-specific primers (Table 1). The PCR reaction mixture for each gene was prepared in a total volume of 25  $\mu$ L consisting of 12.5  $\mu$ L of master mixture (GoTaq<sup>®</sup> Green Master Mix; Promega, USA), 1.5  $\mu$ L of 50  $\mu$ M forward and reverse primers (IDT, Singapore), 2.5  $\mu$ L of genomic DNA ( $\leq$ 250 ng) and 7  $\mu$ L of sterile deionized water. Polymerase chain reaction conditions for amplification of *AcrA* were programmed to 1 cycle at 94°C for 1 min; 35 cycles at 94°C for 30 s, at 55°C for 45 s, and at 72°C for 2 min; and 1 cycle at 72°C for 10 min. For amplification of *AcrB* and *TolC*, the PCR cycle was programmed to 1 cycle at 94°C for 1 min; 35 cycles at 94°C for 30 s, at 65°C for 45 s, and 72°C for 2 min; and 1 cycle at 72°C for 10 min. The PCR products were purified and sequenced as described previously.<sup>37</sup> The sequence was analyzed by a free computer program BioEdit 7.2.5. The similarities of the sequences were searched by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) search program, and nucleotide sequences were submitted to DNA Data Bank of Japan (DDBJ).

#### Detection of mutation in *AcrR* by ARMS PCR

Tri primer amplification refractory mutation system polymerase chain reaction (ARMS PCR)<sup>38</sup> was done by using 1 set of gene-specific primer and 1 mutation-specific primer (Table 1) for determining the mutation in *AcrR* gene. The amplification efficiency of each pair of primers: (1) *AcrR* FP and *AcrR* RP and (2) inner FP and *AcrR* RP was tested. The PCR reaction mixtures were prepared as aforementioned concentration. The PCR condition was programmed to 1 cycle at 94°C for 1 min; 35 cycles at 94°C for 30 s, at 62°C for 45 s and at 72°C for 1 min; 1 cycle at 72°C for 10 min. For ARMS PCR, 40  $\mu$ L reaction mixture consisting of 20  $\mu$ L of master mixture (GoTaq<sup>®</sup> Green Master Mix; Promega, USA), 1  $\mu$ L of *AcrR* FP (1  $\mu$ M), 1  $\mu$ L of inner FP (1  $\mu$ M) and 2  $\mu$ L *AcrR* RP (2  $\mu$ M; IDT, Singapore), 3.5  $\mu$ L of genomic DNA ( $\leq$ 250 ng), and 12.5  $\mu$ L of sterile deionized water was prepared. The ARMS PCR was programmed to 1 cycle at

94°C for 2 min; 35 cycles at 94°C for 30 s, at 62°C for 30 s, and at 72°C for 1 min, and final extension at 72°C for 2 min.

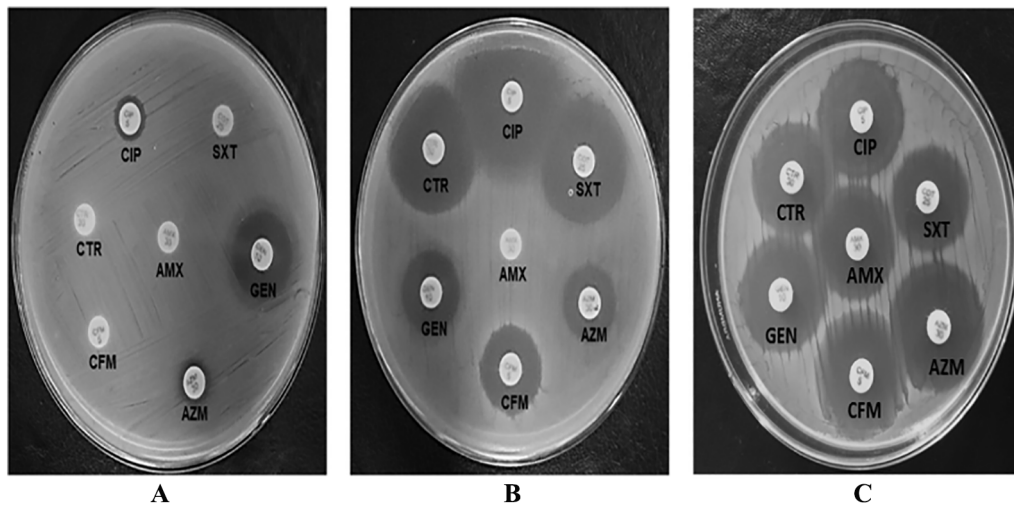
The PCR amplicons were subjected to electrophoresis and visualized in 1% agarose. Polymerase chain reaction products were purified from agarose gel and sequenced as described previously.<sup>39</sup> The sequences were analyzed as aforementioned.

## Results and Discussion

About 90% of the *E. coli* isolates causing UTI in the North-East region of Bangladesh were reported as MDR, and the multidrug resistance in *E. coli* might be either plasmid or chromosomal DNA mediated or the both.<sup>2,13</sup> In this study, the efflux pump system has been discussed as one of the reasons for multidrug resistance in the MDR *E. coli* isolates causing UTIs and omeprazole may be an efflux pump inhibitor.

#### Antibiotic susceptibility in the presence of omeprazole

The efflux systems play important role in antibiotic resistance in MDR bacteria,<sup>40</sup> and omeprazole is reported as an efflux pump inhibitor.<sup>23,31</sup> To investigate the presence of efflux pump, we randomly selected 18 MDR *E. coli* isolates causing UTI.<sup>2</sup> Their susceptibility to AMX, AZM, CFM, CIP, CTR, GEN, and SXT was assayed in the presence and absence of omeprazole (Figure 1 and Table 2). In the presence of omeprazole, the diameter of zone of inhibition by at least 1 of the 7 antibiotics increased against all MDR *E. coli* isolates (Table 2). The result indicated that the omeprazole, an efflux pump inhibitor,<sup>23</sup> increased the antibiotic susceptibility, supporting the notion of the presence of efflux pump in the MDR *E. coli* isolates. The percentage of the MDR *E. coli* isolates that showed efflux activity to AMX, AZM, CFM, CIP, CTR, and SXT was about 22, 44, 72, 66, 38, and 66, respectively. About 77% of the MDR *E. coli* isolates showed efflux activity against 3 or more antibiotics. This result further indicated that efflux activity might be one of the mechanisms of multidrug resistance.



**Figure 1.** Representative antibiogram of *Escherichia coli* isolate 15 in the absence (A) and presence (B) of omeprazole, and *E. coli* ATCC 25922 in the absence of omeprazole (C).

**Table 2.** Increase in zone of inhibition of *Escherichia coli* isolates to different antibiotic disks in the presence of omeprazole.<sup>a</sup>

ISOLATES	AMX		AZM		CFM		CIP		CTR		GEN		SXT	
	C	O	C	O	C	O	C	O	C	O	C	O	C	O
02	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	14	14	13	13
06	NZ	NZ	16	22	16	20	22	26	20	27	18	25	16	21
08	NZ	NZ	22	24	16	17	23	27	19	21	20	24	18	24
11	NZ	NZ	14	20	NZ	22	NZ	22	NZ	22	14	20	16	19
14	NZ	15	14	20	NZ	22	NZ	27	NZ	17	14	16	18	27
15	NZ	NZ	9	15	NZ	19	10	31	NZ	29	18	18	NZ	25
16	NZ	NZ	26	30	14	19	25	27	25	28	20	26	23	25
17	NZ	NZ	22	25	NZ	NZ	NZ	25	NZ	NZ	14	16	22	26
18	NZ	18	NZ	19	NZ	NZ	NZ	NZ	NZ	NZ	NZ	16	18	25
19	NZ	NZ	18	22	NZ	NZ	30	32	24	26	14	17	21	27
20	NZ	NZ	15	18	22	26	18	22	22	29	16	22	16	22
22	NZ	NZ	22	26	18	22	NZ	19	25	27	34	41	12	15
26	NZ	NZ	23	25	18	27	25	26	26	27	18	25	19	24
27	NZ	NZ	20	22	NZ	NZ	NZ	16	NZ	NZ	18	27	NZ	NZ
29	NZ	NZ	18	20	NZ	15	NZ	18	NZ	NZ	15	17	NZ	14
30	NZ	22	17	19	NZ	18	NZ	17	24	25	14	16	17	25
31	NZ	NZ	14	17	18	20	22	24	19	24	17	20	23	27
32	NZ	16	19	21	NZ	19	NZ	NZ	NZ	22	15	18	11	15

Abbreviations: AMX, amoxicillin; AZM, azithromycin; C, absence of omeprazole; CFM, cefixime; CIP, ciprofloxacin; CTR, ceftriaxone; GEN, gentamicin; NZ, no zone of inhibition; O, presence of omeprazole; SXT, sulfamethoxazole.

<sup>a</sup>≥4 mm increase in zone of inhibition in the presence of omeprazole compared with that in the absence of omeprazole was considered as the increase in antibiotic susceptibility.

The efflux pump inhibition by omeprazole was further investigated through determining the MIC of each antibiotic against the *E. coli* isolates as well as the *E. coli* ATCC 25922. Omeprazole treatment reduced the MIC of almost all antibiotics being investigated for the MDR *E. coli* isolates by 2-fold

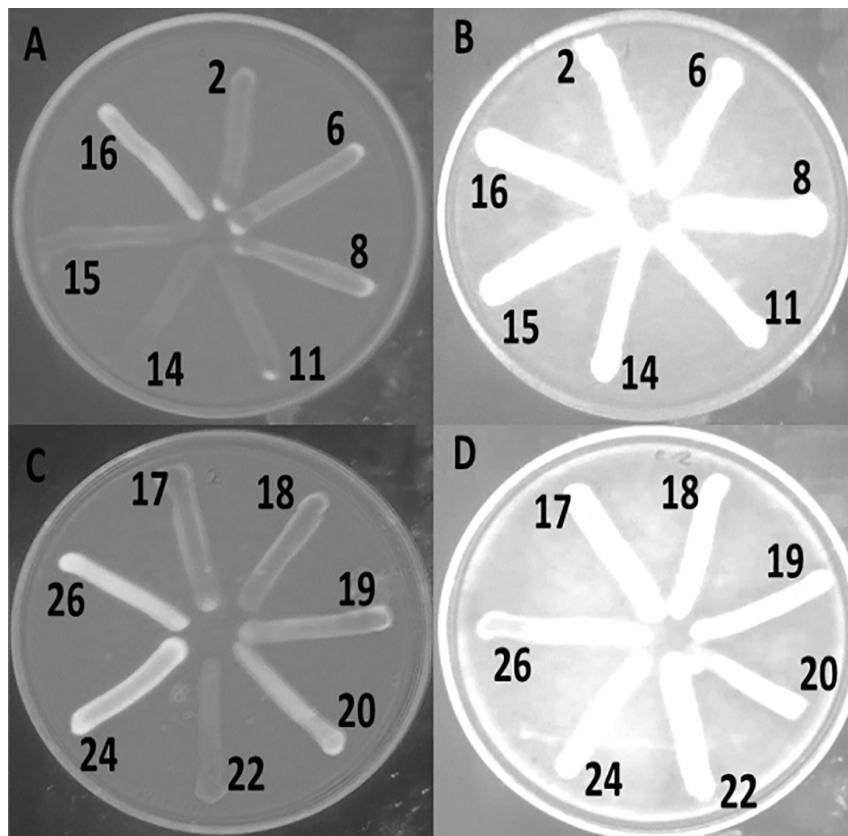
to 8-fold (Table 3). Minimum inhibitory concentration of the same antibiotic varied up to 2-fold based on different *E. coli* isolates. However, the MIC of the same antibiotic for the *E. coli* ATCC 25922 was at least 2-fold to 4-fold less than that found against the *E. coli* isolates treated with omeprazole.

**Table 3.** MIC of MDR *Escherichia coli* isolates in the absence and presence of omeprazole.<sup>a</sup>

ANTIBIOTICS	MIC OF ANTIBIOTICS AGAINST <i>E. COLI</i> ISOLATES		MIC REDUCED IN THE PRESENCE OF OMEPRAZOLE (FOLD)	MIC OF ANTIBIOTICS AGAINST <i>E. COLI</i> ATCC 25922 ( $\mu\text{G}/\mu\text{L}$ )
	IN THE ABSENCE OF OMEPRAZOLE ( $\mu\text{G}/\mu\text{L}$ )	IN THE PRESENCE OF OMEPRAZOLE ( $\mu\text{G}/\mu\text{L}$ )		
AMX	4-8	2-4	2	0.5
AZM	4-8	1-2	4	0.5
CFM	2-4	0.25-0.5	8	0.125
CIP	4-8	0.5-1	8	0.5
CTR	2-4	0.5-1	4	0.5
GEN	1-2	0.125-0.25	8	0.0625
SXT	4-8	2-4	2	2

Abbreviations: AMX, amoxicillin; AZM, azithromycin; CFM, cefixime; CIP, ciprofloxacin; CTR, ceftriaxone; GEN, gentamicin; MIC, minimum inhibitory concentration; MDR, multidrug resistant; SXT, sulfamethoxazole.

<sup>a</sup>Every experiment was repeated at least 3 times.



**Figure 2.** Efflux of EtBr from some *Escherichia coli* isolates in the absence (A and C) and presence (B and D) of omeprazole. EtBr indicates ethidium bromide.

Nevertheless, the MIC of the *E. coli* ATCC did not further change in the presence of omeprazole. This result indicated the presence of active efflux pumps in the *E. coli* isolates but not in the *E. coli* ATCC 25922, which was susceptible to all the antibiotics being investigated (Figure 1C). In addition, this result suggests that omeprazole can act as an efflux pump inhibitor in agreement with other studies.<sup>23,25,26</sup> Molecular docking shows that omeprazole has significant interaction to NorA, an efflux pump in *Staphylococcus aureus* with low binding energy.<sup>24</sup>

However, further study is necessary to clarify which efflux pump(s) in *E. coli* isolates is inhibited by omeprazole.

The presence of efflux pump was further tested by using EtBr. In the absence of omeprazole, many of the MDR *E. coli* isolates grown in the media containing EtBr failed to produce the characteristic intense orange fluorescence, indicating the possible efflux of EtBr from the cell (Figure 2A and C). However, when these strains were grown in the presence of omeprazole, strong fluorescence was observed indicating possible inhibition

**Table 4.** Omeprazole-mediated efflux inhibition of EtBr and increase in antibiotic susceptibility.

ESCHERICHIA COLI ISOLATES	NAME OF RESISTANT ANTIBIOTICS	INCREASE IN ANTIBIOTIC SUSCEPTIBILITY IN THE PRESENCE OF OMEPRAZOLE	ETBR FLUORESCENCE IN THE PRESENCE OF OMEPRAZOLE
02	CTR, CIP, AMX, CFM, AZM, NA, F	No	Moderate
06	AMX, NA, F	Yes	Strong
08	AMX, NA, F	Yes	Strong
11	CTR, CIP, NA, AMX, CFM, F	Yes	Strong
14	CTR, CIP, AMX, CFM, NA, F	Yes	Strong
15	CTR, CIP, AMX, CFM, AZM, SXT, NA	Yes	Strong
16	AMX, CFM, NA	Moderate	Strong
17	CTR, CIP, CFM, NA, AMX	Yes	Strong
18	CTR, CIP, AMX, NA, CFM, GEN, AZM	Moderate	Strong
19	AMX, NA, CFM	Yes	Strong
20	AMX, NA	Yes	Strong
22	CIP, AMX, NA	Moderate	Strong
26	AMX, NA, F	Yes	Strong
27	CTR, CIP, NA, AMX, CFM, SXT	Moderate	Strong
29	CTR, CIP, AMX, NA, CFM, SXT, F	Moderate	Strong
30	CIP, AMX, CFM, NA, F	Yes	Strong
31	AMX, NA, F	Yes	Strong
32	CTR, CIP, AMX, CFM, NA	Moderate	Moderate

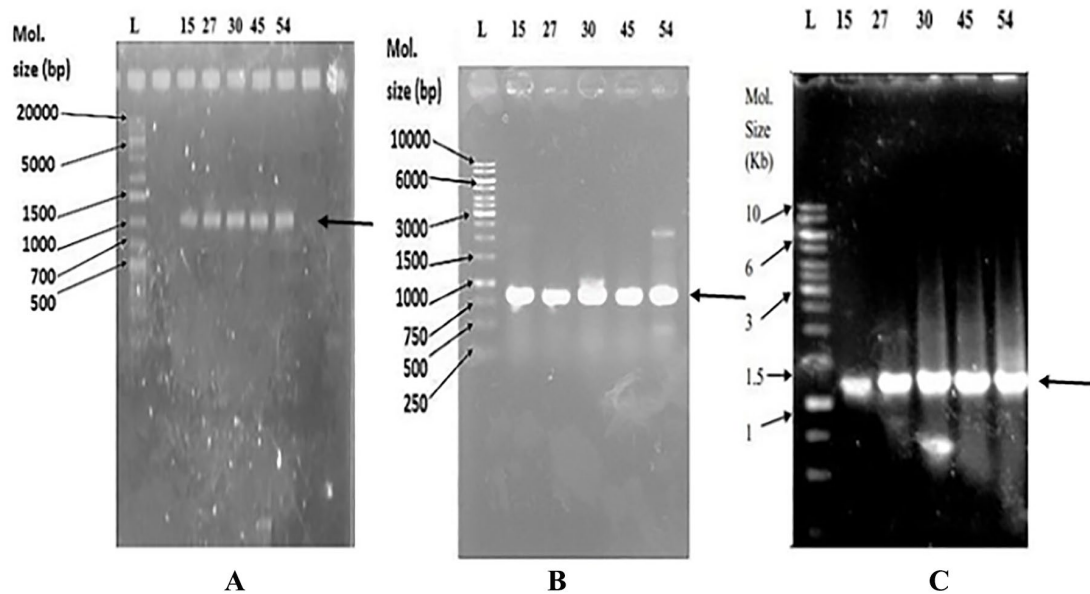
Abbreviations: AMX, amoxicillin; AZM, azithromycin; CFM, cefixime; CIP, ciprofloxacin; CTR, ceftriaxone; EtBr, ethidium bromide; GEN, gentamicin; SXT, sulfamethoxazole.

of the efflux process by omeprazole (Figure 2B and D). In the presence of omeprazole, EtBr could not come out from more than 70% of the bacterial isolates, and therefore, retained in the cell. Consequently, strong fluorescence was observed under a UV transilluminator and the antibiotic susceptibility increased (Table 4). This result supports the correlation between the efflux pump and the AMR of the *E. coli* isolates. The efflux system might be largely responsible for intrinsic resistance of some *E. coli* isolates to antibiotics.<sup>23</sup> Therefore, antibiotics combined with efflux system blockers may increase the susceptibility of bacteria to a given antibiotic to a clinical relevant level.<sup>41,42</sup> However, although a few bacterial isolates showed strong fluorescence under a UV transilluminator, they moderately increase susceptibility to antibiotics in the presence of omeprazole.

#### *Molecular detection and in silico characterization of AcrA, AcrB, and TolC genes in E. coli isolates*

The association of the AcrAB-TolC efflux pump with antibiotic resistance is reported in bacteria.<sup>43</sup> As more than 70% of the *E. coli* isolates have been assayed to have the efflux pump, an attempt was made to isolate and identify *AcrA*, *AcrB*, and *TolC*

genes in their chromosomal DNA. In the presence of omeprazole, the MDR *E. coli* isolates that showed increased susceptibility to antibiotics and those that showed no increased susceptibility were selected for molecular detection of *AcrA*, *AcrB*, and *TolC* through PCR and DNA sequencing. The presence of *AcrA*, *AcrB*, and *TolC* genes with the size of 1194, 761, and 1170 bp, respectively, were found in both groups of *E. coli* isolates (Figure 3). The similarity search with the BLAST revealed that *AcrA*, *AcrB*, and *TolC* of *E. coli* isolates were 99%, 99%, and 95% identical, respectively, to those of *E. coli*. The nucleotide sequences of *AcrA*, *AcrB*, and *TolC* had been deposited to DDBJ under the accession number LC385717, LC385718, and LC271678, respectively. The amino acid sequences of *AcrA*, *AcrB*, and *TolC* were deduced by GENETYX-SV/RC version 7.0 program (Genetyx Corp., Japan) for further structural proteomics analysis. The physicochemical properties of these 3 deduced proteins investigated by ProtParam tools (<https://web.expasy.org/protparam/>) of the ExpASY server revealed that the theoretical pI of *TolC* is basic in nature and that of *AcrA* and *AcrB* is slightly acidic (Table 5). The extinction coefficient that is necessary during protein purification is different for *AcrA*, *AcrB*, and *TolC*. *AcrAB* proteins



**Figure 3.** PCR products of *AcrA* (A), *AcrB* (B), and *TolC* (C) genes in 1% agarose gel.

L indicates the DNA ladder; PCR, polymerase chain reaction. *Escherichia coli* isolate numbers are indicated above the image of the gel with PCR products shown by an arrow.

**Table 5.** Physicochemical properties of *AcrA*, *AcrB*, and *TolC* proteins of the *Escherichia coli* isolates.

PARAMETERS	VALUES		
	ACRA (397 AA)	ACRB (253 AA)	TOLC (389 AA)
Molecular weight (Da)	42,283.78	27,093.00	42,602.59
Theoretical pI	6.06	5.54	10.05
Extinction coefficients ( $M^{-1} cm^{-1}$ )	0.447	0.275	1.205
The estimated half-life ( <i>E. coli</i> , in vivo)	>10h	3min	>10h
Instability index	29.46	24.35	43.51
Aliphatic index	91.44	119.49	69.07
Grand average of hydropathicity (GRAVY)	-0.266	0.690	-0.587

Abbreviation: aa, amino acids.

are much stable than *TolC*, and their aliphatic index indicated them as thermostable proteins (Table 5). The value of GRAVY (grand average of hydropathicity) calculated using the hydropathy value from Kyte and Doolittle<sup>44</sup> is low for *AcrA* and *TolC*, indicating the possibility of their better interaction with water than that of *AcrB*. The pI and the hydropathy values are important for subcellular localization of channel forming membrane proteins. Recently, the in situ structure and assembly of the *AcrAB-TolC* efflux pump have been reported.<sup>17</sup> Therefore, it will be interesting if molecular docking of omeprazole and other efflux pump inhibitors<sup>14,24</sup> is done to predict the inhibitor profiles of the *AcrAB-TolC* efflux pump.

The *AcrB* and *TolC* are localized on inner and outer parts of the membrane, respectively, and *AcrA* protein performs as an adapter of *AcrB-TolC* on the periplasmic region.<sup>45-47</sup> Both *AcrB* and *TolC* comprise membrane domain as well as transmembrane region.<sup>48,49</sup> The similar result was found in this

study by computational analysis of *AcrA*, *AcrB*, and *TolC* with Pfam (<https://pfam.xfam.org/>) and TMpred ([https://embnet.vital-it.ch/software/TMPRED\\_form.html](https://embnet.vital-it.ch/software/TMPRED_form.html)). Three transmembrane helices were predicted in *AcrB*, 1 in *TolC* but not in *AcrA* (Table 6). Domain and transmembrane analyses indicate that *AcrB* is a member of integral membrane protein and cooperates with a fusion membrane protein *AcrA* and an outer membrane channel *TolC*. Two domains found by Pfam analysis may make the *TolC* unique as the outer membrane efflux protein through which antibiotic may be expelled.

#### Detection of mutation in *AcrR*

The ARMS-PCR assay used 3 primers in a single PCR reaction tube. Two outside primers designed to amplify a common fragment of 805 bp flanking the mutation site served as an internal amplification control. The inner primer was specific

for the mutation at position 133 of the gene and the amplicon was 530-bp long (Figure 4A). The both amplicons were subjected to DNA sequencing and the result of sequencing confirmed the substitution of cytosine by thymine at position 133, which resulted a cysteine instead of an arginine at amino acid position 45 of AcrR (Figure 4B and C). The substitution of arginine to cysteine at position 45 of AcrR was observed only

**Table 6.** Transmembrane regions of AcrA, AcrB, and TolC of the *Escherichia coli* isolates.

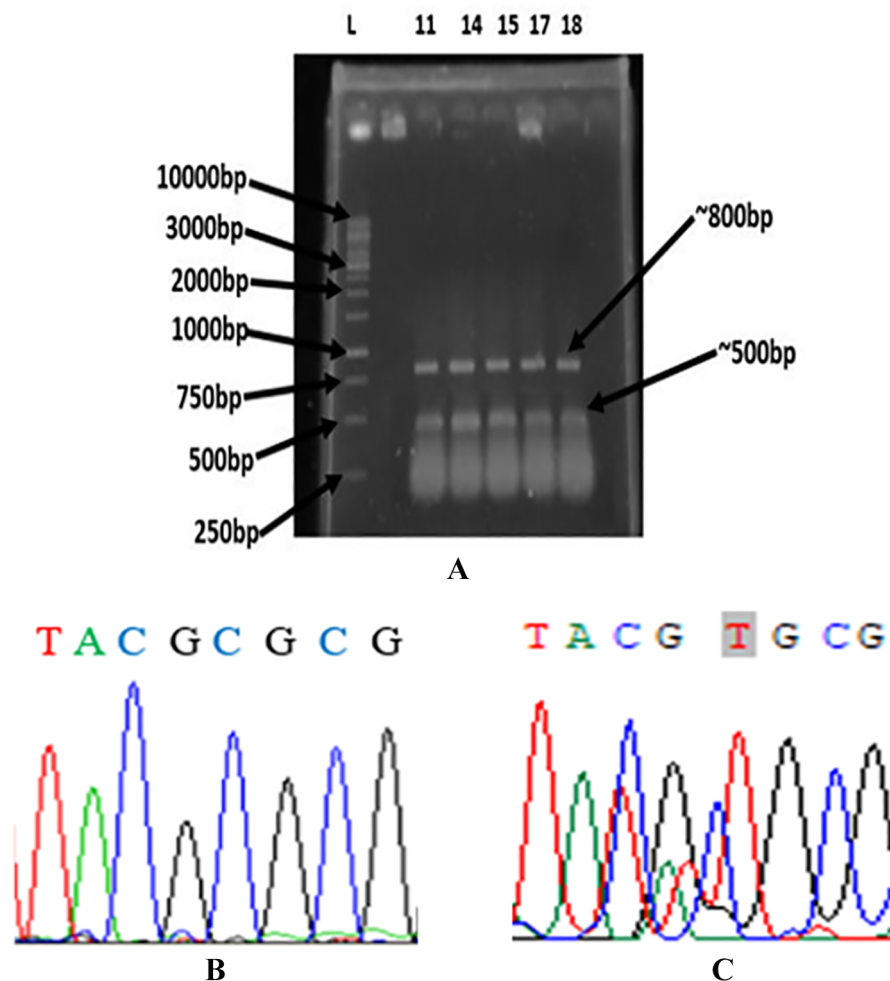
PROTEINS	FROM	TO	LENGTH	ORIENTATION
AcrA	–	–	–	–
AcrB	119	137	19	i-o
	145	163	19	o-i
	213	235	23	i-o
TolC	114	138	25	o-i

Abbreviations: i-o, inner to outer; o-i, outer to inner.

in the *E. coli* isolates that were assayed to have efflux pump (Figure 2, Tables 2 and 4). However, no substitution of arginine to cysteine at position 45 of AcrR was observed in the *E. coli* isolates showing no EtBr fluorescence and no increased susceptibility to antibiotics in the presence of omeprazole. Similarly, this mutation was not observed in *AcrR* of *E. coli* ATCC 25922 that was susceptible to all antibiotics investigated.<sup>2</sup> These results suggested that the substitution of arginine to cysteine at position 45 of AcrR might be responsible for activation of the AcrAB-TolC efflux system.

## Conclusions

This study revealed that omeprazole increased the antibiotic susceptibility of the MDR *E. coli* isolates causing UTI in the North-East region of Bangladesh. AcrAB-TolC, one of the efflux pumps, has been partially characterized in the MDR *E. coli* isolates, which might be important in future molecular docking study to predict and evaluate the inhibitor profiles of the AcrAB-TolC efflux pump. The efflux pump might be active only when substitution of arginine to cysteine occurs at position 45 of AcrR.



**Figure 4.** Agarose gel electrophoretogram and sequence output of ARMS PCR product for *AcrR*. All representative 5 isolates showed positive result for the presence of mutation (A). Normal *AcrR* has cytosine (B)<sup>19</sup> and mutant *AcrR* sequence has thymine (C) at position 133. L indicates the DNA ladder; ARMS PCR, amplification refractory mutation system polymerase chain reaction. *Escherichia coli* isolate numbers are indicated above the image of the gel with PCR products ~800 and 500bp.



## Author contributions

NC and SS equally contributed to experiments, data analysis, and preparing the draft manuscript. AP performed experiments for TolC. MKB and TR performed experiments for screening efflux pump and antibiotic susceptibility. MJA and KI participated in manuscript revision. AKA conceived and designed the experiments, analyzed and interpreted the data, and wrote the paper. All the authors have approved the final article.

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