

Plasmid-Borne Drug Resistance Elimination Potential of Ethidium Bromide and Acridine Orange in Multidrug Resistant and Extensive Drug Resistant *Escherichia Coli*

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ABSTRACT: Multidrug resistant (MDR) and extensively drug resistant (XDR) *E. coli* strains generate diverse and severe infections like bacteremia and urinary tract infections (UTIs) worldwide. They mostly carry antibiotic resistance markers or genes on mobile plasmids making the treatment and eradication of such infections more problematic. Plasmid eliminating agents (Ethidium bromide and Acridine orange) could be advantageous in the expulsion of resistance bearing plasmids and eventually helpful in extermination of MDR and XDR-*E. Coli*.

Objective: This study was designed to determine the antibiotic resistance patterns of *Escherichia coli* isolates from patients with urinary tract infections and bacteremia. We also detected the antibiotics resistance profiles and compared the potential of curing agents in eliminating plasmid mediated antibiotic resistance.

Methods: Three hundred and fifty (350) *E. coli* isolates from patients diagnosed with urinary tract infections and bacteremia were taken. Antibiotic sensitivity testing was accomplished by following CLSI (2015) protocol. Sub-minimum inhibitory concentrations (SICs) of Ethidium bromide and Acridine orange were determined by broth dilution method in Luria Britani (LB) broth to find curing concentrations for resistance plasmids.

Results: *E. coli* which were (100%) resistant to Amoxicillin-clavulanate, Cefaclor, Cefuroxime, Cefixime, Ceftazidime, Cefepime, Cefotaxime, Ceftriaxone, Ciprofloxacin and Pipidemic acid (urine only) and were selected for curing analysis. About 46%, 16%, 14% and 14% of *E. coli* (blood isolates) were resistant to Cefoperazone-sulbactam, Imipenem, Meropenem and Amikacin, respectively. About 34%, 13%, 12%, 10%, 9% and 6% of *E. coli* from urine were resistant to Cefoperazone-sulbactam, Amikacin, Nitrofurantoin, Meropenem, Imipenem and Fosfomycin, respectively. Sub-minimum inhibitory concentrations (SICs) of Ethidium bromide and Acridine orange were effective between 125µg/ml to 1000µg/ml for both curing agent but the most impressive resistance plasmid curing concentrations were 500-1000µg/ml and 500µg/ml of EthBr and AO, respectively. These both curing agents were able to displace Imipenem, Meropenem, Cefoperazone-sulbactam, Nitrofurantoin, Fosfomycin, Amikacin, Cefotaxime, Ceftazidime, Ceftriaxone, Ciprofloxacin and Cefepime resistance in *E. coli*. The most prevailing eliminated resistance was of Imipenem and Meropenem. The study proposes that Ethidium bromide and Acridine orange are pivotal in eradication of plasmid mediated antibiotic resistance in MDR and XDR-*E. coli*.

Key words: MDR-*E. coli*, XDR-*E. coli*, Plasmid Curing, Ethidium bromide, Acridine orange

Introduction

Escherichia coli can produce varied and severe infections like bacteremia and urinary tract infections (UTIs).^{1,2,3}

Treatment of such ailments by antimicrobial drugs has become a comprehensive matter globally due to the expansion and evolution of drug-resistant *E. coli* strains.^{4,5,6} The expanding antimicrobial-resistance (AMR) in Gram negative bacteria especially *E. coli* has been a great challenge for society and human medicine. The important means which are responsible for boosting overall propagation of multidrug-resistant (MDR) and all antibiotic resistant i.e. extensive drug resistant (XDR) *E. coli* are chromosomal

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mutations and portable antimicrobial resistance genes (ARGs). Such genes are usually placed on plasmids.⁷ Furthermore, resistance genes on plasmids (R-plasmids) are found in association with virulence genes and are also conjugative.^{8,9,10} These resistance plasmids (R-plasmids) accommodate extended spectrum beta-lactamase ESBLs genes (CTX-M, SHV and TEM), Colistin and Polymyxin resistance gene (MCR-1) and Carbapenemases genes (OXA, KPC and NDM).¹¹ Moreover, plasmids can also bear *qnr* genes (quinolone resistance genes) and efflux pump genes.¹² Plasmid curing is the plasmid deportation mechanism from bacterial culture. This is an alluring approach to encounter AMR because it has the capacity to abolish ARGs from the population of bacteria instead of killing bacteria.¹³ A plasmid curing agent could be administered to a patient before surgery, to diminish resistant nosocomial infection and to international tourist to lessen the worldwide escalation of AMR. Regrettably, no such regimens are in practice.¹⁴ Anti-R-plasmid approach will never fix AMR problem in solo, but it could be crucial act to minimize international resistance emergence and long-term load of AMR. For instance, plasmid curing can be adopted to eliminate resistance from *E. coli* in wastewater prior to discharge in the environment.¹⁵ Ethidium bromide (EthBr) and Acridine orange (AO) being DNA intercalating agent are of plasmid curing worth. Anti-plasmid activity of both compounds has been found imperative in many bacteria including *E. coli*.¹⁶ EthBr has been benefited to cure plasmids having blaTEM-1, blaKPC-3 and pKpQIL-like plasmid.¹⁷ The above-mentioned curing agents have also been found equally competent in excluding Gram-positive bacterial plasmids e.g. plasmid hosting Staphylococci.¹⁸ This study has been modulated considering the location of antibiotic resistance whether plasmid borne or chromosomally mediated and to evaluate the potential of EthBr and AO to eliminate plasmid born multidrug resistance (MDR) in *E. coli* strains of our region.

Methods

Collection of *E. coli* isolates and identification: Three hundred and fifty (n=350) clinical isolates of MDR and XDR-*E. coli* from blood and urine were gathered from different diagnostic laboratories of Karachi. The isolates were reconfirmed by Gram staining and inoculating on various microbiological media including MacConkey's agar, Triple sugar iron agar

(TSI), Urea agar, Simmon's Citrate agar and Sulfide indole motility (SIM) agar. All the media were incubated at 37°C for 24 hours.¹⁹

Antibiotic sensitivity assay: Antibiotic screening of all *E. coli* isolates (n=350) was conducted by disk diffusion method by following the instructions of Clinical and Laboratory Standards Institute.²⁰ Bacterial suspensions were adjusted to 0.5 McFarland by diluting the cultures in sterilized normal saline. The bacterial suspension was layered over Mueller Hinton Agar (MHA) plate by sterile swab and antibiotic disks were deposited. Then, the plates were placed for overnight at 37°C. Throughout, sixteen antibiotics disc; Amikacin (AK), Amoxicillin-clavulanate (AMC), Cefaclor (CEC), Cefuroxime (CXM), Cefixime (CFM), Ceftazidime (CAZ), Cefepime (FEP), Cefotaxime (CTX), Ceftriaxone (CRO), Ciprofloxacin (CIP), Cefoperazone-sulbactam (SCF), Fosfomycin (F), Imipenem (IPM), Meropenem (MEM), Nitrofurantoin (F) and Pipidemic acid (PIP) were utilized. By using the reference values of CLSI (2015), the resistance (R) and sensitive (S) were ruled out by measuring the zone of inhibition dimension around every disc.²⁰ Plasmid curing test: Out of 350 *E. coli* isolates, (n=25) that were 100% resistant to AMC, CEC, CXM, CFM, CAZ, CTX, CRO, FEP and CIP were selected for plasmid curing trial. These MDR *E. coli* were treated with various concentrations (62.5µg/ml, 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml) of EthBr and AO in Luria Bertani (LB) broth and were incubated at 37°C for 24 hours. After incubation, sub-inhibitory concentration (SIC) of EthBr and AO were determined by plating a loopful culture on MHA. The LB broth showing sub-inhibitory concentration (of EthBr and AO) was blended to mix the content and antibiotic sensitivity was performed directly.^{21,22}

Statistical analysis: The resistance patterns were calculated in percentage and bar diagrams, line diagrams and pie chart were designed by using Excel MS office.

Results

E. coli (n=350) isolates of urine and blood samples were collected from various hospitals and diagnostic laboratories of Karachi. The 84% (n=293) of *E. coli* isolates belong to urine specimens whereas 16% (n=57) are from blood (Fig. 1). In this study, all *E. coli* isolates resistant to Cephalosporins and Ciprofloxacin were selected.

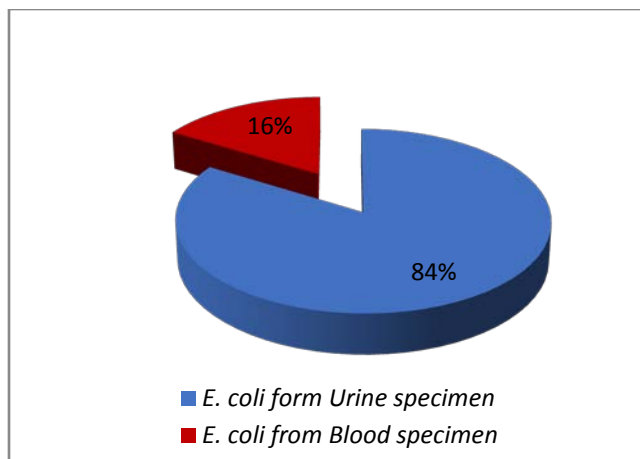


Fig. 1 Frequency of *E. coli* in urine and blood specimens

Blood Isolates:

All *E. coli* (100%, n=57) were resistant to AMC, CEC, CXM, CFM, CAZ, FEP, CTX, CRO and CIP. However, less than 50% of *E. coli* were found resistant against some antibiotics such as SCF (46%, n=26), IPM (16%, n=9), MEM (14%, n=8) and AK (14%, n=8) (Fig. 2).

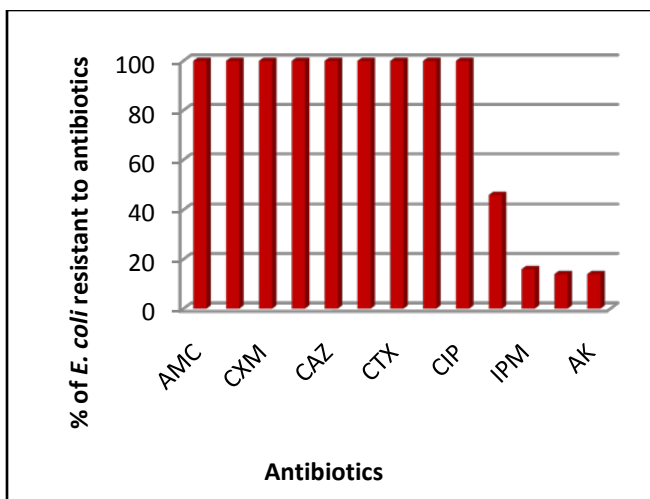


Fig. 2 Antibiotic resistance in *E. coli* (n=57) isolated from blood

Urinary *E. coli* isolates:

All *E. coli* (100%, n=293) were found resistant to AMC, CEC, CXM, CFM, CAZ, FEP, CTX, CRO, CIP and PIP. However, less than 50% of *E. coli* were found resistant against some antibiotics such as SCF (34%, n=100), AK (13%, n=38), F (12%, n=35), MEM (10%, n=28), IPM (9%, n=27) and FOS (6%, n=16) (Fig. 3).

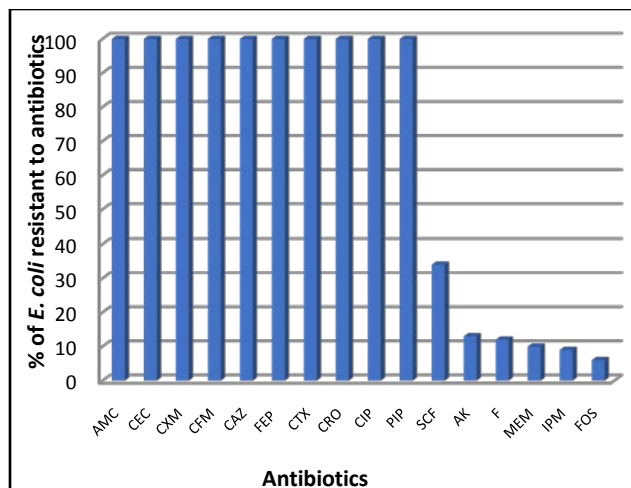


Fig. 3 Antibiotic resistance profiles in percent of *E. coli* (n=293) isolated from urine specimens

Comparison of antibiotic resistance between urinary and blood isolates:

Similar resistance ratios and patterns were recorded both in urinary and blood isolates of *E. coli*. Both isolates were 100% resistant to AMC, CEC, CFM, CXM, CAZ, FEP, CTX, CRO and CIP. However, resistance in SCF, IPM, MEM and AK was found with minor difference (Fig. 4).

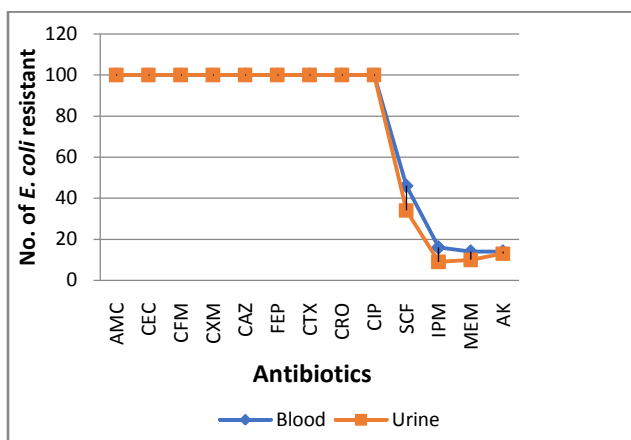


Fig. 4 Comparison of resistance patterns of Urinary and Blood *E. coli*

Resistance patterns of *E. coli* selected for curing study:

From 350 *E. coli* isolates of urine and blood, 25 isolates were selected for curing study showing various resistance patterns in Fig. 5. Accordingly, all were resistant to AMC, CEC, CXM, CFM, CAZ, FEP, CTX, CRO and CIP, while 21, 16, 16, 16, 10, 8, and 2 were resistant to SCF, IPM, MEM, PIP, AK, F and FOS respectively.

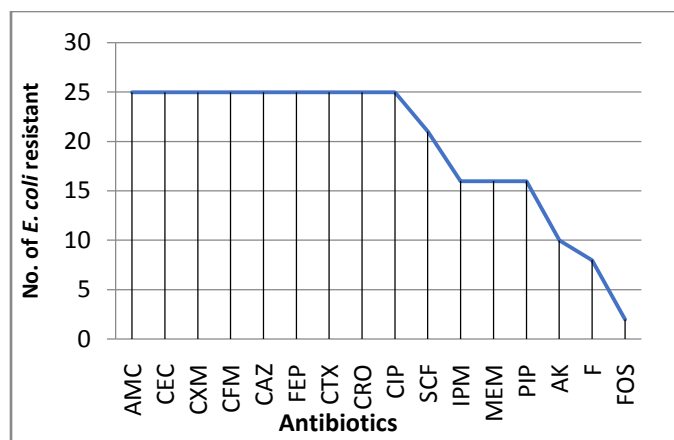


Fig. 5 Resistance patterns of *E. coli* (n=25) selected for curing study

Plasmid curing:

Ethidium bromide (Eth Br) and Acridine orange (AO) chemical agents were checked for plasmid curing efficiency. Sub-minimum inhibitory concentrations (SICs) were found from 125µg/ml to 1000µg/ml for both curing agents. Out of n=25 *E. coli*, n=3, n=4, n=9 and n=9 showed SICs at 125µg/ml, 250µg/ml, 500µg/ml and 1000µg/ml respectively, for Ethidium Bromide. For Acridine orange, n=4, n=5, n=6 and n=10 showed SICs 250µg/ml, 1000µg/ml, 125µg/ml and 500µg/ml respectively. Higher SICs (1000µg/ml) in *E. coli* n=9 were observed against EthBr as compared to AO i.e. *E. coli* n=5 showed SICs at 1000µg/ml (Fig. 6).

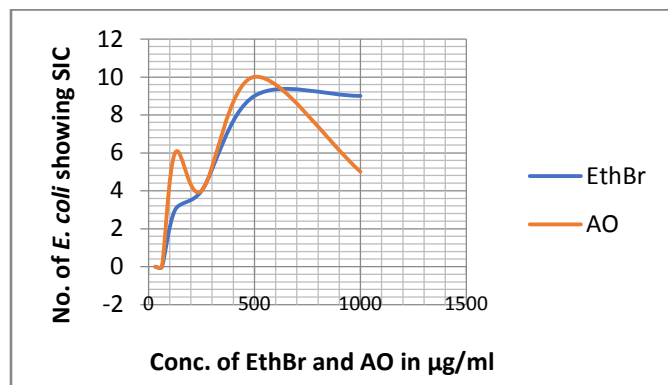


Fig. 6 Sub-minimum inhibitory concentration SICs of EthBr and AO against *E. coli*

Change in antibiotic sensitivity patterns after curing

Change in the antibiotic sensitivity (removal of plasmid mediated antibiotic resistance) was noticed after the treatment of Ethidium bromide and Acridine orange. Accordingly, *E. coli* n=13 isolates showed increase in sensitivity to various antibiotics after treating with EthBr while, n=7 isolates showed

enhanced antibiotic sensitivity after treatment with Acridine orange. Seven of the 13 *E. coli* showed change in antibiotic sensitivity in common with EthBr and AO, while 12 of *E. coli* isolates showed no change in antibiotics sensitivity (Fig. 7).

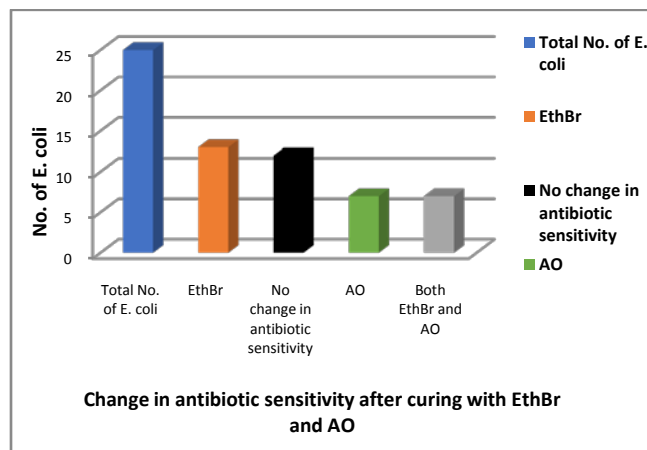


Fig. 7 Change in antibiotic sensitivity patterns after treating with EthBr and AO

Change in antibiotic sensitivity pattern after treatment with Ethidium bromide (EthBr)

E. coli (U=88) was originally sensitive to AK and FOS became sensitive additionally to SCF, IPM and MEM when treated with EthBr at 125µg/ml. *E. coli* (U=129) was initially sensitive to AK, SCF and FOS and came into sensitive to IPM and MEM as well when exposed to EthBr at 500µg/ml. *E. coli* (U=135) was originally sensitive to AK, FOS and F after treatment with EthBr at 250µg/ml turned in sensitive to SCF, IPM and MEM too. *E. coli* (B=5) was initially resistant to all antibiotics (XDR) and became sensitive to FOS, SCF, IPM and MEM after the EthBr treatment at 125µg/ml. *E. coli* (U=219) was only sensitive to FOS but at 250µg/ml showed sensitivity to CAZ, FEP, AK, SCF, IPM and MEM as well. *E. coli* (U=253) was sensitive to FOS only at 1000µg/ml of EthBr became sensitive to F too. *E. coli* (B=44) was originally sensitive to AK and MEM, at 250µg/ml of EthBr it turned sensitive to AK, IPM and SCF, additionally. *E. coli* (U=38) was initially sensitive to only FOS but at 1000µg/ml EthBr it became sensitive to AK as well. *E. coli* (U=237) was susceptible to AK, FOS and F, it showed sensitivity also to CRO, CTX, CIP, CAZ, MEM, IPM, FEP and SCF at 500µg/ml EthBr. *E. coli* (B=46) was initially sensitive to AK only but became sensitive to F, SCF, MEM and IPM as well when treated with EthBr at 1000µg/ml. *E. coli* (B=55) was XDR but at 1000µg/ml of EthBr turned in sensitive to AK, F, FOS, IPM and MEM. *E. coli* (B=30)

was originally susceptible to IPM and MEM but became sensitive to F, FOS, SCF and AK too at 1000µg/ml of EthBr. *E. coli* (U=89) was initially sensitive to AK, SCF, FOS, IPM and MEM but turned into F sensitive additionally, when treated at 1000µg/ml of EthBr (Table. 1).

Change in antibiotic after treatment with Acridine orange (AO)

E. coli (B=5) was XDR but at 125µg/ml of AO became sensitive to SCF, IMP, FOS and MEM. *E. coli* (U=253) was sensitive previously but additionally turned in sensitive to F after treatment at 500µg/ml of AO. *E. coli* (U=237) was susceptible to AK, FOS and F but

developed sensitivity to CRO, CTX, CAZ, CIP, FEP, SCF, IPM and MEM at 250µg/ml of AO. *E. coli* (B=46) was initially sensitive to AK only but at 125µg/ml of AO showed sensitivity to MEM, IPM and F as well. *E. coli* (B=55) was XDR but after treatment at 500µg/ml of AO, it became sensitive to AK, F, FOS, IPM and MEM. *E. coli* (B=30) turned in sensitive to F, FOS, SCF and AK at 500µg/ml AO. *E. coli* (U=89) was originally sensitive to AK, SCF, FOS, IPM and MEM but after treatment at 500µg/ml of AO it became sensitive to F as well (Table. 1).

Table. 1 Change in the antibiotic sensitivity patterns after curing with EthBr and AO

Sr. #	Specimen	Antibiotic sensitivity before curing	Antibiotic sensitivity after curing with EthBr	Antibiotic resistance lost after curing with EthBr at conc. (µg/ml)	Antibiotic sensitivity after curing with AO	Antibiotic resistance lost after curing with AO at conc. (µg/ml)
1	U: 88	AK, FOS	AK, FOS, SCF, IPM, MEM	SCF, IPM, MEM (125 µg/ml)	AK, FOS (no change)	No
2	U: 129	AK, SCF, FOS	AK, SCF, FOS, IPM, MEM	IPM, MEM (500 µg/ml)	AK, SCF, FOS (No change)	No
3	U: 135	AK, FOS, F	AK, FOS, F, SCF, IPM, MEM	SCF, IPM, MEM (250 µg/ml)	AK, FOS, F (No change)	No
4	B: 5	Not sensitive to any antibiotic (XDR)	SCF, IPM, MEM, FOS	SCF, IPM, MEM, FOS (125 µg/ml)	SCF, IPM, MEM, FOS	SCF, IPM, MEM, FOS (125 µg/ml)
5	U: 219	FOS	FOS, CAZ, FEP, AK, SCF, IPM, MEM	CAZ, FEP, AK, SCF, IPM, MEM (250 µg/ml)	FOS (No change)	No
6	U: 253	FOS	FOS, F	F (1000 µg/ml)	FOS, F	F (500 µg/ml)
7	B: 44	AK, MEM	AK, MEM, IPM, SCF	IPM, SCF (250 µg/ml)	AK, MEM (No change)	No
8	U: 38	FOS	FOS, AK	AK (1000 µg/ml)	FOS (No change)	No
9	U: 237	AK, FOS, F	AK, FOS, F, CRO, CTX, CIP, CAZ, MEM, IPM, FEP, SCF	CRO, CTX, CIP, CAZ, MEM, IPM, FEP, SCF (500 µg/ml)	AK, FOS, F, CRO, CTX, CIP, CAZ, MEM, IPM, FEP, SCF	CRO, CTX, CIP, CAZ, MEM, IPM, FEP, SCF (250 µg/ml)
10	B: 46	AK	AK, F, SCF, IPM, MEM	F, SCF, IPM, MEM (1000 µg/ml)	AK, MEM, IPM, F	IPM, MEM, F (125 µg/ml)
11	B: 55	Not sensitive to any antibiotic (XDR)	AK, F, FOS, IPM, MEM	AK, F, FOS, IPM, MEM (1000 µg/ml)	AK, F, FOS, IPM, MEM	AK, F, FOS, IPM, MEM (500 µg/ml)
12	B: 30	IPM, MEM	IPM, MEM, F, FOS, SCF, AK	F, FOS, SCF, AK (1000 µg/ml)	IPM, MEM, F, FOS, SCF, AK	F, FOS, SCF, AK (500 µg/ml)
13	U: 89	AK, SCF, FOS, IPM, MEM	AK, SCF, FOS, IPM, MEM, F	F (1000 µg/ml)	AK, SCF, FOS, IPM, MEM, F	F (500 µg/ml)

Keys: U= Urine specimen, B= Blood specimen, AK= Amikacin, CAZ= Ceftazidime, CRO= Ceftriaxone, CTX= Cefotaxime, CIP= Ciprofloxacin F= Nitrofurantoin, FEP= Cefepime, FOS= Fosfomycin, IPM= Imipenem, MEM= Meropenem, SCF= Cefoperazone-sulbactam

Discussions

The occurrence of *E. coli* and its antibiotic resistance is on the verge in our area due to indefinite use of antimicrobials in hospitals and community.^{23,24} A typical resistance patterns were recorded in *E. coli* isolated from blood and urine (Fig. 2 and Fig. 3). Accordingly, Augmentin (AMC), Cefaclor (CEC), Cefuroxime (CXM), Cefixime (CFM), Ceftazidime (CAZ), Cefepime (FEP), Cefotaxime (CTX), Ceftriaxone (CRO) and Ciprofloxacin (CIP) were found ineffective in these isolates. Whereas Fosfomycin (FOS), Imipenem (IPM), Meropenem (MEM), Nitrofurantoin (F) and Amikacin antibiotics were impressive against *E. coli*. The results agreed with the findings of Rasool et al. (2019).¹⁹ No important variation was observed in the resistance patterns of urine and blood isolates of *E. coli* in our study and other relevant studies (Fig. 4). This resemblance in resistance patterns of both types of isolates may be due to existence of similar type of *E. coli* strains in our region.^{25,26,27}

Dilutions at 100µg/ml and 125µg/ml of Ethidium bromide (EthBr) and 50µg/ml and 75µg/ml of Acridine orange (AO) were set to cure plasmids in *E. coli*, which show low frequency of cured *E. coli* colonies in a study conducted in Bangladesh.²⁸ Whereas, in our study, 500µg/ml and 1000µg/ml of EthBr were found effective to remove resistance plasmids in *E. coli* and 500µg/ml of (AO) was able to dismiss resistance plasmids effectively. This could be due to difference in resistance profiles, plasmids and types of strains used in this study and the other study.²⁹ An increase was occurred in antibiotic sensitivity after treatment with Ethidium bromide and Acridine orange. However, Ethidium bromide was found more efficient than Acridine orange (Fig. 7) which agrees with another study.³⁰ Both Ethidium bromide and Acridine orange treatment revealed the similar resistance pattern removal. But Ethidium bromide was noticed more competent in resistance elimination than Acridine orange as it has deported resistance in 13 strains of *E. coli*, while Acridine orange could eradicate resistance in 7 strains only. This indicates the presence of resistance marker on same plasmid. These results are in coordination with the findings of Otokunefor et al., (2019).³² Both curing agents were able to displace plasmid bearing resistance markers against IPM, MEM, SCF, F, FOS, AK, CTX, CAZ, CRO, CIP and FEP. However, most common eradicated resistance was of IPM and MEM.

Therefore, this finding could be helpful for the expulsion of such resistance and applicable to suppress the MDR threat in clinical setup.^{31,32}

Conclusions

Rising antibiotic resistance in *E. coli* was marked. Several antibiotics has been ineffective to treat MDR and XDR strains. Only Fosfomycin (FOS), Imipenem (IPM), Meropenem (MEM), Nitrofurantoin (F) and Amikacin antibiotics were potent against MDR-*E. coli*. The Ethidium bromide and Acridine orange showed capability at various concentrations to eradicate resistance against many antibiotics from *E. coli* (MDR and XDR). Ethidium bromide was the most impressive curing agent. The most common dismissed antibiotic resistance by curing was against IMP and MEM. The study suggests that Ethidium bromide and Acridine orange could be crucial in eradication of resistance in MDR and XDR-*E. coli*.

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