IRSTI 31.23.25 https://doi.org/10.26577/ijbch.2020.v13.i1.08

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Responses of some *Escherichia coli* clinical isolate strains with multiple drug resistance and overexpressed efflux pumps against efflux pump inhibitors

Abstract. Antibiotics are compounds, which inhibit the growth of or destroy microorganisms. But for some reason, microorganisms may develop resistance to antibiotics. One of the reasons for resistance to antibiotics is efflux pumps. By means of these pumps, microorganisms export antibiotics into the bacterial cell; consequently, they cannot inhibit the growth of or destroy bacteria. This situation decreases the spectrum of antibiotic activity and increases the antibiotic resistance. Thus, microorganisms could develop multiple drug resistance against not only the antibiotic used, but also for many other antibiotics and rapidly spread in the environment. Antibiotic resistance has become a major global issue, new antibiotics and efflux pump inhibitor studies are continuing rapidly. The aim of the present study is to observe the response of some *Escherichia coli* clinical isolate strains with multiple drug resistance and overexpressed efflux pumps against efflux pump inhibitors to use these strains for further studies. Ethidium bromide (EtBr)-agar cartwheel method was firstly used to identify *E. coli* strains with overexpressed efflux pumps by transferring inoculum of all strains to EtBr containing agars, and overexpressed efflux pumps were determined by exposing agar plates to UV light. Verapamil, phenyl arginine beta naphthylamide (PAβN) and thioridazine hydrochloride were used as efflux pump inhibitors (EPIs), and the lowest concentration of EPIs, which inhibit the growth of *E. coli* strains were determined by minimal inhibition concentration (MIC) test. Responses of microorganisms against efflux pump inhibitors were determined with a microplate-based assay, by using EPIs below their MIC values. According to the results of this study, effects of verapamil and thioridazine were observed to be more effective than PAβN. As a result, 98.41% of *E. coli* strains used in the study was found to be affected by Verapamil, where 96.82% by thioridazine and only 38.09% were affected by PAβN inhibitor.

Key words: multiple drug resistance, antibiotics, efflux pumps, efflux pump inhibitors, *E.coli*.

Introduction

Antibiotics are compounds, which inhibit the growth of or destroy microorganisms, thus are used to treat and prevent infections [1-3]. But for some reason, microorganisms in time develop resistance to antibiotics. One of the reasons for resistance to antibiotics is efflux pumps.

Efflux pumps were first described in 1980, as it was observed that tetracyclines were exported out of the cell [4; 5]. Efflux pumps, are known as active pumping systems, which are membrane transport proteins and remove toxic substances from inside to the outside of the cell [6-8]. These transport proteins are known to be found in all Gram-positive and Gram-negative bacteria, prokaryotic organisms and in eukaryotic cells as well [9; 10].

Efflux pump systems can be specific to a single compound or can transport a group of compounds, such as detergents, disinfectants, cancer drugs, antibiotics, antimicrobial peptides, biocides and certain dyes, and this situation causes multiple drug resistance (MDR) [11]. Efflux pumps classified as 5 superfamily; ABC (ATP Binding Cassette); MATE (Multidrug and Toxic Efflux), MF (Major Facilitator), RND (Resistance-nodulation-division) and SMR (Small Multidrug Resistance) [10; 12]. Acr AB-Tol C, AcrEF-TolC, EmrB and EmrD efflux pumps, which are examples of some of these superfamilies, are found commonly in *E.coli* [9; 10; 13-21].

Some compounds are known to inhibit the activity of efflux pumps. The efflux pump inhibitors used in Gram-negative bacteria are Phenyl Arginine Beta Naphthylamide (PAβN) (against RND pump), Carbonyl Cyanide m-Chlorophenyl Hydrazone (CCCP) (against RND, MFS and MATE pumps), 1-(1-Naphthylmethyl) Piperazine (NMP) (against RND pump), Verapamil (against MFS and ABC pumps), Paroxetine (against MFS and RND pumps), Milbemycin (against ABC pump), Biricodar (against VX-710-MFS and ABC pumps), Timkodar (against VX-853-MFS and ABC pumps) and Thioridazine (against EmrE family belonging to small multidrug resistance (SMR) pump) [22-34].

The aim of the present study is to observe the response of some *Escherichia coli* clinical isolate strains with multiple drug resistance and overexpressed efflux pumps against efflux pump inhibitors to facilitate the use of these strains for further studies.

Materials and methods

Objects of study. Eleven clinical isolate multi drug resistant *Escherichia coli* strains were chosen after screening of 100 isolates, and one standard strain (*E. coli* ATCC 25922) were used in the present study. All clinical *E. coli* isolates and the standard ATCC strain were taken from the Kastamonu University, Department of Biology, Microbiology and Biochemistry Laboratory culture collection. The clinical isolates were previously isolated from different clinical resources and identified by BD Phoenix system (Becton Dickinson, USA). All microorganisms were activated in LB Broth (Merck, Germany) and Tryptic Soy Broth (TSB) (Merck, Germany). Single pure colonies were obtained by transferring *E. coli* strains to LB Agar and TSB Agar. The bacterial identification and susceptibility testing for all clinical *E. coli* isolates were done by BD Phoenix system (Becton Dickinson, USA). Single colonies were transferred into sterile saline solution (0.9%) and adjusted to 0.5 McFarland standard to standardize the inocula used in the study to contain about 10^8 cfu/mL [35; 36].

Ethidium bromide-agar cartwheel method. TSB agar plates containing ethidium bromide (EtBr) (Merck, Germany) ranging between 0 and 2.5 mg/L (0, 0.5, 1, 1.5, 2 and 2.5 mg/L) prepared by serial dilution were used to identify *E. coli* strains with overexpressed efflux pumps as defined by Martins et al. (2010) previously [37]. Inoculum of all strains was transferred to EtBr containing agars and incubated at 37ºC for 24 h. After incubation, microorganisms presenting overexpressed efflux pumps were distinguished from others by exposing TSB agar plates to UV light. The EtBr-agar cartwheel method [37] was applied as two replicates for each *E. coli* strain (Figure 1).

Minimal inhibition concentration (MIC) test for efflux pump inhibitors. Verapamil (Sigma Aldrich, USA), phenyl arginine beta naphthylamide (PAβN) (Sigma Aldrich, USA) and thioridazine hydrochloride (Sigma Aldrich, USA) were used as efflux pump inhibitors (EPI). To identify the lowest concentration of EPIs that inhibits the growth of *E. coli* strains minimal inhibition concentration (MIC) test was used. EPI stock solutions were prepared as 512 µg/mL and a serial microdilution was applied in 96-well microplates containing Mueller Hinton Broth (Merck, Germany) to obtain a range of EPI concentrations (0.5-256 µg/mL) [37]. The MIC values were defined as the lowest concentration of EPI inhibiting visible *E. coli* growth. All tests were conducted in triplicate [38].

Efflux pump inhibitor-based microplate assay. One milliliter of Mueller Hinton Broth (MHB) was pipetted into each wells of a 24-well microplate. Antibiotic discs (Cefixime (CFM)-5µg, Ceftazidime (CAZ)-10µg, Ceftriaxone (CRO)-30µg, Cefepime (FEP)-30µg, Aztreonam (ATM)-30µg, Ampicillin (AMP)-10µg, Amoxicillin-Clavulanate (AMC)-30µg, Ciprofloxacin (CIP)-5µg, Norfloxacin (NOR)-10µg, Piperacillin-Tazobactam (TZP)-36µg, Gentamicin (CN)-10µg, Trimethoprim-Sulfamethoxazole (SXT)-25µg, Ertapenem (ETP)-10µg, Oxoid) were distributed to the wells of the plate and the plate incubated at 37°C during 1 h, to allow the diffusion of the antibiotic to the medium. After the incubation, the EPIs were also distributed to the corresponding wells of the microplate at half of their MIC values (MIC/2). The wells were inoculated with 0.1 mL of the *E. coli* suspensions. The microplate was incubated for 24 h at 37ºC. All tests were conducted in triplicate [37].

Statistical analysis. One-way analysis of variance (ANOVA), which is a parametric method was performed (P = 0.05) to determine whether difference between replicates is significant or not. R Studio, version 3.3.2 was used for statistical analysis [39].

Results and discussion

In the first part of the study, according to the BD Phoenix data the clinical *E.coli* isolates presenting multidrug resistance were selected and transferred to TSB agar plates containing EtBr to select strains presenting overexpressed efflux pumps (Figure 1).

According to the results, eleven *E. coli* strains and one standard strain were selected for the study (Table 1).

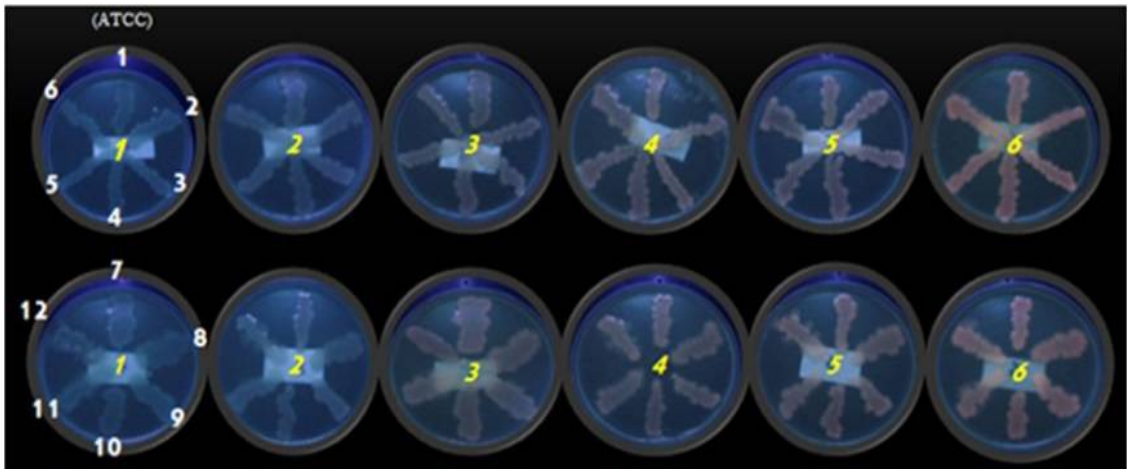

Figure 1 – The efflux capacities of twelve *E. coli* strains for different EtBr concentrations

Table 1 – Susceptibility test results (MIC values (µg/mL) for clinical *E. coli* isolates (bacteria 2 to 12)

Antibiotics	Bacte-ria 2	Bacte-ria 3	Bacte-ria 4	Bacte-ria 5	Bacte-ria 6	Bacte-ria 7	Bacte-ria 8	Bacte-ria 9	Bacteria 10	Bacteria 11	Bacteria 12
Gentamicin	< = 1 (S)	2 (S)	2 (S)	< = 1 (S)	< = 1 (S)	2 (S)	2 (S)	> 4 (R)	2 (S)	< = 1 (S)	< = 1 (S)
Ertapenem	< = 0.25 (S)	< = 0.25 (S)	< = 0.25 (S)	< = 0.25 (S)	< = 0.25 (S)	< = 0.25 (S)	< =0.25 (S)	< = 0.25 (S)	< = 0.25 (S)	> 1 (R)	< = 0.25 (S)
Cefixime	< = 0.5 (S)	> 2 (R)	< = 0.5 (S)	< = 0.5 (S)	1 (S)	< = 0.5 (S)	> 2 (R)	> 2 (R)	> 2 (R)	< = 0.5 (S)	> 2 (R)
Ceftazidime	< = 1 (S)	4 (I)	< = 1 (S)	< = 1 (S)	< = 1 (S)	< = 1 (S)	2 (I)	> 8 (R)	> 8 (R)	< = 1 (S)	> 8 (R)
Ceftriaxone	< = 1 (S)	> 4 (I)	< = 1 (S)	< = 1 (S)	< = 1 (S)	< = 1 (S)	> 4 (R)	> 4 (R)	> 4 (R)	< = 1 (S)	> 16 (R)
Cefepime	< = 1 (S)	2 (I)	< = 1 (S)	< = 1 (S)	< = 1 (S)	< = 1 (S)	4 (I)	> 8 (R)	< = 1 (S)	2 (I)	> 8 (R)
Aztreonam	< = 1 (S)	4 (I)	< = 1 (S)	< = 1 (S)	< = 1 (S)	< = 1 (S)	2 (I)	> 16 (R)	8 (R)	< = 1 (S)	> 16 (R)
Ampicillin	> 8 (R)	> 8 (R)	> 8 (R)	< = 2 (S)	> 8 (R)	> 8 (R)	> 8 (R)	> 8 (R)	> 8 (R)	< = 2 (S)	> 8 (R)
Amoxicillin-Clavulanate	16/2 (R)	4/2 (S)	16/ 2 (R)	4/2 (S)	8/2 (R)	> 32/2 (R)	> 32/2 (R)	> 32/2 (R)	> 32/2 (R)	4/2 (S)	16/2 (R)
Piperacillin-Tazobactam	< = 4/4 (S)	< = 4/4 (S)	< = 4/4 (S)	< = 4/4 (S)	< = 4/4 (S)	> 16/4 (R)	> 16/4 (I)	8/4 (S)	< = 4/4 (S)	< = 4/4 (S)	< = 4/4 (S)
Trimethoprim-Sulfamethox-azole	< = 1/19 (S)	> 4 / 76 (R)	< = 1/ 19 (S)	> 4 / 76 (R)	> 4 / 76 (R)	> 4 / 76 (R)	> 4 / 76 (R)	< = 1/ 19 (S)	< = 1/ 19 (S)	< = 1/ 19 (S)	< = 1/ 19 (S)
Ciprofloxacin	< = 0.25 (S)	> 1 (R)	> 1 (R)	> 1 (R)	> 1 (R)	< = 0.25 (S)	> 1 (R)	> 1 (R)	0.5 (I)	< = 0.25 (S)	< = 0.25 (S)
Norfloxacin	< = 0.5 (S)	> 2 (R)	> 2 (R)	> 2 (R)	> 2 (R)	< = 0.5 (S)	> 2 (R)	> 2 (R)	1 (I)	< = 0.5 (S)	< = 0.5 (S)
Note: (S): Susceptible (R): Resistance, (I): Intermediate Resistance.											

The Petri dishes having number 1 were negative controls, which contained no EtBr. Starting from number 2 to number 6 the EtBr concentration was increasing from 0.5 mg/L to 2.5 mg/L. The plates having number 1 presented no fluorescence under UV, as expected. All *E. coli* strains, except standard strain (bacteria number 1), on plate number 2 removed 0.5 mg/L EtBr to outside of the cell by their efflux pumps. A noticeably light pinkish fluorescence appeared for the standard strain, which indicates that this strain cannot efflux EtBr, although the concentration is as low as 0.5 mg/L. All *E. coli* strains, except the 5th strain, on plate number 3, which contained 1.0 mg/L EtBr, started to display a pinkish fluorescence under UV light. In Petri dishes numbered 4 to 6 a gradually increasing fluorescence was observed.

The susceptibility test results for clinical *E. coli* isolates (bacteria 2 to 12) are given in Table 1. In addition, the responses of clinical *E. coli* isolates against EPIs in combination with antibiotics are given in Tables 2-5.

To observe the responses of clinical *E. coli* isolates against EPIs in combination with antibiotics, only the antibiotics, against which each *E. coli* strain was found to be resistant and intermediate resistant were tested. The antibiotics, against which each *E. coli* strain was observed to be susceptible were not used. According to the data given in Table 2 four different results given as follows can be observed.

– Positive growth for no EPI;
– Negative growth for no EPI;
– Positive growth for EPI and antibiotic combination and
– Negative growth for EPI and antibiotic combination.

A positive growth for no EPI is an expected result, considering the antibiotics used in EPI test are the antibiotics against which each *E. coli* strain are resistant. However, the data presented in Table 2 presents negative growth in some antibiotics with no EPI, in contrary to the expectations, since the strains were resistant to those antibiotics. The reason for this observation can be explained with the final concentration of antibiotic in each well. Since the antibiotic preloaded disks were used in the EPI test, the final concentration of antibiotic in each well was a the same with the antibiotic loaded on each disk, thus the concentration of antibiotic in each well could be higher than their MIC value.

As a result of this there may be a negative control. For these results, in a negative growth in EPI-antibiotic combination, it cannot be possible to understand, whether this negative growth was due to the activity of antibiotic or EPI. For such results, a further research should be conducted by adjusting the final antibiotic concentration to be lower than the MIC values.

In addition to that, in negative growths for some antibiotics with no EPI, the final concentration of the antibiotic used was not higher than the MIC value. For such cases, a further fractional inhibitory concentration (FIC) test should also be conducted to observe, whether an additive or a synergistic interaction is present between the EPI and antibiotic, or not.

A positive growth for EPI and antibiotic combination means that the EPI used in the EPI-antibiotic combination did not block the efflux pump, thus the bacteria could export the antibiotic out of the cell and as the bacteria is resistant to that antibiotic they could still survive. On the other hand, a negative growth for EPI and antibiotic combination can be interpreted as the EPI used in the EPI-antibiotic combination blocked the efflux pump, as a result of that bacteria couldn't export the antibiotic out of the cell and survive.

Table 2 clearly shows that bacteria 2 is resistant against AMP within the concentration tested, but using AMP in combination with verapamil and thioridizin HCl killed bacteria 2. According to Table 2, bacteria 3 was found to be resistant against NOR, CFM and ATM within the concentration tested. Using NOR and ATM in combination with verapamil and thioridizin HCl killed bacteria 3, where PAβN with CFM also killed bacteria 3.

Table 2 also presents that bacteria 4 is resistant against AMP, CIP and NOR, and verapamil and thioridizin HCl in combination with these antibiotics killed this strain, where only AMP and PAβN combination inhibited the growth of bacteria 4. Verapamil and thioridizin HCl, which were used in combination with CIP, NOR and SXT against bacteria 5, were observed to kill this strain too.

Table 3 shows the responses of clinical *E. coli* isolates against EPIs in combination with antibiotics for bacteria 6, 7 and 8. According to Table 3, bacteria 6, 7 and 8 was observed to be resistant against AMP; AMC, TZP, AMP and SXT; and CFM, CAZ, CRO, AMP, AMC, CIP and SXT respectively. But, verapamil and thioridizin HCl, which were used in combination with AMP for bacteria 6, and in combination with AMC, TZP, AMP and SXT for bacteria 7, were killed these two strains. Similar results were also observed for CFM, CAZ, CRO, AMP, AMC, CIP and SXT, and Verapamil and Thioridizin HCl combinations. In addition, the combined use of CFM, CAZ, CRO and AMC, and PaβN were also killed bacteria 8.

Table 2 – Responses of clinical *E. coli* isolates against EPIs in combination with antibiotics (bacteria 2 to 5)

Part 1

Variants	**Bacteria 2**		**Bacteria 3**								
No EPI	AMP (+)	AMC (-)	AMP (-)	NOR (+)	CIP (-)	CFM (+)	CAZ (-)	CRO (-)	FEP (-)	ATM (+)	SXT (-)
Vera-pamil	AMP (-)	AMC (-)	AMP (-)	NOR (-)	CIP (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (-)	SXT (-)
Thio-ridizin HCL	AMP (-)	AMC (-)	AMP (-)	NOR (-)	CIP (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (-)	SXT (-)
PAβN	AMP (+)	AMC (-)	AMP (-)	NOR (+)	CIP (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (+)	ATM (+)	SXT (-)
Note: (+) Bacterial growth, (-) No bacterial growth											

Part 2

Variants	**Bacteria 4**				**Bacteria 5**		
No EPI	AMP (+)	AMC (-)	CIP (+)	NOR (+)	CIP (+)	NOR (+)	SXT (+)
Verapamil	AMP (-)	AMC (-)	CIP (-)	NOR (-)	CIP (-)	NOR (-)	SXT (-)
Thioridizin HCL	AMP (-)	AMC (-)	CIP (-)	NOR (-)	CIP (-)	NOR (-)	SXT (-)
PAβN	AMP (-)	AMC (-)	CIP (+)	NOR (+)	CIP (+)	NOR (+)	SXT (+)
Note: (+) Bacterial growth, (-) No bacterial growth							

Table 4 presents the responses of clinical *E. coli* isolates against EPIs in combination with antibiotics for bacteria 9 and 10. According to the results, bacteria 9 was observed to be resistant against CN, CFM, CAZ, CRO, FEP, ATM, AMP, AMC, CIP and NOR, where bacteria 10 was resistant against CFM, CAZ, CRO, ATM, AMP and AMC.

As it was observed for previous strains, verapamil and thioridizin HCl combined with all antibiotics tested were killed bacteria 9 and 10, except for CRO and ATM against bacteria 9. Only combinations of verapamil, and CRO and ATM killed bacteria 9, but this strain was found to be resistant against the combinations of CRO and ATM, and thioridizin HCl. Also, bacteria 9 was observed to be susceptible against a combination of PaβN and AMC, where this is also true for a combination of PaβN and CAZ against bacteria 10.

Lastly, Table 5 shows the responses of clinical *E. coli* isolates against EPIs in combination with antibiotics for bacteria 11 and 12. Bacteria 11 was observed to be susceptible against all antibiotics within the concentration tested, but bacteria 12 found to be resistant against CFM, CRO, FEP, ATM, AMP and AMC. All antibiotics combined with verapamil, except CRO, were killed bacteria 12. In addition, bacteria 12 was observed to be susceptible against all antibiotics combined with thioridizin HCl, except ATM. Bacteria 12 was also found to be susceptible against the combination of CFM and PaβN.

According to the results, the combinations of verapamil and antibiotics were observed to inhibit bacterial growth with 98.41%, where this value for thioridazine HCl and antibiotic combinations was 96.82% and 38.09% for PAβN (Figure 2).

According to some previous studies [22-34] PAβN, verapamil and thioridazin HCl are active EPIs for *E. coli* strains.

Özer [40], previously conducted a research to present the inhibition potential of PAβN on *E. coli* efflux pumps. In this study, bisbenzimidine, a fluorescent stain for DNA, was used in combination with some efflux pump inhibitors including PAβN. As a result, it was proved that PAβN inhibited the efflux of bisbenzimidine, thus the florescence is higher in bisbenzimidine – PAβN combination. In addition, Çetinkaya [41], also tested the efflux pump inhibition activity of PAβN in ciprofloxacin resistant *E. coli* strains and proved that increasing concentrations of PAβN decreased the MIC values for ciprofloxacin.

Table 3 – The responses of clinical *E. coli* isolates against EPIs in combination with antibiotics (bacteria 6 to 8)

	Bacteria 6				**Bacteria 7**				**Bacteria 8**									
No EPI	AMP (+)	CIP (-)	NOR (-)	SXT (-)	AMC (+)	TZP (+)	AMP (+)	SXT (+)	CFM (+)	CAZ (+)	CRO (+)	FEP (-)	ATM (-)	AMP (+)	AMC (+)	TZP (-)	CIP (+)	SXT (+)
Vera-pamil	AMP (-)	CIP (-)	NOR (-)	SXT (-)	AMC (-)	TZP (-)	AMP (-)	SXT (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (-)	AMP (-)	AMC (-)	TZP (-)	CIP (-)	SXT (-)
Thiori-dizin HCL	AMP (-)	CIP (-)	NOR (-)	SXT (-)	AMC (-)	TZP (-)	AMP (-)	SXT (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (-)	AMP (-)	AMC (-)	TZP (-)	CIP (-)	SXT (-)
PAβN	AMP (+)	CIP (-)	NOR (-)	SXT (-)	AMC (+)	TZP (+)	AMP (+)	SXT (+)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (-)	AMP (+)	AMC (-)	TZP (-)	CIP (+)	SXT (+)
Note: (+) Bacterial growth, (-) No bacterial growth																		

Table 4 – The responses of clinical *E. coli* isolates against EPIs in combination with antibiotics (bacteria 9 and 10)

	Bacteria 9										**Bacteria 10**							
No EPI	CN (+)	CFM (+)	CAZ (+)	CRO (+)	FEP (+)	ATM (+)	AMP (+)	AMC (+)	CIP (+)	NOR (+)	CFM (+)	CAZ (+)	CRO (+)	ATM (+)	AMP (+)	AMC (+)	CIP (-)	NOR (-)
Vera-pamil	CN (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (-)	AMP (-)	AMC (-)	CIP (-)	NOR (-)	CFM (-)	CAZ (-)	CRO (-)	ATM (-)	AMP (-)	AMC (-)	CIP (-)	NOR (-)
Thiori-dizin HCL	CN (-)	CFM (-)	CAZ (-)	CRO (+)	FEP (-)	ATM (+)	AMP (-)	AMC (-)	CIP (-)	NOR (-)	CFM (-)	CAZ (-)	CRO (-)	ATM (-)	AMP (-)	AMC (-)	CIP (-)	NOR (-)
PAβN	CN (+)	CFM (+)	CAZ (+)	CRO (+)	FEP (+)	ATM (+)	AMP (+)	AMC (-)	CIP (+)	NOR (+)	CFM (+)	CAZ (-)	CRO (+)	ATM (+)	AMP (+)	AMC (+)	CIP (-)	NOR (-)
Note: (+) Bacterial growth, (-) No bacterial growth																		

Table 5 – The responses of clinical *E. coli* isolates against EPIs in combination with antibiotics (bacteria 11 and 12)

	Bacteria 11		**Bacteria 12**						
No EPI	ETP (-)	FEP (-)	CFM (+)	CAZ (-)	CRO (+)	FEP (+)	ATM (+)	AMP (+)	AMC (+)
Verapamil	ETP (-)	FEP (-)	CFM (-)	CAZ (-)	CRO (+)	FEP (-)	ATM (-)	AMP (-)	AMC (-)
Thioridizin HCL	ETP (-)	FEP (-)	CFM (-)	CAZ (-)	CRO (-)	FEP (-)	ATM (+)	AMP (-)	AMC (-)
PAβN	ETP (-)	FEP (-)	CFM (-)	CAZ (+)	CRO (+)	FEP (+)	ATM (+)	AMP (+)	AMC (+)
Note: (+) Bacterial growth, (-) No bacterial growth									

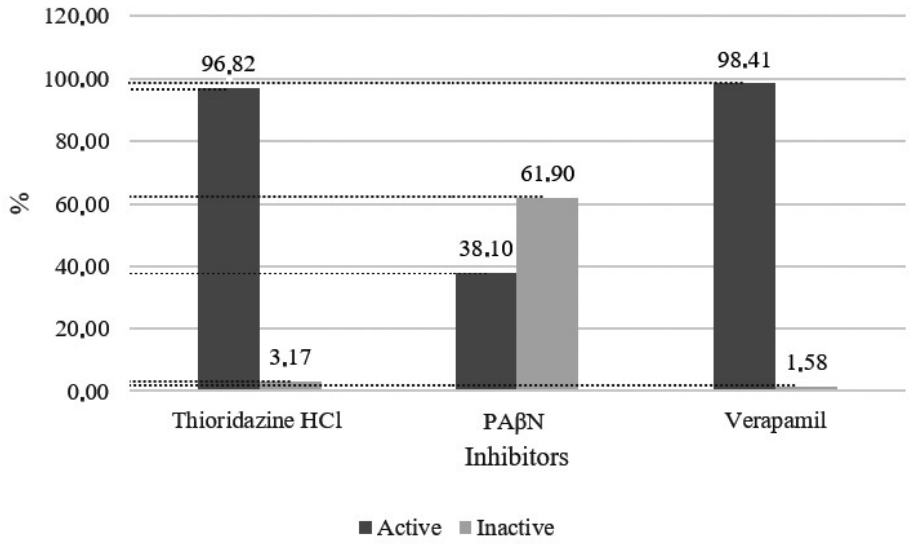

Figure 2 – The efflux capacities of twelve *E. coli* strains for different EtBr concentrations

The study conducted by Çoban et al. [42] also showed that PAβN decreased the MIC values 4 folds in *E. coli* strains presenting high fluoroquinolone resistance. Nevertheless, in the same study two-fold increase in MIC values was noted, when verapamil was used as an efflux inhibitor.

The responses of clinical *E. coli* isolates against EPIs in combination with antibiotics are given in Tables 3-5

Martins et al. [37] used two clinical *E. coli* isolates presenting multi drug resistance and as a result they proved that PAβN used in combination with tetracycline inhibit the growth of bacteria in both *E. coli* strains. Contrariwise, in only one *E. coli* strain thioridazine in combination with tetracycline inhibited the bacterial growth.

Dal [43], used *E. coli* ATCC 25922 as a control strain as we did in our study, and the result showed that the MIC values for ciprofloxacin gentamicin, erythromycin, chloramphenicol, trimethoprim and tetracycline did not affected from using in combination with efflux pump inhibitors, PAβN and 1-(1-naphthylmethyl) piperazine (NMP). According to Dal [43] any decrease was not observed in MIC values for *E. coli* ATCC 25922, because the MIC values were too low already, when compared to the other strains used. Since the *E. coli* ATCC 25922 strain was susceptible against all antibiotics used in this study with very low MIC values, there was no use to try any EPI – antibiotic combinations for *E. coli* ATCC 25922 in EPI test.

As a result, this study clearly presents that bacteria could have been developed resistance to antibiotics in time, which could cause severe problems for public health. In some of our previous studies we have used several multidrug resistant microorganisms, such as *Enterococcus faecalis*, *Enterococcus faecium* [44], *Acinetobacter baumannii*, *Proteus vulgaris* and *Streptococcus pneumoniae* [45; 46]. Time by time we observed an increase both in the number of multi drug resistant microorganisms and the resistance profiles of each strain. The results of the present study clearly indicate that one of the approaches to break their resistance against antibiotics is to use efflux pump inhibitors [47]. One of the approaches to break their resistance against antibiotics is to use efflux pump inhibitors. Studies presenting responses of multi drug resistant clinical isolated strains against EPIs could change the point of view in fight against such dangerous microorganisms [47-49]. Thus, this study extended our knowledge about the EPI use in multi drug resistant *E. coli* strains, which may be used in further researches searching for new efflux pump inhibitors to block efflux pumps present in *E. coli*. Present study also puts forward the responses of some clinical *E. coli* isolates against standard and commercially available EPIs, namely verapamil, phenyl arginine beta naphthylamide (PAβN) and thioridazine hydrochloride in combination with antibiotics, because in such researches the activities of EPI candidate molecules are needed to be compared with standard and commercially available EPIs.

Conclusion

As it was mentioned previously, antibiotics, which are used to treat and prevent infections, are compounds that inhibit the growth of or destroy microorganisms. But microorganisms may develop resistance to antibiotics. One of the reasons for resistance to antibiotics is efflux pumps. Thus, observing the responses of clinical isolated strains with multiple drug resistance and overexpressed efflux pumps against efflux pump inhibitors has great importance, in order to propose further studies about microorganisms with multiple drug resistance and overexpressed efflux pumps.

In the study, ethidium bromide-agar cartwheel method was firstly used to identify *E. coli* strains with overexpressed efflux pumps by transferring inoculum of all strains to EtBr containing agars, and overexpressed efflux pumps were determined by exposing agar plates to UV light. Verapamil, PAβN and thioridazine hydrochloride were used as efflux pump inhibitors (EPIs), and the lowest concentration of EPIs, which inhibit the growth of *E. coli* strains was determined by MIC test. Lastly, responses of microorganisms against efflux pump inhibitors were determined with a microplate-based assay, by using EPIs below their MIC values. The results of this study clearly presented that the combinations of Verapamil and antibiotics were observed to inhibit bacterial growth with 98.41%, where this value for Thioridazine HCl and antibiotic combinations was 96.82% and 38.09% for PAβN.

As a conclusion, it can be proposed that verapamil, thioridazine HCl and PAβN were observed to inhibit efflux pumps in our multi drug resistant clinical *E .coli* isolates at different levels with antibiotic combinations. In addition it can also be concluded that Verapamil and Thioridazine HCl inhibitors are more effective than PAβN inhibitors.

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