Expression of AcrAB Efflux Pump and Role of Mefloquine as Efflux Pump Inhibitor in MDR *E.coli*

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Abstract Multi-drug resistant *Escherichia coli* (MDR*E. coli*), represents a major health problem. The AcrAB pump constitutes a major drug efflux system. Inhibition of efflux pumps is an approach to combat the drug resistance problem. Mefloquine is a bacterial RND efflux-pump inhibitor (EPI). This study aimed to investigate the expression levels of multidrug efflux genes *acrAB*, as well, to study the effect of mefloquine as an efflux pump inhibitor. *acrA* and *acrB* gene expression was measured using real-time PCR. Effect of mefloquine hydrochloride on minimum inhibitory concentration (MIC) of selected antibiotics against the tested isolates was determined using the broth microdilution technique. Overexpression of both genes was detected in all isolates. The differences between expression levels of both genes in MDR strains and an ATCC reference strain were statistically significant ($p <$ 0.001). There was a moderate correlation between *acrA* and *acrB* genes expression levels. (r = 0.593, $p <$ 0.001). The isolates showed a decrease in MIC in presence of EPI. Susceptibility to levofloxacin was recovered in 95% of the tested isolates, 62.5% to ceftriaxone and in 5% each to ciprofloxacin and Gentamicin. In conclusion, Inhibition of *acrAB* efflux pump in MDR *E. coli* by mefloquine could be considered in the design of future antibiotics.

Keywords: AcrAB, Mefloquine, EPI

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1. Introduction

Escherichia coli is an important member of the intestinal microbiota of humans and other mammals, it is a common pathogen linked with community-associated as well as nosocomial infections [10,21,33].

The rise of multidrug resistant (MDR) Gram-negative pathogens poses a significant clinical problem. Multi-drug resistant (MDR) clinical isolates of *E. coli* pathogenic strains are commonly seen today in clinical practice representing a major healthcare problem with increased morbidity and mortality worldwide [9,27,41,42]. Antimicrobial resistance has been found not only in pathogenic strains but also in non-pathogenic ones [18,45].

The mechanisms responsible for increased antimicrobial resistances include biofilm formation, alteration of binding sites, enzymes that can inactivate antibiotics, decreased membrane permeability and active efflux of antimicrobials [3,17].

One of the major contributors to intrinsic resistance in Gram-negative bacteria are efflux pumps. *Escherichia coli* posses different efflux pump systems such as the AcrA, AcrB, and TolC multidrug efflux pump , MdfA multidrug efflux pump , EmrE and SugE multidrug efflux pumps, MdfA multidrug efflux pump as well as MacA and MacB multidrug efflux pumps [32].

The *E. coli* AcrAB–TolC multidrug efflux pump confers resistance to a wide variety of antibiotics including macrolides, β-lactams, tetracycline, chloramphenicol, fusidic acid, and novobiocin [15,31]. The *E. coli* AcrAB–TolC complex consists of the polytopic inner membrane protein AcrB, the periplasmic adaptor protein AcrA, and outer membrane channel TolC, [6,15].

Inhibition of efflux pumps appears to be an attractive approach to overcome the problem of drug resistance. Efflux pump inhibitors can be utilized for increasing the antibiotic concentration inside a pathogenic cell making these drugs more effective [5].

Mefloquine was confirmed to be a strong bacterial RND efflux-pump inhibitor [47]. Mefloquine hydrochloride is a 4-quinolinemethanol derivative. Mefloquine is an antimalarial drug used as a prophylaxis against and treatment for malaria [23]. Mefloquine is bactericidal against Gram positive bacteria, with poor activity against Gram negative enteric bacteria and yeasts.

Therefore, the aim of our work was to screen for the efflux pump mediated resistance among *E. coli* clinical isolates by investigating the expression levels of the multidrug efflux genes *acrAB* and, as well, to study the multi-drug resistance against different families of

antibiotics and the effect of mefloquine hydrochloride as efflux pump inhibitor (EPI).

2. Materials and Methods

2.1. Clinical Isolates and Antibiotic Susceptibility

Forty *E. coli* non-replicate clinical isolates were collected from different clinical specimens (urine, wound swabs, blood) from patients admitted to medical facilities (Medical Research Institute, and Alexandria Main Hospital) over a period of three months, between November 2011 and January 2012. All Isolates were identified by conventional methods, and selected after being tested for their antibiotic susceptibility against the following antimicrobials: amikacin, tobramycin, gentamicin, ofloxacin, ciprofloxacin, norfloxacin; levofloxacin, tetracycline, ceftriaxone, ceftazidime, cefotaxime, ampicillin-sulbactam, amoxicillin-clavulinic acid and trimethoprim-sulphamethoxazol (Oxoid, England) by disc diffusion method [4] according to Clinical and Laboratory Standards Institute (CLSI) 2011 guidelines. *E. coli* ATCC 8739 was used as reference strain.

2.2. Screening for the acrA and acrB GENES in the Clinical Isolates

Screening for *acrA* and *acrB* genes was carried out by PCR amplification among the 40 *E. coli* isolates. For *acrA* gene, the following primers were used: *acrA* -F (5'-GGTCGTTCTGATGCTCTCA-3') and *acrA* -R (5'-GGCTTGCTGGTTATTATCAG-3'). For *acrB* gene, the following primers were used: *acrB*–F (5'-CGTCTAACAGTGACTCCACGG-3') and *acrB*-R (5'-TTCAATCAGACCTTTACCTTC-3') [28]. Amplification of DNA was performed using thermal cycler (Perkin Elmer GeneAmp PCR System 9600) with the following thermal profile: 95°C for 3 min followed by 40 cycles of 94°C for 1min, 52°C for 1 min and 72°C for 2 min; and a final extension at 72°C for 7 min. To amplify a large fragment of approximately 2730 bp, the extension time at

72°C was 3.5 min. [28]. Negative controls were performed in all cases, as well as a positive control with *E. coli* ATCC 8739. PCR products were electrophoresed through 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination (1078 bp for *acrA* and 2730 bp for *acrB*).

2.3. Relative Quantitative Analysis of acrA and acrB Gene Expressions by Real Time PCR

To evaluate the *AcrAB* efflux pump activity genes (*acrA* and *acrB*) relative to the housekeeping gene *rpsL*, quantitative PCR was applied to detect and quantify mRNAs. Relative Quantitation (RQ) using comparative Ct (threshold cycle) determines the change in expression of the targetsequence in a test sample relative to the same sequence in a calibrator sample [24]. For our forty MDR *E. coli* clinical isolates, RNA extracts were stabilized in RNA protect bacterial reagent and isolated using RNeasy minicolumns (Qiagen, Valencia, CA). RNase-free DNase I was incubated on-column for digestion of genomic DNA. RNA concentrations and purities were assessed by using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). cDNA synthesis and amplification were completed using iScriptTM One-Step RT-PCR Kit with SYBR® Green (BIO-RAD) according to manufacturer's instructions. Amplification was done using specific primers (Table 1). Quantitative PCRs (qPCRs) were performed in ABI 7900HT Fast Real Time PCR system (Applied Biosystem, USA) using the optimum thermal profile as follows: 10 min at 50°C (cDNA synthesis), 5 min at 95°C (iScript Reverse transcriptase inactivation), then PCR cycling and detection for 35 cycles(10 sec at 95°C, 30 sec at 56°C) followed by Melting curve analysis (1 min at 95°C, 1 min at 55°C, 10 sec at 55°C). PCR-grade water served as a negative control. RNA extracted from the *E. coli* ATCC 8739 strain was the positive control and sensitive strains were the calibrator. Melting curve analysis ensured that only a single PCR product was amplified.

Table 1. PCR primers used for relative quantitative analysis of *acrA* and *acrB* gene expressions by real time PCR

Primer sequence (5'–3')	Amplicon length (bp)	Refrence
acrA-F CTCTCAGGCAGCTTAGCCCTAA *acrA*-R TGCAGAGGTTCAGTTTTGACTGTT	107	
acrB-F GGTCGATTCCGTTCTCCGTTA *acrB*-R CTACCTGGAAGTAAACGTCATTGGT	107	[a](1)
rpsL-F GCAAAAACGTGGCGTATGTACTC *rpsL*-R TTCGAAACCGTTAGTCAGACGAA	104	

[a](1): [40].

Data were analyzed according to the RQ manager program 1.2 ABI SDS software (ABI 7900HT), data were produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence. The control sensitive samples were used as calibrators, the expression levels are set to 1. But because the gene expression levels were plotted as log10 values, the expression level of the calibrator samples appear as 0 in the graph. Because the relative quantities of the *acrA* and *acrB* genes were normalized against the relative quantities of the endogenous control (*rpsL*) gene, fold expression changes were calculated using the equation $2^{-\Delta\Delta CT}$ [24].

2.4. Evaluation of acrA and acrB Genes Expression before and after Addition of Levofloxacin (3μg/mL) as a Stress Factor

Five samples were selected; each sample represented a category of samples sharing the same MIC against levofloxacin. From each sample, 2μl were taken and added together in a separate tube forming a "Pool" of RNA. Serial dilutions of the pool took place to establish a standard calibration curve. PCR cycles are performed using Rotor-Gene Q real time PCR apparatus. A Melting

curve analysis was performed at the end of the PCR cycles confirming the specificity of the reaction and ensuring that only a single PCR product was amplified. PCR cycles performed for the tested *E. coli* samples, *E. coli* ATCC 8739 (as a positive control) and the different pool concentrations. Water blanks were included as negative controls in every analysis. Readings of the pool were used for establishing a standard calibration curve. Ct readings of both genes *acrA* and *acrB* were normalized with housekeeping gene *rpsL* Ct readings corresponding to each sample. All the genes were amplified for the samples, ATCC standard strain number 8739 and the pool took place at the same run to ensure similar conditions. ΔΔCT method for qRT PCR data analysis was used for calculating the fold difference in gene expression between the samples and the ATCC standard strain [24].

2.5. Effect Mefloquine Hydrochloride, on the MIC of Selected Antibiotics Using Broth Microdilution Technique

To evaluate the contribution of efflux pump activity, the MICs values of the following antimicrobial agents: ciprofloxacin, levofloxacin, ceftriaxone and gentamicin were measured in the presence of the efflux pump inhibitor, mefloquine hydrochloride (Sigma-Aldrich HLPC grade- Germany), final concentration: 50 µM using broth dilution technique. Reduction in MIC of the antibiotic with mefloquine hydrochloride is indicative of the presence of an efflux system mode of resistance against this antibiotic.

2.6. Statistical Analyses

The collected data were statistically analyzed using the Statistics Package for Social Sciences (SPSS) *version, 21*. The results were presented as means ± SD. One Sample t-test was performed. The Pearson's correlation coefficient was used to measure the closeness of a linear relationship between *acrA* and *acrB* genes expression values. The statistics of the evaluation of *acrA* and *acrB* gene expression before and after addition of the antibiotic were calculated using Wilcoxon signed rank test. Differences were considered as statistically significant for a p value of < 0.05.

3. Results

Out of the forty collected isolates, seven (17.5 %) were from blood, twenty one (52.5 %) from urine, four (10 %) from sputum and eight (20 %) from wound. Percentage of different antibiotic resistance among *E. coli* clinical isolates was illustrated in Figure 1.
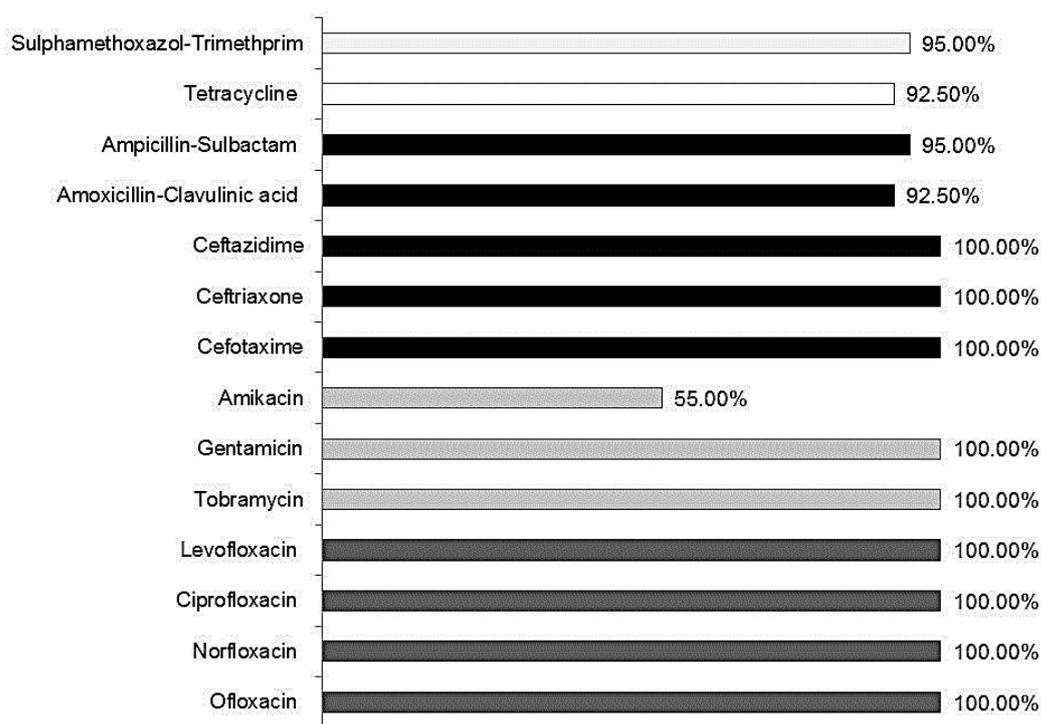

Figure 1. Percentage of different antibiotic resistance among *E. coli* clinical isolates

Primers for amplification of *acrA* amplified fragments of expected length, about 1.1 kb. Primers for *acrB* amplified a fragment of an approximately 2.7 kb, as expected. Regarding the relative quantitative analysis of *acrA* and *acrB* gene expressions by real time PCR, fold increase in gene expression of both *acrA* gene (black) and *acrB* gene (white) for the studied MDR *E. coli* clinical isolates relative to the control group was shown in Figure 2.

Relative to the housekeeping gene *rpsL*, the mean increase in the levels of expression of *acrA* and *acrB* genes in the MDR *E. coli* clinical isolates were (3.9 ± 1.58) and (2.6 ± 1.09) fold respectively compared to those of the ATCC strain. For both genes, the differences between the levels of expression of both genes in MDR strains and ATCC strain were statistically significant ($p < 0.001$) (Figure 2) There was a linear correlation between *acrA* and *acrB* genes expression levels. (r = 0.593, $p < .0001$).
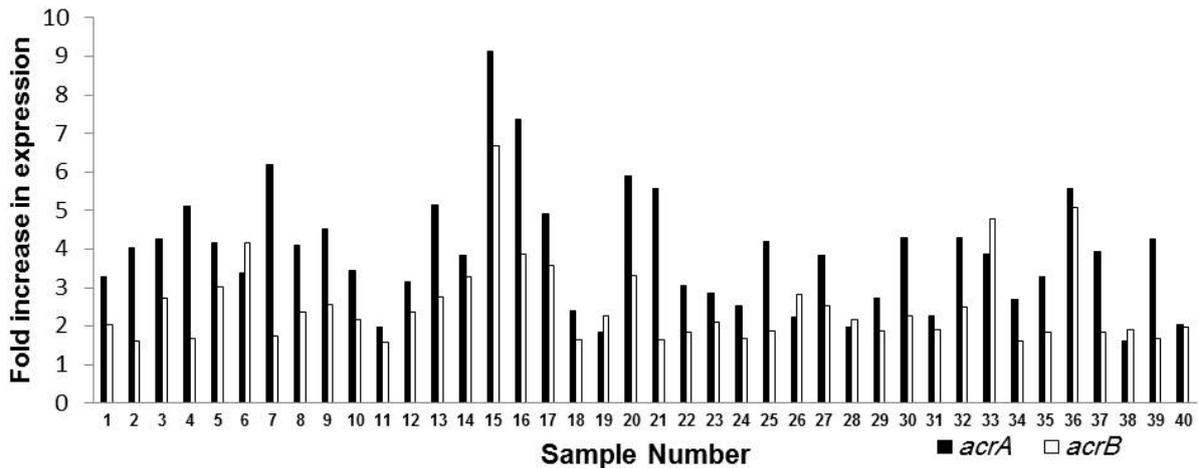
Figure 2. Distribution of fold increase in expression of *arcA* and *acrB* genes for all the studied *E. coli* clinical isolates

The difference in CT readings among the five selected samples, before (samples 14, 15, 18, 21 and 22) and after (14', 15', 18', 21' and 22') addition of the selected antibiotic (levofloxacin 3 µg/ml) where plotted after normalization with respect to the ATCC 8739 as demonstrated in (Figure 3).
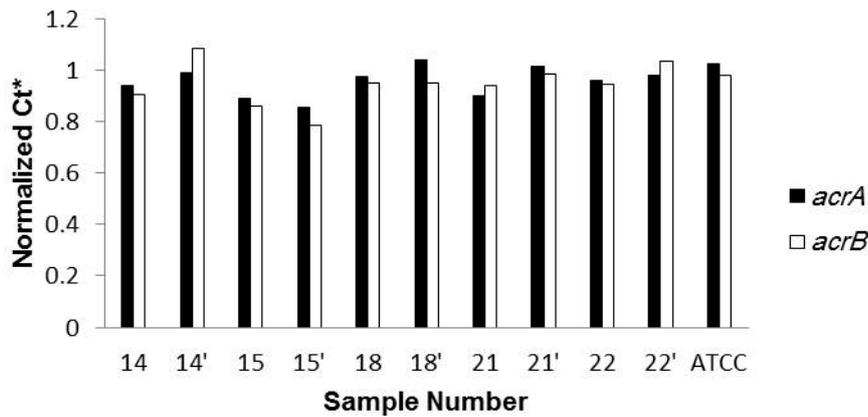

Figure 3. Threshold cycle readings of the selected samples: before (14, 15, 18, 21 and 22) and after adding levofloxacin (14', 15', 18', 21' and 22')

By applying $2^{-\Delta\Delta CT}$ method for calculating fold increase in gene expression relative to the ATCC 8739, before (samples 14, 15, 18, 21 and 22) and after (14', 15', 18', 21' and 22') addition of the selected antibiotic (levofloxacin 3 µg/ml). The difference in gene expressions did not reach the statistical significance ($p > 0.05$), (Figure 4)
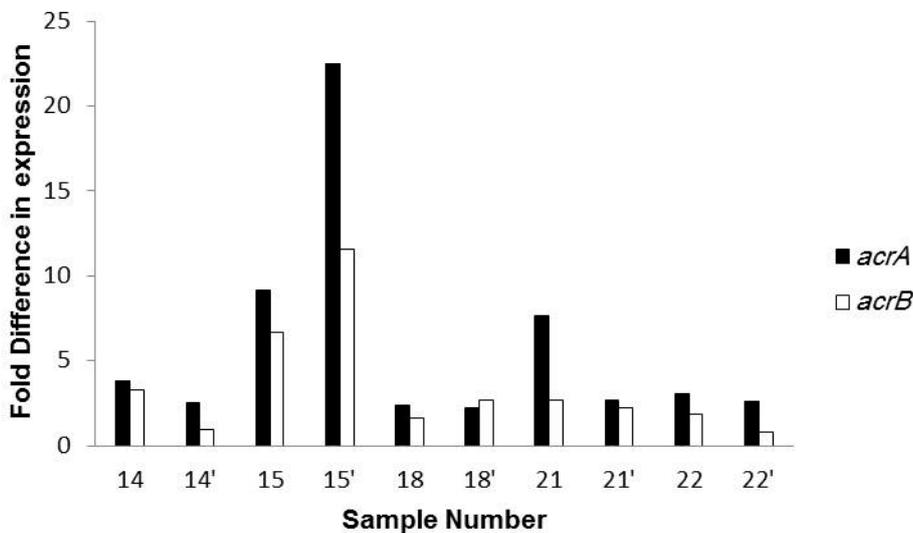

Figure 4. Fold Difference in expression of the selected samples: before (14, 15, 18, 21 and 22) and after adding levofloxacin (14', 15', 18', 21' and 22')

Regarding the MIC values of levofloxacin / ciprofloxacin (quinolones), gentamicin sulphate (aminoglycosides), and ceftriaxone (β- lactams) using broth micro-dilution technique, the *E. coli* isolates showed high levels of resistance against levofloxacin, ciprofloxacin, gentamicin and ceftriaxone with high MIC

values. Breakpoints mentioned in CLSI were (levofloxacin: S: ≤ 2, I: 4, R: ≥ 8 µg/ml, ciprofloxacin: S: ≤ 1, I: 2, R: ≥ 4 µg/ml, gentamicin: S: ≤ 4, I: 8, R: ≥ 16 µg/ml, ceftriaxone: S: ≤ 1, I: 2, R: ≥ 4 µg/ml) (Figure 5).

There was a progressive reduction in MIC values of the selected antibiotics in presence of EPI (mefloquine hydrochloride). The marked decrease in the MIC values was observed with ceftriaxone followed by levofloxacin then ciprofloxacin. The least was observed in case of gentamicin. In case of levofloxacin, in presence of mefloquine hydrochloride, a reduction of the MIC values was observed (Range: 0.25-8) µg/ml. The fold decrease in MIC values ranges were (8-128) fold. While for ciprofloxacin, a reduction of the MIC values was observed (Range: 1- 256) µg/ml. The fold decrease in MIC values ranges were (2- 512) fold. Concerning gentamicin, a reduction of the MIC values was observed (Range: 2- 512) µg/ml. The fold decrease in MIC values ranges were (0- 8) fold. While for ceftriaxone, a reduction of the MIC values was observed (Range: 0.25- 128) µg/ml. The fold decrease in MIC values ranges were (4 -1024) fold.

In presence of mefloquine hydrochloride, 95% out of the levofloxacin resistant isolates recovered susceptibility (MIC ≤ 2µg/ml), while, 5% each of the resistant isolates recovered susceptibility (MIC ≤ 1µg/ml) for ciprofloxacin and (MIC ≤ 4µg/ml) for gentamicin and lastly, for ceftriaxone 62.5 % of the resistant isolates (MIC ≤ 1µg/ml) recovered susceptibility.
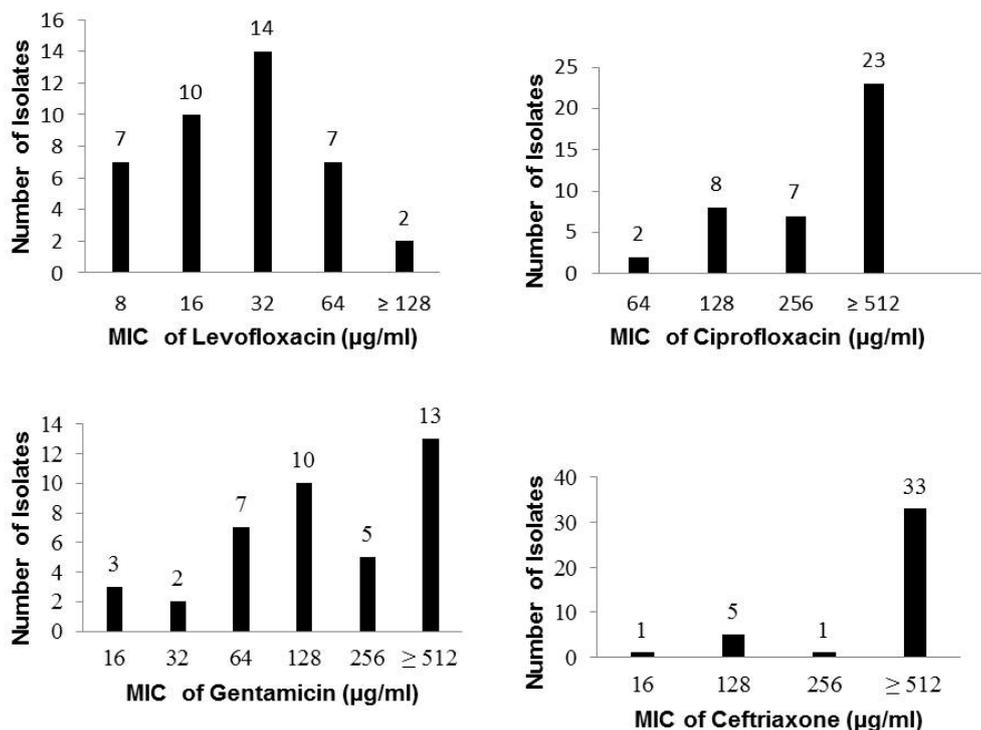

Figure 5. Minimum inhibitory concentration values of selected antibiotics against *E. coli* clinical isolates

4. Discussion

E. coli is reported to be among the most frequent isolates in hospitals [20,29]. The current study included forty MDR *E. coli* clinical isolates from patients admitted to different medical facilities in Alexandria. Selected isolates displayed high-level resistance to different members of antibiotics of different families including fluroquinolones, β-lactams, aminoglycosides, tetracycline and trimethoprim-sulphamethoxazol which could be attributed to widespread abuse of these antibiotics. There was no difference between the different isolate sites regarding their pattern of resistance to the mentioned antibiotics. Our results were in concordance with that of George *et al*. [14] who reported that around 80% of the isolates obtained were found to be resistant to more than two different classes of the reference antibiotics used. Other studies have also reported high level of multidrug resistance among *E. coli* isolates [1,2,34].

Antimicrobial resistance has become a significant public health worldwide problem [51] and especially in developing countries [35]. Bolon *et al*. [7] have reported a consistent stepwise increase in *E. coli* resistance to ciprofloxacin. This problem was more complicated through the emergence of MDR strains

Efflux mechanisms in microorganisms play a major role in resistance to many classes of antibiotics [5,32,38].

Assessment of efflux mediated resistance based only on phenotypic methods is often non-informative due concomitant presence of other mechanisms of resistance affecting same drugs, or to the difficulty of detecting mechanisms conferring low to moderate levels of resistance [16,27,46]. Semi-quantitative reverse transcription PCR (RT-PCR), or quantitative real-time PCR, which directly identify the presence or the expression level of genes of interest, have been successfully used for detecting the expression of *acrAB* efflux pump [8,37,48,49].

In the present study, a combined phenotypic and genotypic approach for the detection of resistance by *acr*-mediated efflux in *E. coli*, concentrating on *acrABtolC* efflux pump was developed. Accordingly a screening step for the DNA of the *acrA* and *acrB* genes was performed using conventional PCR followed by identification of the

expression pattern of the *acrABtolC* efflux pump in the MDR *E. coli* strains using qRT- PCR technique.

The genotypic method confirmed the presence of the *acrA* and *acrB* genes encoding the efflux pump in all clinical isolates (100%) agreed with being as an intrinsic gene. Our results were supported by Moreira *et al*. [28]. Concerning the expression patterns of *acrA* and *acrB* genes, our study revealed the overexpression of both genes was accompanied with MDR phenotype of our MDR *E. coli* clinical isolates. A significant correlation relative to the housekeeping gene *rpsL*, the mean increase in the levels of expression of *acrA* and *acrB* genes in the MDR*E. coli* clinical isolates were (3.9 ± 1.58) and (2.6 ± 1.09) fold respectively compared to those of the sensitive isolates. Thus, our data indicate that *acrAB*overexpression is a biomarker for MDR.

Our data agreed with that of Swick *et al*. [40] who found that 30% of fluoroquinolone-resistant isolates overproduced AcrA, but fluoroquinolone-susceptible isolates had normal AcrA levels. Relative to the housekeeping gene *rpsL*, the average levels of expression of *acrA*and *acrB*in the fluoroquinolone-susceptible strains were 1.8-0.7-fold and 2.0- 0.6-fold, respectively, compared to those of the standard *E. coli* strain. In fluoroquinolone resistant isolates, the expression level of *acrA* averaged 4.5- 2.0-fold and the expression level of *acrB* averaged 4.6- 2.5-fold. Concluding that, the more severe the MDR phenotype, the higher the probability that the isolate overexpressing *acrAB*, consequently increased level of AcrA could be justified which was in concordance with Swick *et al*. [40] who found that 30% of fluoroquinolone-resistant isolates overproduced AcrA, but fluoroquinolone-susceptible isolates had normal AcrA levels.

Keeney *et al*. [22] in a quantitative analysis study revealed the fold increase in *acrA* and *acrB* genes expressions in two different tigecycline resistant clinical isolates of *E. coli*. The expression of *acrA* increased 3 - 3.16 fold than the tigecycline susceptible strains while the expression of *acrB* increased 2.37 - 2.39 fold, suggesting that overexpression of the AcrAB pump is involved in decreased tigecycline susceptibility in *E. coli*. As well, Viveiros *et al*. [48], in a quantitative study reported that the expression of *acrAB-tolC* increases as the organism becomes more and more resistant to the antibiotic. Yasufuku *et al*. [50] investigated the correlation of antibiotic susceptibilities with the expression of the efflux pump genes in *E. coli* of urinary tract infection patients by quantitative real-time reverse transcription-PCR. Their study revealed a significant correlation of the overexpression of *marA* with higher MICs of cefepime, nalidixic acid, ciprofloxacin, and levofloxacin. *marA* overexpression is a key factor leading to the overproduction of the AcrAB.

In our study we found that level of overexpression of acrA gene was higher than that of *acr*B gene. This could be explained by the association of such gene in efflux pumps other than *acr*AB *tol*C such as *acr*AD.

Elkins and Nikaido [12] reported that, in addition to AcrB, AcrA also associates with the aminoglycoside transporter AcrD, which lacks a specific periplasmic accessory protein. Interestingly, association with AcrA enables AcrD to mediate TolC-dependent multidrug efflux. On the other hand, Tikhonova and Zgurskaya [44] explained that AcrA excess could indicate that more than

one AcrA trimer associates with each AcrB monomer. In our study we found that level of overexpression of *acr*A gene was higher than that of *acr*B gene. This could be explained by the association of such gene in efflux pumps other than *acr*AB *tol*C.

Concerning the evaluation of *acrA* and *acrB* genes expression before and after addition of the antibiotic, the non-significant difference in gene expressions may be due to the small sample size involved in the study, however interestingly in the present study we noticed that some of the samples examined showed increase in the expression levels of both genes after addition of the selected antibiotic, while in other samples the expression levels decreased. This could be explained by more than one mechanism involved in the antibiotic resistance. Up-regulation of efflux pump genes could have essential role in the early stages of resistance development until other specific resistance takes place as reported by Singh *et al*. [39].

Efflux pumps or MDR pumps have been recognized as one of the major determinants of the concentration of an antibiotic inside a bacterial cell. Therefore inhibition of efflux pumps can be utilized for increasing the antibiotic concentration inside a pathogenic cell making these drugs more effective [5]. An array of approaches in academic and industrial research settings have generated a number of promising EPIs. This synergistic discovery stage has been exploited in translational directions beyond the potentiation of conventional antimicrobial treatments [43].

In the present study, mefloquine hydrochloride was used as an EPI. By investigating its effect in certain concentration (50 μM) on the MIC values of selected antibiotics. It was observed that the activity of mefloquine was shown to be synergistic to a great extent with that of levofloxacin and ceftriaxone, followed by ciprofloxacin and to a lesser extent with gentamicin. The effect of mefloquine hydrochloride as efflux pump inhibitor in decreasing the MIC of the resistant *E. coli* isolates agrees with that reported by Vidal-Aroca *et al*. [47]. Mefloquine was confirmed to be a strong bacterial efflux-pump inhibitor. In fact, efflux by RND pumps could be one reason why mefloquine displayed much less *in vitro* antibacterial activity against Gram negative bacteria than against Gram-positive bacteria in an earlier study [23].

In conclusion, in the present study, the phenotypic and genotypic diagnostic methods used for detection of *acrABtolC*– mediated MDR in *E. coli* as well as the study of the effect of exposure to antibiotics as a stress factors on the expression of *acrAB* genes need to be considered with caution due to the small number of strains included. Therefore further studies with larger sample size are recommended for investigation of the effect of different factors on the gene expression

The emergence of multiple antibiotic resistant pathogenic bacteria has raised concerns about the future of antibiotics in infectious disease control, it is therefore important to encourage careful and limited use of antibiotics in the health care system to control the spread of these MDR strains. EPIs need to be considered in the design of future antibiotics. Inhibition of *acrABtolC* efflux pump in MDR *E. coli* by mefloquine hydrochloride appears to be an attractive approach to combat the problem of drug resistance. However, further studies

exploring novel strategies to interfere with efflux pump expression and function are warranted.

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