Expression of the Fluoroquinolones Efflux Pump Genes accA and mdfA in Urinary Escherichia coli Isolates

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Abstract

Escherichia coli is one of the most frequent causes of urinary tract infections. Efflux system overexpression is reported to contribute to E. coli resistance to several antibiotics. Our aim in this study was to investigate the relation between antibiotic resistance and the expression of the efflux pump genes accA and mdfA in E. coli by real-time reverse transcription-PCR. We tested the in vitro susceptibilities to 12 antibiotics in 28 clinical isolates of E. coli obtained from urine samples. We also determined the minimum inhibitory concentrations of levofloxacin to these samples. We then revealed significant correlations between the overexpression of both mdfA and accA and MICs of levofloxacin. In conclusion, we demonstrated that the increased expression of efflux pump genes such as mdfA and accA can lead to levofloxacin resistance in E. coli. These findings contribute to further understanding of the molecular mechanisms of efflux pump systems and how they contribute to antibiotic resistance.

Key words: Escherichia coli, accA and mdfA genes, efflux pump, levofloxacin, overexpression

Introduction

Antimicrobial abuse is one of the major factors contributing to the development and maintenance of antimicrobial resistance in bacteria. The issue is of utmost importance in many developing countries, where the sale of antimicrobials is often unrestricted (Okeke et al., 2005). In Egypt, Escherichia coli is among the most common pathogens isolated from patients with urinary tract infections and it has a high prevalence of resistance to antibiotics (Shaheen et al., 2004; Mohamed Al-Agamy et al., 2006).

Bacterial resistance to antimicrobial agents is a threat to public health in Egypt. The consequences of resistance affect not only the ability to treat the infection, but also the cost and duration of treatment (Shaheen et al., 2004; Mohamed Al-Agamy et al., 2006).

Fluoroquinolone resistance is on the rise. The emergence of this resistance in nearly all species of bacteria was documented soon after the introduction of these compounds for clinical use (Acar and Goldstein, 1997).

Levofloxacin resistance has been shown to increase more than 5-fold in 6 years. Rapid emergence of resistance may have been fuelled by the increase in the rate of levofloxacin prescribing (Johnson et al., 2008). A study showed that the annual rates of levofloxacin resistance of E. coli were 29.49% in 2005, 26.51% in 2006, 40.21% in 2007, 43.20% in 2008, and 31.75% in 2009 (Jang et al., 2011). The persistent increase in fluoroquinolone resistance rates influences patient management and demands a change in some existing guidelines for the treatment (Paterson, 2004; Wagenlehner et al., 2011; Han et al., 2012).

The overexpression of efflux pumps contributes to multidrug resistance (MDR) in E. coli. Almost all Gram-negative bacteria have genes for efflux pumps. Two transporters, AcrAB and MdfA, which belong to two distinct families of efflux pumps, have the ability to efflux quinolones in E. coli. Most of these efflux genes are either underexpressed or not expressed at all. When they are overexpressed, they are associated with MDR in E. coli (Yang, 2003; Yasufuku et al., 2011).

The AcrAB-TolC system of E. coli is among the better characterized resistance-nodulation-cell division superfamily (RND) systems. In addition to recognizing many fluoroquinolones, this system exports a diversity of agents including tetracycline, β-lactams, chloramphenicol, erythromycin, rifampicin, disinfectants, dyes, and organic solvents. AcrA is a lipoprotein situated in
the periplasm; the inner membrane protein, AcrB, is a proton-motive-force transporter of the RND type. Strains lacking in the AcrAB proteins are hypersusceptible to many quinolones; on the other hand, fluoroquinolone-resistant efflux mutants frequently overproduce the periplasmic protein AcrA. Such mutants display decreased susceptibility to ciprofloxacin, tetracycline, ampicillin, and chloramphenicol. Removal or inactivation of proteins that suppress expression of AcrAB (AcrR and AcrS) reduces fluoroquinolone susceptibility (Drlica et al., 2012).

MdfA is a paradigm of multidrug-resistance antiporters. It is a member of the major facilitator superfamily (MFS) and is a transmembrane-spanning integral inner membrane protein (Edgar and Bibi, 1997).

In laboratory-generated mutants, overexpression of the efflux pumps AcrAB or MdfA have been shown to cause three to six fold increase in fluoroquinolone resistance (Yang, 2003). The simultaneous overexpression of AcrAB and MdfA results in synergistic increases in resistance to fluoroquinolones (Yang, 2003). The overexpression of AcrA and MdfA in quinolone resistance in E. coli has been thoroughly investigated, yet data about it in Egypt, up until the date this paper has been written, has been really scarce.

Our aim in this study was to investigate the relation between antibiotic resistance and the expression of the efflux pump genes in E. coli by real-time quantitative polymerase chain reaction (RT-qPCR).

Experimental

Material and Methods

E. coli strains and growth conditions. A total of 28 non-duplicate E. coli isolates were used in this study obtained from patients with urinary tract infection (UTI) treated in Damanhour General Hospital, Damanhour, Egypt between the years 2013 and 2014. Post treatment isolates were excluded from this study. The isolates were identified with conventional biochemical tests. All isolates were deemed to be clinically significant (> 10^5 CFU/ml). They were selected according to their sensitivity to levofloxacin in order to correlate their sensitivity profile with the overexpression of efflux pump genes. E. coli isolates with intermediate susceptibility were not classified as being resistant. The strains were propagated at 37°C in Luria-Bertani (LB) broth.

Susceptibility testing. The following 12 antimicrobial discs were selected according to CLSI guidelines (CLSI, 2015): amoxicillin (AML), amoxicillin-clavulanic acid (AMC), cefuroxim (CMX), cefalexin (CL), cefepime (FEP), ceftiraxone (CRO), chloramphenicol (C), gentamycin (CN) tetracycline (TE), levofloxacin (LVX), meropenem (MEM), azithromycin (AZM) (Oxoid Ltd., Basingstoke, United Kingdom) on Mueller-Hinton agar (Oxoid Ltd., Basingstoke, United Kingdom). Zone sizes were measured to the nearest millimetre. Using the published CLSI guidelines, the susceptibility or resistance of the organism to each drug tested were then determined. E. coli ATCC 25922 (LGC Standards GmbH, Wesel, Germany) was used as a control (CLSI, 2015).

Determination of the minimum inhibitory concentration (MIC) to levofloxacin. Determination of the MIC of levofloxacin was done by using the two-fold dilution method according to CLSI guidelines (CLSI, 2015). MICs were performed in 96-well microplates (Greiner, Wemmel, Belgium). Briefly, serial two fold dilutions of 2x strength antibiotic were performed in sterile distilled water. An overnight culture of the tested isolate in Mueller-Hinton Broth (MHB) was diluted in fresh double strength MHB till it reaches the concentration of 10^5 CFU/ml.

Fifty µl suspension of the organism in double strength MHB was added to the well with 0.05 ml of the antibiotic solution, and the microtitre plates were incubated aerobically at 35°C. The MIC was then defined as the lowest concentration of the antibiotic in which there is no visible growth after overnight incubation. Results were recorded in µg/ml after incubation at 35°C for 18 hours (CLSI breakpoints for levofloxacin are ≤ 2 µg/ml for susceptibility and ≥ 8 µg/ml for resistance).

RNA extraction and quantification of RNA expression using RT-qPCR (Taylor et al., 2010). All E. coli isolates were cultured at 37°C for 14 h on MacConkey agar plates (Oxoid Ltd., Basingstoke, United Kingdom), then cultured in 4 ml of Luria-Bertani medium (Oxoid Ltd., Basingstoke, United Kingdom) for 8 h. Total RNA was obtained from mid-logarithmic growth-phase bacterial cultures with Isolate II RNA Micro Kit (Bioline, Michigan, USA) (Pfaffl et al., 2002; Keeney et al., 2008). Genomic DNA contamination was removed by an on-column RNase-free DNase I digestion. RNA purity was assessed spectrophotometrically by measuring the OD_{260}/OD_{280} ratio by NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific – Delaware, USA) RNA integrity was assessed by visualization of one large and one small band of RNA after electrophoresis (Fleige and Pfaffl, 2006).

The quantification protocol was performed immediately following the quality control assessment. The acrA and mdfA genes were quantified by RT-qPCR using a SensiFAST™ SYBR No-ROX One-Step Kit (2x) (Bioline, United Kingdom) in a StepOne™ Real-Time PCR System (StepOne™ system, Applied biosystems, USA). The reaction mixture was prepared in a total volume of 20 µl dispensed in MicroAmp™ optical microplates.
**Table I**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer (5’-3’)</th>
<th>Amplicon length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdfA F</td>
<td>CGGCAACGATATGATCCGCA</td>
<td>523</td>
<td>Yasufuku et al., 2011</td>
</tr>
<tr>
<td>mdfA R</td>
<td>CAGTGACAGTTTCTCGGTAT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>acrA F</td>
<td>CTCTCAGCGGCTAGCCCTAA</td>
<td>107</td>
<td>Swick et al., 2011</td>
</tr>
<tr>
<td>acrA R</td>
<td>TGCCAGGTTCAAGTTGAGTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gapA F</td>
<td>ACTTCGACAAATAGGGGC</td>
<td>215</td>
<td>Yasufuku et al., 2011</td>
</tr>
<tr>
<td>gapA R</td>
<td>CGGGATGATGTTCTGGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(ThermoFisher Scientific, Michigan, USA). Each reaction mixture contained 0.5 µM of both forward and reverse primers, 10 µl of SensiFAST™ SYBR No-ROX One-Step Kit (2x) mix, 0.2 µl of reverse transcriptase, 0.4 µl of Ribosafe RNA inhibitor, 4 µl of template RNA, and nuclease-free water to complete the volume. The oligonucleotide primers for the amplification were adapted from prior studies and are listed in (Table I) (Swick et al., 2011; Y asufuku et al., 2011).

PCR-grade water was used as a negative control. Genomic DNA from the *E. coli* ATCC 25922 strain was the positive control. The melting curve analysis ensured that only a single PCR product was amplified. A no-RT control was included by omitting the reverse transcriptase from the reaction. Each sample was placed in triplicate on a 48-well plate and subjected to one-step reverse transcription at 50°C for 30 min, 40 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 30 s and extension at 72°C for 30 s. The Cq means of almost all samples in our study were less than 30 (Y asufuku et al., 2011).

Using the Applied biosystems stepone™ system software the relative quantification of the expression of the target genes was calculated relative to *gapA*, a housekeeping gene, and then the expression rate of each efflux pump gene was determined by using *E. coli* (ATCC 25922) as the control (Pfaffl et al., 2002; Bohnert et al., 2007).

**Results**

**Susceptibility testing.** Susceptibility testing for the 12 antibiotics was performed according to CLSI guidelines (CLSI, 2015). It showed that 79% of the isolates were MDR. MDR is most commonly defined as resistance to ≥ 3 classes of antibiotics (Magiorakos et al., 2012). The resistance patterns of the isolates (the number of antibiotics to which each isolate is resistant) to the studied antibiotics are shown in (Table II).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>No. (%) of isolates (n = 28)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Susceptible</td>
</tr>
<tr>
<td>AML</td>
<td>0 (0)</td>
</tr>
<tr>
<td>AMC</td>
<td>10 (35.7)</td>
</tr>
<tr>
<td>CMX</td>
<td>6 (21.4)</td>
</tr>
<tr>
<td>CL</td>
<td>6 (21.4)</td>
</tr>
<tr>
<td>FEP</td>
<td>14 (50)</td>
</tr>
<tr>
<td>CRO</td>
<td>10 (35.7)</td>
</tr>
<tr>
<td>C</td>
<td>20 (71.4)</td>
</tr>
<tr>
<td>CN</td>
<td>14 (50)</td>
</tr>
<tr>
<td>TE</td>
<td>10 (35.7)</td>
</tr>
<tr>
<td>LEV</td>
<td>0 (0)</td>
</tr>
<tr>
<td>MEM</td>
<td>28 (100)</td>
</tr>
<tr>
<td>AZM</td>
<td>8 (28.6)</td>
</tr>
</tbody>
</table>

**Correlation of overexpression of efflux pump genes with MICs of each antibiotic.** The overexpression of efflux pump genes was identified as ≥ 2-fold gene expression compared to that of the standard strain *E. coli* (ATCC 25922) in qRT-PCR (Keeney et al., 2008). The correlation of the overexpression of efflux pump genes with the MICs of levofloxacin was determined. The correlations between the pattern of resistance and the expression of both genes together with the MICs of levofloxacin have also been determined.

**Statistical analysis.** Statistical analyses were performed by using Statistical Package for Social Sciences (SPSS) software version 17 with Spearman’s correlation test; *p* < 0.05 was considered statistically significant.

**Minimum Inhibitory Concentration (MIC) and gene expression.** Determination of the MIC of levofloxacin was done by using the two-fold dilution method according to CLSI guidelines (Clinical and Laboratory Standards Institute. 3rd ed. 2015). All the tested isolates (100%) were levofloxacin resistant. Results of determination of MIC of levofloxacin are shown in (Table III).

**Expression of acrA and mdfA genes.** Among the 28 isolates, 23 isolates (82.1%) showed overexpression of *mdfA* ranging from 2–34.47 folds, and 22 (78.6%) showed overexpression of *acrA* ranging from 2–9.52 folds (Table III).
Correlation of overexpression with MIC and patterns of antibiotic resistance. The overexpression of efflux pump genes was defined as ≥ 2-fold gene expression compared to that of the standard strain E. coli (ATCC 25922) in qRT-PCR. A strong positive correlation of the overexpression of both of mdfA and acrA with MICs of levofloxacin was detected (Spearman correlation 0.93 and 0.72 respectively, P < 0.05) by bivariate analysis.

Moderate positive correlation was found between the expression of mdfA and the pattern of antimicrobial resistance of each isolate (Spearman correlation 0.45, P < 0.05). This was not the case with the expression of acrA where no significant correlation with the pattern of antimicrobial resistance was found.

Discussion

Multidrug resistance is a growing public health concern worldwide (Okeke et al., 2005). The increasing incidence of resistance among pathogenic E. coli makes the treatment difficult with an unpredictable outcome (Kariuki et al., 2007). Resistance to quinolones has reached 50% in some parts of the world (Kronvall, 2010). This leads to a challenge in the use of this class of drugs. It is now necessary to investigate the possible molecular mechanisms by which E. coli strains acquire antibiotic resistance in an attempt to prevent further spread of these kinds of resistant strains and prevent the occurrence of new resistant ones.

In the present study we examined 28 E. coli isolates, all isolates (100%) were levofloxacin resistant and 79% were MDR. Similar to our results, Swick et al. (2011) found that no isolate categorized as MDR was fluoroquinolone susceptible. They also suggested the possibility of an underlying correlation between fluoroquinolone resistance and MDR, this was confirmed in our study where moderate positive correlation was found between levofloxacin MIC and the pattern of antimicrobial resistance of each isolate (Spearman correlation 0.45, P < 0.05)

There is a widespread, escalating use of fluoroquinolones. Even contrary to guidelines that recommend them in only select situations, the frequent use of fluoroquinolone antibiotics has been observed in nearly all settings. Quinolone antibiotics now have exceeded sulfa antibiotics in recent years as the most commonly prescribed treatment for outpatient UTIs in women (Kallen et al., 2006).

We quantified the expression of the efflux pump genes known to affect fluoroquinolone resistance in a single quantitative study and we used gapA as a house keeping gene. Housekeeping genes provided internal controls, which were used as a measure of mRNA expression levels irrespective of growth condition (Szabo et al., 2004). Jandu et al. (2009) reported that the expression of gapA was similar when detected under four different growth conditions so it was chosen as an internal control for our study.

We found that most of the levofloxacin resistant isolates overexpressed both mdfA and acrA genes (82.1% and 78.6% respectively). This was with agreement of other studies, where overexpression of acrA together with acrB led to a three- to six-fold increase in fluoroquinolone resistance, while augmenting the level of acrAB through the overexpression of the tran-
scriptional regulator, sdiA, resulted in about the same (approximately six-fold) increase in drug resistance. Yasufuku et al. (2011) revealed a significant correlation of the overexpression of mdfA with higher MICs of levofloxacin (Rahmati et al., 2002; Yang, 2003).

In general the more severe the MDR phenotype, the higher the likelihood that the isolate also overexpressed acrAB (Nikaido and Pagès, 2012). Han et al. (2012) found that out of 89 patients with fluoroquinolone resistant isolates, 49.4% overexpressed acrAB.

A synergy in quinolone resistance was shown when acrAB was overexpressed simultaneously with norE or mdfA. Deletion of acrAB alone and all of the three pumps together had the same effect on the susceptibility of fluoroquinolones. The authors also found that the strain with acrAB deletion were the most susceptible when compared to mutants strains with deletion in norE and/or mdfA. The maximum quinolone resistance mediated by efflux pumps was suggested to be ~10-fold, irrespective of any increase in production of these pumps (Yang, 2003).

Studies conducted with efflux pump deleted (ΔAcrAB) strain delayed the emergence of resistance suggesting that the inhibition of AcrAB efflux pump could be a strong strategy for slowing the development of resistance in clinically important Gram-negative bacteria (Singh et al., 2012).

In our study 17.9% and 21.4% of the levofloxacin resistant isolates didn’t show over expression of mdfA and acrA respectively, thus fluoroquinolone resistance may be contributed to other mechanisms such as gyrase or topoisomerase IV mutations. Morgan-Linnell et al. (2009) reported mutations in gyrase and topoisomerase IV in 100% of the high-level-resistant E. coli clinical isolates, while over production of the efflux pump (AcrA) contributed approximately 33% to the overall quinolone resistance. As to the mdfA gene, one of the levofloxacin susceptible isolates showed very slight overexpression.

It should be noted that expression of pump genes can be induced by natural substrates encountered during infection. For example, indole and bile, which would be encountered in the mammalian gut, induce expression of acrAB in E. coli and Salmonella (Sun et al., 2014).

Though increased efflux pump expression correlates with increase in levofloxacin MIC, we did not compare expression of efflux pumps in levofloxacin sensitive strains. Thus, this has to be investigated as efflux pump expression might be high in these strains too if they are resistant to other antibiotics since both pumps are broad spectrum. Some studies have found that resistance to antibiotics through the same efflux pump is common (Poole, 2005).

An approach of blocking the efflux pump protein or gene has been studied. This falls under the class of biological inhibition of efflux pumps. The efflux pumps could be switched off with the means of specific antibodies (Yoshihara and Inoko, 2011). The genes encoding these pumps or their regulators could be blocked by means of the antisense strategies. The antisense approach has been revealed to work for AcrAB efflux pump in E. coli and has also been patented (Oethinger and Levy, 2001; 2011).

Efflux pump inhibitors can also be used for the evaluation of the effect of efflux pumps on resistance emergence, however there are two major concerns with such inhibitors. First, some of these inhibitors have been found to be substrates of efflux pumps themselves, thus questioning their credibility (Lomovskaya and Bostian, 2006). Second, the concentrations required for efflux pump inhibitory activity are often associated with toxicity and hence, are not clinically relevant (Schmitz et al., 1998).

While, only one Gram-negative bacterial species has been evaluated in our study, it is imperative to investigate other bacterial species to increase the clinical relevance of our results. Different inhibitory techniques can be utilized to study the different efflux pumps. These should not only be effective against multiple efflux pumps in one bacterial species but also against efflux pumps in more than one bacterial species.

In conclusion, we demonstrated a significant positive correlation between the overexpression of AcrA and MdfA efflux pumps and the resistance to levofloxacin in E. coli clinical isolates. These findings contribute to further understanding of the molecular mechanisms of efflux pump systems and how they contribute to antibiotic resistance and suggest that developing new EPI could lead to treatments for fluoroquinolones resistant bacteria.

Resistance emergence presents a debilitating challenge in the management of infectious diseases. Data from this study might add to our understanding of resistance development. Our findings suggest that the inhibition of efflux pumps could be a potential strategy to thwart the problem of antibiotic resistance. Considering the genetic plasticity in the bacteria, these findings just seem to be the tip of the iceberg and much needs to be unravelled in the future.

**Literature**


