The role of AcrAB-TolC efflux pumps on quinolone resistance of *E.coli* ST131

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ABSTRACT

E.coli ST131 is a global concern because of its high multi-drug resistance and several virulence factors. In this study, the contribution of *acrAB-TolC* efflux system of *E.coli* ST131 to fluoroquinolone resistance was evaluated. A total of non-repetitive 111 ciprofloxacin resistant *E.coli* isolates were included in the study. Multi-locus sequence typing (MLST) was used for genotyping. Expressions of *acrA, acrB and tolC* efflux pump genes were measured by RT-PCR. Mutations in *marA*, *gyrA*, *parC*, and *aac(6')-lb-cr* positivity were studied by Sanger sequencing.

Sixty-four (57.7%) of the isolates were classified as ST131 and 52(81.3%) of the ST131 isolates belonged to H30-Rx subclone. In ST131, CTX-M 15 positivity (73%) and *aac(6')-lb-cr* carriage (75%) were significantly higher than non-ST131 (12.8% and 51%, respectively) ($p<0.05$). The ampicillin-sulbactam (83%) resistance was higher, gentamicin resistance (20%) was lower in ST131 than non-ST131 (64% and 55%, respectively) ($p=0.001$ and $p=0.0002$). Number of the isolates with MDR or XDR profiles did not differ in both groups. Multiple in-dels (up to 16) were recorded in all quinolone resistant isolates. However, *marA* gene was more overexpressed in ST131 compared to non-ST131 (median 5.98 vs 3.99; $P=0.0007$). Belonging to H30-Rx subclone, isolation site, ciprofloxacin MIC values did not correlate with efflux pump expression.

In conclusion, the *marA* regulatory gene of AcrAB-TolC efflux pump system has a significant impact on quinolone resistance and progression to MDR profile in ST131 clone. Efflux pump inhibitors might be alternative drugs for the treatment of infections caused by *E.coli* ST131 if used synergistically in combination with antibiotics.

INTRODUCTION

Escherichia coli ST131 clone has been widely disseminated throughout the world and associated with severe infections [1]. Fluoroquinolone resistance in *E.coli* has become a global concern after the emergence of ST131 clone [2]. H30 clonal subset of ST131 contains allele 30 of the type-1 fimbriae adhesin gene *fimH.* Recently described *H*30Rx clade of ST131-H30 is associated with fluoroquinolone resistance and usually produces CTX-M-15[3].

Quinolone resistance is mostly mediated by target site mutations in quinolone resistant determining regions (QRDR) [4], overexpression of efflux pumps [5] and plasmids[6]. AcrAB-TolC pump is the main component of efflux systems in *E.coli* [7]. Several studies previously showed the overexpression of AcrAB-TolC efflux pump among fluoroquinolone resistant *E.coli* [5, 8, 9]. This pump is regulated by MarA, SoxS and Rob proteins [10]. MarA protein is associated with multi-drug resistance (MDR) [11].

E.coli ST131 was shown to have higher quinolone resistance than non-ST131 *E.coli* with high numbers of mutations at QRDR and carriage of plasmid encoding aminoglycoside/fluoroquinolone-agent modifying enzyme (*aac(6')-1b-cr*) [12]. Johnson et al. reported lower efflux pump activity among strains belonged to ST131 compared to the non-ST131 quinolone resistant isolates by using organic solvent tolerance test; however, the role of AcrAB-TolC system in quinolone resistance in ST131 is still unclear [13]. In this study, contribution of AcrAB-TolC pump and regulatory MarA protein to quinolone resistance of *E.coli* ST131 was evaluated among urinary tract and bloodstream isolates.

MATERIAL AND METHODS

Bacterial isolates

A total of non-repetitive 111 ciprofloxacin resistant *E.coli* isolates from Baskent University Hospital (Ankara, Turkey) between 2010 and 2013 were included in the study. Seventy of the isolates were from bloodstream infections and 41 were isolated from the patients with acute cystitis.

ST131 Determination

Multilocus sequence typing (MLST) was used for genotyping according to the protocol published at University College Cork website (http://mlst.ucc.ie/mlst/). Sequences of 7 housekeeping genes (*adk*, *fumC*, *purA*, *recA*, *mdh*, *gyrB* and *icd*) were analyzed by multiple alignment analysis using Applied Maths version 7.0, Bionumerics [14].

Typing of H30 and H30-Rx

A previously described PCR analysis of *fimH* allele was conducted for determination of H30 subclone. H30-Rx subclone was identified based on a single nucleotide polymorphism (SNP) (G723A) within the allantoin-encoding gene, *ybbW* [15].

Antibiotic Susceptibility tests

Disk diffusion test according to CLSI criteria was used for detection of resistance against amikacin (AMK), gentamicin (GEN), ampicillin-sulbactam (SAM), ampicillin (AMP), ertapenem (ETP), cefazolin (CFZ), cefuroxime (CXM), ceftriaxone (CRO), imipenem (IMP), levofloxacin (LVX), norfloxacin (NOR) and trimethoprim-sulfamethoxazole (SXT)[16]. Ciprofloxacin MIC was determined by agar dilution method. *E.coli* ATCC 25922 was used as quality control [13]. The isolate resistant to three or more antibiotic groups were defined as multi-drug resistant and resistance to 5 or more groups were as described extensively drug resistant (XDR) [17]. ESBL production was investigated based on CLSI standards[13]. CTX-M was detected by PCR and amplicons were sequenced by Sanger sequencing with ABI3500 analyzer. The results were analyzed using Applied Maths version 7.0, Bionumerics.

Molecular quinolone resistance mechanisms

QRDR mutations and plasmid mediated resistance

Topoisomerase mutations (*gyrA* and *parC*) and *aac(6')-lb-cr* were detected by PCR and Sanger sequencing using primers described previously [18, 19].

Efflux gene expressions

RNA isolation (Macherey-Nagel GmbH) and cDNA synthesis (Invitrogen, Thermo Fisher Scientific Inc) were done according to manufacturer's recommendations [9, 20]. For expressions of *acrA*, *acrB*, *tolC*, and *marA* genes qRT-PCR was used. Housekeeping gene was chosen as *gapA*. All expressions were normalized with *E.coli* ATCC 25922. Experiments were done in duplicate. Overexpression of efflux pump genes was defined as ≥1 increase in -$\Delta\Delta(c_t)$ value.

Mutations in marRAB operon

The mutations in *marRAB* were studied by Sanger sequencing with ABI 3500 analyzer by using primers previously described by Keeney et al [21]. Quinolone sensitive clinical *E.coli* isolate (No: 95) was used as reference strain.

Statistical analysis

Statistical analysis was performed using the computing environment R[3]. $-\Delta\Delta(c_t)$ values of efflux pump genes were normalized to generate a heat map with dendrogram of rows and columns. We used hierarchical clustering with complete linkage [22]. Wilcoxon rank-sum test (also known as the Wilcoxon-Mann-Whitney test or the Mann-Whitney U test) was used to make statistical comparisons between two samples [23, 24] and test whether null hypothesis that two samples come from the same population against the alternative hypothesis which is they do not come from the same population. Number of isolates which showed overexpression of efflux pump genes were analyzed by Fisher's exact test. The results were given in the supplementary file. (https://midaslab.shinyapps.io/efflux_pumps_analysis/)

RESULTS

In total of 111 fluoroquinolone resistant *E.coli* isolates, 64 (57.7%) were classified in ST131 clone and 52(81.3%) of the ST131 isolates belonged to H30-Rx subclone. Forty-three (67.2%) of the ST131 isolates were obtained from bloodstream and 21 (32.8%) of the isolates were from urinary tract infections. ESBL production was detected in 54 (84.4%) of the ST131 isolates and in 28 (59.6%) that of non-ST131.

Antibiotic susceptibility test results were given in Table 1. None of the isolates were resistant to carbapenems. The resistance of ST131 isolates to ampicillin-sulbactam (83%) was found to be significantly higher than non ST131 group (64%, p=0.01). However, gentamicin resistance of ST131 clone (20%) was lower than non-ST131 (55%, p=0.0002). The proportion of MDR and XDR isolates did not vary between ST131 and non-ST131 (92% vs %98; 52% vs 55%, p=0.2 and p=0.7, respectively). The MIC_{50} and MIC_{90} values for ciprofloxacin in ST131 clone were 64 µg/ml and 512 µg/ml. In non-ST131 isolates, MIC_{50} was 32 µg/ml and MIC_{90} was >512 µg/ml. Forty-seven (73%) of the isolates in ST131 were CTX-M-15 positive whereas it was 13% (n=6) in non-ST131 group (p<0.0001). Forty eight (75%) of ST131 isolates and 24 (51.1%) of non-ST131 isolates harbored *aac(6')-lb-cr* (p=0.016).

Table 1 Antibiotic susceptibility results of ST131 and non-ST131 isolates

	ST131 (n; %) (64; 57.7)	non-ST131 (n; %) (47; 42.3)	 P value
Amikacin	3 (4.7)	2 (4.3)	1,00
Gentamicin	13 (20.3)	26 (55.3)	0,0002
Ampicillin-sulbactam	53 (82.8)	30 (63.8)	0,01
Ampicillin	62 (96.9)	46 (97.9)	1,00
Ertapenem	0	0	
Cefazolin	51 (79.7)	30 (63.8)	0,07
Cefuroxime	22 (34.4)	18 (38.3.)	1,00
Ceftriaxone	37 (57.8)	18 (38.3)	0,12
Levofloxacin	61 (95.3)	44 (93.6)	0,57
Nofloxacin	21 (32.8)	19 (40.4)	0,48

Trimethoprim-sulphamethoxazole	45 (70.3)	37 (78.7)	0,64
MDR	59 (92.2)	46 (97.9)	0,23
XDR	33 (51.6)	26 (55.3)	0.70

All the isolates had at least one mutation in gyrase (*gyrA*) and topoisomerase (*parC*) genes and there was no difference between ST131 and non-ST131 isolates for the number of mutations.

The median level of *marA* expression (5.98) was significantly higher in ST131 than non-ST131 group (3.99), (P=0.0007). The AcrA component was found to be significantly downregulated in non-ST131 isolates compared to ST131 ones (p= 0.0024). There was no significant difference in the expressions of AcrB and TolC in both groups (p=0.24, p=0.75, respectively). (Figure 1). The expressions in ST131 clone did not differ between H30-Rx and non-H30-Rx isolates (supplementary file).

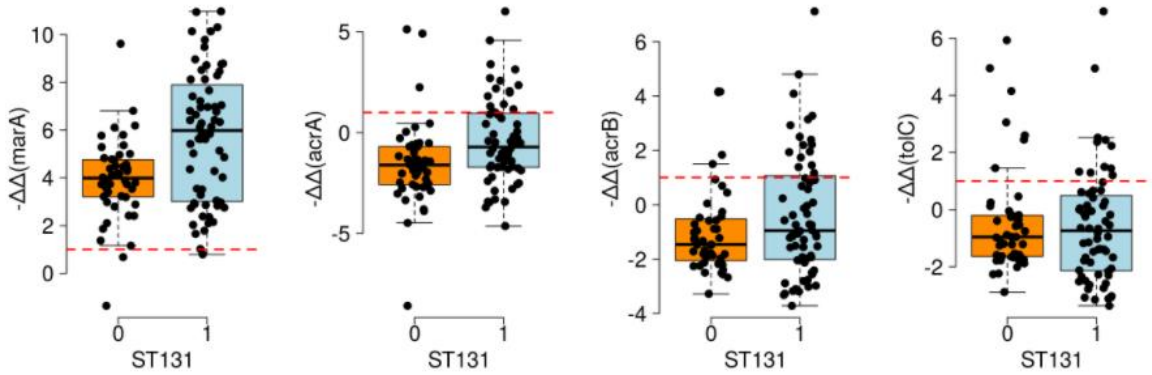

Figure 1 Efflux gene expressions in ST131 and non-ST131 isolates

The proportion of acrA overexpressed isolates ($-\Delta\Delta(c_t)>1$) in ST131 was 23.4% and it was 6.4% in non-ST131 group (p=0.02).

In mutation analysis of *marA*, alignment of sequence reads of susceptible strains were identical, therefore one strain (No= 95) was selected as reference. No missense mutation was detected in QR ST131 and non-ST131 groups. Multiple in-dels (up to 16) were recorded in all quinolone resistant isolates.

A heat map was generated to demonstrate the expression of efflux pump genes in correlation with ST131 or H30-Rx status, ciprofloxacin MIC, and isolation site (bacteremia or UTI). AcrA was found to be closely associated with marA, while AcrB was with TolC pump. The ciprofloxacin MIC's were elevated with the increased expressions of *marA* and *acrA*; however, the correlation was not statistically significant. There was no significant difference of efflux expression levels between the bloodstream and urinary isolates (https://midaslab.shinyapps.io/efflux_pumps_analysis/).

DISCUSSION

Emergence of *E.coli* ST131 has been associated with increased fluoroquinolone resistance [12, 25, 26] and has arisen the need for new treatment options. Furthermore, ST131 clone is known to be more virulent than other

E.coli isolates because of its several virulence genes [2]. For development of new targeted therapy approaches, it is sorely needed to understand mechanisms of the resistance in ST131.

In this study, higher expression levels of *marA* in ST131 clone (5.98 fold) compared to non-ST131 (3.99 fold) was detected among quinolone resistant *E.coli* isolates. Moreover, the proportion of isolates with overexpressed *acrA* was significantly higher in QR ST131 than QR non-ST131 *E.coli*. The role of efflux pumps in quinolone resistance of *E.coli* has been demonstrated in previous studies [4, 8, 19, 20]. In one study focusing on ST131, efflux pump activity of H30-Rx was found to be lower than the isolates belonging to non H30-H3Rx [12]. Yasafuku et al. reported 25.8-fold increased expression of regulatory gene *marA* in quinolone resistant UTI isolates and recorded significant correlation of *marA* overexpression with ciprofloxacin MIC values [20] . Singh et al. showed approximately 4-fold increase in *acrA* and *acrB* gene expressions and elevated quinolone MIC's when *E.coli* isolates were exposed to sequentially increasing levels of levofloxacin [4]. In our study, ciprofloxacin MIC's did not correlate with the expression of efflux pump genes; however, significantly higher *marA* overexpression in QR ST131 than other QR isolates suggested us that these pumps may support quinolone resistance of ST131.

Mutations in *marA* or overexpression of the gene stimulate *acrAB* efflux system [8, 27, 28]. Pantel et al. showed that a 1-bp deletion in repressor gene (*marR*) of *marRAB* system caused a significant increase in the activity of the AcrAB-TolC pump[29]. However, in another study, acrB-overexpressing fluoroquinolone-resistant clinical and veterinary *E. coli* isolates without mutations in *marRAB* region were reported [30]. In our ST131 and non-ST131 groups, multiple in-dels in *marA* gene were detected and number of mutations were not different between two groups.

Inactivation of *acrB* gene of ST131 *E.coli* caused significant increase in susceptibility of the isolates to several antibiotics when there was no mutation in the regulatory gene [13]. In our study, *acrB* of the ST131 isolates were found to be downregulated (median $\Delta\Delta(c_t)$ -0.95).

Efflux pumps contribute to quinolone resistance along with QRDR mutations and plasmid mediated resistance mechanisms [20]. These pumps are known to be associated with low level of quinolone resistance and high level resistance is usually acquired with QRDR mutations. QRDR mutations dominates over efflux pumps once high level resistance occurs [4]. Johnson et al. showed more synonymous QRDR mutations in H30 isolates than non-H30 ST131 *E.coli* in correlation with higher quinolone MICs[12]. In our study, ciprofloxacin MIC values of ST131 and non-ST131 isolates were very high and number of mutations in *gyrA* and *parC* regions did not differ between these two groups. The other quinolone resistance mechanism, carried by *aac(6')-lb-cr*, was found to be significantly higher in ST131 isolates compared to non-ST131 group ($p<0.05$). This result supports findings of Periano et al. higher presence of *aac(6')-lb-cr* in ST131 *E.coli* isolates compared to non-ST131 group [31].

A recent study has shown that ST131 isolates overproducing efflux pump genes can survive in stress conditions, and may pose serious threat in terms of host colonization and virulence [29]. The increased activity of efflux pumps of ST131 isolates in our study might be another reason of selective expansion of ST131 clone throughout the world. Therefore, inhibition of these pumps could be a new option for prevention of dissemination of this clone. Effect of efflux pump inhibitors were analyzed in recent studies [32, 33]. Several efflux pump inhibitors such as phenyl-arginine beta-naphthylamide (PAβN) reduced MIC values in gram negative bacteria[32] but further clinical investigations are required.

In conclusion, the *marA* regulatory gene of AcrAB-TolC efflux pump system has a significant impact on quinolone resistance. These systems contribute to development of MDR profile in ST131 clone, as well. Efflux pump

inhibitors might be alternative drugs for the treatment of infections caused by *E.coli* ST131 if used synergistically in combination with antibiotics.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

1. Lopez-Cerero, L., et al., *Escherichia coli belonging to the worldwide emerging epidemic clonal group O25b/ST131: risk factors and clinical implications.* J Antimicrob Chemother, 2014. **69**(3): p. 809-14.
2. Rogers, B.A., H.E. Sidjabat, and D.L. Paterson, *Escherichia coli O25b-ST131: a pandemic, multiresistant, community-associated strain.* J Antimicrob Chemother, 2011. **66**(1): p. 1-14.
3. Can, F., et al., *The clinical impact of ST131 H30-Rx subclone in urinary tract infections due to multidrug-resistant Escherichia coli.* J Glob Antimicrob Resist, 2016. **4**: p. 49-52.
4. Singh, R., et al., *Temporal interplay between efflux pumps and target mutations in development of antibiotic resistance in Escherichia coli.* Antimicrob Agents Chemother, 2012. **56**(4): p. 1680-5.
5. Swick, M.C., et al., *Expression of multidrug efflux pump genes acrAB-tolC, mdfA, and norE in Escherichia coli clinical isolates as a function of fluoroquinolone and multidrug resistance.* Antimicrob Agents Chemother, 2011. **55**(2): p. 921-4.
6. Karah, N., et al., *Plasmid-mediated quinolone resistance determinants qnr and aac(6')-Ib-cr in Escherichia coli and Klebsiella spp. from Norway and Sweden.* Diagn Microbiol Infect Dis, 2010. **66**(4): p. 425-31.
7. Nikaido, H. and Y. Takatsuka, *Mechanisms of RND multidrug efflux pumps.* Biochim Biophys Acta, 2009. **1794**(5): p. 769-81.
8. Yamasaki, E., et al., *Expression of marA is remarkably increased from the early stage of development of fluoroquinolone-resistance in uropathogenic Escherichia coli.* J Infect Chemother, 2015. **21**(2): p. 105-9.
9. Paltansing, S., et al., *Exploring the contribution of efflux on the resistance to fluoroquinolones in clinical isolates of Escherichia coli.* Microb Drug Resist, 2013. **19**(6): p. 469-76.
10. Ruiz, C. and S.B. Levy, *Many chromosomal genes modulate MarA-mediated multidrug resistance in Escherichia coli.* Antimicrob Agents Chemother, 2010. **54**(5): p. 2125-34.
11. Tavio, M.M., et al., *Quorum-sensing regulator sdiA and marA overexpression is involved in in vitro-selected multidrug resistance of Escherichia coli.* J Antimicrob Chemother, 2010. **65**(6): p. 1178-86.
12. Johnson, J.R., et al., *Intensity and Mechanisms of Fluoroquinolone Resistance within the H30 and H30Rx Subclones of Escherichia coli Sequence Type 131 Compared with Other Fluoroquinolone-Resistant E. coli.* Antimicrob Agents Chemother, 2015. **59**(8): p. 4471-80.
13. Schuster, S., et al., *Contribution of AcrAB-TolC to multidrug resistance in an Escherichia coli sequence type 131 isolate.* Int J Antimicrob Agents, 2017. **50**(3): p. 477-481.
14. Can, F., et al., *Emerging Escherichia coli O25b/ST131 clone predicts treatment failure in urinary tract infections.* Clin Infect Dis, 2015. **60**(4): p. 523-7.
15. Banerjee, R., et al., *Molecular epidemiology of Escherichia coli sequence type 131 and Its H30 and H30-Rx subclones among extended-spectrum-beta-lactamase-positive and -negative E. coli clinical isolates from the Chicago Region, 2007 to 2010.* Antimicrob Agents Chemother, 2013. **57**(12): p. 6385-8.
16. *Clinical and Laboratory Standards Institute . Performance standards for Antimicrobial Susceptibility Testing: 20th Informational Supplement. Wayne, PA: CLSI, 2010.*

17. Magiorakos, A.P., et al., *Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.* Clin Microbiol Infect, 2012. **18**(3): p. 268-81.
18. Cremet, L., et al., *Prevalence of plasmid-mediated quinolone resistance determinants in ESBL Enterobacteriaceae clinical isolates over a 1-year period in a French hospital.* Pathol Biol (Paris), 2011. **59**(3): p. 151-6.
19. Shigemura, K., et al., *Does mutation in gyrA and/or parC or efflux pump expression play the main role in fluoroquinolone resistance in Escherichia coli urinary tract infections?: A statistical analysis study.* Int J Antimicrob Agents, 2012. **40**(6): p. 516-20.
20. Yasufuku, T., et al., *Correlation of overexpression of efflux pump genes with antibiotic resistance in Escherichia coli Strains clinically isolated from urinary tract infection patients.* J Clin Microbiol, 2011. **49**(1): p. 189-94.
21. Keeney, D., et al., *MarA-mediated overexpression of the AcrAB efflux pump results in decreased susceptibility to tigecycline in Escherichia coli.* J Antimicrob Chemother, 2008. **61**(1): p. 46-53.
22. Hartigan, J., *Clustering Algorithms*. 1975: Wiley.
23. Wilcoxon, F., *Individual Comparisons by Ranking Methods.* Biometrics Bulletin 1945. **1**(16): p. 80–83
24. Mann, H. and R. Donald, *On a Test of Whether one of Two Random Variables is Stochastically Larger than the Other.* Annals of Mathematical Statistics 1947. **18**(1): p. 50-60.
25. Stoesser, N., et al., *Evolutionary History of the Global Emergence of the Escherichia coli Epidemic Clone ST131.* MBio, 2016. **7**(2): p. e02162.
26. Kim, S.Y., et al., *Prevalence and characteristics of Escherichia coli sequence type 131 and its H30 and H30Rx subclones: a multicenter study from Korea.* Diagn Microbiol Infect Dis, 2016. **84**(2): p. 97-101.
27. Ruiz, C. and S.B. Levy, *Regulation of acrAB expression by cellular metabolites in Escherichia coli.* J Antimicrob Chemother, 2014. **69**(2): p. 390-9.
28. Aly, S.A., D.M. Boothe, and S.J. Suh, *A novel alanine to serine substitution mutation in SoxS induces overexpression of efflux pumps and contributes to multidrug resistance in clinical Escherichia coli isolates.* J Antimicrob Chemother, 2015. **70**(8): p. 2228-33.
29. Pantel, A., et al., *Modulation of Membrane Influx and Efflux in Escherichia coli Sequence Type 131 Has an Impact on Bacterial Motility, Biofilm Formation, and Virulence in a Caenorhabditis elegans Model.* Antimicrob Agents Chemother, 2016. **60**(5): p. 2901-11.
30. Webber, M.A. and L.J. Piddock, *Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli.* Antimicrob Agents Chemother, 2001. **45**(5): p. 1550-2.
31. Peirano, G. and J.D. Pitout, *Fluoroquinolone-resistant Escherichia coli sequence type 131 isolates causing bloodstream infections in a canadian region with a centralized laboratory system: rapid emergence of the H30-Rx sublineage.* Antimicrob Agents Chemother, 2014. **58**(5): p. 2699-703.
32. Opperman, T.J. and S.T. Nguyen, *Recent advances toward a molecular mechanism of efflux pump inhibition.* Front Microbiol, 2015. **6**: p. 421.
33. Ni, W., et al., *Effects of Efflux Pump Inhibitors on Colistin Resistance in Multidrug-Resistant Gram-Negative Bacteria.* Antimicrob Agents Chemother, 2016. **60**(5): p. 3215-8.