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Brief Communication

YnfA, a SMR family efflux pump is abundant in *Escherichia coli* isolates from urinary infection

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Abstract

A quantitative study was undertaken to determine the expression level of different efflux pumps in multi-drug-resistant (MDR) *Escherichia coli* isolates from urinary infection. We have determined the presence of different efflux pumps and measured the expression level of *tolC, mdfA, norE* and *ynfA* genes among 48 isolates by quantitative real-time PCR. The expression level of *tolC* and *ynfA* was constantly high and observed among 75-80% of isolates, whereas *mdfA* and *norE* were expressed occasionally. Our findings suggest that *ynfA*, a new SMR efflux pump gene family member increases the antibiotics' resistance in *E. coli.*

Key words: *Antibiotic resistance, efflux pumps, small multi-drug resistance family, ynfA*

Introduction

Escherichia coli is one of the most common human pathogens of urinary tract infections (UTI). There is a whole spectrum of antimicrobials available for the treatment of *E. coli* infections, but nowadays, most of them are ineffective to the multi-drug-resistant (MDR) pathogenic isolates. Several recent reports pointed out different emerging group of *E. coli* strains, which are both highly virulent and resistant to almost all group of antibiotics.[1] Now, it is hard to treat these resistant *E. coli* strains cases of UTIs.[2] *E. coli* becomes resistant to individual class of antibiotics by developing specific defense mechanisms. First, the organism may acquire genes-encoding enzymes, such as beta-lactamases, that inactivate the beta-lactam antibiotics before it can have an effect. Second, bacteria may acquire efflux pumps that extrude the antibacterial agents from the cell before it can reach its target site and exert its effect. Third, bacteria may acquire several genes for a metabolic pathway that altered bacterial cell walls and no longer contain the binding site of the antimicrobial agent, or altered the genetic sequence by mutations that limit the access of antimicrobial agents.[3]

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Of these resistance mechanisms, the upregulation of efflux pump systems in *E. coli* plays a vital role by decreasing the intracellular concentration of antibiotics and reducing their clinical efficacy. Presence of the efflux pumps are also explains high-level intrinsic resistance found in specific organisms. Physiologically, it appears to be part of the natural defense mechanisms of bacteria against toxic compounds that exist in the environment.[4] The overproduction of these efflux pumps are generally accompanied by an increase in resistance to two or more structurally unrelated antibiotics and significantly contributes to the emergence of MDR pathogens. Efflux pump systems are set of cytoplasmic membrane-bound proteins that pump out the incoming drug molecules by a cascade of action. There are five major families of efflux transporters reported including, major facilitator superfamily (MFS), multi-drug and toxic compound extrusion (MATE), resistance nodulation cell division (RND), small multi-drug resistance (SMR) and ATP-binding cassette (ABC).[5] In Gram-negative bacteria, the majority of multi-drug transporters share a common three-component organisation, a transporter located in the inner membrane (IM) functions with an outer membrane (OM) channel and a periplasmic accessory protein.[6] All three components of the efflux pumps, observed, belong to the same gene cluster, e.g. MexAB-OprM efflux complex from *Pseudomonas aeruginosa*, AcrAB-TolC efflux complex of *E. coli*. One of the most important efflux pump system present in *E. coli* is AcrAB-TolC, which is a three-component proton motive force-dependent multi-drug efflux system, function as active efflux pump that confers resistance to several antimicrobial agents, like solvents, dyes and detergents as well as antibiotics.[5] This study examines the influence of other efflux pumps along with AcrAB-TolC are responsible to develop high level of antibiotic-resistance mechanism in *E. coli*. To our knowledge, this is the first report on the involvement of

SMR family efflux pump to increasing MDR in clinical isolates of *E. coli*.

Materials and Methods

We determined the history of urinary-infected patients as suggested by physician at Sanjiban Hospital in Howrah district, India between Jan' 2012 to Dec' 2012. Patients were positive with other symptoms and urine characteristics of UTI. Isolation of clinical strains (total 48 strains) were done at Sanjiban Hospital and characterised biochemically. The reagents, media and antibiotics used in this study were procured from Himedia Pvt. Ltd (India). Post-biochemical characterisation, the antibiotics sensitivity test (AST) was performed using disk-diffusion method following CLSI guidelines.[7] During AST for each strain, a single colony was isolated from a pure culture and suspended in normal saline uniformly. The antibiotic disks were then dispensed using disk dispenser mark IV (Himedia) and incubated for 16-18 h at 37ºC aerobically. The inhibition zone were measured (diameter of the cleared zones) using Hiantibiotic ZoneScale-C (Himedia). In order to determine the involvement of different efflux pumps systems in isolated strains, primer were designed from three specific corresponding genes as *tolC*, *norE* and *mdfA*, which have the major roles in the regulation of three efflux pump families as RND MATE and MFS, respectively, and *ynfA*, new gene of SMR family efflux pump. All primer sets are used in this study listed in supplementary file [Table 1]. We have also determined the expression level of individual gene using semi-quantitative real time PCR (RT-PCR) from their cDNA. The conditions of normal PCR and RT-PCR are given details in Table 2. The analysis and quantification of RT-PCR data were made as described earlier.[8] For RT-PCR analysis, RNA was isolated using RNeasy minicolumns (Qiagen, Valencia, CA) following the guidelines provide with kit. Reverse transcription was completed using the ABI high-capacity reverse transcription kit (Applied Biosystems, CA). RT-PCRs were performed in triplicate on a 7000 sequence detection PCR system from Applied Biosystems using 2X power of SYBR green chemistry. The primer concentrations were equaled to 200 nM and melt curve analysis ensured that only a single PCR product was amplified.

In order to sequence the *ynfA* gene, PCR was carried out using the primers as forward: GCCCCCAGCCCGCAACAATG and reverse: TTGGATGCTTTTTGCCCTGGCTGT following the gene sequence of NCBI Reference no, NC_011601.1. PCR was performed with an initial denaturation for 1 min and 30 sec, followed by 30 cycles of denaturing at 94ºC for 45 sec, annealing at 55ºC for 30 s and extension at 72ºC for 1 min and a final extension step at 72ºC for 10 min. PCR products were analysed by 1.0% agarose gel electrophoresis, stained with ethidium bromide and visualised under UV-transilluminator. The PCR amplified DNA was eluted from gel, purified by QIA quick gel extraction kit (QIAGEN), sequenced using Bigdye terminator kit (ABI) in an automated DNA sequencer (ABI model 3100, Hitachi). The obtained sequence was aligned with ynfA gene sequence (NCBI Reference no, NC_011601.1) and confirmed the correct amplification [Figure 1].

Results

AST analysis of all strains revealed that almost 97% strains are resistant to beta-lactam antibiotics. In case of other structurally unrelated antibiotics, the resistance pattern of these isolates were found 60% to aminoglycosides, 65-72% to fluoroquinolones (except levofloxacin),

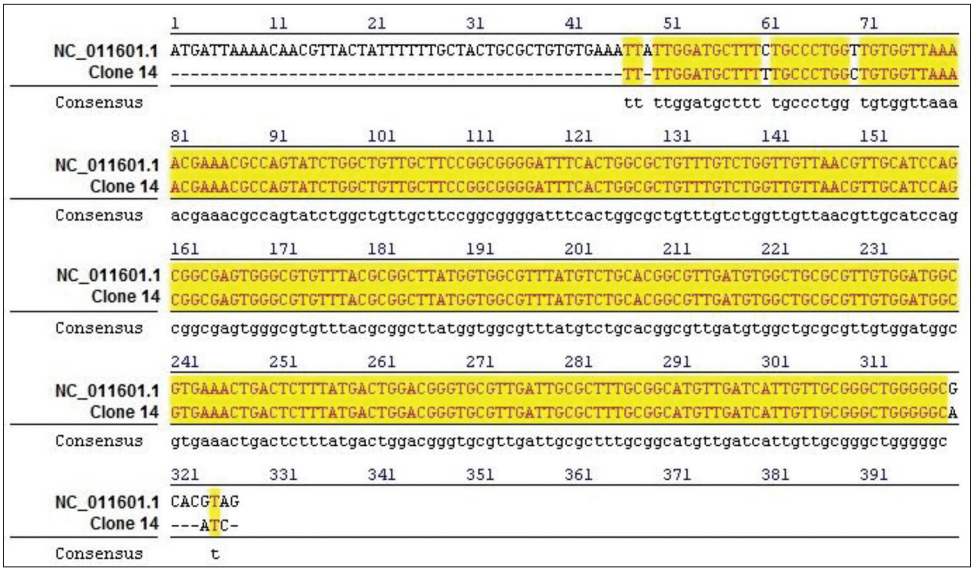

Figure 1: Alignment of ynfA obtained sequences from one clone with respective known sequence in NCBI database (NC_011601.1)

Table 1: List of primers used in this study

Primers to ensure the amplification of respective genes
tolC F: CGCCGATCGTGATGCTGCCT
tolC R: TCTGTTCCGGCGTTTGCGGT
mdfA F: ACCCGGTATGTTGGCCGTGG
mdfA R: GTCCGTTGCCCCCGTTCAGC
ydhE F: GATACCGTGATGGCGGGCGG
ydhE R: GCTGCCGACGTCAGGCCAAT
ynfA F: TGCTACTGCGCTGTGTGAAA
ynfA R: TGATCAACATGCCGCAAAGC
Primers to determine the semi quantitative expression level of respective genes
rpsL F_rt: GCAAAAACGTGGCGTATGTACTC (house keeping gene)
rpsL R_rt: TTCGAAACCGTTAGTCAGACGAA (house keeping gene)
mdfA F_rt: CATTGGCAGCGATCTCCTTT
mdfA R_rt: TTATAGTCACGACCGACTTCTTTCA
norE F_rt: CTGGCGGCAGCGGTAA
norE R_rt: TGCCATACAGACACCCACCATA
tolC F_rt: AAGCCGAAAAACGCAACCT
tolC R_rt: CAGAGTCGGTAAGTGACCATC
ynfA F_rt: TCAGTTTCACGCCATCCACA
ynfA R_rt: GCGCTGTTTGTCTGGTTGTT

Table 2: Amplicon length, primer efficiency and primer length of used primers

Primers name	Primer length (bp)	Amplicon length (bp)	Primer efficiency (%)
tolC F	20	1272	96
tolC R	20		
mdfA F	20	542	94
mdfA R	20		
ydhE F	20	1192	87
ydhE R	20		
ynfA F	20	275	95
ynfA R	20		
rpsL F_rt	23	104	97
rpsL R_rt	23		
tolC F_rt	19	100	95
tolC R_rt	21		
mdfA F_rt	20	103	97
mdfA R_rt	25		
norE F_rt	16	108	94
norE R_rt	22		
ynfA F_rt	20	101	97
ynfA R_rt	20		

65% to sulfadrug, 55% to Chloramphenicol, 55% to tetracyclines and 3-5% to carbapenems. The antibiotics resistance can arise in different ways in *E. coli*, such as alteration or preventing the antibiotic permeability into the cell, production of antibiotic hydrolyzing or modifying enzymes, modifications or alterations of the normal target of antibiotics, and ability to pump out the antibiotics after its entry into the cell.[8]

Initially, we have checked the normal amplification pattern of different genes corresponding to their relevant efflux pumps in all *E. coli* isolates. Further, gene expression level was quantified following RT-PCR analysis. The 30S ribosomal subunit gene, *rpsL* (housekeeping gene) was used as positive control [Table S1] and PCR-grade water served as a negative control. The threshold cycle (Ct) was determined for both housekeeping gene and genes of interest (efflux pump genes) from the same amount of template. Fold change of gene expression was determined as $2^{-\Delta\Delta Ct}$. The level of ynfA gene expression was observed between 2-6 folds equivalent to tolC gene. However, the expression level of norE lies between 1-2 fold. Interestingly, ynfA (SMR family member) and tolC (RND family member) genes are detected in all cases except very few strains. But *norE* (MATE family member) and *mdfA* (MFS family member) are detected in very selective cases those are resistant to floroquinolone antibiotics along with other classes which is usual.[9] Whereas, the AST data gives an unusual spectrum that strains are highly resistant to floroquinolones, aminoglycosides, beta-lactam and cotrimoxazole, where neither *norE* nor *mdfA* were detected, but the expression level of both, *ynfA* and *tolC* was very high. It seems that *ynfA* might have an additional role along with *tolC* or alone to increase the antibiotics resistance. The relative expression level of all tested genes are plotted, *ynfA* shows more prominent or upregulated simultaneously along with *tolC* [Figure 2]. Overall, the average *ynfA* expression levels of the fluoroquinolone-susceptible or fluoroquinolone-resistant isolates did not differ too much, but a real correlation was observed with *tolC* in expression pattern, which indicates a complex regulation between *tolC* and *ynfA* expression when required. The gene, *ynfA* of *E. coli* is the newest member of small multi-drug-resistance (SMR) gene family, identified in both Gram-negative and Gram-positive bacterial species. A *ynfA* in frame deletion mutant of an isogenic wild-type *E. coli* MG1655 exhibited no growth defect compared to its wild-type isogenic pair, but displayed antibiotic resistance mechanism. Moreover, the *ynfA* deletion mutant revealed the increase the carbenicillin susceptibility in *E. coli*. It was also hypothesised that *ynfA* expressed *E. coli* gains antibiotic resistance to penicillines and cephalosporins.[10] Our observations are also in same agreement that *ynfA* might play an important mechanism for the rapid acquired resistance of *E. coli* pathogens over-prescribed antibiotics.

Conclusions

It might be concluded that occasionally the ability to acquire antibiotic resistance in *E. coli* is efflux pump mediated and an excellent example of bacterial evolution. The

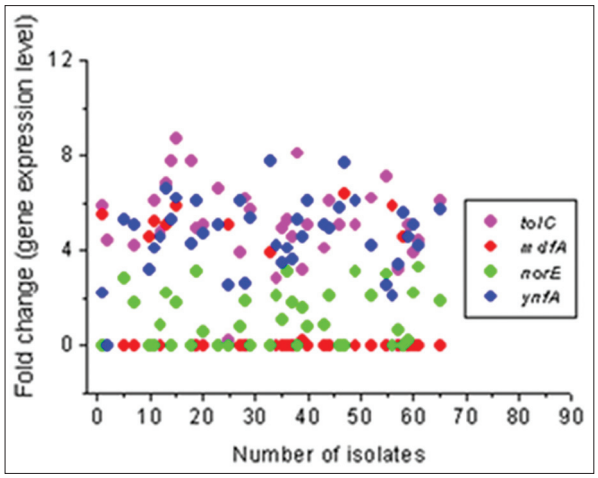

Figure 2: Correlation of expression level of representative genes from each efflux pump of isolate. The level of each gene was determined by qPCR and normalised to their expression level using housekeeping gene, rpsL to calculate the relative expression. Each point is the average of three experiments

gene, *ynfA*, a SMR gene family member, might be involved in alone or with *tolC* or any other way by complex regulation in which the initial susceptible bacteria become resistant.

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