Isolation, identification and detection of some acriflavine (Acr) Efflux pumps genes in uropathogenic E. coli isolates isolated from Iraqis patients

Ali R Mulakhudair¹, Khalid Abdulkadim Hadi²

¹ Department of Food Health and Nutrition, College of Food Sciences, Al Qassim Green University, Babil, Iraq.
² Department of Medical Laboratory techniques, Al-Safwa University College, Holy Karbala, Iraq

*Corresponding author:
Ali R Mulakhudair
Armulakhudair1@gmail.com

Abstract
Urinary Tract Infections (UTIs) are one of the most prevalent bacterial infections. During this study, which was extended from November 2018 to April 2019, 30 samples were collected from patients with confirmed UTIs at Al-Husseini Teaching Hospital, Karbala, Iraq. The results show that 24 samples of them were caused by E. coli, while the rest were caused by Staphylococcus aureus, Enterobacter aerogenes, Acinetobacter spp and Serratia liquefaciens. Antibiotic sensitivity test for the isolated E.coli strains using Vitek 2 compact system showed that all the isolates were sensitive to imipenem and meropenem, Carbapenems antibiotics. However, PCR results interestingly showed that all the isolates have AcrA and AcrB genes, important genes in the acriflavine (Acr) Efflux System. In other words, these bacterial isolates have the capability to shift to resistance ones when suitable conditions become available. The ramifications of results are vast and can help to explain the increasing frequency of carbapenems-resistance strains.

Keywords: carbapenems-resistance, acriflavine (Acr) Efflux System and uropathogenic E. coli.

How to cite this article: Mulakhudair AR, Hadi KA (2020): Isolation, identification, and detection of some acriflavine (Acr) efflux pumps genes in uropathogenic E. coli isolates from Iraqi patients, Ann Trop Med & Public Health; 23(S19): SP232119. DOI: http://doi.org/10.36295/ASRO.2020.232119

Introduction
Urinary Tract Infection (UTI), is one of the main causes of morbidity and mortality in the world and international organizations such as WHO and CDC, are continuously expressed their deepest concern regarding the ongoing increase in emerging of multidrug-resistance (MDR) bacteria. It has been recommended that investment in the discovery and development of new antibiotics and therapeutic agents to treat these MDR bacteria is immediately needed (1). The extensive use of antibiotics and antimicrobial substances is considered one of the main causes for the emergence of multi-drug resistant bacteria in diverse environments. Indeed, the induced selection has led bacteria to develop new defence strategies such as enzymatic inactivation of the target therapeutic compounds and decreasing the cell permeability. Also, the innate ability of bacteria to use a mechanism to extrude a wide range of substrates through expression-overexpression of efflux pumps has recently been intensively researched (2). Of interest, efflux pumps have drawn much attention in the recent years. Generally, efflux pumps are classified into five different family: the ATP Binding Cassette (ABC) superfamily; the major facilitator superfamily (MFS); the Multidrug and Toxic Compound Extrusion (MATE) family; the small multidrug resistance (SMR) protein family and the Resistance-Nodulation-Division (RND) family. These pumps can be specified for a single substrate or multiple substrates such as antibiotics, biocides, and heavy metals by facilitating the extrusion of these compounds out the bacterial cells (3-5).

During bacterial growth, E.coli generates secondary metabolites that can be toxic to the bacterial cells. To eliminate their toxic effects, bacteria have three ways to do that, which are inclusions these metabolites into a second metabolic pathway, degradation to non-toxic metabolites or extrusion of these metabolites into the external environments. Usually, Acriflavine (Acr) efflux system contributes to extrude toxic secondary metabolites and compounds that have a signaling role (6).
One group of antibiotics that can be extruded out of the bacterial cells using efflux pumps, is Carbapenems. Carbapenems are β-lactams with the broadest spectrum of activity against Gram-positive and Gram-negative bacteria; therefore, they are often used to treat multidrug-resistant infections and have been considered as “last-line agents” or “antibiotics of last resort” (7). Unfortunately, the recent emergence of multidrug-resistant (MDR) pathogens has frequently isolated worldwide and seriously threaten this class of lifesaving drugs (8). To overcome the microbial resistance caused by these active efflux pumps, concerted efforts are required to better understand the fundamentals of antibiotics efflux mechanisms. Understanding the distribution of the RND pump among carbapenems-sensitive bacteria represents a serious attempt to predict the development of bacterial resistance to the carbapenems and would help to rationalize the prescription of this group of antibiotics. The aim of this study was to isolate the pathogenic bacteria from the urine samples of patients, who were diagnosed with urinary tract infections with different bacterial species. The study also aims to map the antibiotic sensitivity profile and detect the existence of Acriflavine (Acr) efflux pump genes in the isolated strains.

**Materials and methods**

**Sample collection**

A total of 30 urine samples collected from patients with confirmed UTIs at Al-Husseini Teaching Hospital, Karbala, Iraq. Patients were asked to clean their external genitalia with disinfectant and collect midstream urine in the sterilized cap. Samples were kept in an ice bag and directly transported to the microbiological laboratory.

**Urine culturing**

Urine samples were cultured on Nutrient agar and blood agar and incubated overnight at 37°C. Significant growth was evaluated as ≥ 105 colony-forming units CFU/mL of midstream urine.

**Bacterial Identification**

Nutrient agar and 5% sheep blood agar were used to select the pure colonies of the isolates. Preliminary identification of each isolate was done using gram stain, catalase, and oxidase. Subsequently, selective media such as Eosin methylene blue agar (EMB) was used if the isolates were suspected as *E. coli*. Further, Identification was performed with the Vitek 2 compact (bioMerieux Inc. USA) system using GP ID REF21342 (identification-Gram-positive bacteria) and GN ID REF21341 (identification-Gram-negative bacteria) cards. All the test procedures were followed according to the manufacturer’s instructions. AST-N291 (Gram-negative bacilli) Cards were used to determine antibiotic susceptibility and the results were interpreted using Vitek 2 compact software version 07.01.

**PCR detection**

The DNA was extracted by GeNet Bio company (Korea, Cat. no. K-3000) and used as a template for polymerase chain reaction (PCR). The acrA and acrB genes were amplified for all *E. coli* strains by means of PCR using the primer sets and thermal cycling conditions described in Tables 1.

Table 1: List of PCR primers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′-3′)</th>
<th>Length of amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>acrA</td>
<td>F: TTGAAATTACGCTTCAGGAT R: CTTAGCCCTAACAGGATGTG</td>
<td>189</td>
<td>(9)</td>
</tr>
<tr>
<td>acrB</td>
<td>F: CGTACACAGAAAGTGCTCAA R: CGCTTCAACTTTGTTTTCTT</td>
<td>183</td>
<td>(9)</td>
</tr>
<tr>
<td>gapA</td>
<td>F: ACTTAGGAGCATCAAGAC R: AGTTTCACGAAGTGTGCTT</td>
<td>170</td>
<td>(9)</td>
</tr>
</tbody>
</table>

**Results and discussion**

**Isolation and Identification**

The results show that urinary tract infections are mainly caused by gram-negative bacteria, but gram-positive pathogens may also be involved. According to the results, *E. coli* was isolated from 80% of the samples, which matches with global numbers (10). More than 95% of uncomplicated UTIs are mono-bacterial infections. *E. coli* is the common pathogen for uncomplicated UTIs (75%–95%), followed by *Staphylococcus aureus, Enterobacter faecalis*, and *Proteus mirabilis* (10) (Table 2).
According to statistics from the Center for Disease Control (11), up to 60% of women have at least one symptomatic UTI during their lifetime. Young, sexually active women 18–24 years of age have the highest incidence of UTIs. In men, however, the prevalence of UTIs is significantly lower than in women and it can primarily happen in males with abnormalities in their urological structure or elders. This could be due to the anatomy of women where they have a short distance from the urethra to the anus and the urethral opening to the bladder. In addition, sexual intercourse can increase the possibility of cystitis (bladder infection). The later comes in line with the results were 20 samples out of 24 cases who had E. coli infections, were women (Table 3).

**Table 2**: Bacteria distribution and their prospective frequency.

<table>
<thead>
<tr>
<th>Causative bacteria</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td>24 (80%)</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>6.6%</td>
</tr>
<tr>
<td><strong>Enterobacter aerogenes</strong></td>
<td>6.6%</td>
</tr>
<tr>
<td><strong>Acinetobacter SPP</strong></td>
<td>3.3%</td>
</tr>
<tr>
<td><strong>Serratia liquefaciens</strong></td>
<td>3.3%</td>
</tr>
</tbody>
</table>

**Table 3**: E coli infection distribution according to gender.

<table>
<thead>
<tr>
<th>UTIs infections caused by E.coli</th>
<th>Gender</th>
<th>Sample</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Female</td>
<td>20</td>
<td>(83.3%)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>4</td>
<td>(16.7%)</td>
</tr>
</tbody>
</table>

Vitek 2 compact was used to identify bacterial species as well as screen the susceptible of the isolated bacteria for the commonly used antibiotics in Iraqi hospitals and private clinics. The tested antibiotics range from Beta-lactams, macrolides, aminoglycosides, and Extended-spectrum beta-lactam, carbapenems. Figure 2 shows the microbiological report of the Vitek2 compact for the covered antibiotics and their obtained interpretation, R for resistance and S for sensitive.
Figure 2: Vitek 2 compact report, showing the identified E. coli and its carbapenem-sensitive bacteria

Figure 2 shows an example of the isolated strain of E.coli and it can clearly be seen that the strains are resistant to Ticarcillin, Piperacillin, Ceftazidime, and Aztreonam, while, it is sensitive for both Imipenem and Meropenem, carbapenems antibiotics. The sensitivity to Imipenem and Meropenem was further confirmed using the disc diffusion method, using 10 mcg concentration for both antibiotics. It would be expected that such a strain does not have mechanisms to resist these antibiotics, therefore, it was sensitive! But is it? Therefore, the next question asked in the study, was that are there any genes from Acriflavine (Acr) efflux pump that exist in these strains? The reason to seek for these genes is that acriflavine efflux pumps can provide resistance to a broad range of antimicrobials such as antibiotics, biocides, and heavy metals by facilitating the extrusion of these compounds out the bacterial cells and one of the most important groups of these antibiotics is carbapenems, “last-line agents” or “antibiotics of last resort” (7). Hence, the next section was dedicated to detecting the existence of Acr efflux pump genes in the isolated E.coli strains. gap gene is a housekeeping gene in E.coli that is used in this study to confirm the identification of the Vitek 2 compact system that these isolates belong to E.coli (Figure 4).

Detection of Acriflavine (Acr) efflux pump genes in the isolated strains

The current study focused on exploring the potential mechanism that can be developed to resist carbapenems through Acriflavine (Acr) efflux pump. B-lactams and carbapenems can be overcome by antimicrobial inactivation by β-lactamase enzymes; and extended-spectrum beta-lactamase (ESBL), but importantly, bacteria can also resist these groups by Acriflavine (Acr) Efflux System (11). Therefore, as a part of the current study, acrA and acr B, are two of the main genes in the Acr efflux pump, are detected. Detection of the Acr efflux pump genes is very important as it can help to understand the resistance mechanism to last-line agents” or “antibiotics of last resort” (7). This pump is composed of AcrA and AcrB and TolC as functional genes, while AcrR, Acr S are used to regulate expression of Arab (12). All the isolated E. coli strains interestingly show a sensitive profile for carbapenem antibiotics. These sensitive isolates have the probability to become resistance ones and most of the naturally sensitive bacteria become resistant due to certain genetic chromosomal changes (in 10-20% of the cases) or extrachromosomal (less than 80% of the resistance cases), a process that is called genetic resistance (12).

Both genes (acrA and acrB) genes present in all the isolates (Figure 3). These genes are part of essential genes (chromosomal) that situated in the structure of the chromosome and therefore, the acquired antimicrobial resistance are emerged by the selection, consequently to the exposure to antibacterial drugs or due to the emergence of variants resistant inside some previously sensitive species. The exposure to antibiotics is not the cause in itself for the manifestation of the bacterial resistance to drugs. The expression of this efflux system in
E. coli is strongly regulated on transcription levels to facilitate the adaptation of bacteria to external stimulation \cite{13}. Both AcrR and AcrS are local transcription factors, regulate the expression of the AcrAB-TolC efflux system, and loss of AcrAB-TolC efflux system function could be a consequence of overexpression of either of these genes \cite{14,15}.

The bacterial changes allowing the bacteria to resist antibiotics naturally appear as a consequence of the mutation or as a result of the genetic combination. The importance of the current study is to follow the evolution of antibiotic resistance in the bacterial population in order to prevent and repress the emergence of multidrug-resistant strains of E. coli that can still be treated with antibiotics.

\textbf{Figure 3:} PCR products. (A) acrA gene in the isolated bacteria. (B) acrB gene. First lane shows the ladder and other lanes show PCR products.
Conclusion
The widespread use of antimicrobial agents has increased the pressure exerted in various environmental fields. The most common pathogen for uncomplicated UTIs is E. coli (75%–95%), followed by Staphylococcus aureus, Enterobacter faecalis, and Proteus mirabilis. The prevalence of UTIs in men is significantly lower than in women and more than 80% of E. coli isolated in this study was from females in their reproductive age, while only 16% was isolated from males. The data collected so far allow us to assume that the resistant profile is not only due to the intensive use of antimicrobial but also because of the innate ability of bacteria to use a mechanism to extrude a wide range of substrates. It is therefore important to characterize the flow pumps and their mechanism of operation in different bacteria to draw a conclusion about the mechanism of action of this pump and the environmental conditions that help to make it function properly in the resistant bacteria. Understanding how to flow mechanisms contribute to quorum sensing is critical to developing new molecules that can target key genes or proteins in this chain.

References


