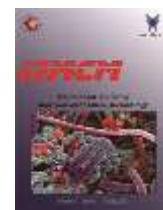




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### Evaluation of Efflux pump activity among Uropathogenic *Escherichia coli* and *Klebsiella pneumonia* multiple- Drug Resistance isolates

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#### ABSTRACT

Antibiotic resistance is a phenomenon in which antibiotic used to treat bacteria becomes useless due to resistance mechanism. Increased drug resistance and occurrence of Multiple Drug Resistance in bacteria specificity nosocomial and Urinary Tract Infection bacteria has reduced the possibilities of treating these infectious diseases. Efflux pumps are one of the major mechanisms of MDR in bacteria which effluxes out the drugs accumulated. Various MDR phenotypes that confer active protection against environmental toxic compounds by efflux mechanisms have been described in Enterobacteriaceae. The aim of this study was evaluation of efflux pumps in Uropathogenic *Escherichia coli* and *Klebsiella pneumonia* MDR isolates. 41 UPEC and 37 *Klebsiella* MDR strains that were isolated from urine samples and identified by biochemical tests were determined by disc diffusion method according to CLSI. The ETBr-agar cartwheel method with different concentrations of Ethidium bromide was used to assess the presence of efflux activity. Our results showed that among UPEC isolates 39%, 9.8% and 51.2% had High, moderate and no efflux activity. Among *klebsiella pneumonia* isolates 27%, 24.32% and 48.64 % had High, moderate and no activity, respectively. Also the frequency of UPEC bacteria with over expression of efflux pumps was 80% but for *Klebsiella pneumonia* isolates 52% was reported. UPEC isolates had more actively efflux pumps than *Klebsiella* isolates. Both bacterial isolates had ability to use efflux antibiotics out. The Et-Br cartwheel method could be used as a simple method for determining the phenotypic activity of efflux pump in UPEC and *K. pneumoniae* MDR isolates.

#### 1. Introduction

Bacterial Antibiotic resistance is the most important health problems around the world from previous years. Increased bacterial resistance to antibiotics makes complicate successful treatment of infections (Henriques Normark & Normark, 2002; Piddock, 2006; Theuretzbacher, 2012). So that in many cases recurrence of infections is occurred after treatment. Multi-drug resistance (MDR) is a common form of clinical resistance and is

defined as the ability of organism to survive in a variety of lethal dose of different drugs or chemicals. Today, gram negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Escherichia coli* are important MDR bacteria (Okusu et al., 1996; Piddock, 2006; Viveiros et al., 2005). Studies have shown that the emergence of multi-drug resistant strains of uropathogenic *E. coli* (UPEC) and *K. pneumonia* that cause urinary tract infections

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are increasingly. Multi-drug resistance can be created by the simultaneous presence of several unique mechanisms of resistance that the efflux pumps are one of them. Efflux pumps that are capable of extruding one or more unrelated antibiotics before reaching their targets have been demonstrated in all bacterial species studied to date (Bohnert & Kern, 2005). In most gram-negative bacteria efflux pumps are energy-dependent membrane that effectively efflux antibiotics out of bacteria via Triple Complex include inner membrane pump, periplasmic protein and outer membrane channel (Nikaido, 1994; Saier Jr & Paulsen, 2001) and their Multi drug resistance mechanism is due to the over expression of these efflux pumps such as the AcrAB-Tol C dependent RND family (Pumps et al., 2011).

The most antibiotics that are effluxed by these systems are fluoroquinolone, tetracycline, Chloramphenicol and beta-lactams (Mazzariol et al., 2002). There is a need to develop and implement new and improved methods for a real-time and quick identification of efflux mediated multi-drug resistant (MDR) phenotypes (Viveiros et al., 2008a; Viveiros et al., 2008b). There are number of different classes of such efflux pumps that most of them have utilized ethidium bromide (EB) as a substrate. Consequently, demonstration and evaluation of efflux pump activity of over twenty-two species of bacteria have involved the extrusion of EB when the pump is active (Katz, 2005; Li & Nikaido, 2004; Truong-Bolduc et al., 2005). Other methods, based on radio-labeled antibiotics, have also been employed for the demonstration of efflux pumps which extrude the given antibiotic when active and retain it when their activity has been inhibited (Ghisalberti et al., 2005). Cartwheel method is a fluorometric assays that is the passage of EtBr across the cytoplasmic membrane and its subsequent intracellular accumulation inside the bacterial cell (Pumps et al., 2011). EtBr traverses the bacterial cell wall (in the case of Gram-negative bacteria *via* porin channels) and once inside, it can be concentrated to a point where it fluoresces when excited by ultraviolet (U.V.) light. Efflux pumps of MDR bacteria recognize this substrate and are able to extrude it to the medium (Amaral et al., 2011). The cartwheel method, is instrument free, agar-based method utilizes EtBr for the

demonstration of efflux pump activity in bacteria (Martins et al., 2011). The aim of this study was conduction of cartwheel method for Comparison of phenotypic activity of efflux pump among uropathogenic *E. coli* and *K. pneumonia* multi-drug resistant isolates.

## 2. Materials and Methods

### 2.1. Clinical strains and bacterial diagnosing

The study was performed on 89 uropathogenic *E. coli* and 57 *K. pneumonia* isolates from urine specimen of patients with UTI who were admitted from May 2015 during 60 days to the Milad hospital in Tehran. The specimens were directly inoculated on MacConkey agar and EMB agar plates and after overnight incubation at 37°C, the biochemical identification was carried out by bacterial culturing on TSI agar, SIM medium, Simon Citrate agar and MRVP broth.

### 2.2. Determination of MDR strains

At first Antibiotic susceptibility of all strains was conducted by the Kirby-Bauer method, following the Clinical and Laboratory Standards Institute (CLSI 2015) guidelines. Antibiotics selected was Ampicillin (Am; 10 µg), Trimethoprim Sulfamethoxazole (SXT; 25 µg), Ciprofloxacin (CP ;5 µg), Tetracycline (TE, 30 µg) and Nalidixic acid (NA; 30µg ). Results were evaluated as resistant (R), Intermediate (I) and Sensitive (S) according to the CLSI breakpoints. The MDR isolates was defined as resistance to three or more different classes of antibiotics. *E. coli* isolates inserted in this study have a confirmed MDR phenotype (Maleki et al., 2016). *E. coli* ATCC 35218 and *K.pnoemoniae* ATCC 700603 were used as quality control strains.

### 2.3. Evaluation of efflux activity by EtBr-agar cartwheel method

All the MDR isolates were evaluated by the EtBr-agar cartwheel method. In a first approach it is necessary to establish the conditions in which the assays should be performed. Therefore, minimum fluorescence values shown by the bacterial isolates should be determined. Reference strains were used in each of the assays for comparison terms and to help

determine the fluorescence base-line of each isolate. An EtBr stock solution was prepared in distilled water at a concentration of 50 mg/ml, stored at 4°C and protected from light. Overnight culture of bacterial strains was grown in 5 ml of appropriate liquid broth until adjusted with PBS to 0.5 of a McFarland standard. Agar plates containing EtBr concentrations ranging from 0 to 2.5 mg/l were prepared on the same day of the experiment and protected from light.

Cultures were swabbed on EtBr-agar plates starting from the centre of the plate and spreading towards the edges, as indicated by the arrowheads shown in Figure 1. Each plate that consists of 12 swabbed isolates (8 swabbed isolates in this study) and included at least one reference strain that served as a comparative control (Figure1). Positive control had highest rate of fluorescence (indicated Inactivity efflux) and negative control had no fluorescence rate (indicated activity efflux) (Peleg et al., 2007). The swabbed plates were then incubated at 37°C for 16 h and examined under a suitable source of UV transilluminator. The concentrations of EB present in the agar that is to be streaked or swabbed with the bacterial inoculum must be well below those that have an effect on growth. Furthermore, because EB will fluoresce under

UV light, the selection of the maximum concentration of EB in the agar must be one that is below the resolution of the UV detector employed. *Staphylococcus aureus* ATCC25923 used as negative control strain.

### 3. Results

#### 3.1. Antibiotic susceptibility of Uropathogenic *E. coli* and *Klebsiella pneumonia* isolates and MDR isolates determination

The results of antibiotic susceptibility among 89 Uropathogenic *E. coli* strains showed that highest resistance was belong to ampicillin (98.2%) and Nalidixic acid (68.53%) respectively. Antibiotic resistance to Co-trimoxazol (52.8%), tetracycline (49.43%) and ciprofloxacin (48.31%) was reported (Table 1). Among 57 *Klebsiella pneumonia* isolates the highest rate of resistance was to ampicillin (95.87%), tetracycline (86.73%) and Nalidixic acid (64.91%). Antibiotic resistance to Co-trimoxazol 54.38%) and ciprofloxacin (52.63%) was reported (Table 1). The frequency of multiple drug resistance (MDR) among Uropathogenic *E. coli* and *K. pneumonia* isolates was 41(46%) and 37(64.9%).

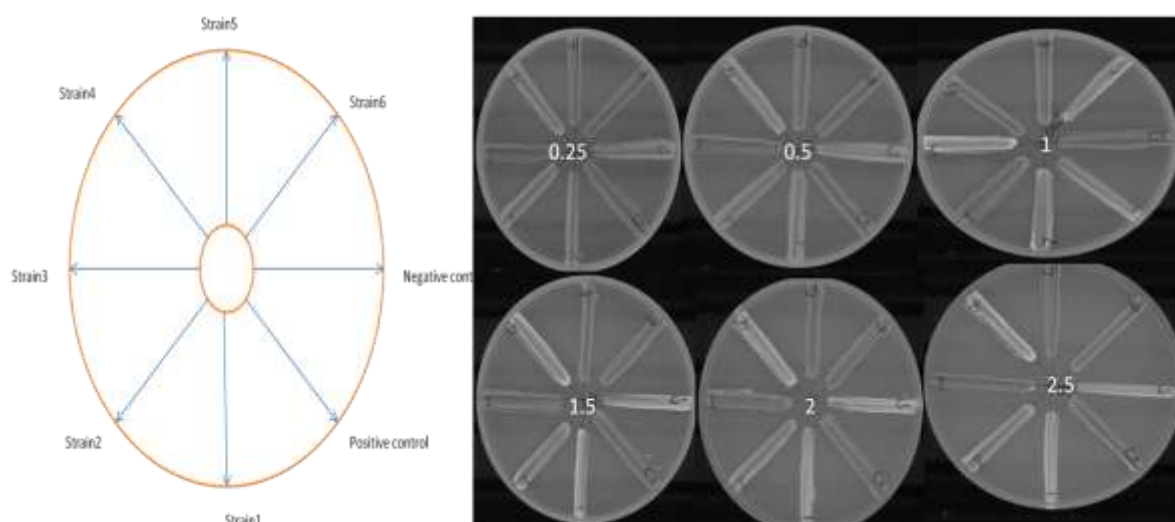
**Table 1.** Antimicrobial susceptibility patterns of Uropathogenic *E. coli* and *Klebsiella pneumonia* isolates

Antibiotic	Uropathogenic <i>E. coli</i> (N=89)			<i>Klebsiella pneumonia</i> (N= 57 )		
	Sensitive (%)	Intermediate (%)	Resistance (%)	Sensitive (%)	Intermediate (%)	Resistance (%)
AM (Ampicillin)	1.72%	0%	98.2%	4.22%	0%	95.87%
SXT(Co-trimoxazole)	47.19%	0%	52.8%	45.61%	0%	54.38%
CP (Ciprofloxacin)	48.31%	3.37%	48.31%	47.36%	0%	52.63%
TE (Tetracyclin)	40.44%	10.11%	49.43%	31.6%	0%	68.4%
NA( Nalidixic acid)	20.22%	11.23%	68.53%	35.08%	0%	64.91%

#### 3.2. Efflux pump activity by cartwheel method

After illumination of agar plates containing EtBr concentrations ranging from 0 to 2.5 mg/l by UV transilluminator the minimum concentration of EtBr (MCEtBr) that produced fluorescence of the bacterial mass were recorded. The strains that its Minimum concentration of ethidium bromide produced

fluorescence was 0.25 had inactive efflux pumps (Figure1). The strains that its Minimum concentration of ethidium bromide produced fluorescence was not 0.25 considered as moderate activity and strains that in none of ethidium bromide concentrations were fluorescent had over expression of efflux activity (Figure1).



**Figure 2.** Fluorescent Pictures of agar plates containing 0.25-2.5 mg/l ethidium bromide concentration with various bacterial strains, C-: Control negative, C+: Control positive.

Results showed that among 41 UPEC MDR isolates 16 (39.02%) had high or over expression efflux activity, 4 (9.75%) had moderate efflux activity and 21 (51.21%) had no efflux activity (Figure2,a). Among 37 *K. pneumonia* MDR isolates 10 (27.03%) had high or over expression efflux activity, 9 (24.32%) had moderate efflux

activity and 18 (48.64%) had no efflux activity (Figure2,b). Among 20 UPEC MDR isolates, 16 (80%) had efflux pumps with over expression but for 19 *K. pneumonia* MDR isolates 10 (52%) had efflux pumps with over expression.

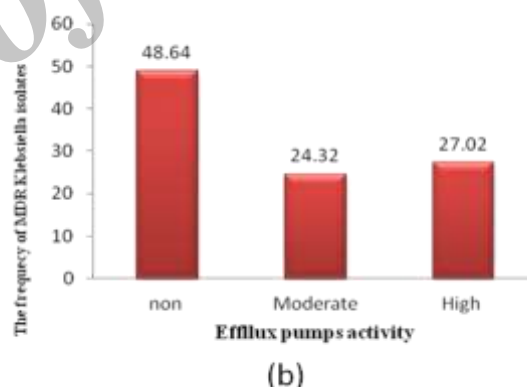
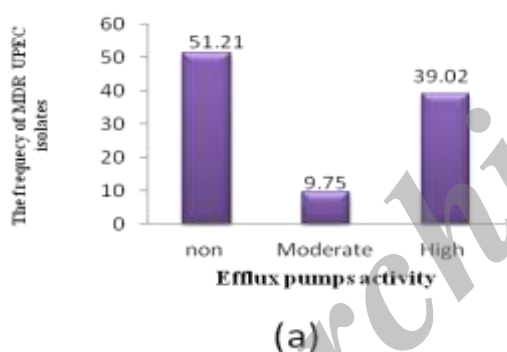


Figure2: The frequency of UPEC (a) and *K. pneumonia* (b) MDR isolates according to their efflux pump activity

#### 4. Discussion

The role of bacterial efflux systems in MDR occurrence is an important subject that has been widely discussed in recent years (Piddock, 2006; Tenover, 2006; Theuretzbacher, 2012). RND efflux pump family is the most important efflux systems which play an important role in the development of multi-drug resistance in bacteria by releasing different classes of antibiotics to outside of the bacterial cell (Peleg et al., 2007).

Since bacterial multi-drug resistance could be result of over expression of their efflux systems which are used for disposal of unwanted antibiotics before to reach the target location, it is necessary to development and implement of innovative and approved methods for rapid identification of resistant phenotypes by efflux (Viveiros et al., 2008a; Viveiros et al., 2008b). The cartwheel based on EtBr- agar is a simple method for rapid screening of MDR bacteria upon efflux pump (Martins et al., 2006) EtBr is a compound that inserted to the bases in DNA and

routinely used as a marker fluorescence in molecular biology (Martins et al., 2011). In the present study, The frequency of multiple drug resistance (MDR) among Uropathogenic *E. coli* and *K. pneumonia* isolates was 41(46%) and 37(64.9%). cartwheel method was used for determining the efflux pump activity for identify the phenotypic drug-resistant UPEC and *K. Pneumoniae* isolates. The results showed that among 41 UPEC MDR isolates, 16 (39%) isolates which were not fluorescent in all EtBr concentrations considered as high active based on efflux pumps, 4 (9.8%) isolates active and 21 (51.2%) isolates that fluorescent in 0.25 EtBr minimum concentration considered as non active based on efflux pumps. Among 37 *Klebsiella pneumonia* isolates, 10 (27%) were considered as high active, 9 (24.32%) active and 18 (48.64%) non active based on efflux pumps. Also totally among 20 active UPEC MDR isolates, 16 (80%) were over expression based on efflux pumps that was more than 10 (52%) *K. pneumonia* MDR isolates among 19 active isolates. The absence of EtBr fluorescence among some MDR isolates and control resistant isolate suggested that strains contains efflux pump and pheotypically active, effluxed out EtBr from bacterial cell. AlsoThe relative similarity between the frequency of MDR isolates and isolates with actively pumps in both bacteria showed that the cartwheel is a simple method for determining the phenotype of MDR.

Marta Martins in 2006 during a study of Et-Br agar method on six standard strains (*E. coli* K-12 AG100, *E. coli* K-12 AG100-Tet, *Enterobacter aerogenes* EA27, MRSA COL 10, MRSA HPV107 and *S. aureus* ATCC25923) reported that there is a different between minimum concentration of Et which fluoresced among different bacteria that is depend on the activity of efflux pumps (Martins et al., 2006). In 2013, Martins showed that the cartwheel is a suitable method for determining efflux pumps activity in both gram negative and gram positive bacteria (Martins et al., 2011). In a study by Ana Martins et al in 2012, they used Acridine Orange for determining of efflux pump activity like cartwheel method instead of Ethidium bromide because it's non toxicity (Martins & Amaral, 2012). Costa et al in a study through 2011 and 2013 years, evaluated the efflux pump activity of 52 ciprofloxacin resistant *S.aureus* isolates.

12 isolates that showed fluorescent in highest Et concentration had more activity than ATCC 25923 control negative strain. 31 isolates that showed fluorescent in minimum Et concentration were not active and 7 isolates had intermediate efflux pump activity (Costa et al., 2011). In a study by Rana 2015 by cartwheel assay, a total of 8 MDR *K. Pneumoniae* strains were analyzed for the presence of efflux pumps. EtBr fluorescence was not observed among selected resistant isolates of *K. pneumonia*, control strains of *K. pneumoniae* that had AcrAB efflux pump overexpresse and also in *K. pneumonia* wild type strains with AcrAB efflux pump. Similar results were observed for *P.aeruginosa* (Rana et al., 2015). Pirbonye In 2016 evaluated Phenotypic activity of efflux pumps in *P.aeruginosa* isolates by Ethidium bromide (Et-Br) agar based Cartwheel method .Based on the results of cartwheel method 44 (78.6%) isolates were detected positive for efflux pump activity (Pirbonyeh et al., 2016).

Totally in this study it was shown that the Et-Br cartwheel method could be used as a suitable and reliable method for determining the phenotypic activity of efflux pump in UPEC and *K. pneumoniae* MDR isolates. Also results showed that UPEC MDR isolates maybe have stronger activity based on over expression efflux pumps than *K. pneumonia* MDR isolates. In this study it was not shown that this activity is used for bacteria to extrude which antibiotics? For this purpose, Further studies are recommended.

## Conclusions

Although high-level resistance of bacteria may not occur as a result of efflux pumps alone, but the association of over-expression of specific genes among highly resistant clinical isolates cannot be ignored (O'Regan et al., 2009). So we must notice that the intrinsic resistance to antibiotics of some isolates may be extremely due to their efflux systems. Therefore the role of efflux pumps in resistance of some clinical strains should be considered as one of the parameters in the design of future antimicrobial or other active ingredients.

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