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Phenotypic Diagnosis of Efflux Pump of *Escherichia coli* Isolated from Urinary Tract Infections

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Abstract: This study aimed to phenotypically detection of efflux pump in *Escherichia coli* isolated from urinary tract infections of Iraqi women and their relationship with antibiotic resistance. This study included taking 100 samples of urinary tract infection in Iraqi women. All samples were subjected to primary identification test by using various methods **(**cultural characteristics, biochemical test, VITEK device 2, molecular method). Only 53 sample (58.09%) were specific for *Escherichia coli* while other bacterial isolates were *Staphylococcus aureus* 20 isolates (22.2%), *Klebsiella Pneumonia* 9 isolates (10 %), *Proteus mirabilis* 6 isolates (6.66%), *Pseudomonas aurogenosa* 2 isolates (2.22%) and 10 sample did not have any growth on culture media. The phenotype of the efflux pump in *Escherichia coli* was detected using ethidium bromide (EtBr-CW) method, and the results showed that 25 isolates (47.2%) were contained efflux pump. The antibiotic sensitivity test used to detect the sensitivity of bacteria to nine antibiotics showed that *E. coli* isolates were more resistant to the antibiotic Erythromycin with a percentage of (100%), while the bacteria were sensitive to both antibiotics Nitrofurantoin, Ceftriaxone with a percentage of (52.83%), (50.94%) respectively. The EtBr- agar Cartwheel method is a good screening marker for efflux activity. It is easy to perform, less time-consuming and can be used to screen large numbers of bacterial strains, thereby facilitating the rapid identification of isolates displaying an MDR phenotype.

Keywords: *E.coli*, EtBr agar, efflux pump.

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Introduction

Urinary tract infections occur as a result of the growth of many microbes and they are common in humans, the microbes cause such infections included bacteria, fungi, and parasites (1, 2). *Escherichia coli* are a Gram-negative, rod-shaped, flagellated and facultative anaerobic bacterium of the family *Enterobacteriaceae* responsible for urinary tract infections (UTIs) in human. Beside that it is responsible for many other serious infections including diarrheal infections, intra-abdominal and soft tissue infections, meningitis, pneumonia and rarely endocarditis (3). *E. coli* bacteria resistance that causes urinary tract infections represents a major threat to public health because it is resistant for different groups of antibiotics (4). Various mechanisms of resistance developed by the bacteria target one or more of these abilities to prevent the antibacterial from exerting its inhibitory or bactericidal action. Among these mechanisms, the over-expression of efflux pump systems, which consists of extruding the

antibacterial molecules out of the bacterial cell, thereby reducing their concentrations to an insufficient value for a proven effect, is the main mechanism giving rise to multi drug resistance (MDR) (5). All microorganisms, with a few exceptions, have highly conserved DNA sequences in their genome that are transcribed and translated to efflux pumps. Efflux pumps are capable of expelling a variety of different toxic compounds out of cells,such as antibiotics, heavy metals, organic pollutants, plant-produced compounds, quorum sensing signals, bacterial metabolites and neurotransmitters via active efflux, which is vital part for xenobiotic metabolism (6).The first line of protection for antimicrobial bacteria is called active efflux (7).Some efflux proteins bestow resistance to just a narrow spectrum of antibiotics (e.g., *TetM* in *E. coli* against tetracyclines or *MexCD-OprJ* of *Pseudomonas aeruginosa* against fourth generation cephalosporins) while others expel out a wide range of antimicrobials (e.g., *AdeABC* of *Acinitobacter baumanii*, *NorA* of *Staphlococcus. aureus* or *AcrAB-TolC* of *E. coli*) (8). Efflux pumps have been categorized into five different families (9) based on three criteria: the amino acid sequence identity, the energy source required to drive export and the substrate specificities (9,10). The flow pumps are divided into five families. Major Family Facilitator Super Family (MFS), Small Multidrug family resistance family (SMR), Multidrug and Toxic Efflux Family (MATE), Family ATP-Binding Cassette Family (ABC) and Resistance - Nodulation - Division (RND) Family. Resistance - Nodulation - Division Family including *AcrAB-ToIC*, which is most common in *E. coli* bacteria that consists of three proteins, the inner membrane protein *AcrB*, which is encoded by the gene *acrB* and proteins Scattered in the plasma vacuum *acrA* encoded by the *acrA* gene and the *ToIC* channel located in outer membrane (11). To investigate the prevalence of efflux pumps in multidrug resistant *E. coli*.

Materials and methods

Bacterial isolates collection

From November 2021 to March 2022 a total of 53 samples of *E. coli* were collected from urinary tract infection of Iraqi women after performing culturing method, biochemical test, VITEK2 system and molecular method.

Antibiotic susceptibility test

E. coli isolates were transferred with sterile swabs to tubes of sterile saline to achieve turbidity equal to that of a 0.5 McFarland standard. Cell suspensions and sterile swabs were used to inoculate the surface of Mueller-Hinton agar plates. Antibiotic resistance was determined by the Kirby-Bauer method. Inoculated plates were incubated for 24 h at 37°C, after which the diameters of inhibition zones were measured in millimeters following the manufacturer's instructions to assess resistance, intermediate, or susceptibility.

Molecular identification of *E. coli*

The genomic DNA of *E. coli* isolates was extracted by using a commercial genomic DNA purification kit (Promega, USA) In this study, DNA sample of *E. coli* isolates has been selected to detect the *uidA* diagnostic gene. The PCR products have been confirmed by the analysis of the bands on gel electrophoresis and by comparing their molecular weight DNA ladder.

Primer selection

The primer was used for detection of *uidA* gene. Table (1) (12).

Table (1): Primer sequences *uidA* gene

Primer name	Sequence (5^{-} - 3^{-})	Product (bp)
uidA	F-CATTACGGCAAAGTGTGGGTCAAT	658
	R-CCATCAGCACGTTATCGAATCCTT	

The EtBr-agar cartwheel method for morphological detection of efflux pumps

The dilution of all bacterial isolates was prepared using sterile physiological salt solution and measure turbidity with Instrument Mcfarland Standard. This examination was carried out on bacterial isolates that possess characterization of antibiotic resistance by adopting the method cartwheel agar-EtBr, using the medium of trypton soy agar and ethidium bromide dye in different concentrations according to mentioned in (13) as follows:

1. Various concentrations of ethidium bromide dye (5, 10, 15, 20, 25) (µg/ml) were prepared by adding to the medium of the trypton soy agar after sterilization and cooling it
2. The media was shaken well, and after sterilization, it was poured into sterile dishes that were previously divided radially store at refrigerator temperature until use.
3. The bacterial suspension is then swabbed on the EtBr- TSA plates starting from the center of the plate to the edge margin. The dishes were then incubated at 37 °C for 16 hours.
4. The plates were examined under UV light. Fluorescence of isolates at different concentrations of EtBr were noted. Isolates without fluorescence indicated active efflux pump activity while those that fluoresced lacked efflux pump activity.

Statistical analysis

The Statistical Analysis System-SAS (2018) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study (14).

Result and discussion

Biochemical tests

Manual biochemical tests

For biochemical diagnosis of *E. coli* isolates of the present study, the manual biochemical tests were carried out for the isolates which include oxidase test, catalase test, Indole test, Methyl red test(MR-T) Vogas–proskuar test (VP-T), Simmon Citrate test, Motility test and Urease test as in table (2) and figure(1).

The results of manual biochemical tests of *E. coli* isolates showed all isolates were positive results for Catalase test, Indole test Methyl red test(MR-T) and motility test, while the negative results for Oxidase test, Vogas–proskuar test (VP- T), Simmon Citrate and urease production. Catalase is an enzyme that catalyzes the decomposition of hydrogen peroxide (H2O2) to water and gaseous oxygen that prevent the accumulation of toxic metabolites (15). *E. coli* isolates gave positive results in the indole production test due to the ability of bacteria to hydrolyze tryptophan to indole by the production of tryptophanase enzyme. Also, *E. coli* isolates gave positive results for motility, the movement of the growth away from the stab line or a hazy appearance through the semisolid medium indicates that the bacteria are motile (16). The positive result of methyl red test (MR) test for the production of sufficient acid

during the fermentation of glucose. Some bacteria have the ability to utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product. The observed phenotypic biochemical characteristics isolates of the present study are in agreement with those characteristics of the E. coli described in the Bergey's Manual of Determinative Bacteriology (17).

Automated biochemical test (Vitek 2 system)

The vitek-2 system used for further diagnosis of *E. coli* isolates figure (2).This system used in many previous studies and gave good results for identification and confirmation the biochemical tests**..**

The Vitek-2 System is an identification system, which depends on the biochemical reactions between the bacterial isolates suspended in their solutions and the media in the Vitek-2 identification Cards, to identify the isolates, For more accuracy bacterial isolate diagnosis was confirmed by Vitek 2 system and the results showed that the isolates exhibited phenomenal probability (95%- 99%), it has a very good specificity (95.9%), fast preparation and interpretation of results, makes an easy adaptability during routine of clinical laboratories (18).

Molecular identification of *E. coli* by detection *uidA* gene

In this study, DNA sample of *E. coli* isolates has been selected to detect the *uid A* gene. The PCR products have been confirmed by the analysis of the bands on gel electrophoresis and by comparing their molecular weight DNA ladder. The results of PCR reaction showed that *uidA* gene (658bp) exists in *E.coli* isolates as figure (3). *UidA* gene was detected in DNA samples of UPEC by PCR amplification and specific primer. The results of this study are in agreement with the findings of (19, 20).

Antibiotic susceptibility testing

The antimicrobial susceptibility profiles to the main classes of antibiotics to the 53 of *E.coli* isolated were determined by using disk diffusion method (DD method) (Kirby–Bauer method) as described by(21).Resistance of *E.coli* isolates were tested against several types of antibiotic including: Ampicillin (AMP); Amoxicillin-clavulanic acid (AMC); cefotaxime (CTX); Ceftriaxone (CRO); cefixim (CFX); Cefepime (FEP); Erythromycin (E); Nitrofurantoin (NFR); and Piperacillin (PR L) as shown in table (3).In the present study all *E.coli* isolates have shown highest resistance to Erythromycin was recorded (100%) the resistance rate was similar with other study reported by (22) who revealed that (100%) of the *E.coli* isolates resistant to this antibiotic (23) who found the percentage of bacterial resistance to this antibiotic was (96.34%). However, the findings of *E. coli* antibiogram in the present study disagree with a study done by (24) in Iran that has found *E.coli* isolates have low resistance to Erythromycin (52.8%).The results of the current study showed resistance to Ceftriaxone, (47.16%), while (25,26,27) found that the resistance rate was (7.6%, 32.2%, 31%) respectively and the study by (28,29,30)found that the resistance rate (44%, 89%, 100%) respectively.

In this study, the dominant isolate *E. coli* was sensitive to Nitrofurantoin (52.83) %. The study by (31) in Zakho City in Iraq related the antibiotics sensitivity of *E. coli* were extremely sensitive to Nitrofurantoin (82.4%). The other study was done in Addis Ababa,

Ethiopia that showed highly sensitive to nitrofurantoin (93.1%) (32).

Efflux pump activity by EtBr cartwheel method

All *E. coli* isolates (53) were submitted to the ethidium bromide cartwheel method to assess the activity of efflux pump. Depending on the ethidium bromide stain as a guide for phenotypic detection, the results showed that(E3-E8-E12-E13-E14-E15- E16-E17-E18-E19-E20-E23-E26-E27-E34-E35- E36-E38-E39) of bacterial isolates phenotypically displayed efflux pump activity at concentration (5,10µg / ml) while bacterial isolate (E24) phenotypically displayed efflux pump activity at concentration (5,10,15,20,25µg / ml) and bacterial isolates (E45-E47-E48-E51-E53) phenotypically displayed efflux pump activity at concentration (5,10,15 µg/ ml) table (4) as they did not fluoresce under UV light since they did not retain ethidium bromide within their cells as shown in figure (4). The result by (33) in Baghdad, Iraq found the efflux pumps were detected in (70%) of *E. coli* isolates tested in tryptic soy agar containing EtBr. The result of (34) which showed that (27.90 %) of isolates were positive for phenotypic detection of efflux pumps. By (35) showed that bacterial isolates possess different types of efflux pump, as the percentage of positive bacterial isolates for phenotypic detection of effluent pumps was (40.54%). The study of (36) in Egypt has been showed that (92%) of *E. coli* isolates were positive for phenotypic detection of efflux pumps. The Kuala Lumpur found all isolates with the same efflux activity (37, 38). This method is the most simple and important method due to the speed of containing results, in addition, to relying on the EtBr as in guide for phenotypic detection (13).

Table (2): Results of manual biochemical tests for *E.coli* isolates.

Biochemical test	Result
Oxidase test	–
Catalase test	+
Indole test	+
Methyl red test (MR)	+
Vogas–proskuar test (VP)	–
Simmon Citrate test	–
Motility	+
Urease test	–

bioMérieux Customer: Microbiology Chart Report Printed January 30, 2022 11:54:24 AM CST

Patient Name: Patient ID:
Location: Physician:
Lab ID: zena hh 39 Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source: **Collected:**

Comments:	

Identification Information	**Analysis Time:**	3.87 hours	**Status:**	Final
Selected Organism	99% Probability	**Escherichia coli**		
	Bionumber:	0405611540525611		
ID Analysis Messages				

Biochemical Details

2	APPA	-	3	ADO	-	4	PyrA	-	5	lARL	-	7	dCEL	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	-	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	+
33	SAC	-	34	dTAG	-	35	dTRE	+	36	CIT	-	37	MNT	-	39	5KG	-
40	lLATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	+	47	ODC	-	48	LDC	+	53	lHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	lMLTa	-	62	ELLM	+	64	lLATa	-			

Figure (2): Diagnosis of *E. coli* by Vitek 2 system.

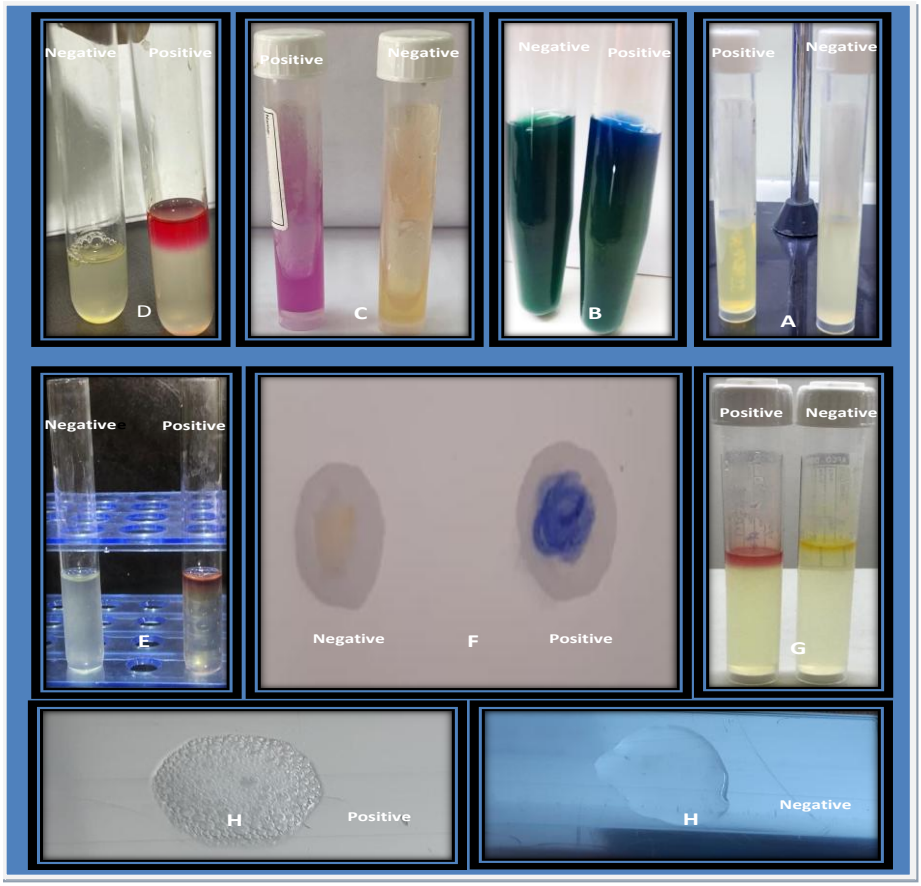

Figure (1): Biochemical tests for Identification of Bacterial Isolate, (A) Motility test, (B) Simmons Citrate test, (C) Urease test, (D) Methyl Red test (E) VogesProskauer test, (F) Catalase test, (G)Indole test, (H) Oxidase test.

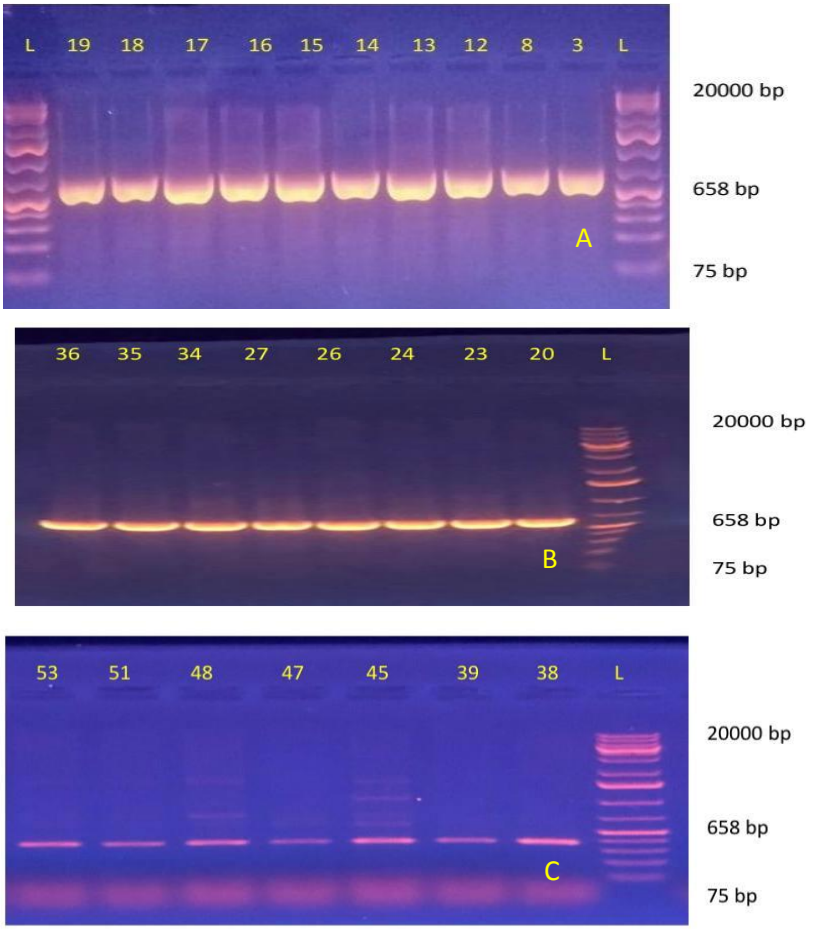

Figure (3): (A, B, C): Gel electrophoresis of PCR products of *UidA* gene using 1 % agarose gel at 70V for 1 hr. Lane (L): DNA Ladder.

Table (3): Antimicrobial sensitivity test results for isolated UPEC from urine specimen.

Classes of Antibacterial drugs	Tested antibiotics	Antibiotic Generation	Sensitivity n (%)	Intermediate n (%)	Resistance n (%)	P-value
B - lactamase	Ampicillin	3rd	9(16.98)	0 (0)	44(83.01)	0.0001 **
	Amoxicillin-clavulanic acid	4rd	7(13.20)	0 (0)	46(86.79)	0.0001 **
	Piperacillin	4th	12(22.64)	0 (0)	41(77.35)	0.0001 **
	cefotaxime	3st	4(7.54)	0 (0)	49(92.45)	0.0001 **
	Ceftriaxone	3rd	27(50.94)	1(1.88)	25(47.16)	0.0001 **
	Cefixime	3rd	13(24.52)	2(3.77)	38(71.69)	0.0001 **
	Cefepime	4th	17(32.96)	1(1.88)	35(66.03)	0.0001 **
Macrolide	Erythromycin	NCG	0 (0 %)	0 (0)	53 (100 %)	0.0001 **
Nitrofurans	Nitrofurantoin	NCG	28(52.83)	0 (0)	25(47.16)	0.0001 **
P-value			0.0001 **	0.823 NS	0.0001 **	---
** (P≤0.01).						

NCG: No classified generation.

Table (4): Results of phenotypic detection of pumps efflux of *E. coli* using different concentrations of ethidium bromide dye in A tryptic soya agar.

Isolate code	No	Ethidium bromide dye concentrations used (µg / ml)				
		5	10	15	20	25
E1,E2,E6,E9,E10,E11,E44,E28,E33,E52, E4,E5,E21,E22,E30,E25,E29,E31,E32,E40, E42,E43,E46,E50, E37,E41,E49,E7.	28	-	-	-	-	-
E3-E8- E12-E13-E14- E15- E16- E17- E18-E19-E20- E23- E26- E27-E34-E35- E36-E38- E39	19	+	+	-	-	-
E24	1	+	+	+	+	+
E45-E47-E48-E51-E53.	5	+	+	+	-	-

Positive result is no florescence(+), Negative result is florescence.

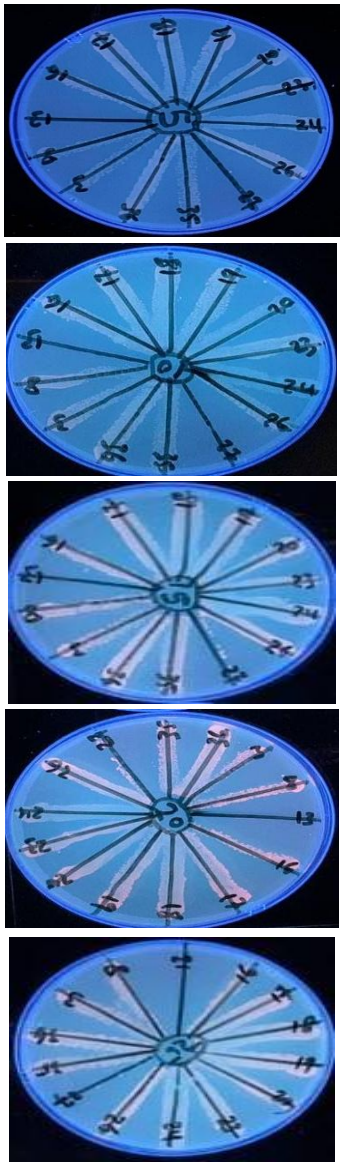

Figure (4): Phenotypic detection of effluent pumps using Cart-Wheel method.Fluorescent and non-fluorescent bacterial isolates of *E. Coli* at different concentrations of ethidium bromide dye under ultraviolet light.

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