REVIEW ARTICLE

Detection of Plasmid Mediated Efflux Pump Genes in Multidrug-resistant *Escherichia coli* isolated from Clinical Samples

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ABSTRACT

Introduction: Multidrug-resistant (MDR) *Escherichia coli* becomes an important problem that threatens life because it plays a vital role in disseminating antibiotic resistance genes.

Objective: Present study aimed to investigate the prevalence of plasmid-mediated efflux pump genes among MDR *E. coli* isolated from clinical samples in Iraq.

Methods: Fifty MDR *E. coli* isolates were isolated from clinical samples, then identified by the biochemical and microbiological methods, API-20 E system, and VITEK-2 System. All isolates were tested their susceptibility against 12 antibiotics by disk diffusion method, then the minimum inhibitory concentration (MIC) for 16 antibiotics and extended-spectrum beta-lactamase (ESBL) production were determined by VITEK-2 Compact/AST-GN69 card.

Results: Out of 50 MDR *E. coli* isolates, the isolation rate from urine was higher with 78%(39 isolates), while from infected wounds and blood were 14% (7 isolates) and 8% (4 isolates). All *E. coli* isolates were showed resistance to ciprofloxacin (MIC ≥ 4 µg/mL), levofloxacin (MIC ≥ 8 µg/mL), norfloxacin and nalidixic acid, ampicillin (MIC ≥ 32 µg/mL), ampicillin/sulbactam (MIC ≥ 32 µg/mL), cefazolin (MIC ≥ 64 µg/mL), ceftazidime (MIC ≥ 64 µg/mL) and ceftriaxone (MIC ≥ 64 µg/mL). Most MDR *E. coli* isolates were produced extended spectrum beta-lactamases (ESBL) with rate 60% (30 isolates). Out of 23 representative MDR *E. coli* isolates (ESBL positive), five isolates harbored two plasmid-mediated efflux pump genes (*qepA* and *oqxA*) and two isolates had only *qepA*, while *oqxB* gene was not detected in any isolate. All isolates that carried *qepA* and *oqxA* showed pan drug-resistant (PDR) patterns that revealed resistance to 20 tested antibiotics.

Conclusions: The study concluded that the spread of *oqxA* gene among *E. coli* isolates refer to a greater risk on mankind.

Keywords: ESBL, *Escherichia coli,* MDR, Plasmid-mediated efflux pump genes.

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INTRODUCTION

Multidrug resistance (MDR) in *E. coli* represents the important problem that threatens human and animal health.[1,2] This return to *E. coli* becomes an indicator of antimicrobial resistance in bacterial communities because it is considered a reservoir of antimicrobial resistance genes.[3] Fluoroquinolones are drugs utilized to treat gram-negative and gram-positive bacterial infections. Since 1960, these antibiotics had become predominant in the treatment of different infections caused by *E. coli,* including; urogenital tract infection, urinary tract infection (UTI), skin infection, gastrointestinal tract infection (GTI), respiratory tract infection (RTI), and intra-abdominal infection (IAI).[4] Since 1990, fluoroquinolone-resistant *E. coli* increased due to increased utilization of these antibiotics.[5]

Three routes developed fluoroquinolone resistance; the accumulation of point mutations in (quinolone-resistance-determining-region) of deoxyribonucleic acid (DNA) gyrase and topoisomerase IV genes decreases the entrance of fluoroquinolones intracellular by modulations in porins and efflux pumps and the acquisition of plasmid-mediated quinolone resistance (PMQR) genes.[6,7] The PMQR genes include; *qnr* genes (encode the Qnr proteins), *aac(6')-Ib-cr* gene (encode the aminoglycoside-acetyltransferase), *qepA* gene (encode the QepA (specific quinolone efflux pump)) and *oqxAB* genes (encode the OqxAB (multidrug resistance pump)).[2]

Efflux pumps define as transport proteins present in the cytoplasmic membrane of both gram-positive and gram-negative bacteria. These proteins extrude antibiotics and

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toxic substrates from the inside of bacterial cell into the outside of it. The cytoplasmic membrane of *E. coli* contains efflux pumps called AcrAB and QepA. They discharge the antibiotics to the extracellular environment, causing antibiotic resistance by decreasing its concentration inside cell.[8,9] The *qepA* is a plasmid-mediated efflux pump gene that belongs to a superfamily of proton-dependent transporters. It reduces susceptibility to hydrophilic fluoroquinolones, including; ciprofloxacin, norfloxacin, and enrofloxacin, leading to increased MICs with a 32 to 64 fold.[10] The *oqxA* and *oqxB* genes are encoded OqxAB (efflux pump transporter). The OqxAB overexpression leading to MDR because it extrude different antibiotics (chloramphenicol, nitrofurantoin, trimethoprim, tigecycline, quinoxalines, quinolones such as ciprofloxacin, norfloxacin and nalidixic acid), disinfectants and detergents including (triclosan, benzalkonium chloride, and SDS), and it disseminates multiple drugs resistance by horizontal transfer.[11] This study aimed to investigate the prevalence of plasmid-mediated efflux pump genes among *E. coli* isolates that were isolated from Iraqi patients.

MATERIALS AND METHODS

Data Collection and Bacterial Isolation

Fifty MDR *E. coli* isolates were isolated from clinical samples (39 urine, 4 blood and 7 infected wounds) from inpatients at Baghdad Teaching Hospital during the period from October 2017 to January 2018. All isolates were identified depending on the standard biochemical and microbiological methods, API-20 E[12] and VITEK-2 Systems.

Antimicrobial Susceptibility Testing

The disk diffusion method was used for detection of the resistance profile for all isolates against 12 antibiotics; ampicillin (AMP) 10 µg, chloramphenicol (CHL) 30 µg, nitrofurantoin (NIT) 300µg, sulfamethoxazole-trimethoprim (SXT) 25 µg, ciprofloxacin (CIP) 5 µg, levofloxacin (LVX) 5 µg, norfloxacin (NOR) 10 µg, nalidixic acid (NAL) 30 µg, ceftazidime (CAZ) 30 µg, ceftriaxone (CRO) 30 µg, gentamicin (GEN) 10 µg and tetracycline (TET) 30 µg. VITEK-2 compact/AST-GN69 card was used to determine MIC for 16 antibiotics and Screening for ESBL production. The results were interpreted depending on the CLSI, 2014.[13]

Molecular Methods

Alkaline lysis Method utilized for plasmid DNA extraction for 23 representatives MDR *E. coli* isolates that appeared ESBL positive.[14] The *qepA*, *oqxA* and *oqxB* genes were screened using specific primers. A 199 bps fragment of *qepA* was amplified using the primers (*qep*F: 5'-GCAGGTCCAGCAGCGGGTAG-3') and (*qep*R: 5'-CTTCCTGCCCGAGTATCGTG-3').[15] The thermal cycling program of PCR was carried out as the following: 96°C for 1-minute as initial denaturation; followed by 30 cycles (1-minute at 96°C for denaturation, 1-minute at 60°C for annealing and 1min at 72°C for extension); 5 minutes at 72°C for final extension. A fragment of *oqxA* (392bps) and fragment of *oqxB* (512bps) were amplified using the primers (*oqxA* F: 5'-CTCGGCGCGATGATGCT-3'), (*oqxA* R:5'-CCACTCTTCACGGGAGACGA-3'), (*oqxB* F: 5'-TTCTCCCCCGGCGGGAAGTAC-3') and (*oqxB* R: 5'-CTCGGCCATTTTGGCGCGTA-3'), respectively.[16] The amplification of *oqxA* and *oqxB* genes were done by the following thermal cycling conditions: initial denaturation at 95°C for 12 minutes; followed by 32 cycles consisting of [denaturation at 94°C for 45 seconds, annealing at 64°C for 45 seconds and extension at 72°C for 60 seconds]; finally, followed by a final extension at 72°C for 5 minutes. Amplified DNA products were resolved by electrophoresis on 1.5% agarose gels containing 5 µL/100 mL of Diamond™ Nucleic acid dye.

RESULTS

Bacterial Isolation

Fifty MDR *E. coli* isolates were isolated from clinical specimens during October 2017 to January 2018. Out of 50 MDR *E. coli* isolates of the current study, Table 1 indicated that a higher percentage of MDR *E. coli* isolates were reported from a urine sample with 78% (39 isolates), and infected wound came in second-step with 14% (7 isolates). The lower isolation rate of MDR *E. coli* was from blood samples 8% (4 isolates).

Antimicrobial Susceptibility Patterns

The results of study indicated that all isolates (50, 100%) showed resistance to ciprofloxacin (MIC ≥4 µg/mL), levofloxacin (MIC ≥8 µg/mL), norfloxacin, nalidixic acid, ampicillin (MIC ≥32 µg/mL), ampicillin/sulbactam (MIC ≥32 µg/mL), cefazolin (MIC ≥64 µg/mL), ceftazidime (MIC ≥64 µg/mL) and ceftriaxone (MIC ≥64 µg/mL), as shown in Table 2. Additionally, high resistance patterns showed to sulfamethoxazole-trimethoprim (≥320 µg/mL), cefepime (≥64 µg/mL), gentamicin (MIC≥16 µg/mL), amoxicillin/clavulanic acid (MIC≥32 µg/mL), tetracycline, gentamicin and chloramphenicol with (42, 84%), (41, 82%), (37, 74%), (36, 72%), (40, 80%), (37, 74%) and (30, 60%) respectively. Moderate resistance level was noted against nitrofurantoin (MIC≥128 µg/mL) and piperacillin/tazobactam (MIC≥128 µg/mL) with (24, 48%) and (20, 40%). On the other side, low resistant profile to carbapenems (ertapenem MIC≥64 µg/mL and imipenem MIC≥16 µg/mL) and tobramycin (MIC≥8µg/mL) was recorded with resistance rate [13 (26%), 10 (20%) and 11 (22%), respectively].

Detection of Extended-Spectrum Beta-Lactamase (ESBL) among MDR *E. coli*

A total of 50 MDR *E. coli* isolates were screened for ESBL production by VITEK-2 Compact/AST-GN69 card.

Table 1: Multidrug resistant *E. coli* isolates among clinical samples.

	E. coli isolates	
Clinical samples	*No.*	*(%)*
Urine sample	39	78
Infected wound swab	7	14
Blood sample	4	8
Total	50	100

The results showed that most studied MDR *E. coli* isolates was produced ESBL with rate 30 (60%) of tested isolates, as shown in Figure 1.

Detection of Plasmid-Mediated Efflux Pump Genes

Out of 23 representative MDR *E. coli* isolates (ESBL positive), five isolates harboured (*qepA* and *oqxA*) and 2 isolates harboured only *qepA*, Figure 2. polymerase chain reaction (PCR) products corresponding to *qepA* were 199bps in size, while the PCR products corresponding to *oqxA* were 392 bps. On the other side, *oqxB* gene was not detected in anyone of the studied isolates.

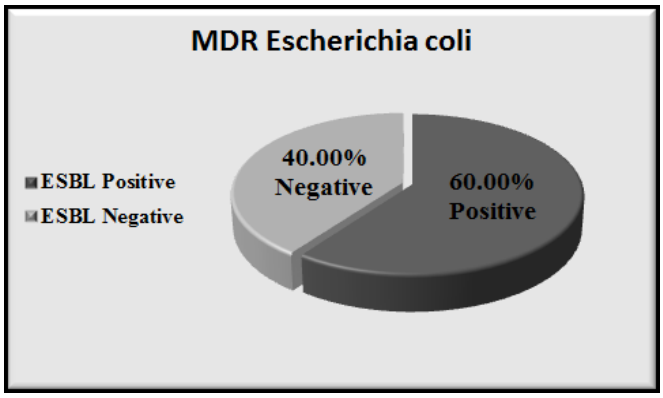

Figure 1: ESBL for Multidrug-Resistant *E. coli* isolates to group of antibiotics using VITEK-2 System (n = 50).

Table 2: Antibiotic susceptibility profile by disk diffusion method, and MIC values (µg/mL) and Extended-spectrum beta-lactamase (ESBL) by VITEK-2 System for MDR *E. coli* isolates (n = 50).

Antibiotics	*MIC value*	*Resistant E. coli isolates*	
		No.	*(%)*
Ciprofloxacin	(≥4)	50	100
Levofloxacin	(≥8)	50	100
Norfloxacin	-	50	100
Nalidixic acid	-	50	100
Ampicillin	(≥32)	50	100
Amoxicillin/Clavulanic Acid	(≥32)	36	72
Ampicillin/Sulbactam	(≥32)	50	100
Piperacillin/Tazobactam	(≥128)	20	40
Chloramphenicol	-	30	60
Nitrofurantoin	(≥128)	24	48
Sulfamethoxazole-Trimethoprim	(≥320)	42	84
Cefazolin	(≥64)	50	100
Ceftazidime	(≥64)	50	100
Ceftriaxone	(≥64)	50	100
Cefepime	(≥64)	41	82
Gentamicin	(≥16)	37	74
Tobramycin	(≥8)	11	22
Ertapenem	(≥64)	13	26
Imipenem	(≥16)	10	20
Tetracycline	-	40	80

Table 3 showed the results of phenotypic and genotypic characteristics of 23 MDR *E. coli* isolates. These results showed that each isolate showed a resistance profile against many antibiotics, while the isolates that harbored two plasmid-mediated efflux pump genes (*qepA* and *oqxA*) were PDR isolates because they showed resistance to all 20 examined antibiotics (ciprofloxacin, levofloxacin, norfloxacin, nalidixic acid, ampicillin, ampicillin/ sulbactam, cefazolin, ceftazidime, ceftriaxone, cefepime, amoxicillin/clavulanic, sulfamethoxazole-trimethoprim, gentamicin, tobramycin, chloramphenicol, tetracycline, piperacillin/tazobactam, nitrofurantoin, ertapenem, and imipenem). On the other side, the rate of *qepA* was 7 (30.4%), and the rate of *oqxA* was 5(21.7%), as appeared in Table 3.

DISCUSSION

E. coli is the most important pathogen that plays an essential role in causing serious infections to humans.[17] Out of 50 MDR *E. coli* isolates, the higher isolation rate was recorded from urine samples with 78%. This result was near another local study in Baghdad city that reported a higher isolation rate (65.45%) of *E. coli* isolates from urine samples.[18]

Although, MDR *E. coli* causes wound infection.[19] The results of study revealed low isolation in infected wounds. This result is close to that reported by another study that showed an isolation rate of 17.3%.[20] Although; *E. coli* considered a most common causative agent of bacteremia,[21] the isolation of *E. coli* from blood was low 8%, which is near the isolation rate 9% reported by previous study.[22]

E. coli may play a fatal role in disseminating resistance phenomena within the community,[23] because they are considered the reservoir of antibiotic resistance genes and can transmit them to another pathogenic organism.[24] In the present study, all studied *E. coli* isolates were MDR and (flouro) quinolone resistant which showed resistance against (ciprofloxacin, levofloxacin, norfloxacin and nalidixic acid), in addition to resistance against ampicillin, ceftazidime and ceftriaxone, (Table 2). In the last years, ampicillin has become ineffective in treating *E. coli* infections.[25] This was evident in our result and in another study that showed also documented high resistance rate to ampicillin 96.2%.[26] The resistance pattern against ceftazidime, ceftriaxone, and gentamicin is

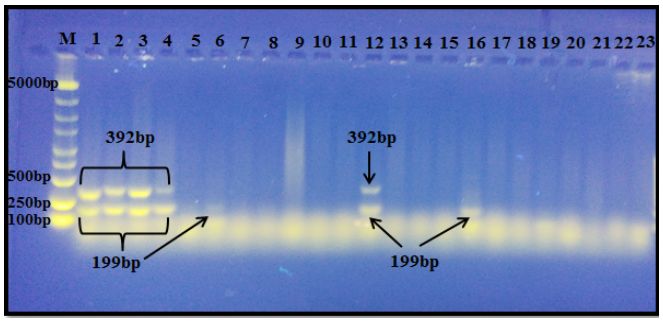

Figure 2: *qepA* and *oqxA* genes of 23 MDR *E. coli*. Lane(M), DNA Marker; Lane(1, 2, 3, 4, 6, 12 and 16), positive isolates for *qepA* generated (199 bp); Lane(1, 2, 3, 4, and 12), positive isolates for *oqxA* generated (392 bp).

Table 3: Phenotypic and genotypic characteristics of 23 MDR Escherichia coli isolates.

Isolate	*Sample source*	*Antimicrobial resistance profile*[a]	*ESBL*[b]	*PMQR genes*		
				qepA	*oqxA*	*oqxB*
Eco U_1	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP,AMC, SXT, GEN, TOB, CHL, TET,TZP, NIT, ETP, IPM	+	+	+	-
Eco U_2	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB, CHL, TET, TZP, NIT, ETP, IPM	+	+	+	-
Eco W_3	Wound	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB, CHL, TET, TZP, NIT, ETP, IPM	+	+	+	-
Eco U_4	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB, CHL, TET, TZP, NIT, ETP, IPM	+	+	+	-
Eco U_5	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB	+	-	-	-
Eco U_6	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB, TET	+	+	-	-
Eco U_7	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB	+	-	-	-
Eco W_8	Wound	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, SXT, GEN,	+	-	-	-
Eco B_9	Blood	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, SXT, GEN,	+	-	-	-
Eco U_{10}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, SXT, GEN, TOB, TET	+	-	-	-
Eco U_{11}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, SXT, GEN, TOB, TET	+	-	-	-
Eco U_{12}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB, CHL, TET, TZP, NIT, ETP, IPM	+	+	+	-
Eco B_{13}	Blood	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, SXT, GEN	+	-	-	-
Eco W_{14}	Wound	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, SXT, GEN	+	-	-	-
Eco U_{15}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, SXT, GEN, TET	+	-	-	-
Eco U_{16}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TOB, TET	+	+	-	-
Eco U_{17}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, SXT, GEN, TET	+	-	-	-
Eco W_{18}	Wound	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN,	+	-	-	-
Eco U_{19}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TET	+	-	-	-
Eco B_{20}	Blood	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN,	+	-	-	-
Eco U_{21}	Urine	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, AMC, SXT, GEN, TET	+	-	-	-
Eco W_{22}	Wound	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, SXT, GEN, TOB,	+	-	-	-
Eco B_{23}	Blood	CIP, LVX, NOR, NAL, AMP, SAM, CFZ, CAZ, CRO, EFP, SXT, GEN, TOB	+	-	-	-
Total N(%)				7 (30.4%)	5 (21.7%)	0 (0%)

[a] CIP = Ciprofloxacin, LVX = Levofloxacin, NOR = Norfloxacin, NAL = Nalidixic acid, AMP = Ampicillin, SAM = Ampicillin/Sulbactam, CFZ = Cefazolin, CAZ = Ceftazidime, CRO = Ceftriaxone, EFP = Cefepime, AMC = Amoxicillin/Clavulanic, SXT = Sulfamethoxazole-trimethoprim, GEN = Gentamicin, TOB = Tobramycin, CHL = Chloramphenicol, TET = Tetracycline, TZP = Piperacillin/Tazobactam, NIT = Nitrofurantoin, ETP = Ertapenem, IPM = Imipenem.

[b] ESBL = extended-spectrum beta-lactamase.

agreed upon within the previous study, demonstrating a high resistance rate.[27] The resistance rate of tetracycline among isolates was high, which agrees with the previous study that revealed a resistance rate of 81.9%.[28]

All MDR *E. coli* isolates (100%) in study were resistant to ciprofloxacin and levofloxacin with (MIC ≥4 µg/mL and MIC ≥8 µg/mL, respectively). This result is corresponding with another study that recorded (92.95%) of isolates were ciprofloxacin resistant with MIC ≥4 µg/mL.[29]

Table 2 showed that all isolates (100%) showed resistance for levofloxacin, ampicillin, ampicillin/sulbactam, ceftazidime, ceftriaxone, and cefazolin was with MIC (≥8 µg/mL, ≥32 µg/mL, ≥32µg/mL, ≥ 64 µg/mL, ≥ 64 µg/mL and ≥64 µg/mL respectively). This increase in resistance profile returns to the use of antibiotics that altered the bacterial evolution by reducing the susceptibility of bacteria against antibiotics.[30]

In this study, a high rate of ESBL production was reported among 30 studied MDR *E. coli* strains (60%). This high result

was also reported in another study conducted in Nibal by Mahato *et al*. (2019), who found that (57.1%) of *E. coli* isolates revealed ESBL production.[31]

E. coli isolate considered MDR when the isolate is resistant to at least 3 or more antibiotic classes, including (penicillins, fluoroquinolones, cephalosporins, tetracyclines, aminoglycosides, sulfonamides, carbapenems, and nitrofurantoin), while PDR isolates are resisting to all antibiotic classes.[32]

Previous studies demonstrated that the MDR, XDR, and PDR strains were increased among gram-negative bacteria, representing a very dangerous threat to public health.[33,34] PDR profile was detected in 5 isolates (*Eco* U_1, *Eco* U_2, *Eco* W_3, *Eco* U_4, and *Eco* U_{12}) among studied MDR *E. coli*, the phenotypic and genotypic characteristics shown in Table 3. These isolates showed resistance to 20 antibiotics (all antibiotic classes). This result may be due to the excessive utilization of antibiotics, resulting in multidrug resistance among pathogenic bacteria.[35]

In our study, the *qepA* gene was detected in 7 (30.4%) isolates. This result is higher than that reported to another study that revealed the incidence rate of this gene (14.4%) among *E. coli* isolates,[36] while in Turkey, the incidence rate of *qepA* gene was (5.7%).[37] We also found that the oqxA gene's prevalence was 5(21.7%) of 23 MDR *E. coli* isolates. This result is higher than that reported by recent study in Cuba, by Quiñones *et al*. (2020), who found low incidence (3.1%) of *oqxA* among *E. coli* isolates.[38] To our knowledge, this is the first report of the presence of *qepA* and *oqxA* genes among MDR *E. coli* isolated from clinical samples in Iraq. Both *qepA* and *oqxA* genes were detected in 5 isolates, these isolates were PDR and ESBL producers, while only *qepA* gene was detected in 2 isolates and these isolates were ESBL producers but not PDR. According to previous researches, ESBL genes and resistance genes to other antibiotic classes such as fluoroquinolones and aminoglycosides are encoding on the same plasmids and regulated by the same promoter, eventually leading to the spread of MDR bacteria.[2,39,40] This phenomenon is frightening because it leading to treatment failure as mentioned previously.[41]

CONCLUSION

The present study concluded that the plasmid-mediated efflux pump genes (*qepA* and *oqxA*) are disseminated among Iraqi *E. coli* isolates. To our knowledge, this study is the first that detect the presence of *qepA* and *oqxA* genes among MDR *E. coli* isolated from clinical samples in Iraq. Additionally, *oqxA* in combination with *qepA* gene plays a vital role in PDR phenomena among *E. coli* isolates. This refers to a real disaster that threatens the human because *oqxA* gene can be disseminated by horizontal transfer among species.

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