Original Article

The Effect of Carbonyl Cyanide m-Chlorophenyl-Hydrazine on Antibiotic Susceptibility in MDR Enterobacteriaceae Isolates in Babylon, Iraq

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Abstract

Background: One of the efflux pump inhibitors is carbonyl cyanide 3-chlorophenylhydrazone (CCCP) that has often been found to increase the susceptibility of a number of multi-drug resistant (MDR) MDR bacteria, isolated from human clinical specimens. **Objectives:** To investigate the role of active efflux system to aminoglycoside and quinolones resistance in clinical isolates of Enterobacteriaceae using the efflux pump inhibitor CCCP. **Materials and Methods:** Enterobacteriaceae isolates were recovered from different clinical samples from hospitalized patients. The minimum inhibitory concentrations (MICs) of antibiotics (levofloxacin, ciprofloxacin, and gentamycin) were compared with and without the efflux pump inhibitor (CCCP) in order to confirm the effective role of the efflux pump in our isolates. **Results:** The results found that out of 280 clinical samples, only 134 (47.1%) isolates belonged to Enterobacteriaceae. Polymerase chain reaction (PCR) results showed that six (42.8%) out of 14 selected MDR isolates were positive for efflux pump gene oqxA. However, no isolates showed positive results for the efflux pump oqxB. The results of MIC for 14 Enterobacteriaceae isolates against these three antibiotics showed that all isolates had MIC ≥128 µg/mL in the absence of CCCP for levofloxacin, ciprofloxacin, and gentamycin. The results showed the MIC of levofloxacin and ciprofloxacin were reduced for isolates, and the growth of bacteria was inhibited in presence of the CCCP. However, all Enterobacteriaceae isolates showed high MIC values (≥128) even in the presence of the CCCP which indicates no effect the inhibitor in reducing the MIC of the isolates for Gentamycin. **Conclusion:** From this study, we can conclude that high prevalence of efflux pumps gene (oxqA) was detected among MDR and XDR Enterobacteriaceae isolates and the efflux pump inhibitor (CCCP) has a positive effect and improves the sensitivity of MDR isolates to ciprofloxacin, levofloxacin but not gentamicin.

Keywords: Aminoglycosides, CCCP, *E. coli*, efflux pump inhibitors, fluoroquinolones, hospitals

Introduction

Certain strains of Enterobacteriaceae are now resistant to many commonly used antibiotics, and multidrug resistance is often responsible for the failure of antibiotic therapy. One of the antibiotic resistance mechanism is reduction in the drug accumulation due to either impermeability of outer membrane or overexpression of active efflux pumps.[1-4]

Drug efflux pumps are important in medicine because of their efficient function in ejecting a variety of different chemical structures unrelated to the use of antibiotics or antimicrobials up to the concentrations needed to kill bacteria that are multi-resistant to more than one antibiotic or antiseptic.[5-7]

Bacteria function in the presence of specific external risks, in particular multiple antibiotic mechanisms that simultaneously compensate and secure these risks; for example, Enterobacteriaceae often use efflux pumps

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to dispose of antibiotics, but under certain conditions they use a different mechanism such as modulating drug targets.[8]

Efflux systems are composed of transport proteins that pump out a broad range of toxic substrates such as antibiotics and biocides from bacteria, in an energy-dependent manner. In these circumstances, the intracellular antibiotic concentration is decreased and bacteria become less susceptible to that compound. The minimum inhibitory concentrations (MICs) of antibiotics for strains overexpressing an efflux pump are usually 2–8 folds higher than those for susceptible strains of that species.[9]

To assess the role of drug efflux mechanism in bacteria, efflux pump inhibitors are widely used to totally abolish the efflux of various molecules. One of these compounds is carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (an uncoupler of oxidative phosphorylation which disrupts the proton gradient of the membranes) that has often been found to increase the susceptibility of a number of MDR bacteria, including members of the family Enterobacteriaceae.[9-11]

In Iraq, no information is available on the role of efflux pump inhibitors on CCCP to increase the susceptibility of a number of MDR bacteria. So, the aim of this study was to investigate the role of active efflux system to aminoglycoside and ciprofloxacin resistance in clinical isolates of Enterobacteriaceae using the efflux pump inhibitor CCCP.

The objective of this study was to investigate the role of CCCP on antibiotic susceptibility in MDR Enterobacteriaceae isolates and genotypic detection of and prevalence of oqxA and oqxB genes of efflux pumps among MDR Enterobacteriaceae isolates in Babylon, Iraq.

Materials and Methods

Study design

This cross-sectional study was prepared to investigate the role of CCCP on antibiotic susceptibility in MDR Enterobacteriaceae isolates in Babylon, Iraq. A total of 300 different clinical samples were collected from patients (females 172: 57.3%; males 128: 42.6%) with urinary tract infection (urine), reproductive system (swabs), patients with bacteremia (blood) and lower respiratory tract infection (sputum) in Al-Mahaweel General Hospital, Marjan Teaching Hospital, and private laboratories during the period from November 2022 to March 2023.

Enterobacteriaceae isolates were isolated and identified based on their morphological characteristics and biochemical tests.[12] Identification was confirmed using Vitek-2 system (BioMérieux, France).

Antibiotic susceptibility testing

The antibiotic susceptibility of Enterobacteriaceae isolates to a variety of antibiotics was tested using Vitek-2 system (BioMérieux) and disc diffusion test (DDT). The results were interpreted based on Clinical and Laboratory Standards Institute documents.[13]

The following antibiotics were purchased (from Oxoid, UK) as standard antibiotic disks as an identified potency for lab use: gentamicin, kanamycin, amikacin, streptomycin, ciprofloxacin, levofloxacin, gatifloxacin, norfloxacin. *Escherichia coli* ATCC 25922 was used as the reference strain for antimicrobial susceptibility testing. Antibiotic resistance patterns [MDR, XDR, and pan drug resistant (PDR)] of all Enterobacteriaceae isolates were detected using definitions of Magiorakos *et al*.[14]

MIC test was performed by agar dilution method, in which Mueller-Hinton agar plates and appropriate concentrations (0.5–128 µg/mL) of antimicrobial agent were prepared, and inoculated with enterobacterial suspensions, and the plates were incubated at 37°C in the same micro-atmosphere for 24 h. The MICs for antimicrobials were scored as the lowest concentration of antimicrobial agent that completely inhibited the growth of Enterobacteriaceae on agar plates.[13]

Phenotypic detection of efflux mechanism by CCCP with antibiotics

The MICs of antibiotics (levofloxacin, ciprofloxacin, and gentamycin) were compared with and without the efflux pump inhibitor (CCCP) in order to confirm the effective role of the efflux pump in our isolates. The previous experiment was repeated in the same way except adding the inhibitor. To confirm the mechanism of efflux pump, the efflux pump inhibitor (CCCP) was supplemented to each of the M-H agar plates containing 128 µg/ml levofloxacin (LE) ciprofloxacin (CIP) and gentamycin (CN). The final concentration of CCCP in the M-H agar was 25 µg/mL and the result was recorded.[15]

DNA extraction and PCR assay

Bacterial isolates were inoculated into 5 mL of Broth Heart Infusion medium and grown overnight at 37ºC. DNA was extracted from bacterial cell using Genomic DNA Extraction Kit (Bioneer, Seoul, Korea). The PCR reactions were performed in a thermal cycler (Clever, UK). For quantification of the DNA (ng μL^{-1}), 1 µL of Genomic DNA was quantified with a NanoDrop™ spectrometer (NanoDrop Technologies), according to the manufacturer's guidelines. DNA concentration was assessed from absorbance at 260 nm. DNA profiles were carried out using bacterial DNA and loading buffer adopting the manufacturer directions.

PCR protocols

The DNA purified from 14 Enterobacteriaceae isolates were exposed to a set of primers for efflux pump genes by PCR. The protocols used were as stated by the manufacturer's directions. A set primers[16] were used (Bioneer) to detect the efflux pump genes oqxA and oqxAB in Enterobacteriaceae isolates [Table 1]. The PCR conditions are illustrated in Table 1. The PCR products were identified by agarose gel electrophoresis. Ethidium bromide dye was used for visualization of the amplicons. The gel images were acquired using a regular geldocumentation system (Consort, Belgium).

Evaluating the efflux pump inhibitor's effect on selected isolates

In order to confirm the effective role of the efflux pump in our isolates, the MICs of antibiotics (levofloxacin, ciprofloxacin, and gentamycin) were compared with and without the efflux pump inhibitor (CCCP). The following criteria were used to select the isolates for studying the effect of Efflux Pump Inhibitor's on the Isolates: the presence of oqxA gene and the high rate of MIC of the isolate (MIC ≥128 μg/mL).

Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) software program, version 27.0 (SPSS, IBM Company, Chicago, Illinois). Categorical variables were presented as frequencies and percentages.

Ethical consideration

Verbal consent was obtained from each patient before taking samples. The study protocol was approved by the committee on publication ethics at College of Medicine, University of Babylon, under the reference No. BMS/427/022.

Results

The results found that out of 280 clinical samples, only 134 (47.1%) samples belonged to Enterobacteriaceae. The following species were obtained: *E. coli n* = 94 isolates (70%), *Klebsiella pneumoniae n* = 24 (17.9%), *Proteus* spp *n* = 7 (5.2%), and *Enterobacter* spp *n* = 9 (6.7%).

Regarding the distribution the Enterobacteriaceae isolates according to patients sex, the results found that out of 134 patients, 82 (61.1%) were female patients versus 52 (38.8%) were male patients.

The results of isolation of Enterobacteriaceae revealed high percentage of *E. coil* isolates distributed as follows: in urinary tract infections (UTIs) (43 isolates), lower respiratory tract infection (RTIs) (20 isolates), Bacteremia (17 isolates), and 14 isolates for bacterial vaginitis. The isolation rate of *K. pneumoniae* 24 isolates was as follows: from UTIs (4 isolates), RTIs (8 isolates), and (12 isolates) for bacteremia. Regarding the distribution of *Enterobacter* spp. among clinical samples, seven isolates from UTIs and two isolates from bacteremia. For *Proteus* spp., six isolates were recovered from UTIs and one isolate from Bacteremia.

The results of the susceptibility of Enterobacteriaceae isolates to fluoroquinolones found that *E. coli* and *K. pneumoniae* isolates were resistant to ciprofloxacin, levofloxacin, norfloxacin, and gatifloxacin. *Enterobacter* spp., were resistant only to ciprofloxacin, levofloxacin, and Norfloxacin. However, *Proteus* ssp., isolates were highly sensitive to all members of fluoroquinolones.

Regarding the susceptibility of Enterobacteriaceae to aminoglycosides, *E. coli* isolates exhibited a low resistance rate against Kanamycin and Streptomycin versus relatively high resistance rates exhibited by *K. pneumoniae* isolates.. *K. pneumoniae* and *Proteus* spp. isolates were highly resistant to kanamycin and streptomycin. Also *Proteus* spp. isolates were resistant to amikacin and gentamycin with 71% and 86%,

Table 1: Sequencing and PCR conditions designed in the present study

Genes	Sequence 5–3	Bp	Conditions
*oqx*A	*oqx*AF-5′-CTCGGCGCGATGATGCT-3′	392	Step 1: 94ºC, 5 min
			Step 2: 94ºC, 45 s by 34 cycles
	*oqx*AR-5′-CCACTCTTCACGGGAGACGA-3′		Step 3: 51ºC, 45 s
			Step 4: 68ºC, 1 min
			Step 5: 72ºC, 10 min
*oqx*B	*oqx*Bs-5′-TTCTCCCCCGGCGGGAAGTAC-3′	512	Step 1: 94ºC, 5 min
			Step 2: 94ºC, 45 s by 32 cycles
	*oqx*Ba2-5′-CTCGGCCATTTTGGCGCGTA-3′		Step 3: 64ºC, 45 s
			Step 4:72ºC, 1 min
			Step 5: 72ºC, 10 min

respectively. However, *Enterobacter* spp. isolates were resistant to kanamycin with percentage of 66.6% but they were sensitive to amikacin, gentamicin, and streptomycin [Table 2].

The results also found that the antibiotics resistance pattern PDR was only detected in *Klebsiella* isolates; XDR was detected only in *E. coli* and *K. pneumoniae,* whereas MDR was detected in all Enterobacteriaceae isolates except *Proteus* spp. isolates (n = 7) which did not show any type of ARP and were all sensitive to all antibiotics tested [Table 3].

Only 14 isolates were selected to determine their antibiotic susceptibility against two classes of antibiotics, fluoroquinolones and aminoglycosides by DDT. The result showed that Enterobacteriaceae isolates had higher resistance to fluoroquinolones than Aminoglycosides,

Analysis of PCR results showed that six (42.8%) out of 14 selected MDR Enterobacteriaceae isolates were positive for efflux pump gene oqxA, and eight (57.1%) of the isolates were negative for oqxA gene. However, no isolates showed positive results for the efflux pump oqxB. The result of the molecular detection of oqxA and oqxB genes among 14 selected clinical isolates of MDR, XDR, and PDR Enterobacteriaceae was as follows: 12 XDR *E. coli,* one PDR *K. pneumoniae*, and one MDR *Enterobacter* spp. isolate.

Table 2: Antibiotic susceptibility of Enterobacteriaceae isolates by DDT for fluoroquinolones and aminoglycosides

Antibiotic class	Antibiotic	Suscept. status	Bacterial isolates				Total
			E. coli n = 94	*K. pneumoniae* n = 24	*Enterobacter* Spp. n = 9	*Proteus* spp. n = 7	
Fluoroquinolones	CIP	R	60 (63.8%)	19 (79.1%)	5 (55.5%)	0 (0.0%)	84 (62.6%)
		S	30 (32.2%)	0 (0.0%)	4 (44.4%)	7 (100 %)	41 (30.5%)
		I	4 (4.3%)	5 (20.8%)	0 (0.0%)	0 (0.0%)	9 (6.7 %)
	LE	R	46 (48.9%)	21 (87.5%)	6 (66.6%)	0 (0.0%)	73 (54.4%)
		S	34 (36.1%)	3 (12.5%)	3 (33.3%)	7 (100 %)	47 (35.0%)
		I	14 (14.8%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	14 (14.8%)
	NX	R	54 (57.4%)	18 (75%)	7 (77.7%)	0 (0.0%)	79 (58.9%)
		S	34 (36.1%)	2 (8.3%)	2 (22.2%)	7 (100 %)	45 (33.5%)
		I	6 (6.3%)	4 (16.6%)	0 (0.0%)	0 (0.0%)	10 (7.4%)
	GAT	R	42 (44.6%)	15 (62.5 %)	0 (0.0%)	0 (0.0%)	57 (42.5%)
		S	40 (42.5%)	2 (8.3%)	4 (44.4%)	7 (100 %)	53 (39.5%)
		I	12 (12.9%)	7 (29.1%)	5 (55.5%)	0 (0.0%)	24 (17.9%)
Aminoglycosides	AK	R	4 (1.0%)	16 (66.6%)	0 (0.0%)	5 (71.4 %)	25 (18.6%)
		S	89 (94.6%)	1 (4.1%)	5 (55.5%)	0 (0.0%)	95 (70.8%)
		I	1 (1.0%)	7 (29.1%)	4 (44.4%)	2 (28.5%)	14 (10.4%)
	CN	R	32 (34.4%)	16 (66.6%)	0 (0.0%)	6 (85.7 %)	54 (40.2%)
		S	54 (57.4%)	2 (8.3%)	5 (55.5%)	0 (0.0%)	61 (45.5%)
		I	8 (8.6%)	6 (25%)	4 (44.4%)	1 (14.2%)	19 (14.17%)
	KAN	R	39 (41.4%)	24 (100 %)	6 (66.6%)	7 (100 %)	76 (56.7%)
		S	40 (42.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	38 (28.3%)
		I	15 (15.9%)	0 (0.0%)	3 (3.33%)	0 (0.0%)	18 (13.4%)
	HLS	R	5 (5.3%)	24 (100 %)	0 (0.0%)	0 (0.0%)	29 (21.6%)
		S	80 (86.0%)	0 (0.0%)	5 (55.5%)	6 (85.7 %)	91 (67.9%)
		I	9 (9.5%)	0 (0.0%)	4 (44.4%)	1 (14.2%)	14 (10.4%)

Table 3: Antibiotic resistance patterns of Enterobacteriaceae isolates based on Vitek-2 system results

Enterobacteriaceae isolates	Type of multidrug resistance pattern (*N*, %)		
	MDR	XDR	PDR
E. coli (n = 44)	33 (75%)	11 (25%)	-
K. pneumoniae (n = 24)	10 (42%)	6 (25%)	8 (33 %)
Enterobacter spp (n = 5)	5 (100%)	-	-
Proteus spp. (n = 7)	-	-	-
Total	48 (35.8%)	17 (12.6%)	8 (5.9%)

Table 4: MIC of levofloxacin and ciprofloxacin among PDR, XDR, and MDR Enterobacteriaceae isolates with and without addition of the inhibitor CCCP

Isolates	Antibiotic resistance patterns	Efflux pumps genotype	MIC (μg/mL) of levofloxacin and ciprofloxacin without CCCP	MIC (μg/mL) of levofloxacin and ciprofloxacin with CCCP
E. coli-47	XDR	*oqx*A	≥ 128	N.G*
E. coli-49	XDR	*oqx*A	≥ 128	N.G
E. coil-63	XDR	*oqx*A	≥ 128	N.G
E. coli-66	XDR	*oqx*A	≥ 128	N.G
E. coli-78	XDR	*oqx*A	≥ 128	N.G
K. pneumoniae-3	PDR	*oqx*A	≥ 128	N.G

PDR: pan drug resistant, XDR: extensively drug resistant

*$P < 0.05$ is considered significant

The results of MIC for 14 Enterobacteriaceae isolates against (levofloxacin, ciprofloxacin, and gentamycin) showed that all isolates had MIC ≥128 μg/mL in the absence of CCCP for levofloxacin, ciprofloxacin, and gentamycin. The results showed the MIC of levofloxacin and ciprofloxacin were reduced for isolates, and the growth of bacteria was inhibited in presence of the CCCP [Table 4]. However, all Enterobacteriaceae isolates showed high MIC values (≥128 μg/mL) even in the presence of the CCCP which indicates no effect the inhibitor in reducing the MIC of the isolates for Gentamycin.

Table 4 shows the effect of the inhibitor in reducing the MIC of levofloxacin among PDR, XDR, and MDR Enterobacteriaceae isolates. The results showed the MIC of levofloxacin was reduced for XDR *E. coli* (5 isolates) and PDR *K. pneumoniae* (one isolate) and inhibition of the growth in presence the inhibitor (CCCP). Also, the results in the showed the effect of the inhibitor in reducing the MIC of Ciprofloxacin among PDR, XDR, and MDR Enterobacteriaceae isolates. The results showed that the MIC of ciprofloxacin was reduced for *E. coli* (5 XDR isolates) and one PDR *K. pneumoniae* isolate, and the results showed the disappearance of the bacterial growth in presence of the inhibitor (CCCP).

Discussion

Regarding the distribution the Enterobacteriaceae isolates according to patients sex, the predominance of females (61.1%) than males (39%) in this study was compatible with the results obtained by several authors worldwide.[17,18]

The results of the current study regarding the resistance to fluoroquinolones were consistent with the results obtained by the Tajbakhsh *et al*.[19] and Malekzadegan *et al*.,[20] who found that the rate of bacterial resistance to ciprofloxacin were 56.25% and 56.5%, respectively. It was shown that the rate of resistance to antibiotics, including this antibiotic, is higher in biofilm-producing bacterial isolates from non-forming bacterial isolates. However, the

Figure 1: Agarose gel electrophoresis of PCR products of oqxA gene (329 bp), among *E. coli* (1, 2, 4, 5, 6, 7, 9,10, 11, 12, 13, and 14) *Enterobacter* spp. (8), and *K. pneumoniae* (3) isolates. M = DNA molecular marker. Isolates1, 2, 3, and 8 are positives to the oqxA gene

results of the current study was not consistent with the results obtained by Al-Musawi *et al*. [4] who revealed that the rate of bacterial resistance to this antibiotic was only 25%.

As the bacterial resistance to antibiotics is related to different agents, in this study, detection of efflux pump genes was performed by PCR on selected 14 isolates to determine whether their resistance to antibiotics, including ciprofloxacin, levofloxacin, and gentamycin, depended on the presence of these genes.

In this study, all oqxA-positive Enterobacteriaceae strains were resistant to ciprofloxacin, and levofloxacin. As some reports have confirmed that oqxA and oqxB efflux pumps confer resistance to multiple antimicrobial agents;[21-25] therefore, in this study also, the presence of oqxA efflux pumps in clinical isolates of *E. coli* (5 isolates) and 1 kp isolate containing oqxA genes has conferred resistance to multiple antimicrobial agents (AMG, Qns, and β-lactams) as shown in Figure 1.

In general, our findings, regarding the utilization of the inhibitor, helped to clarify the important function that pumps conduct and how resistance develops in resistant isolates. The isolates that showed a decrease in MIC were consistent with the study conducted by several authors

worldwide[26-31] who measured MICs for different MDR bacterial isolates in the presence and absence of the efflux pump inhibitor carbonyl cyanide 3-chlorophenylhydrazone and they showed the effect of CCCP was significantly greater on intrinsically antibiotic-resistant bacteria (*Acinetobacter baumanii*, *E. coli*, *K. pneumoniae*, *Proteus* spp., *Serratia marcescens*, *Morganella morganii*, *Brucella intermedia*) than on other Enterobacteriaceae.

The decrease in MIC of the isolates were consistent with the results obtained by El-Mahdy and Mashaly (2022) who measured the MICs for different MDR *E. coli* and *K. pneumoniae* isolates in the presence and absence of the efflux pump inhibitor (CCCP). The result that showed a decrease in MIC are consistent with the studies by Sanchez-Carbonel *et al*.[29] who measured the MICs in clinical strains of *Acinetobacter.baumannii* isolates in the presence and absence of the efflux pump inhibitor (CCCP) and they revealed that the isolates showed a decrease in MICs in the presence of the efflux pump inhibitor (CCCP).

Regarding the effect of the inhibitor in reducing the MIC of gentamicin, the results of the present study were inconsistent with the results obtained by Honarmand Jahromy *et al*.[27] who studied MIC of amikacin and gentamicin before and after treatment by efflux inhibitor CCCP and they found that lowest resistance to gentamycin (62%) and amikacin (74.5%).

Conclusion

From this study, we can conclude that the efflux pump inhibitor CCCP has a significant role on antibiotic susceptibility in MDR Enterobacteriaceae isolates in Babylon, Iraq. A high prevalence of efflux pumps gene (oxqA) was detected among MDR and XDR Enterobacteriaceae isolates, and the efflux pump inhibitor (CCCP) has a positive effect and improves the sensitivity of MDR isolates to ciprofloxacin, levofloxacin, but not gentamicin.

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Conflicts of interest

There are no conflicts of interest.

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