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RESEARCH ARTICLE OPEN ACCESS

Effect of Carbonyl Cyanide M-chlorophenylhydrazone on Ciprofloxacin Resistance and Biofilm Formation in Hospital-acquired Uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*

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Abstract

Antibiotic resistant and biofilm forming uropathogenic Enterobacteriaceae are rising. This study was conducted to evaluate the efflux pump and plasmid mediated efflux genes in ciprofloxacin (CIP) resistant hospital acquired uropathogenic *Escherichia coli* and *Klebsiella pneumoniae*. Also, to assess the anti-biofilm action of carbonyl cyanide m-chlorophenylhydrazone (CCCP). Uropathogenic *E. coli* and *K. pneumoniae* isolates were collected from Mansoura University Hospitals in Mansoura, Egypt. The effect of Sub- minimum inhibitory concentration (MIC) of CCCP on CIP MIC was evaluated and the MIC decrease factor (MDF) was calculated. The presence of *oqxAB* and *qepA* genes was detected by PCR. The effect CCCP on biofilm was detected in strong biofilm formers. 56 and 47 CIP-resistant uropathogenic *E. coli* and *K. pneumoniae* isolates respectively were detected. Significant MDF by CCCP was observed in 55.3% of these isolates. The *qepA* gene was only present in *E. coli*. However, *oqxAB* genes were found only in *K. pneumoniae*. Biofilm formation was detected in 58.9% and 72.3% of CIP-resistant *E. coli* and *K. pneumoniae* isolates, respectively. Biofilm formation was significantly decreased by CCCP. According to these findings, CIP resistance and plasmid-mediated efflux pumps in uropathogenic *E. coli* and *K. pneumoniae* are of rising concern. Efflux pump inhibitor CCCP represents a possible option to decrease the biofilm formation in these resistant urinary pathogens.

Keywords: Ciprofloxacin Resistance, Biofilm, CCCP, Efflux Pump

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INTRODUCTION

Urinary tract infection (UTI) represents a predominant infection in both healthcare settings and community.[1] The most common bacterial cause of UTI is Escherichia coli, but many other *Enterobacteriaceae* such as *Klebsiella pneumoniae* are also present.[2]

The Multidrug-resistant (MDR) *Enterobacteriaceae* is increasing as a cause of UTI.[3] Biofilm formation plays a significant role in UTIs.[4] When these uropathogens colonize the mucous membrane of the urinary tract, they tend to form biofilm, which favors their persistence for a long time. In addition, microbial biofilms are impermeable to many antibiotics that contribute to the development of MDR bacteria.[5]

Fluoroquinolones (FQs) represent one of first-line agents for UTI empirical therapy.[6] Efflux pumps (EPs); *QepA* and *oqxAB* are important contributors of quinolones resistance in *Enterobacteriaceae* including *E. coli, Klebsiella* spp. These pumps actively extrude FQs from the cytoplasm. *QepA* is a proton-dependent transporter that belongs to the superfamily of major facilitators. *QepA* promotes resistance to quinolone,[7] whereas *OqxAB* is a multidrug efflux pump with transmissible resistance-nodulation division that decreases bacterial susceptibility to ciprofloxacin (CIP).[8]

These EPs can be blocked by *in vitro* inhibitors such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The effect of EP inhibitor CCCP against resistant Gram-negative bacteria has been investigated.[9] This study was aimed at finding out the role of EP in CIP resistance of *E. coli* and *K. pneumoniae* causing hospital acquired UTI. In addition, the prevalence of plasmid-mediated EP genes, biofilm, and the anti-biofilm effect of CCCP against these isolates were investigated.

MATERIALS AND METHODS

Bacterial Identification

A Prospective cross-sectional study was carried out for 18 months from March 2019 to August 2020 in Mansoura University hospitals (MUHs), Mansoura, Egypt. The Institutional Research Board at faculty of medicine, Mansoura University has approved this study (with a code number R.21.09.1463). Each patient included in this study signed an informed consent. Urine samples were collected from patients with suspected UTIs. Cystine–lactose–electrolyte-deficient (CLED) agar (Oxoid- UK) plates were used to culture collected urine samples according to method described before.[10]

Routine microbiological methods were used to identify bacterial isolates, biochemical reactions (Kligler iron agar, oxidase test, lysine iron agar, methyl red, Voges-Proskauer test, citrate tests, motility indole ornithine).[10] API 20E (BioMerieux) was used to confirm the bacterial identification.

Antimicrobial Susceptibility Testing

The antibiotic susceptibilities of *E. coli* and *K. pneumoniae* isolates were determined by disc diffusion method according to guidelines of Clinical and Laboratory Standards Institute(CLSI).[11] The tested antibiotics include: gentamicin, ciprofloxacin, cefuroxime, cefotaxime, ceftazidime, cefoxitin, amoxicillin/clavulanic, piperacillin/tazobactam, imipenem, trimethoprim/sulfamethoxazole, and aztreonam. Quality control was done using *E. coli* ATCC®25922TM strain. Criteria for multidrug-resistant (MDR) phenotypes were defined as described before.[12]

Determination of CIP and CCCP Minimum Inhibitory Concentrations

Broth micro dilution assay was done for determining CIP and CCCP minimum inhibitory concentrations (MICs) in *E. coli* and *K. pneumoniae* isolates. The procedure and break points interpretation were done according to CLSI recommendations.[13] Pure powders of CIP and CCCP were acquired from Sigma Aldrich (St. Louis, MO, USA). *E. coli* ATCC®25922TM strain was used as Quality control.

Evaluating the Effect of EP Inhibitor CCCP on CIP Resistance

To detect the EP mechanism, the effect of EP inhibitor CCCP on CIP MIC was determined.[14] CIP MIC in CIP resistant isolates was re-measured in the presence of a sub-MIC of CCCP (i.e. 0.5 × MIC) using serially rising concentrations of the antibiotics.

The MIC decrease factor (MDF) was calculated for each isolate using this formula; MDF = CIP MIC in the absence of CCCP/ CIP MIC in presence of CCCP.[14] With MDF value of 4 or above, significant inhibition of EP by CCCP was considered (in the presence of CCCP, the CIP MIC drops fourfold or more).[14]

Plasmid Mediated EP Genes

Plasmid EP genes were assessed in CIP resistant isolates as previously described by two PCR: one PCR for *oqxAB* genes7 and the other PCR for the detection of qep A gene8 (Table 1).

Detection of Biofilm

Biofilm formation assays were performed for CIP resistant isolates as previously described.[15] Overnight culture of isolates was adjusted to turbidity 0.5 McFarland standard. Then for each isolate, 125 microliters of 1:100 diluted bacterial suspensions were put in sterile flat-bottomed 96-well microtiter plates and incubated at 37°C for 24 hours. The wells were washed thrice with 300 mL of sterile phosphate buffered saline (PH 7.3) and dried inverted at room temperature. For staining of the formed biofilm, 125 µL crystal violet of 0.1 percent was added for 10–15 minutes followed by rinsing with distilled water for three times. Uninoculated medium was used as control. The experiment was done in triplicates. A microtiter plate was read at the optical density (OD) 600 nm. The strains were then categorized using the following criteria: The cut-off OD (ODc)= mean OD of the negative control+ three standard deviations (SD). Biofilm were classified as strong biofilm producer when (OD more than 4×ODc); OD of non biofilm producer (OD equal to or less than ODc); weak biofilm producer (ODc< OD≤2x ODc); moderate biofilm producer (2 x ODc< OD≤4 x ODc).[16]

The Effect of Sub-MICs of CCCP on Biofilm Formation

The effect of CCCP on the formation of biofilm were assessed on strong biofilm producing CIP resistant isolates of *K. pneumoniae* and *E. coli*.

The biofilm assays were done as above but with the addition of sub MIC CCCP concentration of each strain compared to biofilm formation in absence of CCCP as control. The microtitre plate was read at the OD600nm after 24 h incubation at 37°C.

Statistical Analysis

After the KS test for normality, quantitative data will be described as mean (SD) or median (range). parametric tests were used for normally distributed data, while non parametric tests were used for non normally distributed data. A P value is considered statistically significant when it is < 0.05.

RESULTS

Bacterial Isolates

During the study period, 159 isolates (88 *E. coli* and 71 *K. pneumoniae*) were identified from a total of 279 cases of suspected UTIs. These urinary bacterial isolates were obtained from 89 (56%) females and 70 (44%) from males. The age of patients ranged from 19 to 77 years (48.4±13.3). Out of these 159 isolates, 56 isolates *E. coli* and 47 *K. pneumoniae* (64.8%) were CIP resistant (Table 2).

Table 1. Primers of plasmid EP genes

Gene	Sequence of primer	Annealing temp.	Product size	Ref.
oqxA	CTCGGCGCGATGATGCT CCACTCTTCACGGGAGACGA	68°C	392	7
oqxB	TTCTCCCCCGGCGGGAAGTAC CTCGGCCATTTTGGCGCGTA	64°C	512	7
qepA	GCA GGT CCA GCA GCG GGT AG CTT CCT GCC CGA GTA TCG TG	60°C	199	8

Antibiotics Sensitivity Testing

The highest resistance among *E. coli* isolates was cefuroxime (86.4%) followed by amoxicillin / clavulanic acid (76.1%) and the least was gentamicin (27.3%). Regarding *K. pneumoniae*, the highest resistance was found in amoxicillin / clavulanic acid (78.9%) and cefuroxime (76.1%) and the least was in piperacillin-tazobactam (47.9%). CIP resistance was reported in 63.6% of *E. coli* and 66.2% of *K. pneumoniae* isolates (Table 2). 93% of the *E. coli* and 95% of *K. pneumoniae* were MDR.

Table 2. Antibiotic resistance pattern of uropathogenic *E. coli* and *K. pneumoniae*

Antibiotic	*E. coli* Total =88 N (%)	*K. pneumoniae* Total =71 N (%)
Gentamicin	24 (27.3)	41 (57.7)
Ciprofloxacin	56 (63.6)	47 (66.2)
Cefuroxime	76 (86.4)	54 (76.1)
Cefoxitin	46 (52.3)	36 (50.7)
Cefotaxime	66 (75)	51 (71.8)
Ceftazidime	60 (68.2)	55 (77.5)
Amoxicillin / clavulanic acid	67 (76.1)	56 (78.9)
Piperacillin-tazobactam	30 (34.1)	34 (47.9)
Imipenem	38 (43.2)	55 (72.5)
Trimethoprim-sulfamethoxazole	54 (61.4)	47 (66.2)
Aztreonam	46 (76.1)	39 (54.9)

Ciprofloxacin Resistance

Ciprofloxacin MIC values of isolated *E. coli* and *K. pneumoniae* ranged from 0.125 to 256 mg/L using the broth microdilution method. One hundred and three isolates were CIP resistant. CIP MIC estimates ranged from 1–256 mg/L. Sixty-four isolates (62.1%)of the isolates were high-level CIP resistance (MIC ≥ 32 mg/L), the MIC 50 and MIC90 were 32 mg/L and 256 mg/L, respectively.

Activity of EP

Reduction of fourfold or more CIP MIC in the presence of CCCP (MDF value of higher than 4) was detected in 57(55.3%) CIP resistant isolates.

Table 3. Biofilm formation in CIP resistant uropathogenic *E.coli* and *K. pneumoniae*

	E.coli 56 N (%)	*K. pneumonia* 47 N (%)
Strong biofilm formers	6 (10.7)	8(17)
Moderate biofilm formers	16(28.6)	14(29.8)
Weak biofilm formers	11 (19.6)	12(25.5)
Non biofilm formers	23 (41.1)	13(27.7)

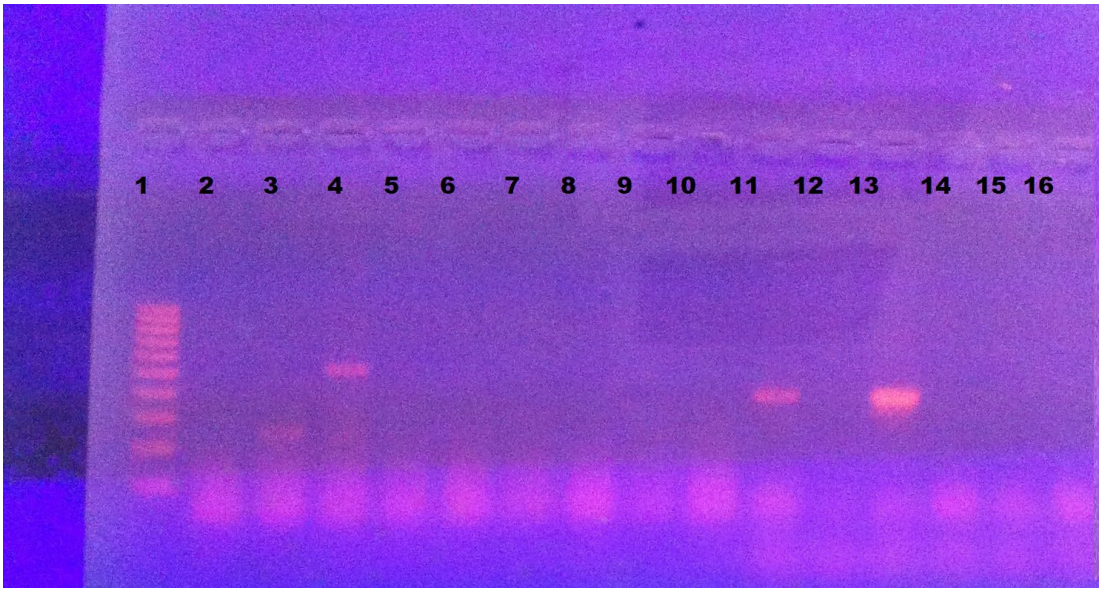

Figure 1. Detection of *oqxAB* genes by PCR to detect *oqxA* (392 bp) and *oqxB* (512bp). Lane 1 showing 100 pb DNA ladder Lane 4 showed *oqxB.* Lane 11 and 13 showed *oqxA*

Plasmid Mediated EP Genes in Quinolone Resistant Isolates

As regard resistant *K. pneumoniae*, both *OqxA* and *OqxB* genes were detected together in 8 isolates, while *OqxA* gene only was detected in 12 isolates. *OqxA* nor *OqxB* was not detected in any of *E. coli* isolates. *qepA* was detected in 12 isolates of *E. coli* (Figure 1, 2).

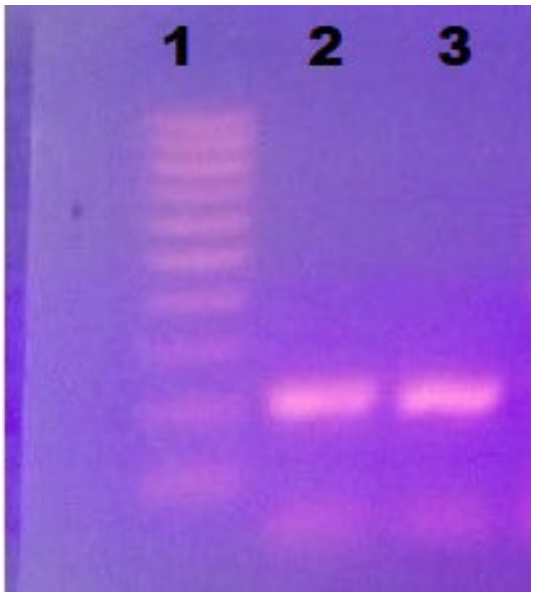

Figure 2. Detection of *qepA* gene (199bp) by PCR. Lane 1 showing 100 pb DNA ladder and ladder 2, 3 showed *qebA*

Biofilm Formation in CIP Resistant Isolates

Among CIP resistant isolates, biofilm formation was detected in 33 (58.9%) of *E. coli* and 34(72.3%)of *K. pneumoniae* isolates. Six of *E. coli* in addition to 8 *K. pneumoniae* isolates expressed strong biofilm production capacity (Table 3). MICs of CCCP, CIP and resistance genes among strong biofilm producers were summarized in Table 4.

Effects of CCCP sub-MIC on Biofilm Formation

The effect of sub-MIC concentration of CCCP was evaluated on biofilm forming capacity of strong biofilm producing isolates (six *E. coli* and eight *K. pneumoniae* isolates). The results showed that sub-MIC of CCCP significantly inhibited the formation of *E. coli* and *K. pneumoniae* biofilms (P equal 0.002 and 0.0001 respectively) (Figure 3).

DISCUSSION

Treatment of MDR bacteria in UTI represents a challenge in healthcare settings.[3] MDR organisms expressed simultaneous resistance to common antibiotics that are used in the treatment of UTI such as aminoglycosides, beta-lactams and FQs that are considered a great concern.[17] Plasmid exchange of resistance aggravates the problem and represents major threat to health care system.[18] In this study, 93% and 95% of isolated urinary *E. coli* and *K. pneumoniae* were MDR, respectively.

Table 4. MIC and plasmid mediated EP genes among strong biofilm producing CIP resistant *E. coli* and *K. pneumoniae*

Strain No.	MIC				*OqxA*	*OqxB*	*qepA*
	Species	CIP	CCCP	CIP+ subMIC CCCP (MDF)			
7	*E. coli*	256.0	32.0	128 (2)	-	-	-
67	*E. coli*	256.0	32.0	32 (8)	-	-	+
70	*E. coli*	256.0	32.0	64 (4)	-	-	-
9	*E. coli*	256.0	16.0	32 (8)	-	-	+
89	*E. coli*	128.0	16.0	128 (0)	-	-	-
81	*E. coli*	128.0	32.0	32 (4)	-	-	-
942	*K. pneumoniae*	128.0	32.0	32 (4)	-	-	-
959	*K. pneumoniae*	64.0	16.0	32 (2)	+	-	-
824	*K. pneumoniae*	4.0	16.0	2 (2)	-	-	-
6	*K. pneumoniae*	256.0	16.0	32 (8)	-	-	
72	*K. pneumoniae*	4.0	32.0	2 (2)	-	-	-
65	*K. pneumoniae*	4.0	64.0	1 (4)	-	-	-
60	*K. pneumoniae*	256.0	32.0	128 (2)	+	-	-
363	*K. pneumoniae*	256.0	32.0	32 (8)	+	+	-

Similar high percentage of MDR in uropathogenic *E. coli* in a study that was conducted in Egypt, 90.85% were MDR,[19] but lower percentage of MDR *E. coli* was reported in previous studies in Saudi arabia[20] and Australia.[21]

Resistance to broad spectrum 2nd generation (Cefuroxime) cephalosporin and amoxicillin-clavulanic acid was found to be the highest in uropathogenic *E. coli* representing (86.4%, 76.1%) and in *K. pneumoniae* representing (76.1%, 78.9%). Resistance to 3rd generation cephalosporin cefotaxime and ceftazidime in *E. coli* was 75%and 68.2%, respectively and in *K. pneumoniae* was 71.8% and 77.5%, respectively. Imipenem resistance was presented in about forty percentages (43.2%) of isolated uropathogenic *E. coli* and about seventy percentages (72.5%) of *K. pneumoniae* isolates.

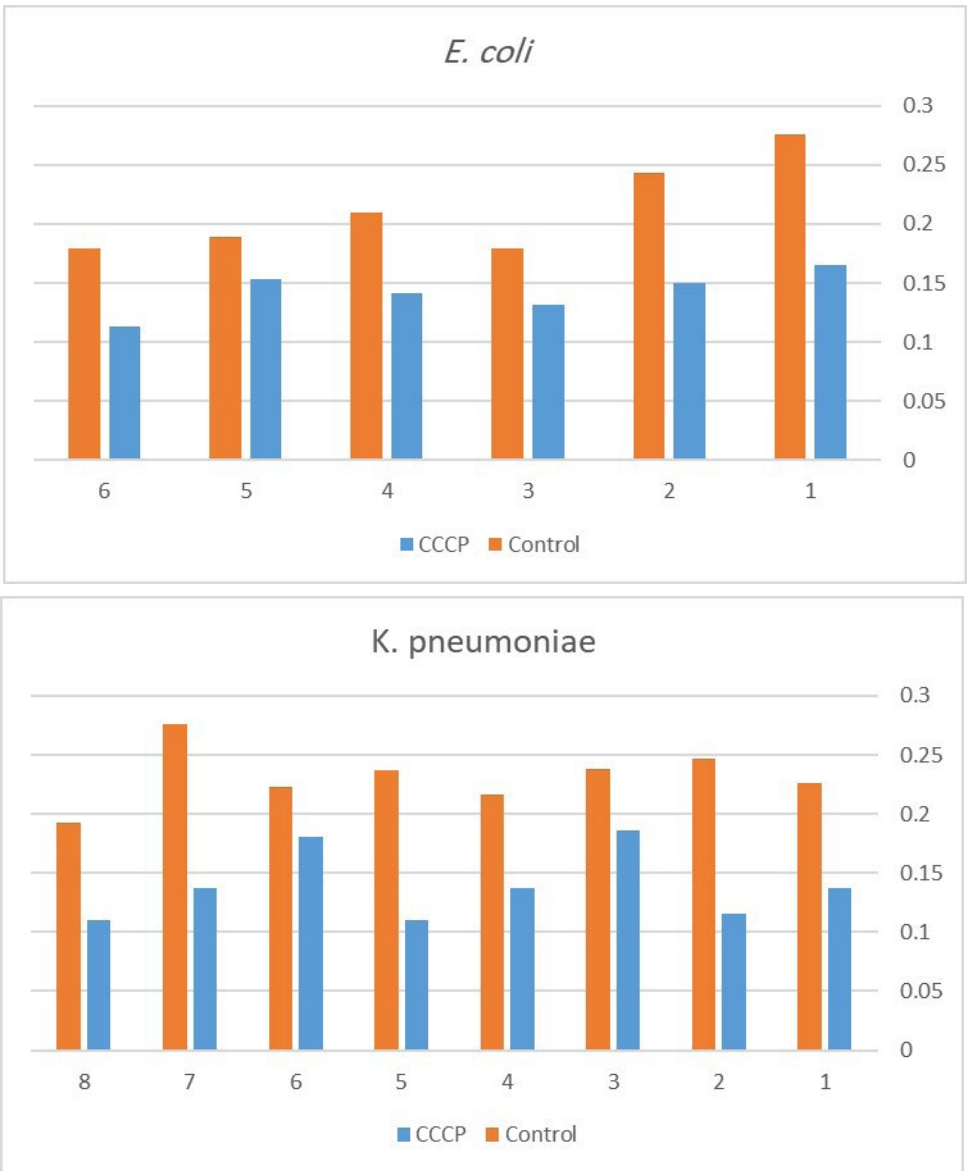

Figure 3. Impact of CCCP on the biofilm formation after 24h of incubation compared to control (a) *E. coli* (b) *K. pneumoniae*

Resistance to beta lactam antibiotics is a global phenomenon, but occurrence rates are variable. Matching with the result of this study, high resistance rate to 2nd and 3rd generations cephalosporin was found by Tandogdu[22] in *Klebsiella* and by Esmaeel et al.[23] in uropathogenic *E. coli* isolates in Egypt. Similarly, high prevalence of amoxicillin–clavulanic acid resistance was detected in *K. pneumoniae,*[24] and in *E. coli* in Asian area.[22] However, lower rate of resistance was detected in *K. pneumoniae* in Tunisia.[25]

About 64.8% of the isolates were CIP resistant including 62.1% of the isolates that showed high CIP resistance (MIC >32). This result consistent with previous report.[23] High rates of CIP resistance 75% and 91.4% was reported in urinary isolates of *E. coli* in other studies in tertiary hospitals. High CIP resistance was described previously.[26] Lower rate of CIP resistance (33%) was reported in urinary *K. pneumoniae*.[24]

This high rate of resistance in this study to beta-lactam antibiotics and FQ could be due to their wide use as an empirical therapy for community acquired UTIs and gastrointestinal infection representing a selective pressure for development of resistance in patient's flora, which represents the main source of UTI infection. In addition, the use of these agents empirically to treat UTIs with the absence of antibiotic policy in the hospital may represent another factor for this high rate of resistance.[27]

Numerous mechanisms are responsible for FQ resistance for example decreases in membrane permeability, efflux systems, and topoisomerase mutations.[28] EP genes that transfer quinolones outside the bacterial cytoplasm are involved in plasmid-mediated quinolone resistance. Two of them are well known; *qepAB* and *oqxAB*.[29] By transporting antibiotics across the membrane, these EP systems *OqxAB* increased CIP and norfloxacin MICs 32- and 64-fold, respectively, above the control strain. Because *OqxAB* conferred CIP resistance on other *Enterobacteriaceae*, the spread of this FQ resistance mechanism is very likely.

The over expression of *OqxAB* and *QepA* mediates resistance to many antibiotics including quinolone. This plasmid-borne MDR-EP may represent a major problem, as it may pose resistance to many antimicrobial agents in addition to the spread of this resistance via horizontal transfer.[30]

The efflux systems as a cause of drug resistance in *Enterobacteriaceae* is growing.[9] EP inhibitor CCCP was used to reveal the contribution of EPs in CIP resistance and MIC of CIP was assessed in the presence of sub-inhibitory concentrations of CCCP. Reduction in CIP MIC (MDF ≥ 4) was as a principle for significance, efflux activity was present in 57 (55.3%) of CIP resistant isolates. Likewise, the role drug EP was detected in 47.5% of CIP resistant isolates of *K. pneumoniae*.[31] While the role of EP was at lower rate that range from 12.1% and 18.3% of isolates of *Enterobacteriaceae* in other study.[14] Higher rate of EP inhibition by CCCP was previously reported with CIP MIC reduction ranged from 2-64 fold in 93% of MDR *k. pneumoniae*.[32]

As regard plasmid-mediated EPs, *QepA*-was found in *E. coli* , while *oqxAB* was detected in *K. pneumoniae* only. *OqxA* gene was the highest prevalence in *K. pneumoniae* (20 isolates) including eight isolates had both *OqxA* and *OqxB* genes. *qepA* was found in 12 isolates of *E. coli*. The same was reported in a study conducted in CIP resistant isolates in Egypt.[14] Also efflux pump *qepA* was detected in 12.3% of CIP resistant bacteria isolated from UTI.[33] Higher rate of *oqxAB* was detected in *K. pneumoniae* but it was not detected in *E. coli*. Moreover, *QepA* was not detected in any of Enterobacteriaceae species in a study conducted in South Africa.[34] Also, *QepA* was not detected in other studies in India and South Africa.[26,35]

In this study, biofilm was produced in 60% and 75.5% of quinolone resistant *E. coli* and *K. pneumoniae*, respectively. A similar higher rate was reported in Egypt,[19] Ethiopia[36] and Iran,[37] while, a lower rate of biofilm production *Klebsiella* and *E. coli* was also described.[38,39]

This study assessed the ability of EP inhibitor CCCP to inhibit biofilm formation in strong biofilm forming isolates with EP activity. Biofilm formation was inhibited by sub-MICs of CCCP in both *E. coli* and *K. pneumoniae*. The same was previously in a study showed inhibition of biofilm formation of *Salmonella typhimurium* by CCCP.[40] Also, a study investigates *K. pneumoniae* biofilm showed CCCP inhibition of biofilms in

a dose-dependent effect. With the effective inhibitory concentration of CCCP towards *K. pneumoniae* EP was 10 µg/mL.[41]

CONCLUSION

CIP resistance in hospital acquired uropathogenic *E. coli* and *K. pneumoniae* is of high prevalence and plasmid mediated EP is of rising concern. Biofilm development is a critical virulence determinant in these organisms. EP inhibitor CCCP represents a possible option to decrease the biofilm formation in these resistant urinary pathogens.

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None.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

RE designed the study. RE and GM conducted experiments. RE wrote the draft manuscript. RE and GM wrote and revised the manuscript. Both authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

The Institutional Research Board at faculty of Medicine, Mansoura University approved this study with code number R.21.09.1463.

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