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Fluoroquinolones resistance and its relationship to extended-spectrum β-lactamases production in *Proteus species* isolated from some Egyptian Patients

Mohamed Maher*, Al-Saied A-H. M. Helal, Moselhy S. Mansy, Kamel A. Al-Ghareeb

Department of Microbiology and Immunology, Faculty of Pharmacy, Al- Azhar University, Cairo, Egypt.

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***Corresponding author:**

Email : m_maher872010@yahoo.com
Tel.: 01111261040

ABSTRACT

Beta-lactams and fluoroquinolones antibiotics are widely used in treatment of bacterial infections including those caused by *Proteus species*. Wild-type strains of *Proteus spp.* are usually susceptible to these antibiotics. Development of resistance to such antibiotics severely limits therapeutic options and complicates the treatment process. This study aimed to investigate the prevalence of some genes that confer resistance to beta-lactams and fluoroquinolones among clinical isolates of *Proteus spp.* A total of 220 *Proteus* isolates were selected from 1547 clinical specimens collected from patients attended 4 Egyptian local hospitals during the period from October 2014 till March 2017. Fifty one of the *Proteus* isolates exhibited multi-drug resistance (MDR). Those MDR *Proteus* isolates were resistant to extended spectrum cephalosporins; such resistance was revoked on the use of β-Lactamase inhibitor such as clavulanic acid. These isolates were tested phenotypically for the production of extended spectrum beta-lactamases (ESBLs) and genotypically for detection of bla_{TEM} and *aac(6')Ib-cr* genes. Results: 33/51 (64.7%) of isolates showed phenotypic ESBL production by double disc diffusion synergy test; whereas 36/51 (70.58%) of isolates showed identical results on using the combination disc method. Genotypically: Among the 25 tested MDR isolates, 11 isolates (44%) showed positive bla_{TEM} gene encoding and 5 isolates (20%) showed the presence of *aac(6')Ib-cr* gene.: Beta-lactams and fluroquinolones co-resistance due to encoding of *bla*TEM and *aac(6')Ib-cr* genes has been well identified among clinical isolates of *Proteus spp.* in Egypt.

INTRODUCTION

Proteus spp. are among the common etiological agents of complicated infections in the urinary tract, respiratory tract, ear, burns and wounds [1]. Although wild-type strains of *Proteus spp*. are usually susceptible to beta-lactams and fluoroquinolones, a marked increase in resistance to such antibiotics has been reported in clinical isolates of these species[2,3]. The relationship between fluoroquinolone resistance and extended spectrum β-Lactamase(ESBL) production in some members of the family *Enterobacteriaceae* is now well-known, however, the epidemiology of fluoroquinolone resistance and its relationship to ESBL production in *Proteusspp*.have not yet been clarified[3,4].

MATERIALS AND METHODS

Bacterial isolates

A total of 220 *Proteus* isolates were obtained from 1547 clinical specimens (14.22%) (including urine, sputum, ear swabs and wound swabs)collected from in- and out-patients at the ICU, surgery, out-clinics and chest departments of Al-Hussein teaching hospital, Health insurance hospital, Qasr Al-Ainy teaching hospital, and Sayed Galal teaching hospital (Cairo/Egypt) during the period from October 2014 till March 2017 (Table 1).The isolates were identified by the conventional methods according to Collee *et al*. (1996), Konemanet *al*. (2006) and Brooks *et al*. (2007)[5-7] and by using the API 20E system kits (Biomerieux, France).

Table 1 : Clinical specimens collected from patients at different Egyptian hospitals :

Specimen type / Hospital	Ear discharge		Sputum		Urine		Wound		Total	
	T	P	T	P	T	P	T	P	T	P
Al-Hussein	35	2 (5.71%)	53	3 (5.66%)	56	16 (28.57%)	97	8 (8.24%)	241	29 (12%)
Health insurance	42	3 (7.14%)	65	2 (3.07%)	120	26 (21.66%)	169	41 (24.26%)	396	72 (18.18%)
Qasr Al-Ainy	34	0 (0%)	57	8 (14.03%)	274	47 (17.15%)	104	19 (18.26%)	356	74 (20.78%)
SayedGalal	44	6 (13.63%)	68	3 (4.41%)	195	23 (11.79%)	134	13 (9.7%)	441	45 (10.2%)
Total	155	11 (7.09%)	243	16 (6.58%)	645	112 (17.36%)	504	81 (16.07%)	1547	220 (14.22%)

T= total number of clinical specimens.
P= number of *Proteus* isolates.

Antimicrobial susceptibility test:

The antimicrobial susceptibility tests for the isolates were performed by the disc diffusion method according to Kirby-Bauer protocol (2003)[8] and the interpretation of the antibiogram was done according to CLSI (2014)[9]. The tested antimicrobial agents (concentration/disc) included amikacin (30µg), amoxycillin/clavulanic acid "20/10" (30µg), ampicillin (10µg), ampicillin/sulbactam "20/10" (30µg), cefotaxime (30µg), cefoxitin (30µg), ceftazidime (30µg), ceftriaxone (30µg), ciprofloxacin (5µg), doxycycline (30µg), gentamicin (10µg), imipenem (10µg), levofloxacin (5µg), meropenem(10µg), nitrofurantoin (300µg), norfloxacin (5µg), piperacillin (100µg), and sulfamethoxazole/trimethoprim "1.25/23.75" (25µg) products of Oxoid, UK.

Primers:

The following primers, (Table 2) were obtained from (Invetrogen, UK).

Phenotypic detection of ESBLs

Fifty one MDR isolates which were resistant to extended spectrum beta-lactams but sensitive to beta-lactams when combined with clavulanic acid were selected for further testing of ESBLs production phenotypically by combination disc method (CDM) and double disc diffusion synergy test[10,11].

Combination disc method (CDM) for detection of ESBLs

An overnight culture of the test isolate was suspended to the turbidity of 0.5 McFarland and used to swab a Muller-Hinton (MH) agar plate. Discs of ceftazidime 30 µg, ceftazidime-clavulanate (30 µg/10 µg), cefotaxime 30 µg and cefotaxime-clavulanate (30 µg /10 µg) were placed on MH agar. Isolates were considered ESBL positive if the inhibition zone measured around one of the combination disks after an overnight incubation was at least 5 mm larger than that of the corresponding cephalosporin disk (CLSI(2014) and Garrec*et al.* (2001)[9,10].

Double disc diffusion synergy test for detection of ESBLs

An overnight culture of the test organism on MH agar was suspended to the turbidity of 0.5 McFarland in 5.0 mL of saline. An aliquot of 500 µL of this suspension was streaked for a confluent growth on a MH agar plate. A disc of amoxicillin/clavulanic acid "20/10 µg" (30 µg) was placed at the center of the plate and the discs of aztreonam, ceftazidime, ceftriaxone, and cefepime were placed in close proximity of 20 to 30 mm distance. Clear extension of the edge of the inhibition zone of cephalosporins toward the amoxicillin/clavulanic acid disc was interpreted as ESBL producer and considered as positive test[11].

DNA extraction

Total crude DNA was extracted from isolates by heating

Table 2 : Primers used in the present study

Target gene	Primers sequence (5' – 3')
bla_{TEM}	Forward primer: 5'-TCCGCTCATGAGACAATAACC-3' Reverse primer: 5'- TTGGTCTGACAGTTACCAATGC-3'
aac(6')Ib-cr	Forward primer: 5'- TTGCGATGCTCTATGAGTGGCTA-3' Reverse primer: 5'-CTCGAATGCCTGGCGTGTTT-3'

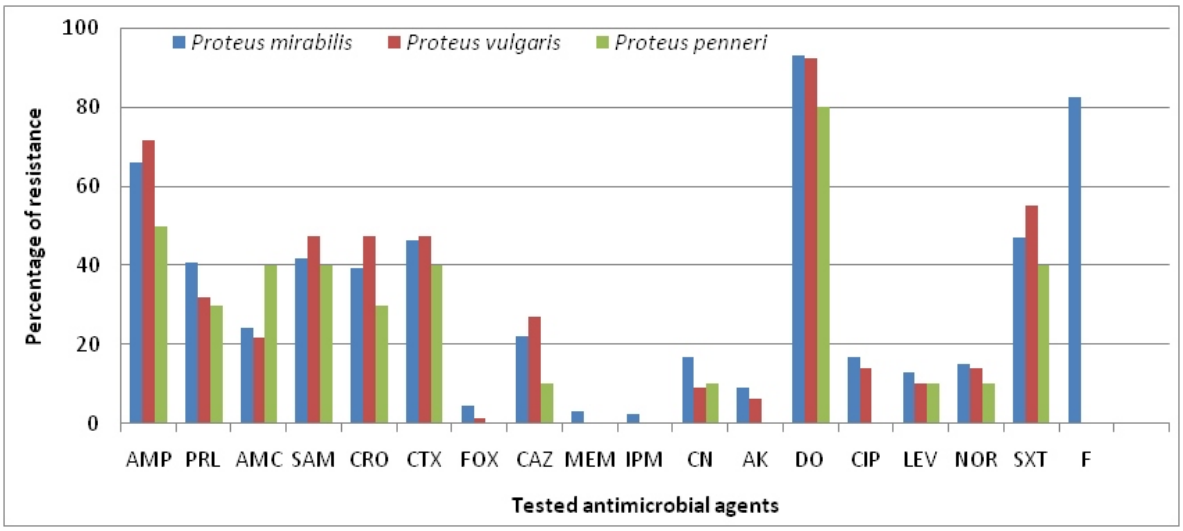

Figure 1 : Antibiotic resistance profile of *Proteus* isolates

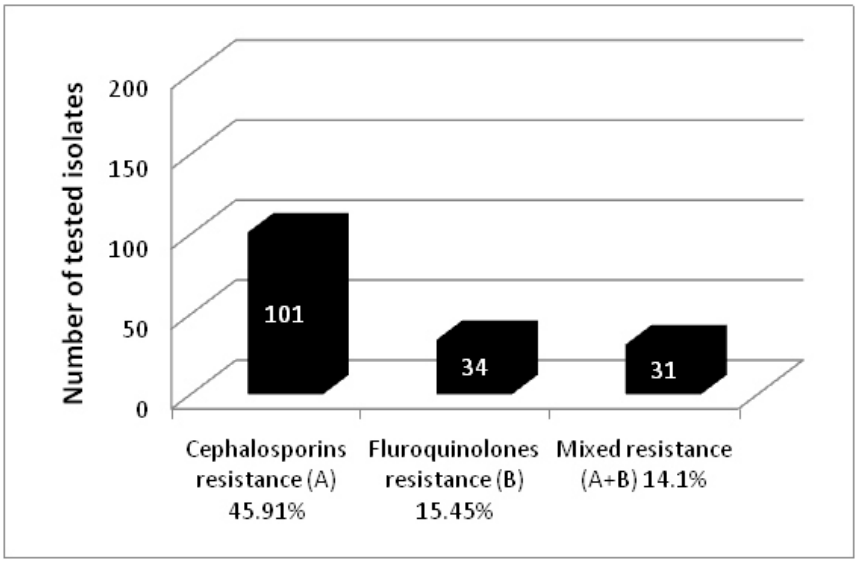

Figure 2 : Resistance to cephalosporins and fluroquinolones among *Proteus* isolates

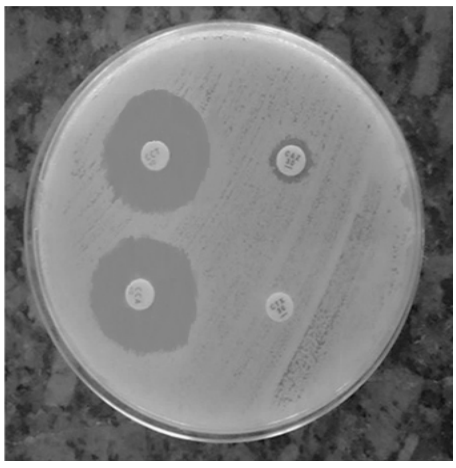

Figure 3 : CDM for detection of ESBLs

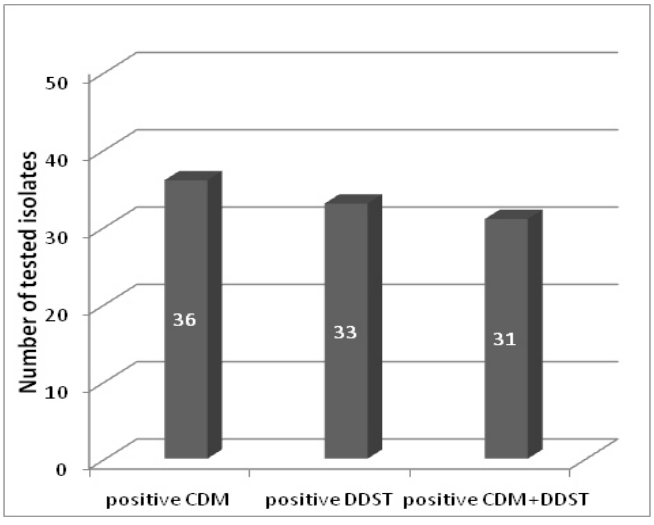

Figure 4 : Phenotypic tests results

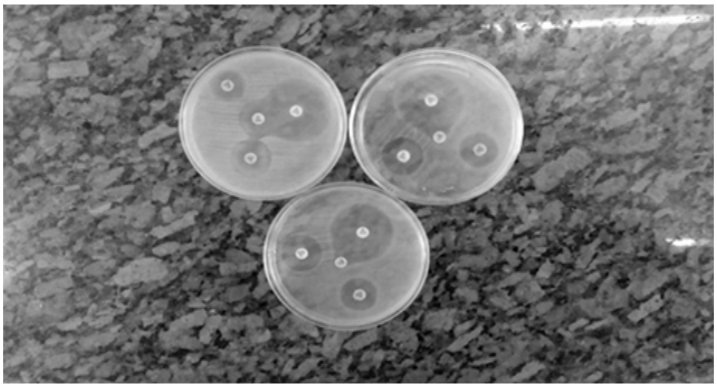

Figure 5 : Double disc diffusion synergy test for detection of ESBLs

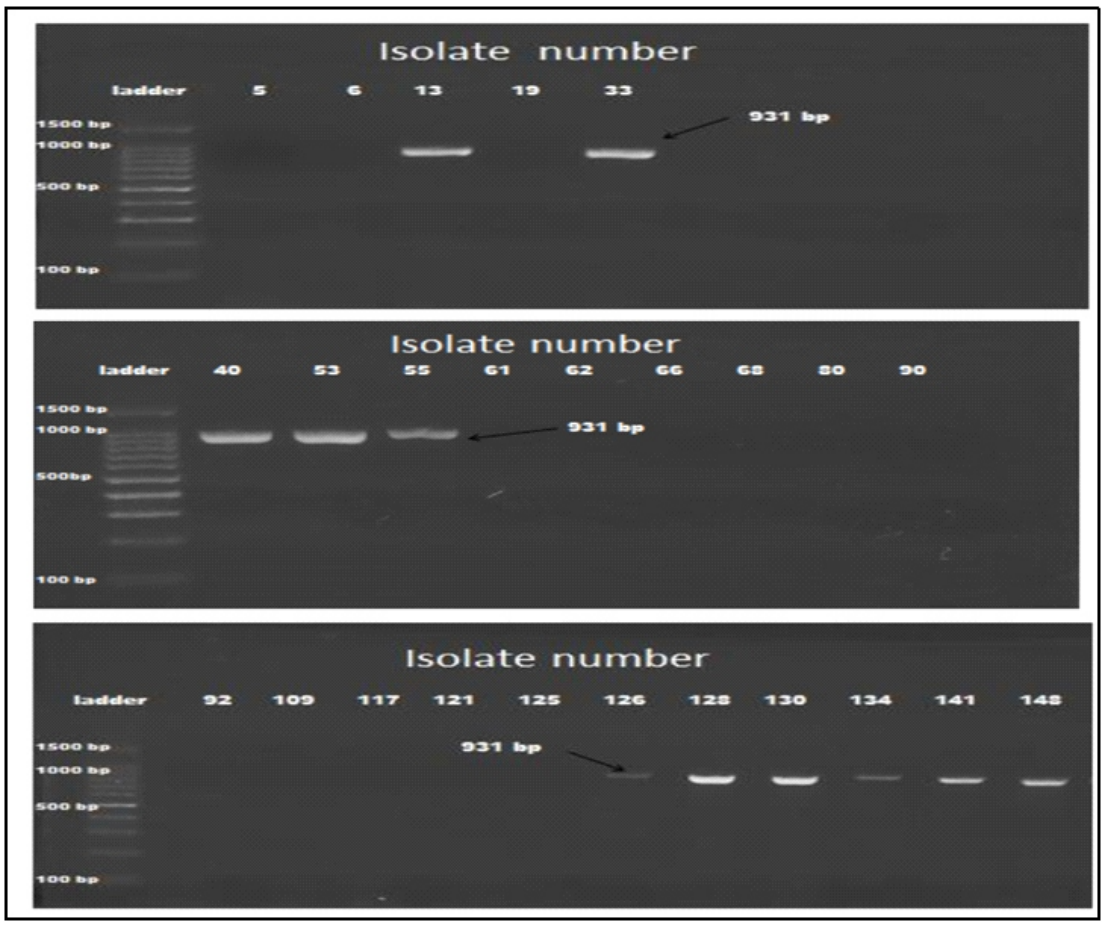

Figure 6 : PCR amplification of *bla*$_{TEM}$in some *Proteus isolates*

bacterial suspension in sterile distilled water at 95°C for 10 min, followed by removal of cellular debris by centrifugation at 14,000 rpm for 1 min. The supernatant was collected and used as a template DNA[12].

Detection of bla$_{TEM}$ and aac (6') Ib-cr

1-Preparation of the specimens

The lyophilized primers (Table 2) were reconstituted in nuclease free water and the concentration of the primers was adjusted to10 picomole/µL. The Polymerase Chain Reaction (PCR) was set up in a PCR tube (total volume 20 µL) by adding 10 µL of the double strength master mix (2X), 1µL of the forward and reverse primers and 1µL of the template DNA and the volume was completed to 20 µL by addition of nuclease free water 8µL. The PCR reaction was performed in GeneAmp thermal cycler[13].

2-Detection of bla$_{TEM}$

The specimens were subjected to initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds and extension at 72°C for 1minute. A final extension procedure was carried out at 72° C for 7 min. Gel electrophoresis was then carried out using 1% agarose gel. Gel was viewed in a UV transilluminator and the bands pattern was observed[12,13].

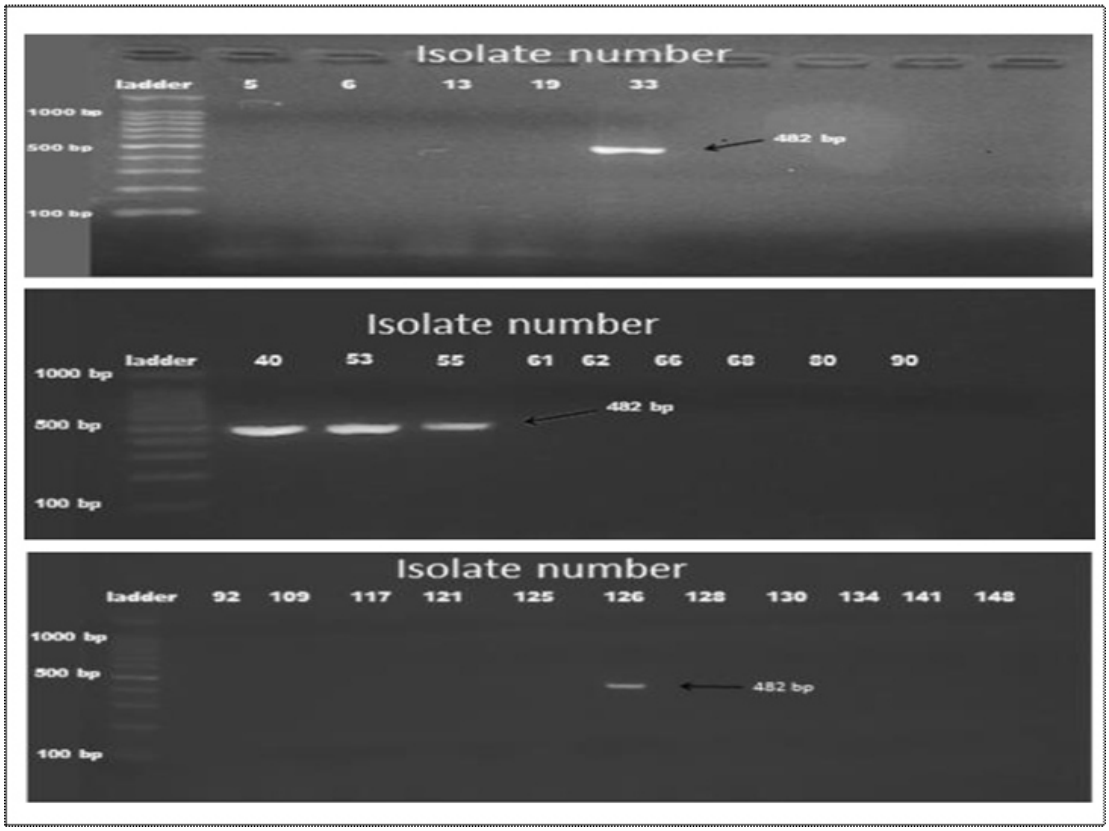

Figure 7 : PCR amplification of *aac (6') Ib-cr* in some *Proteus* isolates

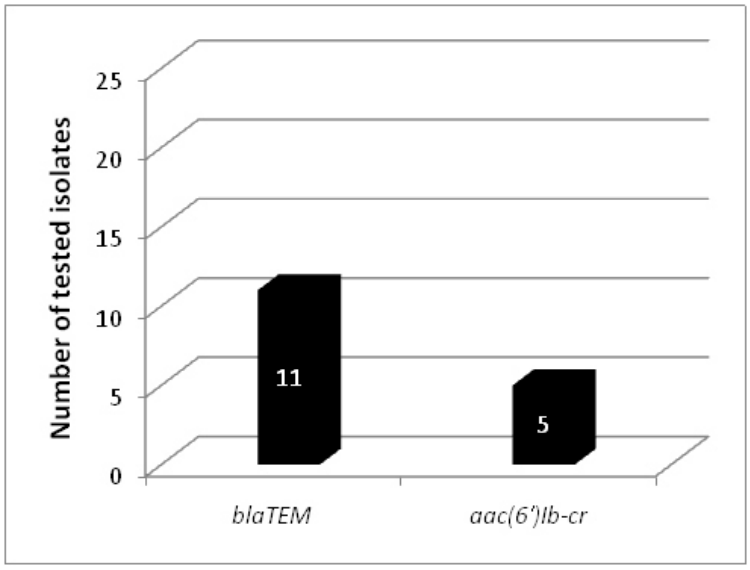

Figure 8 : Genotypic tests results

3-Detection of aac (6') Ib-cr

Similar procedures to that used for detection of bla_{TEM} were carried out to detect *aac (6') Ib-cr* gene except that annealing was done at 56°C instead of 53°C in the bla_{TEM} procedures. Gel was viewed in a UV transilluminator and the bands pattern was observed [12,14].

RESULTS

Antimicrobial susceptibility testing of Proteus isolates

Of the tested 220 *Proteus* isolates, 34 isolates showed resistance to hydrophilic fluroquinolones; 31 of these isolates were additionally resistant to 3rd generation cephalosporins. The antibiogram of all the tested *Proteus* isolates is illustrated in figure (1).

Phenotypic detection of ESBLs:

Combination disc method (CDM) for detection of ESBLs

Among the 51 MDR *Proteus* isolates which were resistant to third generation cephalosporins but sensitive when clavulanic acid is combined, 36 isolates (70.58%) were positive for CDM

and 15 isolates (29.41%) were negative for this test, positive results are shown in figures (3 and 4)

Double disc diffusion synergy test for detection of ESBLs

Among the tested 51 isolates, 33 (64.7%) isolates showed positive ESBL production by DDST; while 18 isolates (35.29%) were negative for this test, results are shown in figures (4 and 5).

PCR detection of bla_{TEM} gene

Twenty five isolates that showed phenotypic ESBLs production by both CDM and DDST were further tested for the presence of bla_{TEM} gene using the PCR technique. Eleven of these isolates 44% (6 isolates from urine, 4 isolates from wound and 1 isolate from ear swab clinical specimens) showed the presence of bla_{TEM} gene at 931 bp as illustrated in figure (6).

PCR detection of aac (6') Ib-cr gene

Twenty five isolates that showed phenotypic ESBLs production by both CDM and DDST were further tested for the presence of *aac (6') Ib-cr* gene using the PCR technique. Five of these isolates 20% (3 isolates from urine, 1 isolate from wound and 1 isolate from ear swab clinical specimens) showed the presence of *aac (6') Ib-cr* gene at 482 bp as illustrated in figure [7].

DISCUSSION

Over the past three decades, bacterial resistance to quinolones has widely increased among clinical isolates[15]. In the present study the percentages of resistance against norfloxacin and ciprofloxacin among isolates were 32/220 (14.54%) and 33/220 (15%) respectively which are slightly higher than the percentage reported by El-Sokkary *et al.*(2015)[16]which was11.2% resistance against ciprofloxacin. Higher resistance rates against ciprofloxacin; 4/15 (26.66%) and 25/49 (51%) were reported by Abbas *et al*. (2013) and Kamel *et al*. (2014) respectively[17,18]. These differences in the resistance rates may be attributed to the difference in number of tested isolates or higher antibiotic abuse in the areas from which those isolates were collected.

According to the current results, fluoroquinolone-resistant isolates showed significantly higher frequency of resistance to ampicillin, piperacillin, ampicillin/sulbactam, ceftriaxone and cefotaxime. In the present study 32/220 (14.54%) of isolates were norfloxacin resistant from which 90.62% and 93.75% were resistant to ceftriaxone and cefotaxime respectively. In addition 33/220 (15%) of isolates were ciprofloxacin resistant from which 96.96% were resistant to ampicillin and 87.87% were resistant to cefotaxime and ceftriaxone. In Egypt, Dwedar*et al.*(2015)[19] investigated 31 *Proteus* isolates obtained from diabetic-foot infections; their results revealed that 12% of those isolates were ciprofloxacin resistant where as 6.5%, 12%, 58% and 100 % of isolates showed resistance against augmentin, amikacin, cefotaxime and ampicillin respectively. In Japan Saito *et al.* (2007) studied 13 ciprofloxacin resistant *Proteus mirabilis* isolates, 85% of their isolates were resistant to ampicillin and ampicillin-sulbactam whereas 62% of isolates were resistant to piperacillin and cefotaxime[3]. Those results agree with the results of the current study in respect of fluoroquinolone resistance which is usually accompanied with higher frequency of resistance to beta-lactams including broad spectrum cephalosporins. In the present study 36/51 (70.58%) and 33/51 (64.7%) of tested isolates were ESBLs producers as confirmed phenotypically by combination disc method (CDM) and double disc diffusion synergy test (DDST) respectively. Kamel *et al.* (2014) reported 26/33(78.7%) ESBL production among *Proteus* isolates from diabetic-foot infections by DDST[18]; whereas Saito *et al.* (2007) results showed 61.5% ESBL production by CDM[3]. Those results showed a fair agreement with the results of the current study. The prevalence rate of the bla_{TEM} gene among the tested isolates in this study was 11/25 (44%) as confirmed by PCR technique. The bla_{TEM} gene has been reported by Kamel *et al*. (2013) in 50% of their *Proteus* isolates in Egypt[18]. In a concurrent clinical study in India (2013) 8/24 (33.33%) of *Proteus spp*. isolates were found to encode the bla_{TEM} gene which was reported as the only beta-lactamase gene responsible for its extended spectrum beta-lactamase activity in that study[20]. Those results show a fair agreement with the current results. In the present study 5/25 (20%) of the tested isolates were found to encode the *aac(6')Ib-cr* gene which reflects their resistance to hydrophilic quinolones. In China Hu *et al.*(2012) reported 12/19 (63.15%) *P. mirabilis* isolates encoding this gene[21]. The *aac(6')Ib-cr* gene was reported by Mahrouki *et al*. (2013) in Tunisia in 6/50 (12%) of *P. mirabilis* quinolone resistant strains[22]. Also Majlesi *et al*. (2016) identified (61.5%) *P. mirabilis* isolates that encode the *aac(6')Ib-cr*gene in Iran[15]. Those results indicated that *aac(6')Ib-cr* gene spreads worldwide among *Proteus spp*. and reflected its contribution in the resistance behavior against quinolones. The differences between results may be due to strain differences. In the present study, among the 33/220 (15%) fluoroquinolones resistant isolates, eleven isolates (33.33%) produced a TEM-type ESBL from which 5 isolates (45.45%) encoded also *aac(6')Ib-cr* gene. Saito *et al*. (2007) demonstrated that among 13 ciprofloxacin resistant *Proteus* isolates 8(62%) were ESBL producers[3]. Also Sohn *et al*. (2011) reported 60% of ESBL production among their ciprofloxacin resistant isolates[23]. Abreu *et al.*(2011) reported 88.9% ciprofloxacin resistance among 18 ESBL producing *Proteus mirabilis* and one *Proteus vulgaris* isolates[24]. All these results give a great cause for concern since there is a marked increase in the incidence of *Proteus* isolates that are resistant to fluoroquinolones and broad-spectrum cephalosporins.

CONCLUSION

Co-existence of some genes that confer resistance to fluoroquinolones and broad-spectrum cephalosporins has been well identified among clinical isolates of *Proteus* species in Egypt. This association is of great concern because ESBL-producing *Proteus* isolates are usually resistant to penicillins and cephalosporins. Thus, ciprofloxacin resistance severely limits already restricted treatment options.

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