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Full Length Research Paper

The emergence of plasmid mediated quinolone resistance genes qnrA, qnrB, and qnrS among Egyptian clinical isolates of Enterobacteriaceae

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Quinolone resistance is emerging in Gram-negative pathogens worldwide. In the present study, the prevalence of this problem was determined for different species of Enterobacteriaceae that were isolated from clinical specimens mainly from Ain Shams University Hospitals at Cairo, Egypt. *Escherichia coli* had the largest number of isolates, and relatively showed the highest resistance among Enterobacteriaceae but not as compared with other non-enterobacterial species such as *Acinetobacter* sp. Selected isolates of quinolone resistant Enterobacteriaceae were investigated for qnrA, qnrB and qnrS genes in single reaction by multiplex polymerase chain reaction (PCR). The results showed that 41.2% of the tested isolates were positive for one at least of these genes, which indicates the role of other qnr genes or chromosomal mutations in the process of quinolone resistance. Since, the first reported case of qnrA in *Providencia stuartii* from clinical Egyptian isolate at 2001, the current study may be the first Egyptian study which reports the detection of qnrA, qnrB and qnrS in *E. coli* and *Klebsiella pneumoniae* and qnrA and qnrS in *Klebsiella* sp and qnrS in *Enterobacter* sp from clinical specimens. Additionally some isolates harbored more than one of these genes simultaneously. The presence of more than one of the investigated genes in the same isolate did not give a noticeable additional effect on the quinolone resistance.

Key words: qnr, quinolone, multiplex PCR, Enterobacteriaceae, plasmid.

INTRODUCTION

Quinolone resistance in Enterobacteriaceae mostly results from chromosomal mutations in genes coding for DNA gyrase and topoisomerase IV, changes in outer membrane and efflux proteins or in their regulatory mechanisms (Ruiz, 2003). Recent findings indicate that plasmid-mediated resistance mechanisms might also play a significant clinical role related to pentapeptide proteins of the qnr family (Nordmann et al., 2005). Plasmid-mediated resistance to quinolone (related to protein qnrA) was reported first in 1998 for a *K. pneumoniae* isolate from the United States (Poirel et al., 2006).

The qnrA gene responsible for this resistance (now termed qnrA1) codes for a 218 amino acid protein belonging to the pentapeptide family that protects DNA from quinolone binding to gyrase and topoisomerase IV (Cattoir et al., 2007b), from the inhibitory activity of quinolones (Poirel et al., 2006).

QnrA confers resistance to Nalidixic acid and increases MIC values of fluoroquinolones (Siu et al., 1999). The QnrA determinant has been reported worldwide from unrelated enterobacterial species and six variants of qnrA are known (QnrA1 to QnrA6) (Cattoir et al., 2007b).

Recently, two other plasmid-mediated quinolone resistance genes, namely, qnrB and qnrS, have been identified that code for qnrB (six variants) and qnrS (two variants) (Cattoir et al., 2007).

QnrB-like determinants were identified in *Citrobacter koseri*, *Escherichia coli, Enterobacter cloacae,* and *K.*

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pneumoniae from the United States and India (Jacoby et al., 2006). The QnrS1 determinant was identified in a *Shigella flexneri* isolate from Japan and in an *E. cloacae* isolate from Vietnam (Hata et al., 2005), whereas the QnrS2 variant was identified in non-typhi *Salmonella* isolate from the United States. QnrA1 and QnrS1 determinants share 59% amino acid identity, whereas both determinants share 40% amino acid identity with QnrB1 (Poirel et al., 2006).

MATERIALS AND METHODS

Sample collection

Clinical samples (Urine, stool, pus, blood, chest, Wound, Sputum, Pleural effusion, Synoival, Central line) were collected and randomly chosen. They were collected from hospitals in the greater Cairo region (Ain Shams University Hospitals and Cairo Medical Center) in Egypt, during January 2008 to July 2008.

Isolation of bacteria

The fresh collected samples were inoculated in nutrient agar, MacConkey and blood agar media for 24 h in 37°C, then chick the purity of isolated bacteria.

Identification of bacterial isolates

Morphological tests

Morphological characteristics were analyzed according to the Bergey’s Manual of Systematic Bacteriology (Sneath, 1986).

Physiological and biochemical tests

Standard physiological and biochemical identification tests were carried out as described in Bergey's Manual of Systematic Bacteriology (Sneath, 1986) and Manual automated systems for detection and identification of microorganisms (O’Hara et al., 2003).

Further biochemical tests (API 20E kit and Vacsera Kit)

Commercial Vacsera (Egypt) and API 20E (Biomerieux, France) Kits were used (Smith et al., 1972).

Microscan

Confirmation of the species identification of Gram-negative bacilli was performed with Microscan Walkaway automated System (Dade MicroScan, Inc., W. Sacramento, CA, USA); it was used according to the instructions of the manufacturer.

Antibiotic sensitivity assay by agar diffusion method

Mueller Hinton Agar is used for antimicrobial disc diffusion susceptibility according to Bauer-Kirby method, (Ryan and Kirby, 1970; Barry et al., 1970) as standardized by the National Committee for Clinical Laboratory Standards (NCCLS, 2000).

Antibiotic sensitivity assay by tube dilution method [Minimum Inhibitory Concentration (MIC)]

The method described below is an amended version of the procedure described by National Committee for Clinical Laboratory Standards (1997); Andrews (2002) and Reynolds et al. (2003).

1. Standard powder for antibiotics: Ciprofloxacin, Levofloxacin, Norfloxacin, Ofloxacin, Nalidixic acid were used.
2. Stock solutions were prepared following the manufacturer’s recommendations. Stock solutions were freezed and thawed only once and then discarded.

i. Adjustment of the organism suspension to the density of the 0.5 McFarland standard.
ii. Suspensions should contain between 10^7 and 10^8 cfu/ml.
iii. A final inoculum of 10^5 cfu/ml is required and therefore suspensions should be diluted 1:100 in broth medium used for preparing the antibiotic dilutions.

3. 1 ml aliquots of test organism was added to one set of tubes and 1 ml of control organism to the other.
4. Inoculated and uninoculated tubes of antibiotic-free broth were included (the first tube controls the adequacy of the broth to support the growth of the organism, the second is a check of sterility) (Winstanley et al., 1994).
5. Incubation conditions: Incubate at 35-37°C for 18-20 h in air.
6. Reading and interpretation: MIC endpoint was read as the lowest concentration of antibiotic at which there is no visible growth.

DNA techniques

Extraction of DNA

Enterobacteriaceae isolates were obtained on selective medium (MacConkey agar medium). A loop of 10 µL of overnight culture of Enterobacteriaceae was used.

First, Bacteria were placed in molecular grade water 200 µl and mixed good by Vortex. Then, tube was Boiled for 15 min in Dry bath type at (80- 90°C). then, the tube was Centrifuged by Cooling Microfuge at 10,000 RPM for 5 min. then, supernatant was taken in clean 1.5 ml microcentifuge tubes and 2 volumes from Ethanol (96-100%) was added to precipitate DNA. Then, the precipitated DNA strands were removed and put in a 1.5 ml microcentifuge tubes containing 1 ml of “ice-cold” 70% ethanol. Then, it was centrifuged for 5 min at 12000 RPM. Then, the pellet was dried in air then DNA was resuspended in 60 µl of deionized water.

Integration of DNA

Extracted DNA (15 µl) was examined by 2% (w/v) agarose gel in electrophoresis Submarine Chamber by Power supply (standard Power Pack P25) 80V, 45 min and ethidium bromide (0.05%). Stained gels were visualized under Ultraviolet transilluminator. All images were archived by digital camera.

Multiplex polymerase chain reaction (multiplex PCR)

The PCR conditions were 94°C for 45 s, 53°C for 45 s, and 72°C for 60 s, with 32 cycles. The reaction volume has shown 10 µl of Template DNA, 2 µl of Primer (qnrS, reverse), 2 µl of Primer (qnrS, forward), 2 µl of Primer (qnrB, reverse), 2 µl of Primer (qnrB, forward), 2 µl of Primer (qnrA, reverse), 2 µl of Primer (qnrA, forward) till it completed 50 µl by RNase –free water (Appendix).

Detection of PCR product was done by 2% agarose gel

Table 1. Frequency of bacterial isolates.

Antibiotics	Isolates No. / Total (%)		
	Resistant	Moderate	Sensitive
Ciprofloxacin	116/248 46.80%	46/248 18.50%	86/248 34.70%
Levofloxacin	124/249 49.80%	30/249 12.00%	95/249 38.30%
Norfloxacin	118/249 47.40%	25/249 10.00%	106/249 42.60%
Ofloxacin	109/249 43.80%	21/249 8.40%	119/249 47.80%
Nalidixic acid	154/248 62.10%	14/248 5.60%	80/248 32.30%

electrophoresis.

Sequencing of the PCR products

The dideoxyribonucleoside chain termination procedure originally developed by Sanger et al. (1977). The automated DNA sequencing reactions was performed using ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE applied Biosystems, USA) in conjunction with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the Gene Amp 2400 Thermal Cycler, the reaction was conducted in a total volume of 20 µl containing 8 µl of terminator ready reaction mix, 1 µg of DNA, and 3.2 pmol of M13 universal forward primer (provided with the kit). The cycle sequencing program was 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, repeated for 25 cycles with rapid thermal ramping, the nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 3100 Genetic Analyzer. The data were provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software.

RESULTS

The bacterial isolates in the current study were identified into twenty three different bacterial species. Among the 238 identified bacterial isolates, the highest identified number for a single species was 57 isolates (23.9%), for *E. coli* (Table 1).

Table 2 shows that, among the tested bacterial isolates, the highest percentage of resistant was for Nalidixic acid (62.1%), while the percentages laid at the forties for Ofloxacin, Ciprofloxacin, levofloxacin and norfloxacin.

Table 3 shows the common sensitivity patterns between Nalidixic acid and flouroquinolones among *E. coli* isolates.

Among 57 isolates that identified as *E. coli* and tested for Ciprofloxacin sensitivity, 34 isolates (59.6%) were resistant, 4 isolates (7%) were intermediate and 19 isolates (33.3%) were sensitive. From 29 *K. pneumonia*, 14 isolates (48.3%) were resistant, 8 isolates (27.6%) were intermediate and 7 isolates (24.1%) were sensitive, while for *Klebsiella* sp 33.3% was resistant, 26.7% was intermediate and 40% was sensitive. However, two of the highest resistance percentages (76.5, 75%) were observed for *Acinetobacter baumannii haem* and *Acinetobacter* sp respectively. Only one isolate was identified for each of *Salmonella arigonae* and *Shigella sonnei*, both were resistant to ciprofloxacin (Table 4).

Levofloxacin sensitivity test for different isolates showed that, among 57 *E. coli* isolates, 32 isolates (56.1%) were resistant, 4 isolates (7%) were intermediate and 21 isolates (36.8%) were sensitive. For *K. pneumoniae* and *Klebsiella* sp, the percentages for resistant, intermediate, and sensitive isolates were (34.5, 37.5%) and (17.2, 18.8%) and (48.3, 43.8%) respectively. Among 7 isolates of *Enterobacter* sp, 2 isolates (28.6%) were resistant, 1 isolate (14.3%) was intermediate and 4 isolates (57.1%) were sensitive. Two of the highest resistance percentages (88.2, 75%) were observed for *Acinetobacter baumannii haem* and *Acinetobacter* sp respectively. Only one isolate was identified for each of *Acinetobacter xylosoxidans* and *Shigella sonnei*, both were resistant to levofloxacin (Table 4).

Among 57 isolates that identified as *E. coli* and tested for Norfloxacin sensitivity, 32 isolates (56.1%) were resistant, 4 isolates (7%) were intermediate and 21 isolates (36.8%) were sensitive. From 29 *Klebsiella pneumonia*, 12 isolates (41.4%) were resistant, 2 isolates (6.9%) were intermediate and 15 isolates (51.7%) were sensitive, while for *Klebsiella* sp 31.3% was resistant, 25% was intermediate and 43.8% was sensitive. However, two of the highest resistance percentages (82.4, 75%) were observed for *Acinetobacter baumannii*

Table 2. Sensitivity pattern for all isolates.

Bacterial Species	Number/Total Identified	Percentage
E. coli	57/238	23.9
Pseudomonas aeruginosa	32/238	13.4
Klebsiella pneumoniae	29/238	12.2
Pseudomonas sp.	22/238	9.2
Acinetobacter baumannii heam	17/238	7.1
Klebsiella sp.	16/238	6.7
Citrobacter sp	11/238	4.6
Salmonella sp.	9/238	3.8
Proteus sp	8/238	3.4
Enterobacter sp.	7/238	2.9
Enterobacter cloacae	5/238	2.1
Acinetobacter sp	4/238	1.7
Shigella sp.	4/238	1.7
Pseudomonas fluor/putida	2/238	0.8
Proteus mirabilis	2/238	0.8
Serratia marcescens	2/238	0.8
Kluyvera sp.	2/238	0.8
Acinetobacter xylosoxidans	1/238	0.4
Klebsiella oxyatca	1/238	0.4
Enterobacter aerogenes	1/238	0.4
Enterobacter sakugderia	1/238	0.4
P. penneri	1/238	0.4
C. freundii cplx	1/238	0.4
Salmonella arigonae	1/238	0.4
Shigella sonnei	1/238	0.4
P. vulgaris	1/238	0.4

Table 3. The common sensitivity pattern between nalidixic acid and the tested flouroquinolones among *E. coli* isolates.

		Ciprofloxacin (Isolates number)		
		R	R	R
Naladixic acid (isolates number)	R	32	32	32
	M			
	S	2	2	2
		Levofloxacin (Isolates number)		
		R	R	R
	R	30	30	30
	M			
	S	2	2	2
		Norfloxacin (Isolates number)		
		R	R	R
	R	30	30	30
	M			
	S	2	2	2
		Ofloxacin (Isolates number)		
		R	R	R
	R	28	28	28
	M			
	S	3	3	3

R: Resistant, M: Moderate, S: Sensitive.

Table 4. The isolated bacterial species sensitivity for each quinolone.

Species	Sensitivity to different antibiotics Isolates number / Total (%)														
	Ciprofloxacin			Levofloxacin			Norfloxacin			Ofloxacin			Nalidixic		
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive	Resist-ant	Intermediate	Sensitive	Resist-ant	Intermediate	Sensitive
E. coli	34/57 (59.6)	4/57 (7.0)	19/57 (33.3)	32/57 (56.1)	4/57 (7.0)	21/57 (36.8)	32/57 (56.1)	4/57 (7.0)	21/57 (36.8)	31/57 (54.4)	3/57 (5.3)	23/57 (40.4)	34/57 (59.6)	1/57 (1.8)	22/57 (38.6)
Acinetobacter sp	3/4 (75.0)	1/4 (25.0)	0	3/4 (75.0)	1/4 (25.0)	0	3/4 (75.0)	0	1/4 (25.0)	3/4 (75.0)	0	1/4 (25.0)	4/4 (100)	0	0
Acinetobacter baumannii heam	13/17 (76.5)	2/17 (11.8)	2/17 (11.8)	15/17 (88.2)	1/17 (5.9)	1/17 (5.9)	14/17 (82.4)	1/17 (5.9)	2/17 (11.8)	10/17 (58.8)	3/17 (17.6)	4/17 (23.5)	15/17 (88.2)	1/17 (5.9)	1/17 (5.9)
Acinetobacter xylosoxidans	0	1/1 (100)	0	1/1 (100)	0	0	1/1 (100)	0	0	0	0	1/1 (100)	1/1 (100)	0	0
Klebsiella sp.	5/15 (33.3)	4/15 (26.7)	6/15 (40.0)	6/16 (37.5)	3/16 (18.8)	7/16 (43.8)	5/16 (31.3)	4/16 (25.0)	7/16 (43.8)	4/16 (25.0)	2/16 (12.5)	10/16 (62.5)	7/15 (46.7)	2/15 (13.3)	6/15 (40.0)
Klebsiella pneumoniae	14/29 (48.3)	8/29 (27.6)	7/29 (24.1)	10/29 (34.5)	5/29 (17.2)	14/29 (48.3)	12/29 (41.4)	2/29 (6.9)	15/29 (51.7)	14/29 (48.3)	2/29 (6.9)	13/29 (44.8)	18/29 (62.1)	4/29 (13.8)	7/29 (24.1)
Klebsiella oxyatca	0	1/1 (100)	0	0	1/1 (100)	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)	0	0
Pseudomonas sp.	8/22 (36.4)	5/22 (22.7)	9/22 (40.9)	14/22 (63.6)	1/22 (4.5)	7/22 (31.8)	12/22 (54.5)	1/22 (4.5)	9/22 (40.9)	11/22 (50.0)	1/22 (4.5)	10/22 (45.5)	17/22 (77.3)	1/22 (4.5)	4/22 (18.2)
Pseudomonas aeruginosa	18/32 (56.3)	1/32 (3.1)	13/32 (40.6)	22/32 (68.8)	2/32 (6.3)	8/32 (25.0)	19/32 (59.4)	0	13/32 (40.6)	19/32 (59.4)	0	13/32 (40.6)	25/32 (78.1)	0	7/32 (21.9)
Pseudomonas fluor/putida	1/2 (50.0)	0	1/2 (50.0)	2/2 (100)	0	0	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	2/2 (100)	0	0
Enterobacter sp.	2/7 (28.6)	0	5/7 (71.4)	2/7 (28.6)	1/7 (14.3)	4/7 (57.1)	2/7 (28.6)	0	5/7 (71.4)	2/7 (28.6)	0	5/7 (71.4)	3/7 (42.9)	0	4/7 (57.1)
Enterobacter cloacae	2/5 (40.0)	2/5 (40.0)	1/5 (20.0)	1/5 (20.0)	3/5 (60.0)	1/5 (20.0)	1/5 (20.0)	3/5 (60.0)	1/5 (20.0)	1/5 (20.0)	2/5 (40.0)	2/5 (40.0)	2/5 (40.0)	2/5 (40.0)	1/5 (20.0)
Enterobacter aerogenes	0	1/1 (100)	0	0	1/1 (100)	0	0	0	1/1 (100)	0	1/1 (100)	0	1/1 (100)	0	0
Enterobacter sakugderia	0	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)

Table 4. Contd

P. penneri	0	0	1/1 (100)	0	0	1/1 (100%)	0	0	1/1 (100%)	0	0	1/1 (100%)	1/1 (100%)	0	0
Proteus sp	1/8 (12.5)	4/8 (50.0)	3/8 (37.5)	2/8 (25.0)	1/8 (12.5%)	5/8 (62.5)	2/8 (25.0)	1/8 (12.5)	5/8 (62.5)	0	0	8/8 (100)	3/8 (37.5)	0	5/8 (62.5)
Proteus mirabilis	½ (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)
C. freundii cplx	0	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)
Citrobacter sp	2/11 (18.2)	4/11 (36.4)	5/11 (45.5)	3/11 (27.3)	0	8/11 (72.7)	3/11 (27.3)	2/11 (18.2)	6/11 (54.5)	2/11 (18.2)	2/11 (18.2)	7/11 (63.6)	3/11 (27.3)	2/11 (18.2)	6/11 (54.5)
Salmonella sp.	2/9 (22.2)	2/9 (22.2)	5/9 (55.6)	1/9 (11.1)	2/9 (22.2)	6/9 (66.7)	1/9 (11.1)	2/9 (22.2)	6/9 (66.7)	0	1/9 (11.1)	8/9 (88.9)	3/9 (33.3)	1/9 (11.1)	5/9 (55.6)
Salmonella arigonae	1/1 (100)	0	0	0	1/1 (100)	0	1/1 (100)	0	0	1/1 (100)	0	0	1/1 (100)	0	0
Shigella sp.	1/4 (25.0)	1/4 (25.0)	2/4 (50.0)	1/4 (25.0)	1/4 (25.0)	2/4 (50.0)	1/4 (25.0)	1/4 (25.0)	2/4 (50.0)	2/4 (50.0)	0	2/4 (50.0)	2/4 (50.0)	0	2/4 (50.0)
Shigella sonnei	1/1 (100)	0	0	1/1 (100)	0	0	0	1/1 (100)	0	0	0	1/1 (100)	1/1 (100)	0	0
P. vulgaris	0	1/1 (100)	0	0	1/1 (100)	0	0	0	1/1 (100)	1/1 (100)	0	0	1/1 (100)	0	0
Serratia marcescens	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)
Kluyvera sp.	1/2 (50.0)	0	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)	1/2 (50.0)	0	1/2 (50.0)

haem and *Acinetobacter* sp respectively. Only one isolate was identified for each of *Salmonella arigonae* and *Acinetobacter xylosoxidans* and *Klebsiella oxyatca,* all were resistant to norfloxacin (Table 4).

Among the isolates that identified as *E. coli* and tested for Ofloxacin sensitivity, 31 isolates (54.4%) were resistant, 3 isolates (5.3%) were intermediate and 23 isolates (40.4%) were sensitive. From 29 *Klebsiella pneumonia*, 14 isolates (48.3%) were resistant, 2 isolates (6.9%) were intermediate and 13 isolates (44.8%) were sensitive, while for *Klebsiella* sp 25% was resistant, 12.5% was intermediate and 62.5% was sensitive. However, two of the highest resistance percentages (58.8%, 75%) were observed for *Acinetobacter baumannii haem* and *Acinetobacter* sp respectively. Only one isolate was identified for each of *Salmonella arigonae* and *P. vulgaris* and *Klebsiella oxyatca,* all were resistant to ofloxacin (Table 4).

Among 57 isolates that identified as *E. coli* and tested for Nalidixic acid sensitivity, 34 isolates (59.6%) were resistant, 1 isolate (1.8%) was intermediate and 22 isolates (38.6%) were sensitive. From 29 *Klebsiella pneumonia*, 18 isolates (62.1%) were resistant, 4 isolates (13.8%) were intermediate and 7 isolates (24.1%) were sensitive, while for *Klebsiella* sp 46.7% were resistant, 13.3% were intermediate and 40% were sensitive. However, two of the highest resistance percentages (88.2, 100%) were observed for *Acinetobacter baumannii haem* and *Acinetobacter*

Table 5. Quinolones resistance among Enterobacteriace isolates.

Antibiotics	Resistant No. / Total (%)								
	E. coli	***Klebsiella* sp.**	***Klebsiella pneumoniae***	***Enterobacter* sp.**	***Enterobacter cloacae***	***Proteus* sp.**	***Proteus mirabilis***	***Citrobacter* sp.**	***Salmonella* sp.**
Ciprofloxacin	34/57 (59.60%)	5/15# (33.30%)	14/29# (48.3%)	2/7# (28.60%)	2/5# (40.00%)	1/8* (12.50%)	1/2# (50.00%)	2/11* (18.2%)	2/9# (22.20%)
Levofloxacin	32/57 (56.10%)	6/16# (37.50%)	10/29# (34.50%)	2/7# (28.60%)	1/5# (20.00%)	2/8# (25.00%)	1/2# (50.00%)	3/11# -27.30%	1/9* (11.10%)
Norfloxacin	32/57 (56.10%)	5/16# (31.30%)	12/29# (41.40%)	2/7# (28.60%)	1/5# (20.00%)	2/8# (25.00%)	1/2# (50.00%)	3/11# -27.30%	1/9* (11.10%)
Ofloxacin	31/57 (54.40%)	4/16* (25%)	14/29# (48.30%)	2/7# (28.60%)	1/5# (20.00%)	0/8* (0.00%)	1/2# (50.00%)	2/11* (18.2%)	0/9* (0.00%)
Nalidixic acid	34/57 (59.60%)	7/15# (46.70%)	18/29# (62.10%)	3/7# (42.90%)	2/5# (40.00%)	3/8# (37.50%)	1/2# (50.00%)	3/11# (27.30%)	3/9# (33.30%)

#: P > 0.05 compared to *E. coli;* *: P < 0.05 compared to *E. coli*.

sp respectively. Only one isolate was identified for each of *Acinetobacter xylosoxidans, Enterobacter aerogenes, Salmonella arigonae, P. vulgaris, Klebsiella oxyatca, P. penneri* and *Shigella sonnei*, all were resistant to Naladixic acid (Table 4).

Comparing the quinolones resistance of other Enterobacteriaceae isolates with that observed in *E. coli* (as it showed the highest percentage of resistance), it showed that, for ciprofloxacin *Proteus* sp and *Citrobacter* sp were lower in resistance than *E. coli* with statistically significant values. For levofloxacin and norfloxaicn, isolates of *Salmonella* sp had significant lower percentage of resistance compared to *E. coli*. Statistically, for ofloxacin, *klebsiella* sp*, Proteus* sp*, Citrobacter* sp and *Salmonella* sp had lower percentage of resistance compared to *E. coli*. For nalidixic acid, there were no statistically significant difference in resistance between each of the tested Enterobacteriaceae isolates and *E. coli*. Moreover, there were no statistically significant difference in resistance between most of Enterbacteriaceae isolates and *E. coli* (Table 5).

On the other hand, by comparing the quinolones resistance of selected non Enterobacteriaceae isolates (that showed high degree of resistance) with that observed in *E. coli*. It was found that, *Acinetobacter baumannii haem* had resistance percentage of 88.2%, for both levofloxacin and Nalidixic acid compared to 56.1% and 59.6% respectively in *E. coli* isolates, with statistically significant difference ($P<0.05$). No statistically significant differences were observed by comparing the resistance of *Acinetobacter baumannii haem* and *E. coli* regarding ciprofloxacin, norfloxacin and ofloxacin. Additionally, there were no statistically significant differences between *Pseudomonas aeruginosa* and *E. coli* regarding all the five tested quinolones (Table 6). Figure 1 and Table 7, show the result of multiplex PCR performed for detection of the genes qnrA, qnrB and qnrS. The number of isolates that were selected for this experiment was 34 isolates; among them 21 isolates were *E. coli*, 4 isolates were *Klebsiella* sp, 7 isolates were *Klebsiella pneumoniae*, and one isolate from each of *Citrobacter* sp and *Enterobacter* sp. In addition to one Enterobacteriaceae isolate that was sensitive to all quinolone antibiotics and one non Enterobacteriaceae isolate that was resistant to all quinolone antibiotics. The PCR products for these genes were at 517 bp, 469 bp and 417 bp for qnrA, qnrB and qnrS respectively.

From the PCR results in Table 7, it was concluded in Table 8, that the number and (%) of detected qnrA, qnrB and qnrS were : 3 (14.3%), 3 (14.3%) and 8 (32.1%) respectively among the 21 *E. coli* isolates and 1 (25%), 0 (0%) and 1 (25%) respectively among the 4 *Klebsiella* sp isolates and 1 (14.3%), 1 (14.3%) and 2 (28.6%) respectively among the 7 *K. pneumoniae* isolates and 0 (0%), 0 (0%) and 1 (100%) respectively

Table 6. Quinolones resistance of *E. coli* compared to other selected Non-Enterobacteriace isolates.

Antibiotics	Resistant No. / Total isolated numbers (%)		
	E. coli	***Acinetobacter baumannii heam***	***Pseudomonas aeruginosa***
Ciprofloxacin	34/57 (59.60%)	13/17 # (76.50%)	18/32 # (56.30%)
Levofloxacin	32/57 (56.10%)	15/17 * (88.20%)	22/32 # (68.60%)
Norfloxacin	32/57 (56.10%)	14/17 # (82.40%)	19/32 # (59.40%)
Ofloxacin	31/57 (54.40%)	10/17 # (58.80%)	19/32 # (59.40%)
Nalidixic acid	34/57 (59.60%)	15/17 * (88.20%)	25/32 # (78.10%)

(#) P > 0.05 compaired to *E. coli;* (*) P < 0.05 compaired to *E. coli.*

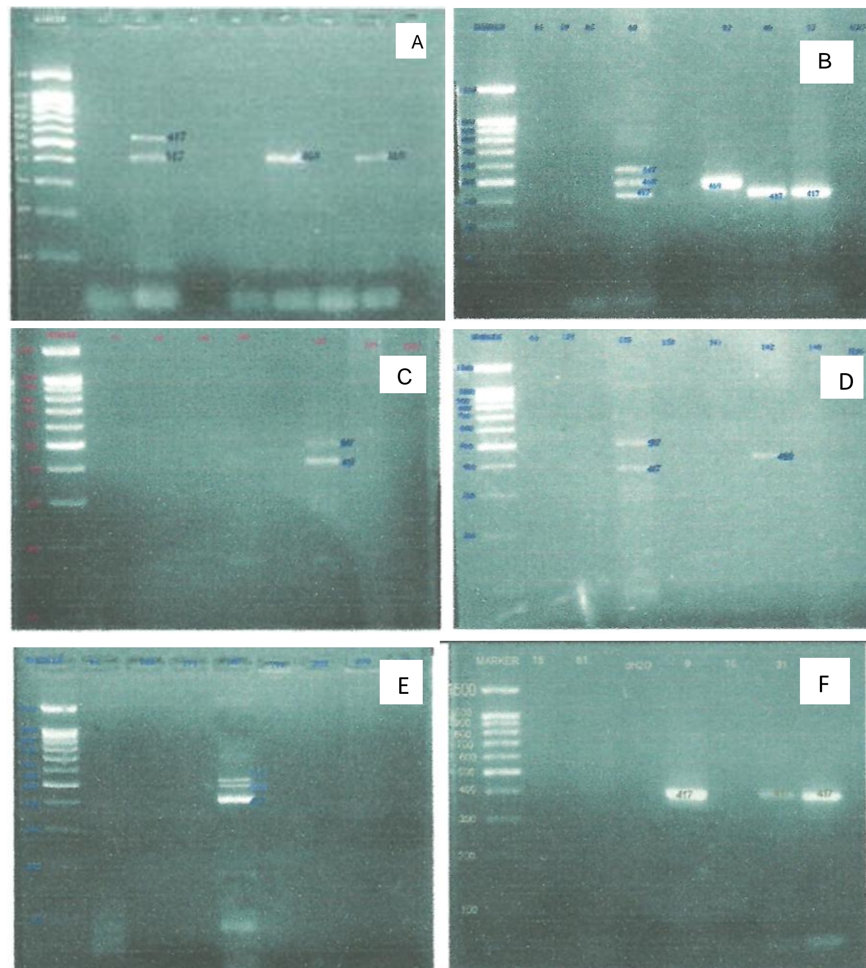

Figure 1. Multiplex polymerase chain reaction for detection of qnr genes among enterobacterial species.
A: Lane 1: Marker, then lanes 2-9: samples 61, 43, 45, 46, 47, 49, 52, water.
B: Lane 1: marker, then lanes 2-9: samples 61, 59, 63, 68, 82, 86, 93, water.
C: Lane 1: marker, then lanes 2-9: samples 61, 69, 102, 109, 122, 125, water.
D: Lane 1: marker, then lanes 2-9: samples 61, 129, 135, 138, 141, 142, 146, water.
E: Lane 1: marker, then lanes 2-9: samples 61, 168, 171, 187, 192, 202, 203, water.
F: Lane 1: marker, then lanes 2-9: samples 15, 61, water, 9, 10 31, 38.

Table 7. Multiplex PCR results for individual isolates.

Sample	Species	DNA Bands		
		Qnr A (517pb)	Qnr B (469 pb)	Qnr S (417pb)
15	*Non enterbacteriaceae resistant control*	Negative	Negative	Negative
61	*Enterobacteriacea sensitive isolate*	Negative	Negative	Negative
H_2O		Negative	Negative	Negative
9	*E. coli*	Negative	Negative	Positive
10	*E. coli*	Negative	Negative	Negative
31	*E. coli*	Negative	Negative	Positive
38	*Enterobacter* sp.	Negative	Negative	Positive
43	*Klebsiella* sp.	Positive	Negative	Positive
45	*E. coli*	Negative	Negative	Negative
46	*Klebsiella pneumoniae*	Negative	Negative	Negative
47	*E. coli*	Negative	Negative	Positive
49	*E. coli*	Negative	Negative	Negative
52	*E. coli*	Negative	Negative	Positive
59	*E. coli*	Negative	Negative	Negative
63	*Klebsiella* sp.	Negative	Negative	Negative
68	*Klebsiella pneumoniae*	Positive	Positive	Positive
82	*E. coli*	Negative	Positive	Negative
86	*E. coli*	Negative	Negative	Positive
93	*Klebsiella pneumoniae*	Negative	Negative	Positive
94	*Klebsiella pneumoniae*	Negative	Negative	Negative
96	*Klebsiella pneumoniae*	Negative	Negative	Negative
102	*E. coli*	Negative	Negative	Negative
109	*E. coli*	Negative	Negative	Negative
122	*E. coli*	Positive	Negative	Positive
125	*Klebsiella sp.*	Negative	Negative	Negative
129	*E. coli*	Negative	Negative	Negative
135	*E. coli*	Positive	Negative	Positive
138	*E. coli*	Negative	Negative	Negative
141	*Klebsiella pneumoniae*	Negative	Negative	Negative
142	*E. coli*	Negative	Positive	Negative
146	*E. coli*	Negative	Negative	Negative
168	*Klebsiella pneumoniae*	Negative	Negative	Negative
171	*E. coli*	Negative	Negative	Negative
187	*E. coli*	Positive	Positive	Positive
192	*Citrobacter sp.*	Negative	Negative	Negative
202	*E. coli*	Negative	Negative	Negative
203	*Klebsiella sp.*	Negative	Negative	Negative

among the 1 *Enterobacter* sp isolate and then all were negative for the one isolate of *Citrobacter* sp.

Tables 9 and 10, show that none of the tested isolates had qnrA alone, two isolates had qnrB alone, 7 isolates had qnrS alone, no isolates had (qnrA and qnrB) or (qnrB and qnrS), 3 isolates had (qnrA and qnrS) together and two isolateshad the three genes together (qnrA, qnrB and qnrS).

DISCUSSION

Quinolone resistance in Enterobacteriaceae mostly results from chromosomal mutations (Ruiz, 2003) or plasmid-mediated resistance mechanisms that relates to pentapeptide proteins of the qnr family (Nordmann et al., 2005).

Since the first description, five major groups of qnr determinants qnrA, qnrB, qnrS, qnrC and qnrD have been identified in various enterobacterial species (Jacoby et al., 2006; Robicsek et al., 2006a; Ambrožiè Avguštin et al., 2007).

In the current study multiplex PCR was performed to detect qnrA, qnrB, and qnrS genes in a single PCR reaction, in accordance with Bourouis et al., 2010.

Table 8. Detection of qnr genes by multiplex PCR among Enterobacteriaceae species.

Species	Qnr A positive isolates (%)	Qnr B positive isolates (%)	Qnr S positive Isolates (%)
E. coli	3/21 (14.30%)	3/21 (14.30%)	8/21 (38.10%)
Klebsiella sp.	1/4 (25%)	0/4 (0%)	1/4 (25%)
Klebsiella pneumoniae	1/7 (14.30%)	1/7 (14.30%)	2/7 (28.60%)
Enterobacter sp.	0/1 (0%)	0/1 (0%)	1/1 (100%)
Citrobacter sp.	0/1 (0%)	0/1 (0%)	0/1 (0%)

Table 9. Qnr genes combination Detected by multiplex PCR among enterobacterial isolates.

Detected genes	Qnr A	Qnr B	Qnr S	Qnr A + Qnr B	Qnr A + Qnr S	Qnr B + Qnr S	Qnr A + Qnr B + Qnr S
Number of positive isolates for each one or more genes	0	2	7	0	3	0	2

Table 10. Minimum Inhibitory concentrations of different quinolones for qnr positive PCR isolates.

Isolate	Species	Genes	MIC (ųg/ml)				
			Ciprofloxacin	Levofloxacin	Norfloxacin	Ofloxacin	Nalidixic acid
187	*E. coli*	qnrA,qnrB, qnrS	32	32	32	64	64
122	*E. coli*	qnrA, qnrS	64	64	128	128	128
135	*E. coli*	qnrA, qnrS	32	32	>128	64	128
9	*E. coli*	qnrS	32	64	>128	64	32
31	*E. coli*	qnrS	32	64	>128	128	128
47	*E. coli*	qnrS	64	64	64	64	128
52	*E. coli*	qnrS	128	128	128	64	128
142	*E. coli*	qnrB	64	64	128	64	128
82	*E. coli*	qnrB	32	16	32	32	64
86	*E. coli*	qnrS	32	64	128	64	128
68	*K. pneumoniae*	qnrA,qnrB, qnrS	32	8	64	32	64
93	*K. pneumoniae*	qnrS	64	128	128	128	128
43	Klebsiella sp.	qnrA, qnr S	64	128	>128	128	>128
38	*Enterobacter* sp.	qnrS	32	64	64	32	64

The identified *E. coli* were 57 isolates (23.9%) in the present study. This indicates that *E. coli* is the most common isolated species from clinical specimens among Enterobacteriaceae in agreement with many early workers such as DuPont, 1982 and Orskov et al. (1977), who reported that *E. coli* is the organism most commonly isolated in the clinical microbiology laboratory. It is an important cause of both intestinal and extraintestinal infections.

Among the tested bacterial isolates, the highest percentage of resistance was for Nalidixic acid (62.1%), while the percentages laid at the forties for Ofloxacin, Ciprofloxacin, levofloxacin and norfloxacin. This may be logical as the nalidixic acid is the oldest antibiotic among them and it was reported by Siu et al. (1999) that qnrA confers resistance to quinolones such as nalidixic acid

while increases MIC values of fluoroquinolones up to 20-fold. Additionally, the presence of a single mutation in positions of the quinolone-resistance determining region (QRDR) of *gyrA* usually result in high-level resistance to nalidixic acid, but to obtain high level of resistance to fluoroquinolones, the presence of additional mutation(s) in *gyrA* and/or in another target such as *parC* is required (Vila et al., 1994; Ruiz et al., 2002). Thus, it has been proposed that the MIC of nalidixic acid could be used as a generic marker of resistance for quinolone family in Gram negative bacteria (Ruiz et al., 2002; Hakanen et al., 1999). Yet, nalidixic acid susceptible, ciprofloxacin resistant phenotypes have been described in two laboratory mutants of *E. coli* (Cambau et al., 1993; Truong et al., 1997). Interestingly, the description of this phenotype is also supported by the current study as one *E. coli* isolate was levofloxacin sensitive and naladixic acid resistant, one *E. coli* isolate was norfloxacin sensitive and naladixic acid resistant, and three *E. coli* isolates were ofloxacin sensitive and nalidixic acid resistant. Additionally, similar observations were recorded for other tested species (data are not shown). However, the common sensitive or resistant isolates between nalidixic acid and the tested fluoroquinolones was the majority.

Some Enterobacteriaceae isolates in the current study, show lower resistance to fluoroquinolones compared to *E. coli*. But there was no statistically significant difference regarding nalidixic acid. For nalidixic acid, most Enterobacteriaceae isolates show relatively higher resistance compared to fluoroquinolones. In accordance with same idea that nalidixic acid resistance is easier to occur than fluoroquinolones resistance.

Regarding Ciprofloxacin sensitivity, 59.6% of *E. coli* isolates were resistant, then 48.3, 33.3% for *Klebsiella pneumonia* and *Klebsiella* sp respectively. However, two of the highest resistance percentages (76.5, 75%) were observed for *Acinetobacter baumannii haem* and *Acinetobacter* sp respectively. Similar observation may be noticed for Levofloxacin, norfloxacin and ofloxacin.

However, statistically by comparing the quinolones resistance of selected non Enterobacteriaceae isolates (that showed high degree of resistance) with that observed in *E. coli*. It was found that, *Acinetobacter baumannii haem* had resistance percentage of 88.2%, for both levofloxacin and nalidixic acid compared to 56.1 and 59.6% respectively in *E. coli* isolates, with statistically significant difference ($P<0.05$). No statistically significant differences were observed by comparing the resistance of *Acinetobacter baumannii haem* and *E. coli* regarding ciprofloxacin, norfloxacin and ofloxacin. The elevated resistance of *Acinetobacter baumannii haem* compared to *E. coli* in some cases may be explained on the basis that, the outer membrane composition of some micro-organisms such as *A. baumannii* or *P. aeruginosa*, has been associated with their intrinsic resistance. Wild-type strains of *A. baumannii* show MICs of ciprofloxacin ranging between 0.125 and 1 mg/L (Vila et al., 1995; 1999). In contrast, wild-type *E. coli* strains show MICs of ciprofloxacin ranging between 0.007 and 0.25 mg/L (Vila et al., 1994). This result has been interpreted as intrinsic resistance or due to the overexpression of an efflux pump(s).

Interestingly, in opposition to the current study, some workers reported that, this proportion is not conserved when analyzing the MIC of nalidixic acid (Vila et al., 1999; 1995).

However, there were not statistically significant differences between *Pseudomonas aeruginosa* and *E. coli* in the current study, regarding all the five tested quinolones. Although, it was reported that the outer membrane of *P. aeruginosa* has very low non-specific permeability to small hydrophobic molecules (Angus et al., 1982; Yoshimura and Nikaido, 1982), which may account for the intrinsic resistance of this microorganism against quinolones. In fact the outer membrane of *P. aeruginosa* is 10- to 100-fold less permeable to antibiotics than that of *E. coli* (Yoshimura and Nikaido, 1982). Regarding these reports, it may be considered that the *E. coli* isolates in the current study showed relatively high resistance than expected compared to *P. aeruginosa* isolates.

It was concluded by PCR, that the number and (%) of detected qnrA, qnrB and qnrS were 3 (14.3%), 3 (14.3%) and 8 (32.1%) respectively among the 21 *E. coli* isolates and 1 (25%), 0 (0%) and 1 (25%) respectively among the 4 *Klebsiella* sp isolates and 1 (14.3%), 1 (14.3%) and 2 (28.6%) respectively among the 7 *K. pneumoniae* isolates and 0 (0%), 0 (0%) and 1 (100%) respectively among the 1 *Enterobacter* sp isolate and then all were negative for the one isolate of *Citrobacter* sp. This was in accordance (for qnrA), with Poirel et al. (2006), who mentioned that Plasmid-mediated resistance to quinolone (related to protein qnrA) was reported first in 1998 for a *K. pneumoniae* isolate from the United States.

It was reported that qnrB-like determinants were identified in *Citrobacter koseri*, *Escherichia coli, Enterobacter cloacae,* and *K. pneumoniae* from the United States and India (Jacoby et al., 2006). However, qnrB was detected in the current study in *E. coli* and *Klebsiella pneumoniae*. The one isolate for each of *Enterobacter* sp and *Citrobacter* sp was investigated and was negative for qnrB gene as no enough number of isolates was tested.

The qnrS1 determinant was identified in a *Shigella flexneri* isolate from Japan and in an *E. cloacae* isolate from Vietnam, whereas the QnrS2 variant was identified in a non-typhi *Salmonella* isolate from the United States (Hata et al., 2005).

The current results show that none of the tested isolates has qnrA alone, two isolates have qnrB alone, 7 isolates have qnrS alone, none of the isolates has (qnrA and qnrB) or (qnrB and qnrS), 3 isolates have (qnrA and qnrS) together and two isolates have the three genes

together (qnrA, qnrB and qnrS). Another recent study reported that among qnr positive fluoroquinolones resistant clinical isolates 47.0% housed the qnrA gene only, 1.2% qnrB and 9.6% qnrS only. Another 36.1% possessed both qnrA and qnrS genes (Stephenson et al., 2010). In the two studies, qnrA and qnrB were not detected together while qnrA and qnrS may be detected in the same isolate. In the current study on Egyptian isolates qnrS was the most detected gene while in (Stephenson et al., 2010), qnrA was the most detectable gene among fluoroquinolone resistant enterobacterial isolates from Jamica. In the present study, there was no detected co-existence of qnrB and qnrS on the same isolate, and it was reported that qnrB and qnrS could be detected simultaneously in a few clinical strains (Wu et al., 2007; Cattoir et al., 2007b), while Fu-Pin et al. (2008), detected qnrB4 and qnrS1 on the same isolate of *K. pneumoniae* and was carried out on two different plasmids pHS7 and pHS8, and were 180 and 45 kb in size, respectively. Conjugation experiments by other workers revealed the coexistence of qnrS1 gene and three β-lactamase genes *bla* CTX-M-28, *bla* TEM-1 and *bla* OXA-1 on the same transferred plasmid (Bourouis et al., 2010).

In other words 14 isolates among 34 quinolone resistant enterobacteriacea isolates (41.2%) in the current study were PCR positive for one or more of qnrA, qnrB and qnrS genes. While, Stephenson et al. (2010) detected qnr genes for the first time in Jamica as 32.5% of fluoroquinolone resistant Enterobacteriaceae clinical isolates is qnr-positive. Both studies may also agree with Ruiz, 2003, who reported that quinolone resistance in Enterobacteriaceae mostly results from chromosomal mutations. Additionally, quinolone resistance may result from other qnr genes such as qnr C and qnr D (Jacoby et al., 2006; Robicsek et al., 2006a; Ambrožiè Avguštin et al., 2007). AAC (6')-Ib-cr was also reported (Robicsek et al., 2006b) and Plasmid-Mediated Fluoroquinolone Efflux Pump, QepA, found in an *E. coli* clinical Isolate (Kunikazu et al., 2007). Even in the presence of one or more of the tested qnr genes in the current study, it is not fair to exclude the role of other chromosomal or plasmid determinants in quinolone resistance among these isolates. It is not even clear in this study which gene(s) has the main role or even it is a combined role of all of them and accumulated expression of different genes determines the end result. So, to know if the presence of more than one of the detected genes increases the minimum inhibitory concentration (MIC) of the quinolone to different enterobacterial species. The MICs (for different antibiotics) of the ten *E. coli* isolates tested by PCR were compared and there was no characteristic effect for the presence of more than one of the tested genes (qnrA, qnrB, qnrS). Similar observation was found in case of the two *K. pneumoniae* isolates.

Quinolone resistant isolate (non-Enterobacteriaceae) was PCR negative for qnrA, qnrB and qnrS which means that, this isolate has quinolone resistance gene(s) (either chromosomal or plasmid) other than the tested genes. But, this is only one tested isolate and does not exclude the role of qnrA, qnrB and qnrS in non-Enterobacteriaceae. However, it was reported that, the plasmid-mediated qnr genes have been identified only in Enterobacteriaceae (Nordmann and Poirel, 2005[b]; Robicsek et al., 2006a). Other findings indicated that those genes originate from environmental Gram-negative bacterial species, such as *Shewanella algae,* the progenitor of the *qnrA* genes (Poirel et al., 2005[b]), and *Vibrio splendidus,* the progenitor of qnrS genes (Cattoir et al., 2007a). Many *Vibrionaceae* species may harbor chromosome-encoded qnr-type genes (Poirel et al., 2005[a]).

Quinolone sensitive Enterobacteriaceae isolate was PCR negative for the tested genes, but because no enough of such isolates were tested, it is not possible to exclude the presence of such genes in sensitive Enterobacteriaceae isolates even it is mutated or not expressed genes.

Since, the first reported case of qnrA in *P. stuartii* from clinical Egyptian isolate at 2001 (Wiegand et al., 2004), the current study may be the first Egyptian study which reports the detection of qnrA, qnrB and qnrS in *E. coli* and *K. pneumoniae* and qnrA and qnrS in *Klebsiella* sp and qnrS in *Enterobacter* sp from clinical specimens. This may change the reported geographical distribution of qnr genes worldwide which reported that qnrA is the only qnr gene detected in Egyptian clinical isolates (Robicsek et al., 2006a). Now, it is fair to say that qnrA, qnrB and qnrS is detectable in Egyptian clinical isolates beside that the possibility of the presence of other qnr genes such as qnrC and qnrD (Ambrožiè Avguštin et al., 2007) was not excluded.

Further study may be required to investigate if the detected qnr genes in one isolate are carried out on the same plasmid. The detected genes in the current study may be of the same or similar sequences discovered by other workers or may be unique, sequencing study of these genes may be of a great value.

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Appendix.

Primer	Sequence (5'-3')	product Size
qnrA	5′-ATTTCTCACGCCAGGATTTG and 5′-GATCGGCAAAGGTTAGGTCA	516-bp
qnrB	5′-GATCGTGAAAGCCAGAAAGG and 5′-ACGATGCCTGGTAGTTGTCC	469-bp
qnrS	5′-ACGACATTCGTCAACTGCAA and 5′-TAAATTGGCACCCTGTAGGC	417-bp