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Detection of Quinolones Resistant among Gram Negative Bacteria Isolated From Clinical Specimens

PROF. DR. YAHYA A .ABBAS[1] MOHAMMED MAHDI ABD[2]
1.AL-Nasiriyiah Technical Institute
2.Department of Biology .College of Science.Uuniversity of Thi-Qar

Abstract
The study was conducted through a period from November 2015 to may 2016, a total of 316 samples from various clinical cases of different patients were randomly collected and examined for detection of Gram negative bacteria .These clinical samples included wound swabs, burn swabs, ear swabs, sputum samples, stool, all collected samples were screened for presence of Gram negative bacteria by culturing on appropriate media and 94 Gram negative bacteria were identified via biochemical tests and confirmed by API 20E system. Overall, *qnr*-genes(quinolones resistance genes)were detected by multiplex PCR technique in 28/94 (29.78%) of all clinical isolates. However, the results showed that quinolones-resistance genes gave 25 positive results for *qnrB*, while 9 positive results for *qnrS*, but *qnrA* gene was not detected in any of the clinical isolates.
Keywords: Gram negative bacteria, Antibiotics and *qnr* genes

INTRODUCTION

Quinolones act by inhibiting the action of type II topoisomerases, DNA gyrase and topoisomerase IV (Gellert *et al.,* 1977). Quinolones are broad-spectrum antibacterial agents, commonly used in both clinical and veterinary medicine. Their extensive use has resulted in bacteria rapidly developing resistance to these agents. Two mechanisms of quinolone resistance have been established to date: alterations in the targets of quinolones, and decreased accumulation due to impermeability of the membrane and/or an overexpression of efflux pump systems. Recently, mobile elements have also been described, carrying the *qnr* gene (quinolone-resistance genes), which confers resistance to quinolones (Joaquim Ruiz, 2003). Recently, a plasmid in *Klebsiella pneumoniae* has been described, capable of conferring quinolone resistance when transferred to a recipient strain (Martínez-Martínez *et al.,*1998). Tran and Jacoby, (2002) they reported that the plasmid contains a novel gene, which they named *qnr*, that encodes a protein of 218 amino acids belonging to the pentapeptide repeat family. The product of this gene protects the DNA gyrase from quinolone inhibition, although its effect on topoisomerase IV is unclear. *In vitro* studies demonstrated that Qnr protected the *E. coli* DNA gyrase from inhibition by ciprofloxacin but did not protect topoisomerase IV (Tran *et al*, 2005). Further molecular studies demonstrated that *qnr* was collocated with other resistance determinants on *sul*1-type integrons (Rodríguez-Martinez *et al*, 2007). The presence of *qnr* has frequently been found in strains producing extended spectrum β-lactamases (Hata *et al*, 2005). Three *qnr* genes have been detected, *qnrA* gene, *qnrS*, and *qnrB* gene (Jacoby *et al.,* 2003; Hata *et al*., 2005 ; Cattoir *et al*., 2008). Qnr determinants have then been identified globaly (Touati *et al*., 2008). Some reports proposed that this plasmid-mediated mechanism was broadly distributed and was detected in all clinically related bacteria from *Enterobacteriaceae* family (Cheung *et al*., 2005; Jeong *et al*., 2005). These genes have been detected in some non-fermenting Gram-negative like *qnrS* gene has been detected in *Aeromonas spp* isolated from both clinical and environment samples (Nazik *et al.,* 2005;Cattoir *et al.,* 2008; Sánchez *et al.,* 2008).

The aim of the present study is to detection some quinolones resistance genes (*qnrA* gene, *qnrS*, and *qnrB* gene) in Gram negative bacteria isolated from clinical samples and the antibiotic profile of these bacteria.

MATERIALS AND METHODS

The study was conducted through a period from November 2015 to may 2016, samples collected from patients retened /or admitted to Al Hussein Teaching Hospitals and Public Health Laboratory in Thi-Qar province. A total of 316 non-duplicate samples from various clinical of different patients were randomly collected and examined for detection of Gram negative bacteria. The results showed 94 (29.74%) of samples gave positive for microbial infection by Gram negative. These clinical samples included wound swab (n=56, 17.7%), burn swab (n=52,16.4%), ear swab (n=87,27.5%) sputum (n=34, 10.7%), stool samples (n=44,13.9%) urine of patients with U.T.I (n= 43, 13.6%).

Isolation and identification of bacterial isolates , all specimens were cultured on blood agar and MacConkey agar and incubated overnight at 37°C under aerobic conditions. Depending on morphological features of colonies and microscopical examination with Gram stain then biochemical tests were used to differentiate among Gram negative bacteria. Diagnosis of species was confirmed by API 20E system.

Antibiotic susceptibility test, susceptibility tests were performed for all bacterial isolates against 17 antimicrobial agent (Bioanalyse, Turkey) from different classes have been determined using kirby-Bauer disc

diffusion method (Bauer *et al*.,1966). Inhibition zone around antibiotic discs was measured as in CLSI guidelines (2014) . The agents tested included amikacin (AK: 30 µg), ceftriaxone (CRO: 30 µg), ciprofloxacin (CIP: 5 µg), gentamicin (CN: 10 µg), imipenem (IMP: 10 µg), meropenem (MEM: 10 µg), cefotaxime (CTX: 30 µg), ceftazidime (CAZ: 30 µg), amoxicillin (AX: 10 µg),amoxicillin - clavulanic acid (AUG:30 µg) norfloxacin (NOR:10 µg) ,naldixic acid (NA:30 µg) ,netilmicin (NET:30 µg) ,ticarcillin (TI:75 µg) , nitrofurantion (F:300 µg) , carbencillin (PY:100 µg) and aztreonam (AT:30 µg).

MULTIPLEX PCR TECHNIQUE

Pure colonies of the clinical isolates were used for DNA extraction (using kit of Geneaid, England). Primers of the qnrA-, qnrB- and qnrS-like genes, which designed by cattoir *et al.,*2007 were used with sizes of 580, 264 and 428 bp, respectively. A pair of degenerated primers was specifically designed to amplify the six variants of qnrB, despite the high polymorphism of this gene. Total DNA (2 µL) was subjected to multiplex PCR in a 50 µL reaction mixture containing 1X PCR buffer [10 mM Tris–HCl (pH 8.3), 50 mM KCl], 1.5 mM MgCl2, 200 mM each deoxynucleotide triphosphate, 20 pmol of each of the six primers and 2.5 U of Taq polymerase. Amplification was carried out with the following thermal cycling profile: 10 min at 95°C and 35 cycles of amplification consisting of 1 min at 95°C, 1 min at 54°C and 1 min at 72°C and 10 min at 72°C for the final extension. DNA fragments were analysed by electrophoresis in a 2% agarose gel at 100 V for 1 h in 1X TAE [40 mM Tris–HCl (pH8.3), 2 mM acetate and 1 mM EDTA] containing 0.05 mg/L ethidium bromide.

Primer name	Primer Sequences (5'-3')		Product Size(bp)	Reference
QnrAm-F	F	AGAGGATTTCTCGCCAGG	580	Cattoir *et al.,*2007
QnrAm-R	R	TGCCAGGCACAGATCTTGAC		Cattoir *et al.,*2007
QnrBm-F	F	GGMATHGAAATTCGCCACTG	264	Cattoir *et al.,*2007
QnrBm- R	R	TTTGCYGYYCGCCAGTCGAA		Cattoir *et al.,*2007
QnrSm-F	F	GCAAGTTCATTGAACAGGGT	428	Cattoir *et al.,*2007
QnrSm-R	R	TCTAAACCGTCGAGTTCGGCG		Cattoir *et al.,*2007

Primers for Detection of Quinolones Resistant genes (*QnrA, QnrB, QnrS*)
M=A or C; H=A or C or T; Y=C or T.

RESULTS

A total of 316 non-duplicate samples from various clinical of different patients were randomly selected and examined for detection of Gram negative bacteria .These clinical samples included wound swab(n=56, 17.7%), burn swab (n=52,16.4%), ear swab (n=87,27.5%) sputum (n=34, 10.7%), stool samples (n=44,13.9%) urine from patients who were suffering from symptoms and signs of urinary tract infection (n= 43, 13.6%),of 316 samples from different sources of clinical cases were founded 94 isolates of Gram negative bacteria , out of these 27 (28.72%) were *E. coli* , 12 (12.76%) *Klebsiella spp* , 3 (3.19 %) were *Enterobacter spp*, 2 (2.12%) were *Serrtia spp*, 5 (5.31) were *Proteus spp*, 26 (27.65) were *Pseudomonas spp*,13 (13.82%) were *Acinetobacter spp* and 6 (6.38%) were *Aeromonas spp*. All the Gram negative isolates (n=94) were screened against seventeen antimicrobial agents belonging to several structurally diverse classes and the results showed that isolates varied in their resistance and sensitivity to the antibiotics .

The amplification products were identified as *qnr*-positive from their sizes in stained agarose gels . Overall, *qnr*-genes were identified in 28/94 (29.78%) of all clinical isolates. The results of screened for quinolones-resistance genes gave 25 positive results for *qnrB* that equal to target (264 bp) product size. While 9 positive results for *qnrS* that equal to target (428 bp) product size, but *qnrA* gene was not detected in any of the clinical isolates as found in (Figure 1).

Table (1): Number and Percentage of bacterial isolation from clinical samples.

Source of samples and specimens	cases	positive cases	%	negative cases	%
Ear swab	87	10	11.49%	77	88.51%
Urine samples	43	22	51.1%	21	48.9%
Wound (pus) swabs	56	21	37.5%	35	62.5%
Burn swab	52	21	40.38%	31	59.62%
Sputum samples	34	10	29.4%	24	70.6%
Stool specimens	44	10	22.72%	34	77.27%
Total	316	94		222	

Table (2) Antibiotic susceptibility patterns in all Gram negative isolates (n=94).

Antibiotics	S	%	I	%	R	%
Amikacin	63	67.02%	8	8.51%	23	24.46%
Amoxicillin	0	0%	0	0%	94	100%
Amoxicillin- clavulanic acid	15	15.95%	16	17.02%	63	67.02%
Aztreonam	7	7.44%	13	13.82%	74	78.72%
Carbencillin	5	5.31%	6	6.38%	83	88.29%
Cefotaxime	12	12.76%	17	18.08%	65	69.14%
Ceftazidime	26	27.65%	5	5.31%	63	67.02%
Ceftriaxone	18	19.14%	10	10.63%	66	70.21%
Ciprofloxacin	57	60.63%	8	8.51%	29	30.85%
Gentamicin	28	29.78%	11	11.7%	55	58.51%
Imipenem	68	72.34%	6	6.38%	20	21.27%
Meropenem	73	77.65%	4	4.25%	17	18.08%
Naldixic acid	19	20.21 %	8	8.51%	67	71.27%
Netilmicin	68	72.34%	4	4.25%	22	23.40%
Nitrofurantion	30	31.91%	4	4.25%	60	63.82%
Norfloxacin	56	59.57%	5	5.31%	33	35.10%
Ticarcillin	2	2.12%	4	4.25%	88	93.61%

Table (3): Distribution of quinolones-resistance genes (*qnrA*, *qnrB*, and *qnrS* genes) among Gram negative isolates.

Isolated organism	No. of isolates	*qnrA*	*qnrB*	*qnrS*	*qnrB* + *qnrS*
Escherichia coli	27	0	5	4	3
Klebsiella pneumoniae	12	0	10	0	0
Serratia marcescens	2	0	2	1	1
Pseudomonas aeruginosa	26	0	2	0	0
Proteus mirabilis	5	0	0	0	0
Aeromonas hydrophila	6	0	0	0	0
Acintobacter baumannii	13	0	6	6	4
Enterobacter aerogenes	3	0	0	0	0
Total	94	0	25	11	8

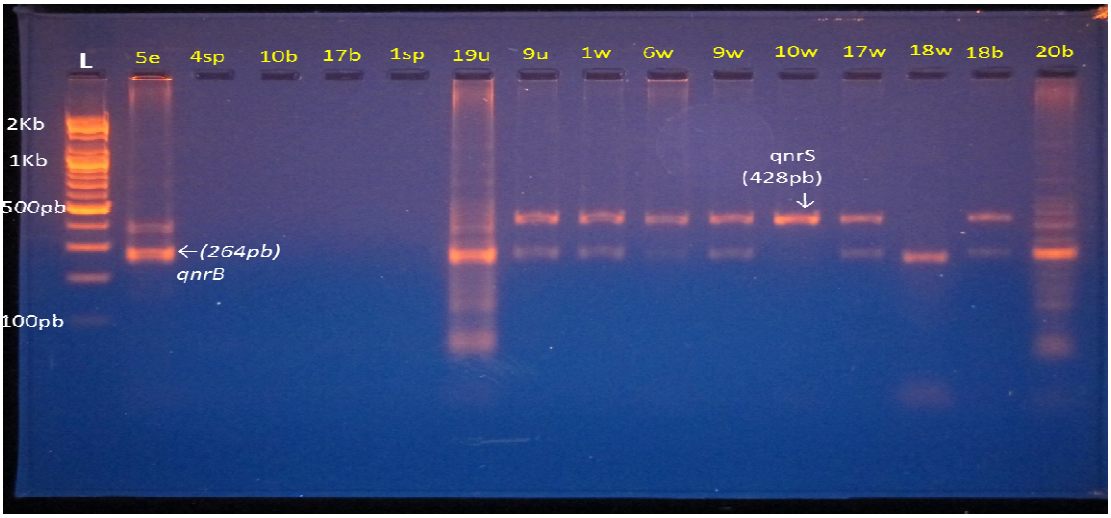

Figure (1): Ethidium bromide-stained agarose gel of multiplex PCR amplified products from extracted Gram negative isolates DNA amplified with primers for quinolones-resistance (*qnrA*, *qnrB* and *qnrS* genes) .lane (L), DNA molecular size marker (2Kb ladder); lane (5e,19e,18w and 20b) shows positive results with *qnrB* (264pb); lane (9u,1w,9w,17w and 18b) shows positive results with *qnrB*(264pb) and *qnrS* (428pb); lane (6w and 10w) shows positive results with *qnrS* (428pb).

DISCUSSION

Quinolones popularly used in both clinical and veterinary medicine. Their extensive use has resulted on bacteria

readily developing resistance to quinolones. However, two mechanisms of quinolone resistance has been established, alterations in site of action of quinolones, and decreased accumulation due to impermeability of the membrane and/or an overexpression of efflux pump systems to microorganisms. Lately, mobile elements have also been described that carrying the *qnr* gene, which confers resistance to quinolones (Joaquim , 2003). Also chromosomal location for *qnr* genes has been noted by some studies (Rodriguez-Martinez *et al*., 2006 and Cavaco *et al*., 2007). In this study, clinical isolates of Gram negative bacteria exhibits relatively low activities for the quinolones, with resistant to ciprofloxacin and norfloxacin, (30.85%) and (35.10%) respectively. Moreover, the percentage of quinolones-resistant in clinical isolates that found in this study is temperately lower than that found in several studies executed in Iraq between 2010-2013, in which resistance range for norfloxacin were 37.8-60% and 40.7-73.4% for ciprofloxacin (Al-Muhannak,2010; Al-Delaimi, 2012; Al-Shara,2013).

A probable reason for the variety of resistance rates in different studies was not understood, but it possibly reflect the amount of antimicrobial that used in various settings. The study prospect that quinolones susceptibility among clinical isolates of Gram negative bacteria appears to be decreasing in Iraq, perhaps due to increasing or cumulative quinolones use and the deficit of adherence to approved infection control practices by hospitals or may be quinolones-resistant isolates are more easily spread in hospitals and community. While, the resistance rates to quinolone antibiotics are higher in other part of the world compared to those in present study. For example, ciprofloxacin resistance more than 50% in Italy (Blandino *et al .,* 2004). But the quinolone resistance is emerging in Gram-negative pathogens, as noted in 2009 by the European Antimicrobial Resistance Surveillance Network that more than 20% of invasive *Escherichia coli* isolates have resistant to quinolones in Europe.

In Spain, Hungary, Portugal, Cyprus and Bulgaria the resistance rates exceeded 25% (European Antimicrobial Resistance Surveillance database,(2009). Ling *et al.*, (2006) noted that, the levels of quinolone resistance in clinical *E. coli* isolates has been noted at 40% in Hong Kong .Qnr determinants have been identified global from America, Europe, Africa, and Asia (Nordmann and Poirel. 2005 and Touati *et al*. 2008). They have been detected in a series of *Enterobacteriaceae* species, including *E. coli*, *Enterobacter spp*, *Klebsiella spp*, *Salmonella spp*, *Providencia stuartii*, *Proteus mirabilis*, and *Serratia marcescens* (Wang *et al*. 2003; Jeong *et al*. 2005; Mammeri *et al*. 2005; Nazic *et al*. 2005;Cambau *et al*. 2006). The screening from *qnr* genes (quinolone resistance determinants) demonstrates a (29.78%) 28/94 of clinical Gram negative isolates as found in (Table 3 and figure 1). These determinants was from isolates were resistant to naldixic acid, norfloxacin and ciprofloxacin. Overall the results of screened for quinolones-resistance genes gave 25 positive results for *qnrB*, while 11 positive results for *qnrS* but *qnrA* gene was not detected in any of the clinical isolates, 8 of all positive isolates had both *qnrB* and *qnrS* genes. However, high detections of *qnr* genes in Gram negative bacteria was noted in several parts of the world like Spain (5 %) (Lavilla *et al*. 2008), China (8 %) (Jiang *et al*. 2008) and USA (15 %) (Robicsek *et al*. 2006). The *qnr* genes were detected in six clinical isolates of *E. coli* and ten clinical isolates of *Klebsiella spp.* The higher prevalence of *qnr* genes in *Klebsiella spp* isolates than in *E. coli* isolates was also observing in other studies conducted in USA (Robiscek *et al*. 2006), Spain (Lavilla *et al*. 2008), and lately China (Jiang *et al*. 2008).

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