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RESEARCH ARTICLE

Occurrence of *qnr* Gene in Diarrheagenic Ciprofloxacin-Resistant *Escherichia coli* Isolated from Patients with Acute Diarrhea

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Abstract

Background: Quinolone resistance *Escherichia coli* have been dramatically increased in last years. This resistance occurs either by chromosomal, or plasmid-mediated. The plasmid-mediated Quinolone resistance (*qnr*) gene which encompasses five different groups including qnrA, *qnr*B, *qnr*C, *qnr*D, and *qnr*S has been shown to aid resistance to fluoroquinolones. Objective: To estimate the occurrence of *qnr* among Diarrheagenic *E coli* strains isolated from patients with acute diarrhea. Methods: This study included 43 *E. coli* clinical isolates recovered from 304 fresh stool specimens which were collected from patients presenting with acute diarrhea. Stool samples were plated onto MacConkey agar for 24 hr at 37ºC. The susceptibility and resistance to Ciprofloxacin and Nalidixic acid for all bacterial isolates were identified by standard procedures such as disk diffusion test and minimum inhibitory concentration. DNA was extracted from plasmids using commercial kits and then, the molecular diagnosis was performed with Multi-Plex polymerase chain reaction (PCR) technique using specific primers for *qnr* genes. Results: Culture result revealed that *E. coli* was isolated from 43(14.1%) of 304 stool samples. Among these, 12 isolates were resistant to both nalidixic acid and ciprofloxacin. There were 18 qnr genes among these isolates. The *qnr*A gene was the most common (8/18, 44.4%) followed by *qnr*B (6/18, 33.3%), whereas qnrS gene represented only 4/18 (22.2%) of the total gens. Conclusion: there is an increase in the occurrence of quinolone resistance among Diarrheagenic *E. coli* which harbored multiple plasmids profile. The most common gene for quinolone resistance in current study was *qnr*A gene.

Keywords: *Diarrheagenic Escherichia coli, Quinolone resistance (qnr) genes, Ciprofloxacin, Nalidixic acid.*

Introduction

Diarrhea is one of the most causes of morbidity and mortality in many countries particularly in developing world [1]. Diarrheagenic *E. coli* which belong to the family Enterobacteriaceae, is one of the most important etiological bacterial agents of diarrheal disease and can affect a variety of age groups mainly children and immunocompromised patients [2].

Six groups from Diarrheagenic *E. coli* have been recognized so far according to the virulence genes. These are enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) [3].

Quinolone resistance *E. coli* has been dramatically increased in last years. This resistance occurs either by chromosomal, or plasmid-mediated. Plasmid mediated Quinolone resistance (qnr) genes which encompasses five different groups including (*qnr*A, *qnr*B, *qnr*C, *qnr*D, and *qnr*S) have been shown to aid resistance to fluoroquinolones [4].

According to the local studies, different strains of *E.coli* showed relatively high

resistance to ciprofloxacin ranged from 45.9% to 90.9% [5, 6].

There for; there is a necessity to investigate the genes responsible for this resistance in the local strains. Thus, this study aimed to estimate the occurrence of *qnr* among DEC strains isolated from patients with acute diarrhea.

Material and Methods

Forty-three *E. coli* non-replicate clinical isolates were recovered from (304) fresh stool specimens which have been collected from patients presenting with acute diarrhea. Those patients were referred to Children Welfare Hospital and Al-Kadhumia Teaching Hospital during the period between the 1st June 2016 to 31st May 2017. The patient's age was ranging from (1 year -64 years).

Isolation of *E.* coli

All stool specimens were collected in appropriate sterile container and transported in Cary-Blair transport medium to the bacteriological laboratory for further isolation and characterization of the causative agents. Tetrathionate broth was cultured by dipping fecal swab and incubated overnight at 37ºC. For isolation and identification of lactose fermenter *E. coli* bacteria, loopful of each culture was plated onto MacConkey agar for 24 hr. at 37ºC. The cultivated bacteria were diagnosed primarily according to morphological characteristics of the colonies.

Diagnosis was confirmed by using Api20E system for *Enterobacteriaceae*, according to instructions of manufacturing company (bio-Merieux/France). Antimicrobial susceptibility of all isolates was determined using the standard Kirby-Bauer disk diffusion method. The diameter of the inhibition zone around the disks was measured and compared with the break points of clinical and laboratory standards institute (CLSI). The minimum inhibitory concentration (MIC) was calculated by a standard agar dilution method and has been applied for determination of the lowest antibiotics concentration that inhibits growth of *E.coli*. The standard isolates from central public health laboratory *E. coli* ATCC25922 was used as negative control.

DNA Extraction and *qnr* Gene Detection

Isolated *E. coli* were sub-cultured overnight in Luria Bertani broth and then agar (Sigma-Aldrich, Germany). Plasmid DNA was extracted from typical colonies using rapid boiling method buffer according to Cabal *et al* [3].The Multiplex-PCR using primer sequences targeting plasmid mediated quinolone-resistance genes (Table 1) including *qnr*A, *qnr*B, and *qnr*S was performed. The thermo cycling conditions with a cleaver scientific thermal cycler (TC 32/80-UK) were as follows: after initial denaturation at 94°C for 7 min, the 35-cycle amplification profile consisted of 94°C for 30 s, 62°C for 30 s and 72°C for 1 min.

Final elongation was occurred at 72°C for 10 min. PCR products were processed into a 2% (wt/vol) agarose gel (Merck-Germany) at 7 V/cm for 1.5 hr. A molecular marker (1-kb DNA ladder; Bioneer) was run concurrently. DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide.

Table 1: Primer sequences for molecular detection of plasmid-mediated quinolone-resistance genes

qnr gene		Nucleotide sequences (5' ⟶ 3')	Products *bp*	References
qnrA	F	GATAAAGTTTTTCAGCAAGAGG	**593**	[7]
	R	ATCCAGATCGGCAAAGGTTA		
qnrB	F	GATCGTGAAAGCCAGAAAGG	**469**	[8]
	R	ACGATGCCTGGTAGTTGTCC		
qnrS	F	TGGAAACCTACAATCATACATATCG	**656**	[9]
	R	TTAGTCAGGATAAACAACAATACCC		

Results

In this study, 304 patients with acute diarrhea ranging from watery diarrhea to severe dysentery were involved. They were 168 males and 136 females. The age was ranged between 1 years to 64 years. Out of 304 stool samples 43(14%) *E. coli* was isolated. Isolation rate of *E. coli* was observed to be high among children 1 - 5 years (53.4%, 23/43), followed by school age 6-10 years (27.9%, 12/43) as shown in Table (2).

Table 2: Distribution of E. coli isolates according to the diarrheal cases, age and gender.

Age group year	Diarrheal cases No.	Gender		*E. coli*
		female	male	
1 - 5	143	63(7)	80(16)	23
6 - 10	85	38(5)	47(7)	12
11- 20	31	17(3)	14(3)	6
21- 30	6	4	2	-
31 - 40	5	0	5	-
41 - 50	15	6	**9**	-
51 - 64	19	8	11(2)	2
Total		136	168	43

The MICs of quinolone (Nalidixic acid and Ciprofloxacin) were determined by an agar dilution method as complementary test to the previous sensitivity test to verify the amount of resistance. An isolate was characterized as resistant if the MIC was greater than the breakpoint MIC defined by CLSI, while it will be susceptible if it is less than the breakpoint. According to susceptibility test, 12 isolates out of 43(27.91%) were found to be resistant to ciprofloxacin and nalidixic acid. Determination of MIC showed that 4 isolates had MIC 64 µg/mL. 2 isolates with MIC 32 µg/mL and 6 isolates with MIC 16µg/mL.

Plasmid Profile

Plasmid DNA of all *E. coli* isolates were extracted and subjected to agarose gel electrophoresis. Analysis of plasmid DNA revealed that all isolates contained multiple plasmids (2-7 plasmid bands), their molecular size ranged from (1 kb to more than 15 kb) and forming a number of unique banding patterns (Figure 1).

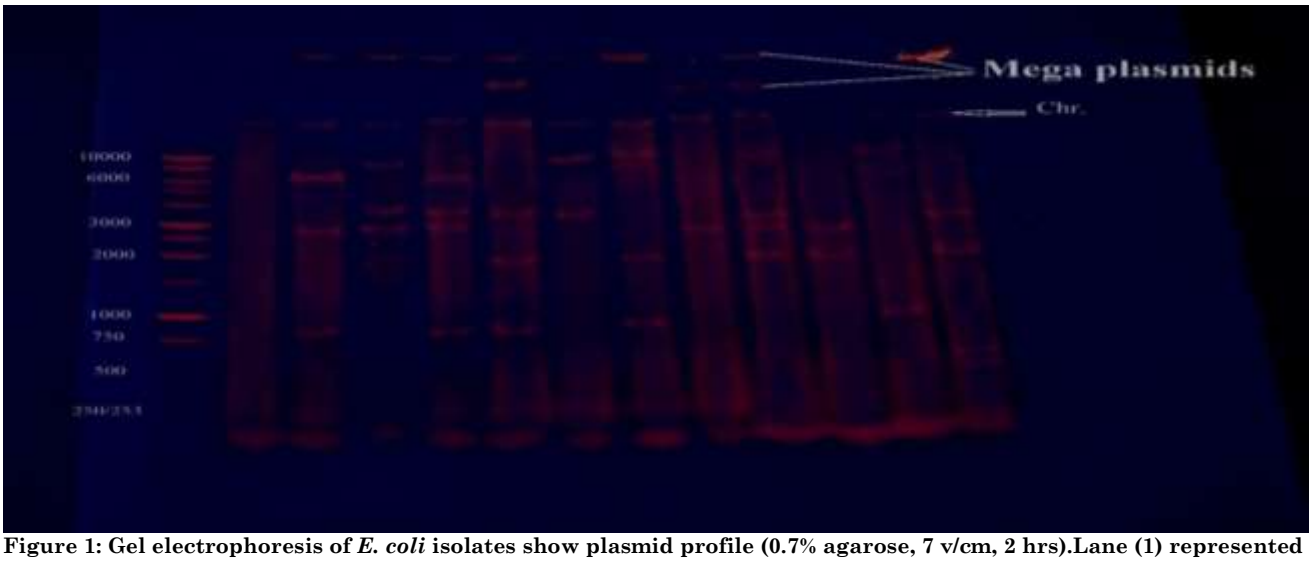

Figure 1: Gel electrophoresis of *E. coli* isolates show plasmid profile (0.7% agarose, 7 v/cm, 2 hrs).Lane (1) represented 1 Kb DNA Ladder

The 12 isolates of *E.coli* which showed resistant to nalidixic acid and ciprofloxacin were screened for the presence of the *qnr*A, *qnr*B, and *qnr*S plasmid-mediated quinolone resistant genes by multiplex PCR (Figure 2). There were 18 *qur* genes among 12 asolates. The *qnr*A gene was the most common (8/18, 44.4%) followed by *qnr*B (6/18, 33.3%), whereas only 4 isolates were positive for *qnr*S (4/18, 22.2%). All isolated had only one *qnr* gene (Table 3).

Table 3: The occurrence of *qnr* genes in quinolone-resistant *E. coli* isolates

Isolate No	*qnr*A	*qnr*B	*qnr*S	Total genes
1	+	+	+	3
2	+	+	-	2
3	+	+	+	3
4	+	-	-	1

5	+	-	-	1
6	-	+	-	1
7	+	-	-	1
8	-	-	+	1
9	-	-	+	1
10	-	+	-	1
11	+	+	-	2
12	+	-	-	1
Total	8	6	4	18

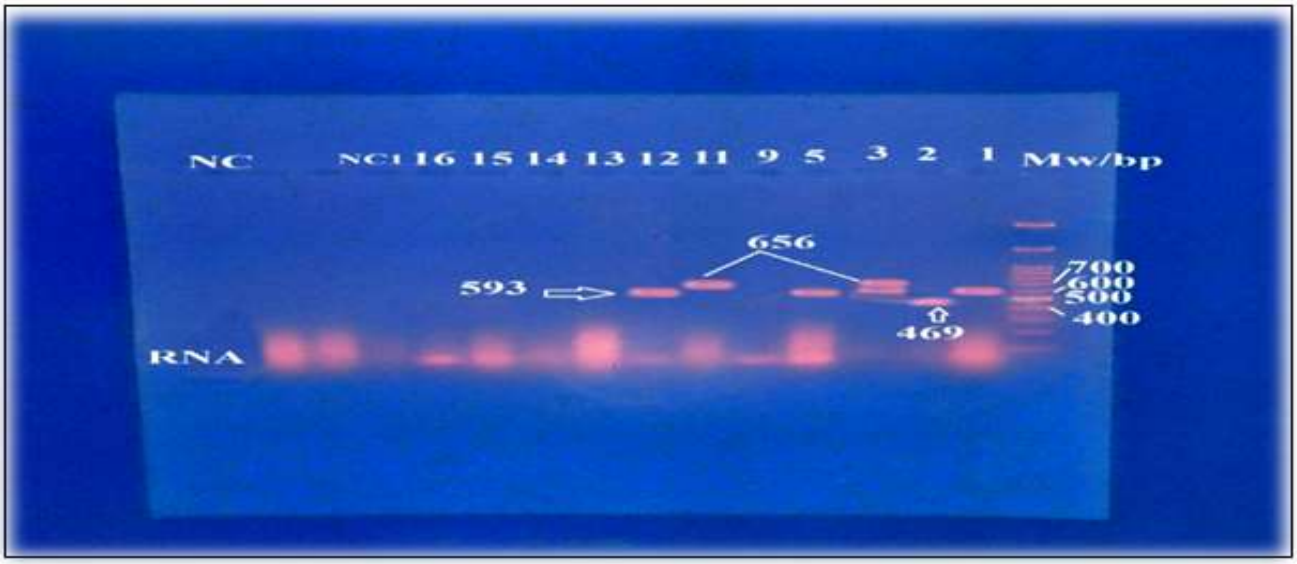

Figure 2: Gel electrophoresis of Multiplex PCR products for *qnr*A, *qnr*B, *qnr*S of *E. coli* positive *isolates*. Lane M/bp: 100bp DNA ladder; lanes 1, 3, 5, 12. *qnr*A gene 593 bp positive isolates; lane (2): *qnr*B 469bp positive isolate; lanes 3,11 *qnr*S 656 bp positive isolates; NC1: Negative control (DW); NC: quinolone negative isolate; (1% agarose, 7 v/cm²,1.5hrs)

Discussion

Diarrheal disease is a major cause of morbidity and mortality in the developing world, including Iraq [2].During the study period, 304 patients with acute diarrhea were involved. The rates significantly higher among children one year -10 years. According to World Health Organization (WHO) Bulletin, diarrheal diseases account for an estimated annual 217 million infections among children younger than five years old in the world. This was attributed to personal hygiene and sanitary conditions which promote spread of organisms like enteric pathogens [10].

In the current study the prevalence of diarrheagenic *E. coli* among diarrheal patients was14.1% which is less than that of previous report from Iraq by Hassan who reported (18.5%) [11] and higher than the rate reported by Ifeanyi *et al*, in India (12.8 %) [12].The current study showed that the infection was most frequent in children.

These results concur with previous study by Malvie *et al*. in which they reported that the prevalence of diarrheagenic *E. coli* in diarrheic children was 45.72% [13]. However, the current results disagreed with the finding of Hassan [11], who reported that the lowest rate of *E. coli* occurrence was in children over two years old (13.6%),.The reason beyond this difference may be attributed to the process of selecting ages in each study. Children within this age-group (5-10) are most susceptible to diarrhea with different etiological agents primarily because of poor resistance and poor personal hygiene [14].

For quinolones group, the MIC values of nalidixic acid and ciprofloxacin in the current study were (16-64 µg/ml). These results are similar to many studies carried out in Arabic countries [15].In the present study, plasmid profile was conducted to identify the genes responsible for resistance. The results showed that all of the isolates contained multiple plasmids (2-8 plasmid bands), their molecular size ranged from (1.0 kb to 15 kb). This result disagrees with many studies such as that conducted by Uma et *al* [16].In which they found that about 67 (64%) strains of *E. coli* isolates harbored plasmids.

The plasmid size ranged from 1.0 to 25 kb. While Jan *et al* [17].Reported that *E. coli* possessed plasmid with different molecular

size ranging from 2-3 kb to 6.5 kb and maximum 26 kb. This discrepancy in such finding may be due to that bacteria isolated from clinical samples are always subjected to the hospital environment where they pick up resistance gene to numerous antibiotics by various mechanisms such as intrinsic resistance or extrachromosomal resistance.

So these bacteria vary in number and size of plasmid profile. In current study, *qnr* gene was detected in about 27% of *E. coli* isolate from stool samples. This percentage is relatively higher than in other investigators [18, 19], while the frequency of *qnr* genes in this study showed that, *qnr*A gene was the most common one (44.4%) followed by *qnr*B (33.3%) whereas *qnr*S (22.2 %). These results disagree with study by Mokhtari *et al* [20] in which they reported that *qnr*A, *qnr*B, and *qnr*S genes accounted for 17.94%, 74.35%, and 64.95%, respectively from the total *qnr* genes. There are some isolates containing more than one *qnr* genes in the current study; two isolates (11.1%) had *qnr*A, *qnr*B, and *qnr*S. these results are in a harmony with study of Mansouri *et al* [21].

Who reported that most isolates had all of three genes and this pattern seems to have a significant resistance to quinolones family. It is noteworthy that, the transferred of resistance gene among *Enterobacteriaceae* bacteria such as quinolones genes is a complex process including many mechanisms for examples plasmid mediated transfer resistance gene and chromosomal mutations.

These mechanisms can be influenced by types of clinical isolate, geographic location and antibiotic consumption rates in each country [22].Collectively, these data indicate that there is an increase in prevalence of quinolone resistance among DEC which harbored multiple plasmids profile.

The most common gene for quinolone resistance is *qnr*A gene. Therefore, the study recommends a restricted use of quinolone may in order to decrease the emergence of resistant bacterial strains.

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