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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF FLUOROQUINOLONE RESISTANCE IN CLINICAL AND ENVIRONMENTAL *VIBRIO CHOLERAE* ISOLATED FROM BAGHDAD, IRAQ

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ABSTRACT

The Gram-negative bacterium Vibrio cholerae is the etiologic agent of cholera. The ability of V. cholerae to colonize and cause disease in hosts requires production of a number of virulence factors during infection. A total of Forty clinical and environmental *V. cholerae* isolates have been isolated from different samples in Baghdad .The susceptibility to different antibiotics was evaluated by disk diffusion method and MICs were determined, All environmental isolates were sensitive (100%) to all antibiotics, while the clinical isolates were shown variable behavior against these antibiotics, these shown (90%) to ciprofloxacin, as a highest value and (50%) for amoxicillin as a lowest value, in other hand, these isolates revealeded resistance (100%) to nalidixic acid, ampicillin and Co-trimoxazole. The MICs values were 128-1024 µg/ml for clinical isolates. Polymerase Chain Reaction (PCR) was performed for detection of *qnr VC3* gene in *Vibrio cholerae* isolates, the results showed that *qnr VC3* gene didn't appear in any isolate. The detection of of plasmid DNA by gel electrophoresis showed that some isolates carried more than one plasmid bands; the curing plasmids for isolates were appeared sensitivity against antibiotics that lead us to conclude that gene was carried on plasmid.

INTRODUCTION

Cholera remains a major public health problem in many areas of the developing world. In addition to maintenance oral rehydration therapy , adjunctive antimicrobial therapy reduces the extent and duration of diarrhea, resulting in reduced fluid requirements and hospitalizations, reductions that are particularly important in resource-limited areas (Zachariah *et al.,* 2002). Clinical microbiology laboratories on most bacterial isolates routinely perform antimicrobial susceptibility testing, the identification of new or usual patterns of antibiotics resistance among bacteria isolated from various patients may raise the suspicion of an outbreak, or presence of a new strain (Russell *et al.,* 1999). *V. cholera* bacteria was sensitive to a large number of antibiotics until the seven the decade of the twentieth century, However, the wide spread and non-programmable using of antibiotics leads to the emergence of resistant strains to many antibiotics (Basu *et al.,* 2000). Until 1970, *V. cholerae* was sensitive to several antibiotics like Tetracycline, Ampicillin, chloramphenicol and fluoroquinolones, such as ciprofloxacin, while during eighties, it emerged resistant isolates to many antibiotics such as Ampicillin, Trimethoprime and Tetracycline (Pan *et al.,* 2008; Faruque *et al.,* 2008). recently, the activity of fluoroquinolones has decreased in some areas (1) The goal of this research is to be able to figure out the genetic factors presence of *V. cholerae* that participated in antibiotic, ciprofloxacin resistance, to do so,simplex PCR has been used to accomplished this goal, and the other goal of current study is determination of genes location (Plasmid DNA).

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MATERIALS AND METHODS

Forty Bacterial isolates belong to *V. cholerae* (clinical and environmental) were obtained from central public health laboratory and that collected from Baghdad and different provinces in Iraq during period (2010-2012). Both clinical and environmental diagnosed in the central public health laboratory. *V. Cholerae* cultured on thiosulfate bile salt. Sucrose (TCBs) agar and incubated at 37 °C for 18-24 hrs.

Antimicrobial susceptibility test

The isolates were subjected to antimicrobial susceptibility testing using Kirby-Bauer disk diffusion method following CLSI guidelines, using commercially available 6mm discs (Bioanalyse/Turkey) The susceptibility of the isolates was determined against 14 antibacterial agents by disc diffusion method, They include: Co-trimoxazol, ampicillin, amoxicillin, tetracyclin, erythromycin, gentamicin, cefalothin, cefotaxime, nalidix acid, ciprofloxacin, norfloxacin, pefloxacin, ofloxacin and amikacin (Bauer *et al.,* 1966).

Determination of (MICs) for antibiotics

The solution of all antibiotics that used in current study were prepared according to Sonnenwirth and Jarret (Sonnenwirth and Jarett 1989). Broth dilution test used to determine minimal

inhibitory Concentration (MIC) of antimicrobial agents as described by (Boyed, 1988).

PCR assay

Specific primers selected for PCR analysis for *qnrVC3* gene (Gene bank accession) is shown in Table (1) according to (Hong Bin Kim *et al.*, 2010; Baranwal *et al.*, 2002). These primers synthesized by Alpha Company, Canada. PCR reaction was conducted in 50 µl of a reaction mixture containing 10µl suspension of few bacterial colonies, 24 µl go tag green master mix, 2 µl $MgCl_2$, 2µl of each primer and 10 µl distilled water. Amplification was conducted using a master cycler (eppendrof) programmed with 1 cycle at 95 for 1 min, 40 cycles of 95 for 1 min, 66, for 1 min, 72 °C for 10 min,72 for 10 min, the amplified product was subjected to 1.8 % agarose gel electrophoreasis and visualized under u.v (image master VDS,pharmacia biotech,USA)after ethidium bromide staining.

Table 1. Sequence and concentration of forward and reverse primer

Primer type	Primer sequence	Concentration In picomles	Product size
Forward primer *qnrVC3*- F	5- AATTTTAAGCGCTCAAACC TCCG- 3	134573	512 bp fragment
Reverse primer *qnrVC3*- R	5- TCCTGTTGCCACGAGCATA TTTT-3	123919	

Plasmid profile

Isolation of plasmid DNA was performed by using Wizard plus SV Minipreps DNA purification system as described by (Sambork *et al.*, 1989; Shetty *et al.*, 1995)

Plasmid curing

Intercalating dys (acridine orange) was used for curing plasmids of the study isolates as described by Carol *et al.* (1984).

RESULTS AND DISCUSSION

Antibiogram pattern of *V. Cholerae* is one of the main goals of this study; therefore, we have done such investigation as shown in Table (2) which gave a clear idea that *V. cholerae* had a variable reaction toward different antibiotics, as far as, clinical isolates are concerned.

Table 2.The resistance percentage of clinical and environmental *V. cholerae* isolates

Antibiotics	Resistance percentage of Clinical isolates%	Resistance percentage of Enviromantal isolates%
Co-trimoxazole	100	0
Amoxicillin	50	0
Ampicillin	100	0
Cefalothin	40	0
Cefotaxime	40	0
Gentamicin	20	0
Erythromycin	40	0
Nalidixicacid	100	0
Ciprofloxacin	10	0
Norfloxacin	20	0
Pefloxacin	30	0
Ofloxacin	30	0
Amikacin	20	0
tetracyclin	20	0

The results illustrated in Table (2) showed that all environmental isolates were sensitive (100%) to all antibiotics that used in the study and all clinical isolates were resistant (100%) to three antibiotics which include, Ampicillin, Co-trimoxazole and Nalidixic acid, also clinical isolates showed a low resistance (10%) to ciprofloxacin, (20%) to norfloxacin, gentamicin, tetracycline and amikacin. A different resistance showed by some of clinical isolates, (30%) to pefloxacin and ofloxacin, (40%) to cephalothin, erythromycin and cefotaxime, (50%) to Amoxicillin. It is interesting to find that the results of the present study were close to the results of other studies reported by several researchers, that *V. Cholerea* isolates showed a high sensitivity to cirprofloxacin, Gentamicin, Norfloxacin and tetracycline, and a high resistance (100%) and (88%) to Nalidixic acid, Co-trimoxazole and Ampicillin (Chatterjiee *et al.*, 2009; Kumer *et al.*, 2009). Our results disagreed with other researchers who founded that *V. Cholerae* isolates showed a high resistance (100%) to tetracyclin, erythromycin and cephalothin (Kitaoka *et al.*, 2011; Noorils *et al.*, 2011; Bhattchaiya *et al.*, 2006). It is a quite interesting to see environmental *V. Cholerae* isolates were complete sensitive to all antibiotics used in this study and to which clinical isolated reacted differently as has been mentioned before, till now, we don't have a decent explanation for these results, it could be in outcome for negative expression imposed by environment on *V. Cholerae* leaving it defenses against antibiotics and that could be due to losing of resistance traits which plasmid, a transposons or bacteriophage (Qu *et al.*, 2003).

Determination of (MICs) for antibiotics

For many infections, the results of sensitivity testing are important in the choice of antibiotic, these results are commonly reported as the minimal inhibitory concentrations (MICs). In this study, minimal inhibitory concentrations were determined for 14 antimicrobial agents, Table (3); which showed that all *V. Cholerea* O1 serotype (Inaba & Ogawa) isolates were more resistant to antibiotics than non/O1 *V. Cholerea* isolates, as far as, clinical isolates are concerned Minimal inhibitory concentration method following (NCCls, 2002). In current study, data from Table (3) showed that there was a relative difference between MIC, results of the microbial agents, tested with serotype and source of *Vbrio cholerea* isolates. All isolates of *V. Cholerea* O1 serotype were resistant to co-trimoxazole, ampicillin,nalidixic acid, nefalothin, Amoxicillin and cefotaxime, while other strains were susceptible to ciprofloxacin, gentamicin, tetracylin, norfloxacin, pefloxacin ofloxacin and amikacin, these results were compatible with Al Simarly and AlKharkhy results which get the same reaction toward antibiotics (AL- Simarly, 2010; AL- Karkhi, 2005). Resistance to antibiotics might be due to mutation since resistance genes were self-transferable by a conjugative plasmid (Falbo *et al.*, 1990). All these differences might be due to geographic regions and seasonal effects. Yochimura and Nikaido reported that the (MIC) value was affected by amount of inoculum, the numbers of copy plasmid that found in the bacterial cell, the outer membrane, the number of channel which is played an important role in the activity of antibiotic resistance (Yochimura and Nikaaido 1982). These

results conducted from this study revealed that this organism had the ability to be resistance to antibiotic due to indiscriminate use of antibiotics in the community, so the use of antibiotics most be strictly controlled. It is a fact that *V. Cholerae* upon releasing from Human to the environment undergo some physical and genitical changes, that could affect the capability of such isolated to resist antibiotics, therefore, the environmental isolated were more sensitive than clinical isolates **(**Najdat 2006; Datta and Bhadra,2003).

Table 3. The MICs (µg/ml) for antibiotics (10 isolates)

Antibiotics	MICs range for Serotyping		
	Inaba	Ogawa	Non/O1 clinical isolates
Co-trimoxazole	(512-1024)	(512-1024)	1024
Amoxicillin	(512-1024)	(512-1024)	512
Ampicillin	(512-1024)	(512-1024)	512
Cefalothin	(512-1024)	(512-1024)	512
Cefotaxime	(256-1024)	(256-1024)	512
Gentamicin	(256-1024)	(256-1024)	256
Erythromycin	512	512	512
Nalidixicacid	(512-1024)	(512-1024)	256-512
Ciprofloxacin	(0.006-0.012)	(0.006-0.012)	0.0006
Norfloxacin	(128-512)	(128-512)	128
Pefloxacin	(128-512)	(128-512)	256
Ofloxacin	(128-512)	(128-512)	256
Amikacin	(128-512)	(128-512)	(128-256)
tetracyclin	(256-512)	(256-512)	256

Simplex PCR

In the current study, simplex PCR has been chosen to be the vehicle to accomplish the goal of this study. Simplex pcr assay was carried out, by using direct DNA samples and use of *V.cholerae* pure culture for comparison the results of two methods, simplex PCR which deal with the detection of *qnrVC3*gene, which encodes apentapeptide repeat protein of the *Qnr* subfamily, a member of the sxt integrating conjugative element family found commonly on the chromosome of multidrug-resistant strains of *V. cholera* and on the chromosomes of *E.coli* transconjugants constructed in the Laboratory **(**Hong Bin Kim *et al.,* 2010; Saha *et al.,* 2006; Strahilevitz *et al.,* 2009). The results presented in Figure (1) revealed that all clinical and environmental isolates of *V. cholera* not contain *qnrVC3* gene, our results obtained in this study by simplex pcr agreed with (Fonseca *et al.,* 2008; Huda *et al.,* 2003) when they found that all clinical isolates were negative to *qnrCV3* gene, similarly (Cattoir *et al.,* 2007; Faruque *et al.,* 2006) have been recorded the same results in Brazile, when they found that all *V. cholerae* of both clinical and environmental isolates were negative to *qnrCV3* gene. Our results came in disagreement with (Baranwal *et al.,* 2002; Strahilevitz *et al.,* 2009), when they found in their work on *V. cholerae* that (20%) of isolates were positive to *qnrVC3* gene. It could be discussed in this results that the absence of *qnrCV3* gene in *V.cholerae* isolates could be one of two reasons, first, the primers which used in this study may be not found their complement sequences on chromosome,

Plasmid Profile

The results of current study showed that only four isolates of *V. Cholerea* that isolated from clinical source indicated the

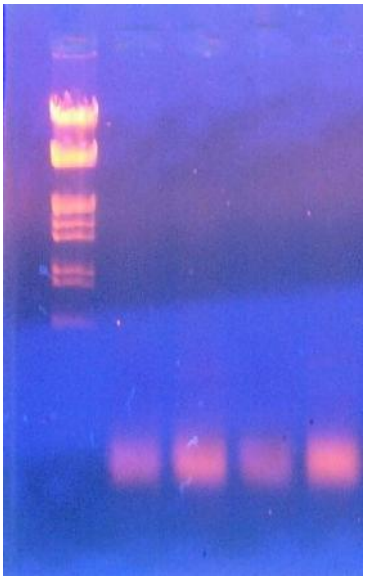

Fig. 1. Agrose gel electrophoresis (1.8 %) of PCR amplicon of *qnr* VC3 gene of *V. cholera* isolate for 1.5 h at 5 volt/cm

presence bands, one isolate Ogawa serotype showed fife plasmid bands, while, the other three were Inaba serotype,one showed four plasmid bands,and the other two showed two plasmid bands, and all environmental *V. cholera* isolates showed no existence for plasmid. Figure (2).

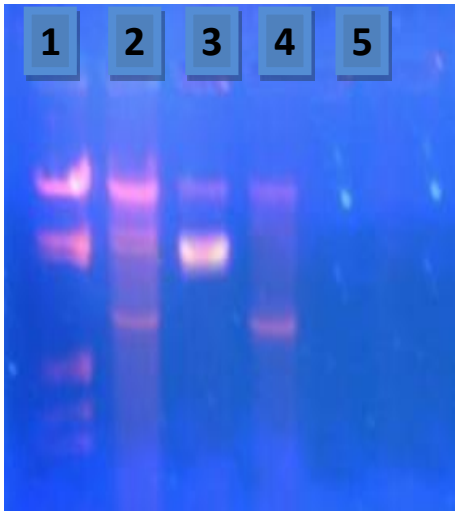

Fig. 3. A plasmid profile of *V. cholerae* isolates, using (1%) agaros for 2 hr at 5 volt/cm in TBE Buffer

Lane 1: *V. cholera*e (Ogawa) (Clinical Source)
Lane 2: *V. cholera*e (Inaba) (Clinical Source)
Lane 3: *V. cholerae* (Inaba) (Clinical source)
Lane 4: *V. cholerae* (Inaba) (Clinical Source)
Lane 5: *V.cholerae*(Non O1)(Clinical source)

It is worthly to mentions that the majority isolates included in this study were plasmid free, our results disagreed with Pourshafie *et al.* who indicated that some of *V.* Cholerae that isolate from Iran possess plasmids of different size (Pourshafie *et al.,* 2002), the results presented here, go along nicely with the results recorded by AL-Janabi and AL- Nadawi (AL- Janbi, 2010; Al-Naddawi 2010). It could be suggested in this aspects that the existence of plasmid free *V. cholera* could be one of two reasons, first, the continuous sub culturing the reserved bacterial isolates, which thought as a routine scheme of work

of the central public Health Laboratories, it is well documented and well know that some of plasmid especially that of copy number may be lost by repeated sub culturing (Bartowsky *et al.,* 1987), and second, the bacterial ancestorlly or genetically has no plasmid. Having mentioned antibiotic resistance among members of *V. cholera*, plasmid content according to the results of this study have no obvious rolein antibiotic resistance, since the plasmid free isolates produce similar pattern of antibiotics resistance comparing with plasmid containing of Vibrio.

Plasmid Curing

An attempt was carried out in order to perform plasmid curing by treating the isolates with various concentration of acridine orange, possibly to know the highest concentration that permit the bacterial growth. 512 mg/L was the highest concentration at which bacterial growth has been noticed. The results of plasmid curing showed that all cured isolates became sensitive to Amoxicillin, cepaltion, cefotaxime, Ampicillin co-trimoxazole, while they conserved with their's resistance completely to other antibiotics. (Cirprofloxacin, Norfloxacin, pefloxcain, ofloxacin nalidixic acid), our results revealed that all cured isolated developed lower resistant (diameter of inhibition zone increase) to tetracyclin, Erythromycin, gentamicin amikacin antibitions in comparision to those obtanited prior to curing,our results were compatable to local studies (Najdat, 2006; AL- Obidi, 2005). Newke (2006) concluded that different sensitivtiel of microbe to antibiotics could be related to the genetic makeup of the organism (Neweke *et al.,* 2006**)**, as far as, curing result were conserved, that the genentic factors which controlled on -cholera resistance were distributed on chromosome and the other on plasmid, therefore, the cured bacterial isolated lost their ability to resist Ampicillin, Amoxicillin, cefotaxime, Cotrimoazol and Cephalothin, while conserved their resistance to other antibiotics. therefore, we need a lot of work to confirm this concept by using other types of primers called *qnr* A, *qnr* B, *qnr* C, *qnr* S, (- mediated quinolinel resistance (PMQR) genes, second, the increases in quinolone resistance in *V. cholera* are linked to mutations in genes encoding the subunits of the quinolone enzymes DNA gyrase (*gyr A and gyr B*) and DNA topoisomerase IV (par C, par E) and the presence of *qnr* and other acquired genes that confer additional resistance to quinolone (Strahilevitz *et al.,* 2009). Some *qnr* gene products have been shown to product gyrase and to pomerase IV from quinolone action in enteric bacteria (Fonseca *et al.,* 2008; Huda *et al.,* 2003) thus, progtassive increases in quinolone resistance *in V. cholerae* are linked cumulative mutations in quinolone targets and most recently to *qnr* on a mobile multi drug resistance elements, resulting in further challenge for the antimicrobial therapy of cholera .

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