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RESEARCH ARTICLE

Detection of qnr resistance genes in Ciprofloxacin and Nalidixic acid resistant *Salmonella* spp. isolated from stool samples.

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Abstract

Gastroenteritis caused by *Salmonella* spp. and increasing of antibiotic resistance of this pathogen is of great concern for public health. This study was designed in order to detection plasmid-mediated quinolone resistance (PMQR) genes in *Salmonella* spp. isolated from stool and theirs correlation with Ciprofloxacin and Nalidixic acid resistance. A total of 40 *Salmonella* spp. isolates recovered from stool samples were examined for antimicrobial susceptibility and the presence of PMQR (qnrA, qnrB, qnrS) genes. Among all isolates, 30 (75%) were multidrug-resistant (MDR) and the majority of them proved to be resistant to Ampicillin, Cephalosporins, Nalidixic acid and Ciprofloxacin. 25 isolates (62.5%) harbored at least one qnr gene. Moreover, two or more PMQR genes coexisted in a 13 (32.5%) isolates. Antimicrobial susceptibility patterns of isolates revealed that 17 (42.5%) exhibited resistance to Nalidixic acid and 25 (62.5%) isolates to Ciprofloxacin. PCR assay detected that 22 of 40 (55%) *Salmonella* spp. carried the qnrS, 15 (37.5%) isolates harbored the qnrB, 11(27.5%) of them contained the qnrA. All the three qnrA, qnrB, qnrS genes were found in 10 (25%) isolates. The study demonstrated that the coexistence of PMQR genes among the *Salmonella* isolates increased the levels of resistance to quinolone antibiotics. Results of this study might improve understanding of the quinolone resistance of *Salmonella* spp.

Introduction:-

Salmonella spp. are an important cause of human infection worldwide. Their resistance to flouroquinolone, quinolone and extended spectrum Cephalosporins (ESR) that are used for treatment is of great concern (1, 2). Non-typhoidal salmonellosis in humans is usually confined to the gastrointestinal tract. However, antibiotic therapy is required because of diarrhea in immunocopromised patients, infants and elderly peoples (3). Plasmid-mediated quinolone resistance (PMQR) was first discovered in a clinical isolate of *Kelebsiella penemoniae* from Birmingham, Albama. Three major groups of qnr determinant were introduced. QnrA with 6 variants, qnrB with 19 variants and qnrS with 3 variants, differ from each other by 40% or more in nucleotide sequences (4). Resistance to quinolone and flouroquinolone arises with mutation within the DNA gyrase (topoisomerase II) and topoisomerase IV gene, especially DNA gyrase and often with decreased expression of outer membrane proteins and overexpression of efflux pump. The qnr gene encodes a pentapeptide repeat protein that protects DNA gyrase against inhibition by quinolone and flouroquinolone (5, 6). In the 1980s, Ciprofloxacin (CIP), a fluoroquinolone with antibacterial activity, particularly against gram negative bacteria, first became clinically available. However, this high level of use, and unnecessary use, or use of quinolones with poor activity in some developing countries, has been blamed for the rapid development of bacterial resistance to these agents (7, 8). The plasmid increasing resistance to both Nalidixic acid and Ciprofloxacin had a wide spread range. The qnr genes especially in gram negative bacteria corroborate resistance to nalidixic acid and ciprofloxacin (9).

In the present study, we investigated the prevalence of PMQR genes(qnrA, qnrB, qnrS) of Ciprofloxacin and Nalidixic acid resistant *Salmonella* spp. Isolated from patients with diarrhea in order to figure out their distribution and significance.

Materials and methods:-

Bacterial Isolates:-

Forte isolates of *Salmonella* spp. were obtained from stool during the October to September at 2015 at different hospitals in Baghdad, Iraq. Each strain obtained from a unique patient in different hospital. Biochemical and serological methods were used to identify each isolates. After performing the biochemical identification by API 20E Kit (BioMerieux, France) about K/A and H2S formation cell group among the colony growing in MacConkey agar plate into the colorless, the *Salmonella* serotypes were determined by slide agglutination according to the Kauffmann-White scheme using O and H-antisera (10) .

Antimicrobial susceptibility testing and minimal inhibitory concentration (MIC) determination:-

Susceptibility of *Salmonella* isolates was determined using a disc diffusion method, according to the guidelines of the Clinical Laboratories Standards Institute (CLSI) (11). All isolates tested with Ampicillin (AMP 10µg), Cefotaxime (CTX 30µg), Ciprofloxacin (CIP 5µg), Streptomycin (S 10µg), Nalidixic acid (NA 30µg), Norfloxacin (NOR 10µg), Ceftriaxone (CRO 30µg), Ceftazidime (CAZ 30µg) and Tetracycline (TE 30µg).. Determination of MIC was performed using E-tests (AB Biodisk, Solna, Sweden) on Mueller Hinton plates following the manufacturer's recommendations with the same antibiotics mentioned above.

PCR (Polymerase Chain Reaction) detection:-

The qnrA, qnrB, and qnrS were detected by PCR in clinical isolates using the following primers: For qnrA-F (GATAAAGTTTTTCAGCAAGAGG) and qnrA-R (ATCCAGATCCGCAAAGGTTA) to give a 700 bp product. Primer for qnrB-F (ATGACGCCATTACTGTATAA) were qnrB- R (GATCGCAATGTGTGAAGTTT) generating a 120 bp fragment. Primer for qnrS-F (ATG- GAAACCTACAATCATAC) and qnrS- R (AAAAA-CACCTCGACTTAAGT) and amplicon size of qnrS was 280 bp. The PCR conditions described previously by Gay et al., 2006 (12).

Results:-

Prevalence of antibiotics Resistance:-

Antimicrobial susceptibility pattern of 40 isolates of *Salmonella* were as a follow: 35 (87.5%) isolates exhibited resistance to Ampicillin, 12 (30%) isolates to Ceftriaxone, 33 (82.5%) isolates to Cefotaxime, 25 (62.5%) isolates to Ciprofloxacin, 17 (42.5%) isolates to Nalidixic acid, 12 (30%) isolates to Norfloxacin, and it was obvious that the minimum levels of resistance were to Tetracyclin (Table 1). Among all the isolates it was found that MIC range of these antibiotics were 2-256 µg/ml , 30 (75%) were multidrug resistant and the high levels of resistance was against Cephalosporins , Ampicillin and quinolones**.** The isolates which resistant to quinolones (25 isolates) were used for detection qnr genes.

Screening of qnr *g*enes by PCR:-

The results of prevalence of qnr genes among quinolones resistant Salmonella spp. isolates were found in Table 2. All 25 isolates which resist to ciprofloxacin were contain one of the three qnr genes. Among all of the quinolone resistance isolates, qnrA,qnrS and qnrB genes were detected in 10 (25%) of them. The most prevalent gene was *qnrS* and identified in 22 (55%) among all of the Ciprofloxacin resistance isolates (Figur 1). Moreover, 15 (37.5%) isolates were found to have qnrB genes (Figure 2). wile the lowest prevalence was found in qnrA gene 11(27.5%) (Figure 3). Our results revealed that the coexistence 2 genes or more may cause increasing the level of resistance especially to quinolones antibiotics.

Discussion:-

The qnr gene, always associated with plasmids genes of quinolones resistance in *Enterobacteriaceae* (13,14) has already been found in *Salmonella* strains with different levels of susceptibility to Ciprofloxacin. However, in this work the presence of this gene confers a high level of resistance to Ciprofloxacin. Chong *et al.* (2010) (15) and Jacoby *et al.* (2003) (16) reported that the increase in Flouroquinolones (FQ) resistance resulting from the presence of qnr genes could reduce the clinical effectiveness of this class of antibiotics. However, according Jacoby *et al.* (2006) (17) the precise level of the involvement of plasmid genes in the resistance to FQs is still poorly understood

when compared with understanding of other mechanisms of resistance. Our results showed the significant of resistance to cephalosporins in Ciprofloxacin resistant *Salmonella* spp., this may be due to the events leads to co resistance of quinolone and extended spectrum Cephalosporins. In another study, we have shown that class 2 integron carrying gene cassettes which confer resistance to different classes of antibiotics such as Aminoglycosides and Trimethoprim are prevalent in *Salmonella* serovars isolated in Iran (18). Although resistance to quinolone and flouroquinolone with qnr genes is few, most of the qnrS positive clinical isolates were found to have high level quinolone resistance in the present study which was usually detected by resistance to Ciprofloxacin and Nalidixic acid. The ability of these genes to supplement resistance is due to mutation in DNA gyrase and topoisomerase IV, porin or efflux mutations and qnrB was more potent than qnrA in blocking the action of Ciprofloxacin. Most of the previous studies indicated to spreading of these genes among *Salmonella* spp., the spread of these gene is depending on the geographical region. The high prevalence of qnr among *Salmonella* spp. isolates has also been described in several countries. For example, the qnrB gene has been found in Senegal, USA and Korea, while the qnrS gene has been found in Enterobacteriacea in Germany, USA, Taiwan, Vietnam, France, Sothern and eastern Asia and Europe (14, 15, 19, 20). Our results disagree with other results which showed that non typhoid *Salmonella* spp. have very low prevalence of qrn genes and the resistance of Nalidixic acid and Ciprofloxacin may be due to other mechanisms such as efflux pumps and function of gyr genes (21, 22). Our results demonstrated that qnrS was sufficient to cause decreased susceptibility to Ciprofloxacin. The highest prevalence of these genes has been found among Enterobacteriaceae, especially in *Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae*, and *Salmonella* spp. The qnr genes have been detected worldwide, with qnrB being the most prevalent variant. However, despite their worldwide spread, the prevalence of the qnr genes is still low in *Salmonella* spp. (0.2–3%, reaching 9.8% among isolates showing decreased susceptibility to fluoroquinolones) (23). The study, performed in South Korea, did not identify the qnrA gene among 261 nalidixic acid resistant and community-acquired Salmonella spp. Isolates (2) In the second study, 10 out of 335 *Salmonella* human clinical isolates from the USA were positive for either qnrB or qnrS but none was qnrA positive . Also was found several qnrB variants were detected in seven *Salmonella* spp. (12). It is important to consider that the *qnr* gene has been reported to be located in a mobile resistance determinant or insertion element that might jump to the chromosome (24)

In conclusion the current study demonstrated the high prevalence of qnr genes in contrast with previous studies, and the presence of these genes is very correlated mainly with the resistance to Ciprofloxacin and also to Nalidixic acid. The cooperation of these genes may responsible for the high levels of resistance to quinolones.

Figure 1. Agarose gel electrophoresis showing positive amplification of 280 bp fragments specific for qnrS Lane M, size marker (100bp DNA ladder); lane 1-4, positive result for *Salmonella* spp., lane 5, negative control.

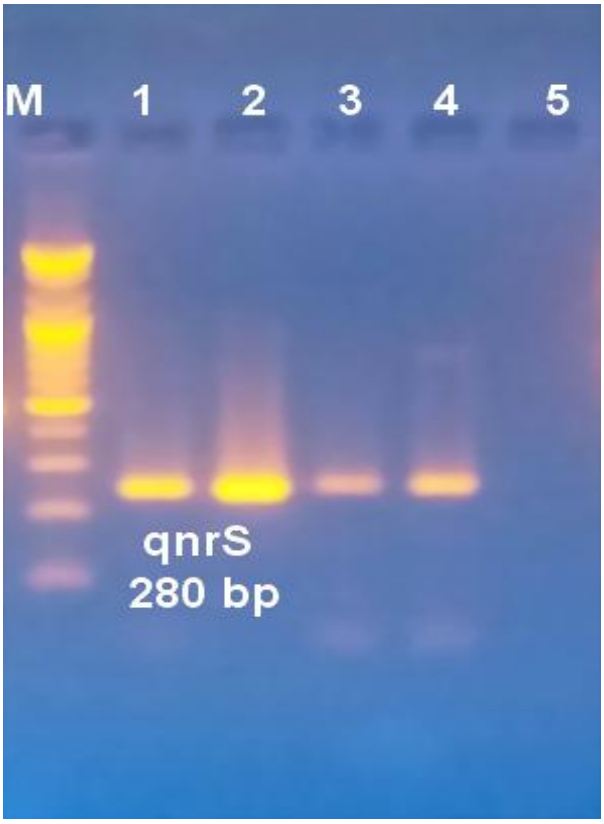

Figure 2. Agarose gel electrophoresis showing positive amplification of 120 bp fragments specific for qnrB Lane M, size marker (100bp DNA ladder); lane 1-2, positive result for *Salmonella* spp., lane c, negative control.

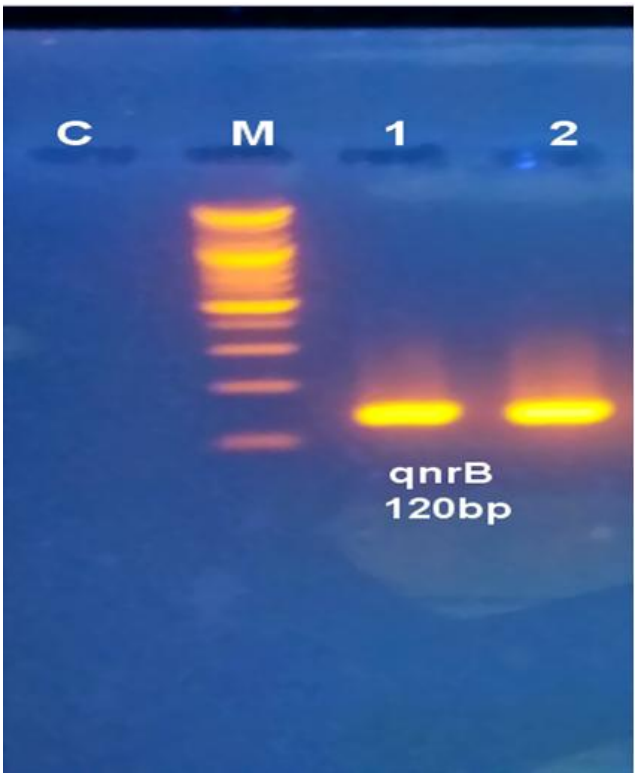

Figure 3. Agarose gel electrophoresis showing positive amplification of 700 bp fragments specific for qnrA Lane M, size marker (100bp DNA ladder); lane 2-4, positive result for *Salmonella* spp., lane 1, negative control.

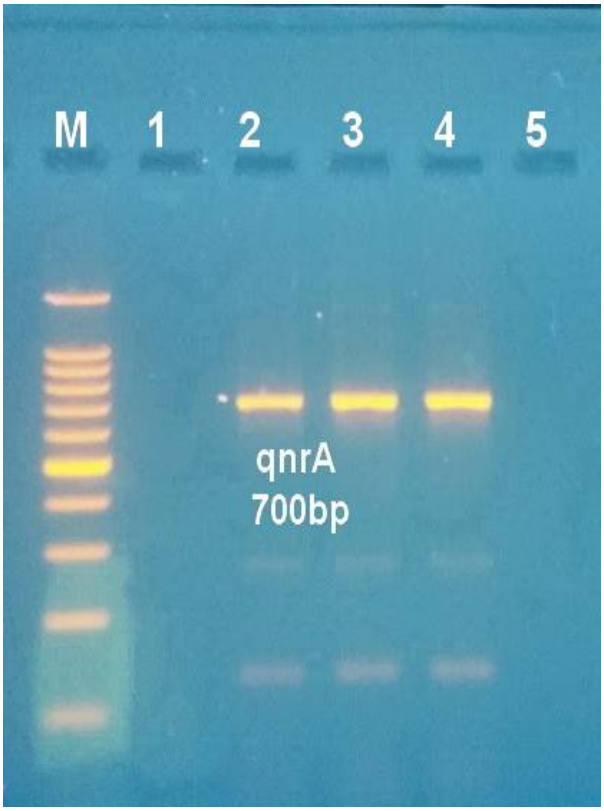

Table 1. MIC range and antimicrobial resistance percentage for 40 isolates of *Salmonella* spp. obtained from stool.

Antibiotic	MIC range in isolates (µg/ml)	Number of resistant isolates 40 (%)
Ciprofloxacin	0.25-32	25 (62.5%)
Norfloxacin	0.5-64	12 (30%)
Nalidixic acid	0.5-128	17 (42.5%)
Cefotaxime	4-256	33 (82.5%)
Ceftazidime	1-128	17 (42.5%)
Ceftriaxone	0.2- 16	12 (30%)
Ampicillin	2-256	35 (87.5%)
Tetracycline	0.5- 16	7 (17.5%)
Streptomycin	0.5- 64	11(27.5%)

Table 2. Distribution of qnrA, qnrB and qnrS genes among quinolones resistant *Salmonella* spp. isolates.

Isolate number	MIC (µg/ml) CIP	MIC (µg/ml) NA	MIC (µg/ml) NOR	qnrA	qnrB	qnrS
Sal1	32	128	16	+	+	+
Sal2	4	16	4	−	−	+
Sal3	16	32	4	−	+	+
Sal4	16	128	8	+	+	+
Sal5	4	32	0.5	−	−	+
Sal6	16	32	4	−	−	+
Sal7	8	0.5	4	+	−	−
Sal8	8	32	8	−	+	+
Sal9	16	16	4	−	−	+
Sal10	32	64	64	+	+	+
Sal11	32	32	8	+	+	+
Sal12	8	32	1	−	−	+
Sal13	2	1	2	−	+	−
Sal14	16	32	32	+	+	+
Sal15	16	32	16	+	+	+
Sal 16	4	4	2	−	−	+
Sal17	8	8	2	−	−	+
Sal18	2	2	1	−	−	+
Sal19	32	128	16	+	+	+
Sal20	4	32	4	−	−	+
Sal21	4	32	8	−	+	+
Sal22	8	64	16	+	+	+
Sal23	2	0.5	2	−	+	−
Sal24	8	64	8	+	+	+
Sal25	16	64	8	+	+	+

NA=Nalidixic acid , NOR= Norfloxacin, CIP=Ciprofloxacin

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