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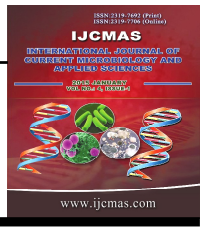

Original Research Article

Prevalence of Plasmid-Mediated Quinolone Resistance Determinants among Clinical Isolates of *Escherichia coli* in a Tunisian Hospital

M. Tarchouna, A. Ferjani*, M, Marzouk, I.Guedda and J. Boukadida

Laboratory of Microbiology - Immunology UR12SP34, CHU Farhat
Hached teaching hospital, Sousse, Tunisia
**Corresponding author*

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Quinolone resistance is traditionally mediated by chromosomal mutations in bacterial topoisomerase genes, genes regulating expression of efflux pumps, or both. In addition, *qnr* genes responsible for plasmid-borne quinolone resistance have been found in clinical isolates of *Enterobacteriaceae*. In this study, we have investigated the frequency of the *qnr* gene and the mutations of gyrase and topoisomerase IV in nalidixic-acid resistant clinical isolates of *E. coli* and we have exanimate the *aac (6')-Ib* gene in qnr-positive strains. A total of 112 *E. coli* quinolone resistant strains (EQRs) isolated from different specimens were screened for the presence of the *qnrA*, *qnrB* and *qnrS* genes by Multiplex PCR. The *aac (6')-Ib* gene was detected in *qnr*-bearing strains by PCR. Presence of the gyrase and topoisomerase IV were analyzed by PCR for all the EQRs of *gyrA, gyrB, parC* and *parE*. Among 112 quinolones *E. coli* isolates, 36 (32%) isolates were positive for the *qnr* gene: 14 strains harbored *qnr*B gene only, 6 *qnr*A only, 4 *qnr*S only but 3 strains had *qnr B+qnr S*, 4 were *qnr A+qnr B*, 2 harbored *qnr A+ qnr S* and 3 contained *qnr A+ qnr B+qnr S* were identified in this population. Detection of the *aac(6')-Ib* gene was showed in 12 (33%) qnr positive strains. The presence of *gyr*A gene were observed in 19 strains (16%), 13 strains containing the *par*E gene (24%), *gyr*B gene was observed of 26% strains and only two strains containing the *par*C gene.

Introduction

Quinolones are widely used to treat clinical infections in both in and out patients; therefore a survey of quinolone resistance would be especially useful (Tie-Li Zhou, 2011). Although quinolone resistance is usually caused by mutations in chromosomal genes or alterations in drug accumulation plasmid mediated quinolone resistance (PMQR) plays an important role because it's horizontal transferability (Hooper, 1999; Hooper, 2000).

This PMQR was originally reported in a *Klebsiella pneumoniae* clinical isolate from the USA in 1998 (Martinez-Martinez l *et al.*, 1998). The gene responsible for this

resistance was the *qnrA* which codes for 218 amino acid protein belonging to the pentapeptide family that protects DNA from quinolone binding to gyrase and topoisomerase IV (Robicsek, 2006b).

Qnr A confers resistance to quinolones and increases MIC values of fluoroquinolones up to 20-fold (Robicsek, 2006a,b). Four main types of the qnr genes, *qnrA*, *qnrB*, *qnrC* and *qnrS* have been identified. (Robicsek, 2006a,b).

Recently, a new mechanism of transferable quinolone resistance was reported: enzymatic inactivation of certain quinolones. The cr variant of *aac (6')-Ib* encodes an aminoglycoside acetyltransferase that confers reduced susceptibility to ciprofloxacin by N-acetylation of its piperazinyl amine (Hooper, 1999).

In the study described here, we have investigated the frequency of the *qnr* gene and the mutations of gyrase and topoisomerase IV in nalidixic-acid resistant clinical isolates of *E. coli* and we have examinated the *aac (6')-Ib* gene in qnr-positive strains; since this species (i) is the most frequent enterobacterial species identified from human specimens and (ii) it is involved in both nosocomial and community-acquired infections.

Materials and Methods

Bacterial isolates:

From November 2012 to April 2013,112 non duplicated *E. coli* clinical isolates were collected from 365 of *E. coli* isolated from all different specimens from Farhat Hached university hospital in Sousse located on the eastern coast of Tunisia, Identification of *E. coli* was performed with the API 20E system (bioMérieux, Marcy-l'Etoile, France).

Antimicrobial susceptibility testing:

Antimicrobial susceptibility was realized according to the recommendations of the French society of microbiology (CA-SFM 2012) performed on Mueller–Hinton agar-containing plates. MICs of fluoroquinolones were determined using the E-test method according to the manufacturer's recommendations (AB Biodisk, Solna, Sweden). MIC breakpoints used for susceptibility and resistance to nalidixic acid and ciprofloxacin were <8 and ≥32 mg/L and <1 and ≥4 mg/L, respectively, as recommended by the Ca-SFM.

Multiplex PCR

A multiplex PCR was used to detect simultaneously detected *qnrA, qnrB and qnrS*. The rapid DNA preparation was performed by a boiling technique that includes a heating step at 100°C of a single colony in a total volume of 100µl of distilled water followed by a centrifugation step of the Cell suspension. The amplified DNA products were examined as described previously; the expected product sizes for *qnrA, qnrB* and *qnrS* were 580, 264 and 428 bp, respectively (Table 1). Total DNA 2µl was subjected to multiplex PCR in 50µl reaction mixture containing 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 (Table 1) and 2.5 U of Taq polymerase (invitrogen, Brasil).

Amplification was carried out with the following thermal cycling profile: 5 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 analyses by electrophoresis in a 2% agarose gel at 100 V for 1 h in TAE [40 mM Tris–HCl (pH8.3), 2 mM acetate and 1 mM

EDTA] containing 0.05 mg/L ethidium bromide.

Detection of *aac (6')-Ib* gene in the qnr positive strains

The variant of aminoglycoside acetyltransferase *aac (6')-Ib* gene of qnr-positive strains were detected by PCR amplification as previously described. The amplified products were visualized. *aac(6')-Ib* was amplified by PCR with primers presents in table 1 to produce a 482-bp product. Primers were chosen to amplify all known *aac(6')-Ib* variants. PCR conditions were 94°C for 45 s, 55°C for 45 s, and 72°C for 45 s for 30 cycles.

Detection of *gyr*A, *gyr*B, *par*C and *par*E

DNA was extracted by boiling as above. The QRDR of *gyrA, gyrB, parC* and *parE* were amplified using the primers showed in table 1. PCR conditions were as follows: initial denaturation step of 5 min at 94C, 45 s at 94 C, 45 s at the annealing temperature (55°C for *gyr*A and *par*E, 44C for *gyr*B and 52°C *par*C) and 45 s at 72°C for 30 cycles, final extension step was 5 min at 72°C. Reaction mixes without a DNA template served as negative controls.

Results and Discussion

this work was conducted on patients having ages ranged from 7 months up to 80 years with a female sex high rate (25 cases) compared to male sex (8 cases), the majority of strains 29 (80%) were obtained from urine. In table 2, community quinolone resistant strains come in (33%) from private hospitals.

Of the 112 *E.coli* quinolone resistant strains collected, 36 (32%) were qnr positive, 24/36 (66%) strains have only one *qnr* gene with the predominance of *qnr*B (38%).

The combination of two qnr or more was seen in 12 (33%) strains (Figure 1).

In this study was obtained 14 *qnr*B, 6 *qnr*A and 4 *qnr*S. The combination of two qnr (4 *qnr*A + *qnr*B, 3 *qnr*B + *qnr*S and 2 *qnr*A+*qnr*S) was obtained as the combinations of these three qnr in three cases, among them are two strains of ESBL (table 2).

Identification of *aac (6')-Ib* gene

Among the 36 qnr positive *E.coli* strains, 12 (33%) contained *acc(6')Ib*, all these strains harbored *qnr*B. All selected strains show a resistance to at least four antibiotics, the most antibiotic resistant were Amoxicillin, ciprofloxacin, Kanamycin and gentamicin (table 2).

Identification of *gyr*A, *gyr*B, *par*C and *par*E

Among 112 *E. coli* qnr positive strains, 19 strains (16%), containing *gyrA* gene, 13 (24%) strains containing the *parE* gene and only two strains containing the *parC* gene. The *gyrB* gene was observed in 30 (26%) (Figures 2 and 3). In this part of work, we identified 15 strains containing the combination of three genes: *gyrA, gyrB and parE.*

Resistance to other antibiotics:

Among the 112 quinolones resistant *E. coli* isolates, we found that the highest rates of resistance (72%) were obtained for amoxicillin. 16 (44%) strains show resistance to third generation cephalosporine with an ESBL phenotype (table II).

The *qnr* genes encode proteins that protect DNA gyrase and topoisomerase IV from inhibition by quinolones (Tran, 2005a,b), and have recently been identified worldwide. The prevalence of the qnr genes in bacterial isolates may range from <1% to >50% (Wang, 2008; Jeong, 2005; Shin *et al.,* 2008; Kim, 2009a,b), depending on the selection criteria and study period for bacterial isolates. In this study, among 112 quinolones resistant *E. coli* isolates, 36 (32%) were positive for the *qnr* gene: 14 strains harbored *qnr*B gene only, 6 for *qnr*A only, 4 *qnr*S only but 3 strains had *qnr B+qnr S*, 4 were *qnr A+qnr B*, 2 contained *qnr A* + *qnr S* and 3 strains harbored *qnr A+ qnr B* + *qnr S*. The prevalence of plasmid-mediated quinolone resistance (PMQR) determinants was investigated in other Tunisian collection of 300 uropathogenic *Escherichia coli*. PMQR genes were detected in 68 isolates (22.7%) as follows: *aac (6')-Ib-cr* (n=66), *qnr*B (n=3), *qnr*A (n=1), and *qnr*S (n=1). Three isolates carried the 2 determinants *aac (6')-Ib-cr* and *qnr*B1 (Sana, 2014). The incidences of *qnr* in China Among ciprofloxacin-resistant *E. coli* is 7.5%. *QnrA, qnrB and qnrS* were detected either alone or in combination in 3.8%, 4.7% and 3.8% of these isolates, respectively (Wang, 2008). In Korea, Shin *et al.* (2008) reported that 5.6% of *E. coli* ciprofloxacin-resistant isolates contained only qnrB. Kim *et al.* (2009a,b) determined that 0.5% of *E. coli* (ciprofloxacin susceptible and resistant) isolates in Korea contained *qnr* (*qnr*B or *qnr*S). Of the qnr variants, qnrA was not detected; *qnr*B was the most common, followed by *qnr*S.

The *aac (6')-Ib-cr* gene, a variant of the gene encoding AAC(6')-Ib, was first described in 2006 (Robicsek, 2006a,b). The *Aac (6')-Ib-cr* enzyme reduces only ciprofloxacin and norfloxacin activity by acetylation (Robicsek, 2006b). Quinolones without piperazinyl nitrogen were not affected by *aac (6')-Ib-cr* (Strahilevitz *et al.*, 2009). However, transconjugants containing only *aac (6')-Ib-cr* also exhibited reduced susceptibilities to levofloxacin in the present study, suggesting it contributes to antimicrobial resistance through additional mechanisms.

The prevalence of *aac (6')-Ib-cr* was lower in our study (33.3%) than in previous studies (Robicsek, 2006a,b; Yang *et al.*, 2014). Among clinical *E. coli* isolates collected in China, 51% had *aac (6')-Ib-cr* (Robicsek, 2006a,b) and in Korea, 73,8% of *E. coli* ciprofloxacin-resistant isolates contained, *aac (6')-Ib-cr*. In the United States, was detected in 32% of *E. coli* and 16% of *K pneumoniae* isolates (Park *et al.*, 2006) however, *aac (6')-Ib-cr* was detected in 3.4% of Enterobacteriaceae (Tran, 2005a,b). In some reports, the presence of *aac (6')-Ib-cr* was prevalent among qnr-positive isolates compared with qnr-negative isolates, suggesting a genetic association of quinolone resistance with aminoglycoside resistance (Yang *et al.*, 2014; Kim, 2009a,b; Ode 2009). Yang *et al.* (2014) also found that the prevalence of *aac (6')-Ib-cr* in qnr-positive isolates [13 of 15 (86.7%)] was slightly higher than in qnr-negative isolates [66 of 87 (75.9%)]. In our study the prevalence of *aac (6')-Ib-cr* was detected in 33.33% of qnr positive isolates.

In *Escherichia coli* and related Gram-negative bacteria, DNA gyrase is the first target for fluoroquinolones. If *gyr*A has resistance-conferring mutations, the primary target of fluoroquinolone switches from DNA gyrase to topoisomerase IV (Hopkins *et al.*, 2005; Hooper, 2001). Studies from other parts of the world have found that resistance-conferring mutations are typically selected in gyrA first, and then parC.

Table.1 Primers used in this study

Primers	Sequence 5'→3'	Size of PCR amplified product (bp)	Reference or source
QNRAF	AGAGGATTTCTCACGCCAGG	580	Vincent Cattoir et al
QNRAR	TGCCAGGCACAGATCTTGAC		Vincent Cattoir et al
QNRBF	GGMATHGAAATTCGCCACTG	264	Cattoir et al
QNRBR	TTTGCYGYYCGCCAGTCGAA		Cattoir et al
QNRSF	GCAAGTTCATTGAACAGGGT	428	Vincent Cattoir et al
QNRSR	TCTAAACCGTCGAGTTCGGCG		Vincent Cattoir et al
aac(6)-Ib F	TTGCGATGCTCTATGAGTGGCTA	482	Park *et al.*, 2006
aac(6)-Ib R	CTCGAATGCCTGGCGTGTTT		
gyrA F	GGATAGCGGTTAGATGAGC	521	Yue *et al.*, 2008
gyrA R	CGTTCACCAGCAGGTTAGG		
gyrB F	CAGCAGATGAACGAACTGCT	376	Yue *et al.*, 2008
gyrB R	AACCAAGTGCGGTGATAAGC		
parC F	AATGAGCGATATGGCAGAGC	376	Yue *et al.*, 2008
parC R	TTGGCAGACGGGCAGGTAG		
parE F	GCTGAACCAGAACGTTCAG		
parE R	GCAATGTGCAGACCATCAGA	426	Yue *et al.*, 2008

F, sense primer; R, antisense primer
M = A or C; H = A or C or T; Y =C or T.

Table.2 Epidemiologic characteristic of qnr-positive isolates

N° of isolates	specimen	sex	Age	Qnr	BLSE	in/out patients	Antimicrobial resistance	Department	Ofloxacine CMI (µ g □ ml)	*acc(6')Ib*
1	**stool**	**m**	**65 year**	***qnrA qnrB***	**-**	**in**	AMX, TIC, CIP, NA, PIP	**Hematology**	**0.125**	-
2	**urine**	**f**	**36 year**	***qnrS qnrB***	**-**	**in**	AMX,TIC,TEC, CIP, NA	**Gynecology**	**0.19**	-
3	**urine**	**f**	**75 year**	***qnrB***	**+**	**in**	AMX,TIC,PIP,FOX,AMC, CTX, FEP,TCC,ATM,CF,SXT,C, TE, MEC,	**Rhumatology**	**0.5**	+
4	**blood**	**m**	**66 year**	***qnrS***	**-**	**in**	OFX, NA, CIP	**Health center**	**0.01**	-
5	**urine**	**f**	**25 year**	***qnrB***	**+**	**in**	AMX,TIC,PIP,AM,FEP,TCC,ATM CF,TE,FT,MEC,K,TOB, CiP,NA	**Gynecology**	**0.01**	+
6	**urine**	**m**	**52 year**	***qnrB***	**+**	**out**	AMX,TIC,PIP,FOX,AMC,TCC, CF,TE,FT,K,TOB	**Health center**	**0.19**	+
7	**urine**	**f**	**64 year**	***qnrA qnrB qnrS***	**+**	**out**	AMX,TIC,MEC,SXT,K,NA	**Health center**	**>0.01**	+
8	**urine**	**f**	**32 year**	***qnrS***	**+**	**out**	AMX,TIC,PIP,TCC,MEC,TGC,TE,C,SXT,CIP	**Health center**	**0.64**	-
9	**urine**	**m**	**7 month**	***qnrB***	**-**	**in**	AMX,TE, C,K,NA,CIP	**Neonatology**	**0.125**	-
10	**urine**	**f**	**1 year**	***qnrB***	**-**	**in**	AMX,TIC,PIP,AMC,TCC, GM,TE,MEC, CIP,NA,OFX	**pediatrics**	**0.125**	+
11	**urine**	**f**	**65 year**	***qnrB qnrS***	**+**	**in**	AMX,TIC,PIP,AMC,FEP,ATM,TCC, CF,TM,K,TE, CIP	**Gynecology**	**>0.01**	-

12	**urine**	**m**	**70 year**	***qnrA qnrS***	**-**	**out**	NA,OFX, CIP	**pediatrics**	**>0.01**	-
13	**urine**	**m**	**55 year**	***qnrB***	**+**	**in**	TIC, C,TE,NA, CIP	**Inner medicine**	**>0.01**	+
14	**blood**	**f**	**2 year**	***qnrS***	**+**	**in**	TCC, PIP, TI C,ME C,TE,NA,SXT	**Pediatrics**	**0.125**	-
15	**blood**	**m**	**80 year**	***qnrA qnrS***	**-**	**out**	AMX,TIC,MEC,TE, TCC, C, CIP	**Gynecology**	**0.125**	+
16	**blood**	**f**	**1 year**	***qnrA qnrB qnrS***	**+**	**in**	AMX,TE,TM,GM,TCC,NA	**Pediatrics**	**0.5**	-
17	**urine**	**f**	**28 year**	***qnrA qnrB***	**+**	**out**	ATM,AMX,FEP,TIC, CIP,OFX,NA	**Health center**	**0.125**	+
18	**urine**	**f**	**80 year**	***qnrS***	**-**	**in**	AMX,TIC,PIP,TGC, CIP,OFX,NA	**infectious Diseases**	**>0.01**	-
19	**urine**	**f**	**48 year**	***qnrB***	**+**	**in**	AMX,TIC,AMC,PIP, CF,FEP,ATM,TM,K,TE, CIP	**Intensive care unit**	**>0.01**	-
20	**urine**	**f**	**52 year**	***qnrA***	**+**	**out**	AMX,TIC,PIP,AMC,ATM, TCC,FEP, CF,TOB,GM,K,MEC,NA, CIP	**Health center**	**>0.01**	-
21	**urine**	**m**	**72 year**	***qnrB***	**-**	**in**	NA,OFX, CIP	**Pediatrics**	**>0.01**	-
22	**urine**	**f**	**60 year**	***qnrA***	**-**	**in**	AMX,TE,K,MEC, TIC,NA,OFX	**infectious Diseases**	**>0.01**	-

23	**urine**	**f**	**33 year**	***qnrA qnrB***	**-**	**in**	AMX,TIC,K, C,TE, CIP	**Gynecology**	**0.125**	-
24	**urine**	**f**	**27 year**	***qnrB***	**-**	**in**	AMX,TIC,TE,FT,IPM, CIP,NA	**Inner Medicine**	**>0.01**	+
25	**urine**	**f**	**80 year**	***qnrB***	**+**	**out**	TE,K,C,SXT,CAZ,CIP,NA	**Health center**	**0.125**	-
26	**urine**	**F**	**78 year**	***qnrA qnrB qnrS***	**-**	**out**	NA,OFX,CIP	**Health center**	**>0.01**	-
27	**urine**	**F**	**57 year**	***qnrB***	**-**	**in**	AMX,TIC,PIP,K,TE,FT,NA ,CIP	**Intensive care Unit**	**0.125**	-
28	**blood**	**f**	**75 year**	***qnrS qnrB***	**+**	**in**	AMX,TIC,PIP,AMC,ATM, TCC,FEP,CF,C,TE,NA,OF X,CIP	**Rhumatology**	**0.5**	+
29	**Stool**	**F**	**33 year**	***qnrB***	**-**	**out**	NA, OFX, CIP	**Health center**	**>0.01**	-
30	**urine**	**F**	**65 year**	***qnrA***	**-**	**out**	NA,OFX,CIP	**Health center**	**0.064**	-
31	**urine**	**F**	**34 year**	***qnrB***	**-**	**in**	AMX,TIC,PIP,TE,CIP	**Gynecology**	**>0.01**	-
32	**urine**	**F**	**67 year**	***qnrB***	**-**	**out**	AMX,TIC,TM,GM,TE,NA, CIP	**Health center**	**0.25**	+
33	**urine**	**M**	**3 year**	***qnrA***	**+**	**in**	AMX,TIC,CAZ,AMC, NA, OFX, CIP,CTX	**infectious Diseases**	**0.125**	-
34	**urine**	**F**	**57 year**	***qnrA qnrB***	**-**	**in**	AMX, TIC,NA, FOS, CIP, TE, MEC	**Intensive care unit**	**0.064**	+
35	**urine**	**F**	**29 year**	***qnrA***	**-**	**in**	NA,OFX, CIP	**Gynecology**	**>0.01**	-
36	**urine**	**F**	**48 year**	***qnrA***	**+**	**in**	AMX, TIC, PIP, CAZ, AMC, TCC, FEP,CF,K, GM, TM	**Inner medecine**	**>0.01**	-

F: female H: male

AMX: Amoxicillin NA: nalidaxic acide G: Gentamicin Cip: Ciprofloxacin K: Kanamycin TM: Tembramycin CTX: Cefotaxim CF: Cefalotin TIC: Ticarcillin PIP: Piperacillin TE: Tetracyclin CAZ: Ceftazidim C: Chloramphenicol FOS: Fosfomycin FEP: Cefepime TCC: Tic Clavulanic Ac MEC: Mecillinam ATM: Aztronam P: Peniciilin FT: Furadoine

Figure.1 Multiplex PCR products *qnr A, qnr B, qnr* S. M, molecular size marker (100 bp ladder; biorad, France) Lanes: 1 *Escherichia coli qnrB*; 2 *qnrA*, *qnrS*, *qnrB*; 3 negative strain; 4 *qnrB*

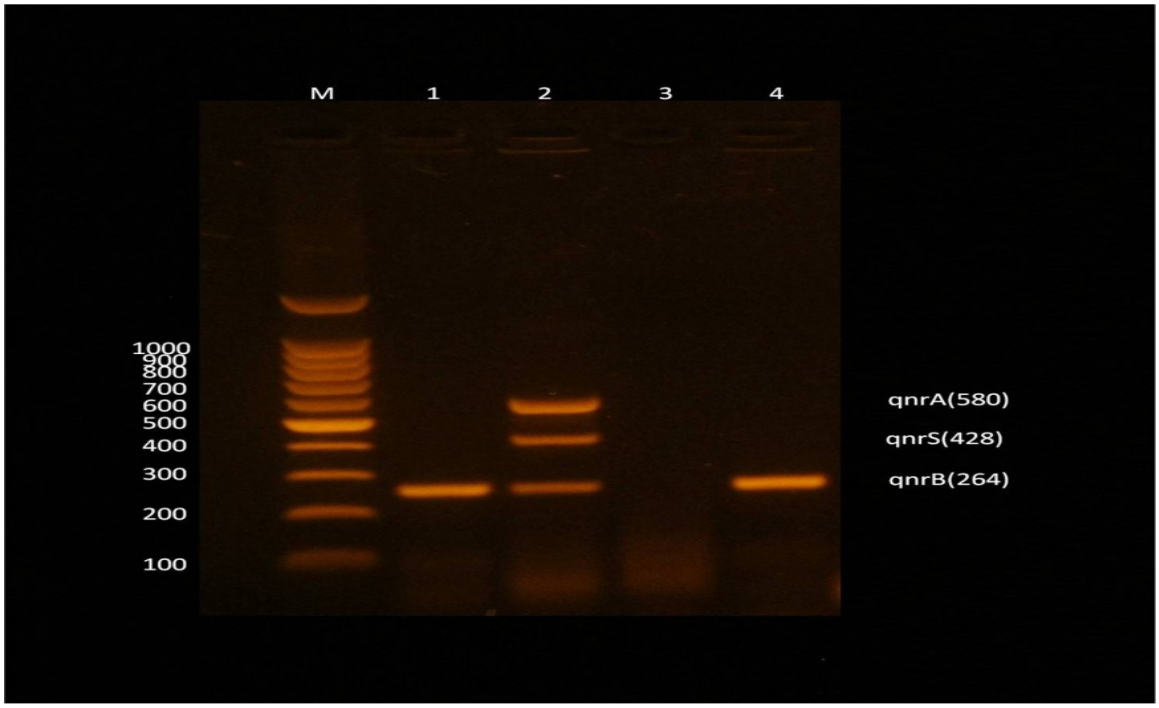
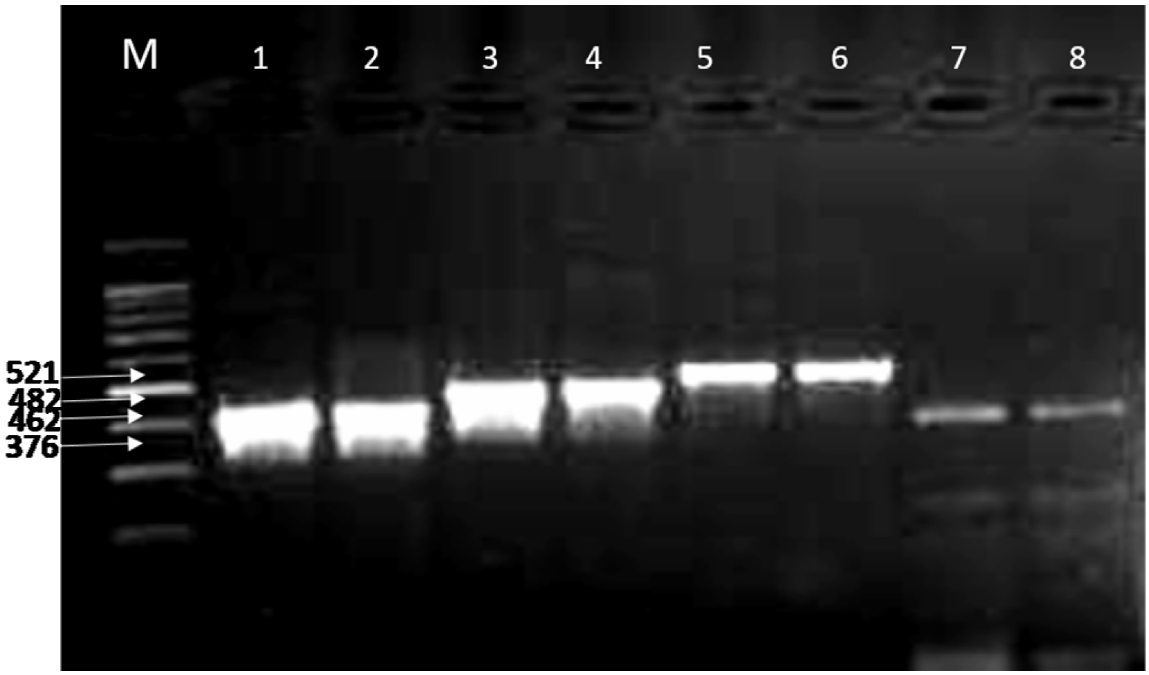
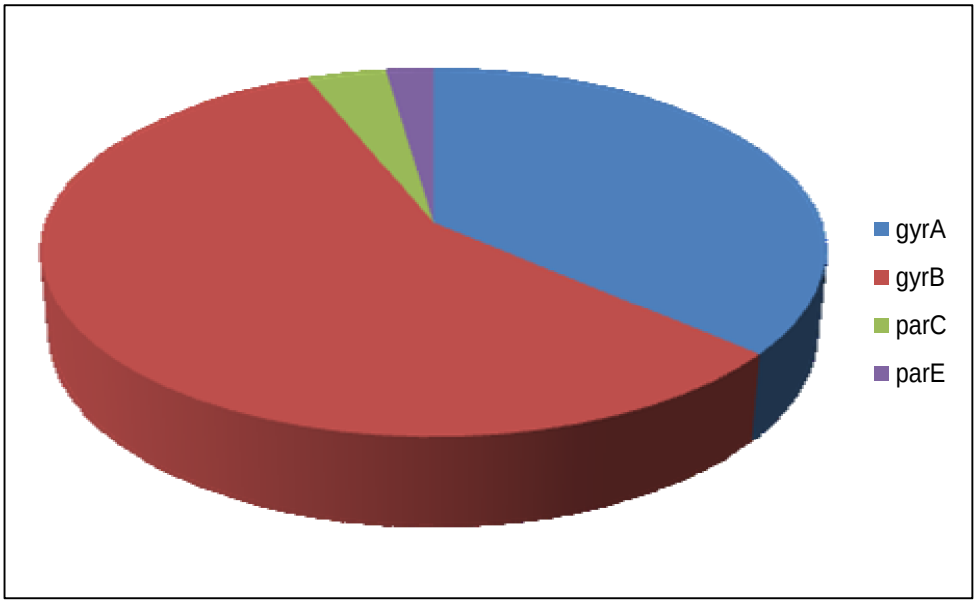


Figure.2 PCR product of *parE, acc(6')Ib*, *gyrA* and *gyrB* genes



M, molecular size marker (100 bp ladder; biorad, France) Lanes: 1-2 *Escherichia coli parE* gene, 3-4 *acc(6')Ib* gene, 5-6 *gyrA* gene and 7-8 *gyrB* gene.

Figure.3 Distribution of QRDRs of gyrase and topoisomerase IV among 112 quinolones resistant *E. coli* isolates



Although mutations in the QRDR of *gyrA* and *parC* are the most commonly documented resistance mechanisms, resistance has also been known to be conferred by mutations in the second topoisomerase gene, *parE*. In the central if India, it is interesting to notice that chromosomal mediated quinolone resistance coded by mutation in QRDR regions of *gyr A* and *parC* genes was present in all multidrug resistant (MDR) *E. coli* isolates found in fecal carriage (Pathak, 2013). A study in Ghana on quinolone-resistant *E. coli* in the faecal flora also reported >90% of *qnr* positive strains had substitutions at *gyrA* and *par C* genes (Namboodiri *et al.*, 2011) and in a Tunisian study conducted on uropathogenic *E. coli*, Substitutions in *gyrA* and *parC* genes were detected in 57.5% of strains. In our study, among 112 quinolone resistant *E. coli*, *gyr*A gene were observed in 19 strains (16%), 13 strains containing the *parE* gene (24%), *gyrB* gene was observed of 26% strains and only two strains containing the *par*C gene.

Quinolone-resistant *E. coli* are commonly present in clinical isolates which have evolved resistance through multiple mechanisms and belong to very few lineages, suggesting clonal expansion. Containment strategies to limit the spread of quinolone-resistant *E. coli* need to be deployed to conserve quinolone effectiveness and promote alternatives to their use.

Acknowledgments

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