Int.J.Curr.Microbiol.App.Sci (2019) *8*(3): 1612-1618

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 8 Number 03 (2019)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2019.803.187

The First Determination of the Plasmid-Mediated Quinolone Resistance Determinants qnrA and qnrB from the Sudan

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Keywords

qnr, plasmid, Genes, Sudan, Ciprofloxacin

Article Info

Accepted:
12 February 2019
Available Online:
10 March 2019

A B S T R A C T

The aim of this study was to determine the prevalence of plasmid-mediated quinolone resistance *qnr* genes (A, B and S) among a collection of enterobacterial clinical pathogens resistant to ciprofloxacin and was ESBL producers from Sudan. Seventy-two isolates that were ciprofloxacin resistant and ESBL producer were screened by PCR for *qnr* genes (A, B and S). *qnr*A positive isolates were tested by PCR for class-1 integrons as well as resistance transfer by conjugation with sodium azide-resistant *Escherichia coli* J53. DNA-relatedness was tested by PFGE. *Qnr* genes were detected among 13% of the test isolates, seven isolates were *qnr*B1 positive, included an *E. coli* and six *Klebsiella pneumoniae* isolates. *qnr*A was detected among two *Enterobacter cloacae* isolates. Genomic analysis by PFGE on the two *qnr*A positive isolates revealed two closely related organisms. Plasmid transfer of quinolone resistance was achieved on the two *qnr*A positive isolates, both plasmids showed part of the 3′-CS with the aminoglycoside-3′-adenyltransferases *aad*A2 and *aac*A4 genes as well as part of the *Intl*1 gene which was In6-like class-1 integrons. This study demonstrated high prevalence of *qnr*B (10%) among the test isolates compared to *qnr*A (3%) and non of the *qnr* S. This is the first report on plasmid-mediated ciprofloxacin resistance genes (*qnr*genes) from Sudan.

Introduction

Quniolones are semi-synthetic antimicrobial agents developed in the 1960s. Fluoroquinolnes are newer synthetic generations with modified antimicrobial activity and broader spectrum of action. They have unsurpassed activity particularly against gram-negative bacteria (Strahilevitz *et al.*, 2009). They act by inhibiting bacterial isomerases enzymes, namely DNA gyrase and topoisomerases IV (Fluit *et al.*, 2001). They are frequently used for the treatment of a variety of infections.

Fluoroquinolone resistance at high levels has been associated with multiple mutations in the chromosomal genes for the target enzymes

and/or changes in expression of efflux pumps and porins (Martinez *et al.,* 1998). However a plasmid-encoded mechanism of resistance of low level, has been described in 1998 (Martinez *et al.,* 1998). Paterson and co-workers reported association between quinolone resistance and production of extended-spectrum β-lactamase (ESBL) (Paterson *et al.,* 2000).

The gene responsible for this resistance, *qnr*, is distinct from previously identified quinolone resistance genes (Tran and Jacoby, 2002). Quinlone-resistance genes, carried by a variety of gram-negative bacteria have been reported from different parts of the world (Wang *et al.,* 2003; Jonas *et al.,* 2005; Corkill *et al.,* 2005; Nazic *et al.,* 2005; Mammeri *et al.,* 2005).

Association of *qnr* gene with ESBL production among enterobacteriaceae has been reported by several workers (Mammeri *et al.,* 2005 and Wang *et al.,* 2003). And the ESBL producing enterobacteriaceae have been reported from different hospitals in the Sudan (Mekki *et al.,* 2010) with high prevalence of *bla*CTX-M gene (unpublished data Malik and Elhag). Therefore I have taken up this study in order to find out the prevalence of plasmid-mediated *qnr* genes (A, B and S) among clinical isolates of ESBL producing enterobacteriaceae from the Sudan.

Materials and Methods

Bacterial isolates

Three hundred twenty four multidrug resistant bacterial isolates from wound and urine samples from patients who presented to the microbiology laboratories in Khartoum and Omdorman hospitals in the Sudan were studied. The strains were identified by standard microbiology methods (Forbes *et al.,* 2007) and were stored at -20°C in cryopreservers until tested.

Susceptibility testing

All 324 strains were tested for antibiotic susceptibilities by disc diffusion according to BSAC standardised methods. (Andrews.J.M, 2004), using Iso-Sensitest agar (Oxoid Ltd, Basingstoke, UK). Minimum inhibitory concentration (MIC) to nalidixic acid and ciprofloxacin were determined by the Etest method (AB Biodisk, Solna, Sweden).

ESBL production

Screening for ESBL production was determined by susceptibility to cefpodoxime and was confirmed phenotypically by the combined disc method (Carter *et al.,* 2000). It was characterized genotypically by the detection of *bla* genes by PCR and nucleotide sequencing.

Strains of *Enterobacteriaceae* that were found to be quinolone-resistant and ESBLs producers were further screened by PCR for the presence of the *qnr* genes.

Plasmid analysis and conjugation studies

Conjugal transfer of resistance determinants was performed on broth culture with *Escherichia coli* J53 as recipient. After 24 hours of incubation at 37˚C, mating mixtures were platted onto agar supplemented with sodium azide (100mg/L) and cefotaxime (1mg/L). Plasmid DNA was prepared from donors and transconjugants using a commercial kit (Plasmid Mini Kit, Qiagen GmbH, Hilden, Germany).

Genomic analysis

Restriction fragment length polymorphism (RFLP) was performed using restriction enzymes *Hin*dIII and *Eco*RI (Roche Diagnsotics Corporation, Indianapolis, USA). Pulsed field gel electrophoresis (PFGE) was performed with a CHEF *DR III* system (Bio-

Rad, Hemel Hempstead, UK). DNA insert blocks were digested overnight with *Xba*I at 37˚C.

PCR and nucleotide sequencing

The DNA was extracted by suspending bacteria in a 5% chelex 100 Resin slurry (Bio-Rad Laboratories) in a sterile distilled water and then was boiled for 10 minutes. PCR was performed to detect the presence of *qnr* genes (A and B) and class 1 integron cassette structures 3′-CS and 5′-CS conserved segments (integron variable region containing gene cassettes), *intl1* integrase gene and s*ul1* conferring resistance to sulphonamides (Corkill *et al.*, 2005). PCR products were detected by electrophoresis on 1% (w/v) agarose gels. Nucleotide sequence analysis was performed using the respective PCR primers for both directions using ABI 3100 automated sequencer (Warrington, UK), nucleotide sequence structures were compared with the data base using GenBank accession numbers: AY070235.

Results and Discussion

Seventy two isolates were ESBL producers and resistant to quinolones. Nine of these isolates (13%) were positive for the *qnr* genes. They included one *E. coli*, six *Klebsiella pneumoniae* and two *Enterobacter cloacae* (table 1).

Sequence analysis of the *qnr* amplicons revealed that seven (10%) of the test isolates were *qnr*B1 positive (Fig. 1) and the remaining two (3%) were *qnr*A1 positive (Fig. 2). *qnr* S was not detected among these isolates.

All the *qnr* positive isolates were positive phenotypically and by PCR for extended-spectrum β-lactamases (ESBL) production, and expressed *bla*SHV and/or *bla*CTX-M. They were negative for Amp-C production.

*qnr*A positive isolates were subjected to conjugation and plasmid transfer experiments as well as integron class-1 studies. Quinolone resistance was detected on both *E. coli* J53 transconjugants (TCJs) crossed with *E. cloacae* 1 and 2 and was transferred on a plasmid with the size of 96.9Kbp (Fig. 3).

Conjugation-experiments were performed on both isolates and each transferred the resistance to nalidixic acid and ciprofloxacin. Other groups of antibiotics were also tested (table 2). Results showed transfer of resistance to amoxicillin, cefpodoxime, gentamicin, trimethoprim, nalidixic-acid, aztreonam, ceftazidime and ciprofloxacin to the recipient organism *E.coli* J53.

The Minimum inhibitory concentration (MIC) for both Nalidixic acid and ciprofloxacin was determined on the two tranconjugant organisms as well as the recipient organism (table 3).

Plasmid extraction was performed on both donor and transconjugant organisms. A plasmid of 96.9kbp in size was responsible for resistance transfer from the donor to the recipient organisms for both isolates (Fig. 3).

Nalidixic acid resistance on *E.coli*-TCJ-1 was increased by 8 folds (MIC 3.0-24mg/L) and for *E.coli*-TCJ-2 increased by 11 folds (MIC 3.0-32mg/L). Resistance to ciprofloxacin for both isolates increased by 16 folds (MIC 0.006-0.094mg/L).

RFLP studies revealed two heterogeneous genotypic patterns. Genomic analysis by PFGE on the two qnrA positive isolates revealed two closely related organisms. Integron studies showed part of the 3′-CS with the aminoglycoside-3′-adenyltransferases *aad*A2 and *aac*A4 genes as well as part of the *Intl1* gene which was In6-like class-1 integrons.

Table.1 qnr and ESBL positive enterobacterial isolates detected within the clinical pathogens from the Sudan

Isolate No.	Bacterial isolate	Source of specimen	ESBL-phenotype	*bla*gene	*qnr*-gene
QNR1	*E.cloacae*-1	Urine	+	SHV5a	***qnr*-A1**
QNR2	*E.cloacae*-2	Urine	+	CTX-M, SHV5	***q*nr-A1**
QNR3	*E.coli*	Urine	+	SHV5	***q*nr-B1**
QNR4	*K.pneumoniae*	Wound	+	CTX-M, SHV11	***q*nr-B1**
QNR5	*K.pneumoniae*	Urine	+	CTX-M, SHV5a	***q*nr-B1**
QNR6	*K.pneumoniae*	Urine	+	CTX-M, SHV5a	***q*nr-B1**
QNR7	*K.pneumoniae*	Urine	+	CTX-M, SHV5	***q*nr-B1**
QNR8	*K.pneumoniae*	Wound	+	CTX-M, SHV11	***q*nr-B1**
QNR9	***K.pneumoniae***	**Urine**	**+**	**CTX-M**	***q*nr-B1**

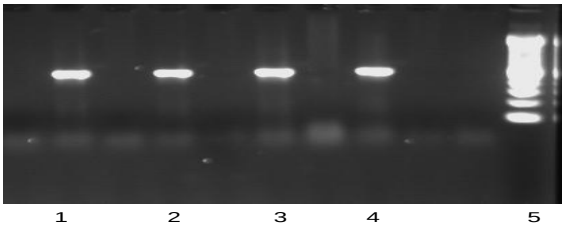
Table.2 Disc-diffusion results for the qnr-A transconjugant organisms, donors and the recipient organism (J53). (T.C= Transconjugant organism, N.R= not recorded)

	Zone Size (mm)					
Antibiotic	**Recipient**	**Donor-1**	**T.C-1**	**Donor-2**	**T.C-2**	**Breakpoints(R≤)**
Ampicillin	23	0	< 6	0	< 6	11
Augmentin	28	12	20	11	18	11
Cefpodoxime	33	10	14	16	16	25
Gentamicin	25	8	8	11	11	16
Ciprofloxacin	34	0	27	0	28	16
Trimethoprim	26	0	< 6	0	< 6	14
Nalidixic-acid	28	0	16	0	15	17
Meropenem	38	30	39	32	40	22
Amikacin	27	18	27	19	25	15
Aztreonam	42	N.R	21	N.R	24	23
Tazobactam	36	N.R	28	N.R	30	22
Ceftazidime	38	10	22	14	22	27

Table.3 MIC results for both qnr-A transconjugants and recipient organism (J53)

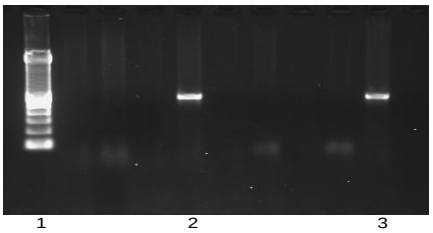
		MIC (µg/L)	
Organism		**Nalidixic-Acid**	**Ciprofloxacin**
E.coli	J53	3	0.006
Entero.cloacae	T.C 1	24	0.094
Entero.cloacae	T.C 2	32	0.094

Fig.1 PCR qnr-B positive isolates (*E.coli* and *Klebsiella* spp).



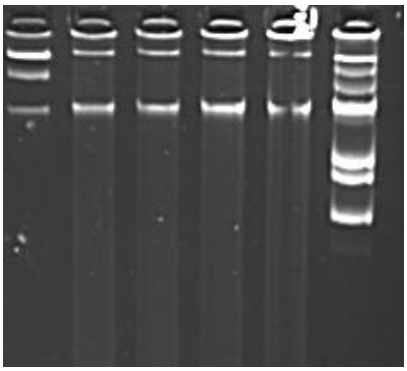
Lane 1: *qnr*B positive *E.coli* isolate. Lane 2, 3: *qnr*B positive *Klebsiella* isolates.
Lane 4: positive control. Lane 5: Lambda DNA of a multiple 100 bp size marker

Fig.2 PCR qnr-A positive isolates.



Lane 1: Lambda DNA of a multiple 100 bp size marker.
Lane 2 and 3: *qnr-A* positive isolates.

Fig.3 Plasmid extraction of the qnrA donor and transconjugants



Lane 2, 3, 4 and 5: plasmid of the size 96.9 Kbp in *qnr-A* positive isolates.
Lane 1 and 6: the markers 39R and V517 respectively.

Fluoroquinolones have been introduced with a broader spectrum of activity, to rival beta-lactams and macrolides in clinical practice (Takahashi *et al.,* 2003). However, in the Sudan quinolones are excessively used for the treatment of a wide variety of infections. Together with cephalosporins they remain the main injectable antimicrobials available for general clinical use. Thus antimicrobial resistance to quinolones and beta-lactams is rising (Ibrahim *et al.,* 2012). No wonder, as these agents have been described as causative of collateral damage (Paterson *et al.,* 2004).

In this study *qnr* genes were detected among 13% of the isolates. *qnr* B genes constitute the majority (10%) as reported by some studies (Jacoby *et al.,* 2006; Tamanag *et al.,* 2008; Leila *et al.,* 2015).

All *qnr* positive isolates from this study were ESBL producers expressing *bla*CTX-M and/or *bla*SHV genes (*bla*SHV5, *bla*SHV5a or *bla*SHV11).

In this study Quinolone resistance transfer was clearly demonstrated by the conjugation study performed on the *qnr*A positive isolates, and was explained by the rise in resistance levels of the transconjugate organisms by 8-11 folds and 16 folds to nalidixic acid and ciprofloxacin respectively. *qnr*A genes on the two test isolates from the present study were located on plasmids of molecular size ~96kbp encoding varying resistance profiles.

Qnr and ESBL resistance determinants were co-transferred on the same plasmid with resistance determinants to other antimicrobials (gentamicin, trimethoprim-sulphamethoxazole, chloramphenicol and tetracycline). This finding also confirms the co-association of plasmid mediated resistance determinants of ciprofloxacin and ESBL resistance determinants that has been reported by many other studies (Paterson *et al.,* 2000; Jonas *et al.,* 2005; Corkill *et al.,* 2005; Nazic *et al.,* 2005; Mammeri *et al.,* 2005).

Integron studies carried-on *qnr*A positive isolates had demonstrated the possibility of carriage of class-1 integron on these plasmid.

The majority of these isolates were uropathogenic isolates collected from two different hospital settings in the capital of the Sudan, where Quinolones are widely used in the treatment of Urinary tract infections. The findings from this study with other few previous studies from the Sudan that demonstrated the rising resistance prevalence to ciprofloxacin and other antimicrobials (Ibrahim *et al.,* 2012), with the development of multidrug resistant pathogens harbouring different genes and mechanisms of resistance to the most essential and lifesaving antimicrobials, clearly reflect the critical situation of the future of antimicrobials in such a developing country.

Acknowledgments

A special thanks to Mr John. E. Corkill and to Professor Ahmed Abdel Mageed for their supervision and help. This work is dedicated to the soul of late Professor C.A. Hart.

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How to cite this article:

Malik, I.A. and Elhag, K.M. 2019. The First Determination of the Plasmid-Mediated Quinolone Resistance Determinants qnrA and qnrB from the Sudan. *Int.J.Curr.Microbiol.App.Sci.* 8(03): 1612-1618. doi: https://doi.org/10.20546/ijcmas.2019.803.187