Polish Journal of Microbiology
2017, Vol. 66, No 4, 529–532

SHORT COMMUNICATION

Analyses of Plasmids Harbouring Quinolone Resistance Determinants in *Enterobacteriaceae* Members

BAYRI ERAC[1], FETHIYE FERDA YILMAZ[1], ISMAIL OZTURK[1], SABIRE SOHRET AYDEMIR[2] and MINE HOSGOR-LIMONCU[1]*

[1] Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ege University, Izmir, Turkey
[2] Department of Medical Microbiology, Faculty of Medicine, Ege University, Izmir, Turkey

Submitted 22 December 2016, revised 14 February 2017, accepted 04 April 2017

Abstract

The aim of this study was to explore the plasmid characteristics of eight clinical *Enterobacteriaceae* strains containing extended broad spectrum beta-lactamases and plasmid-mediated quinolone resistance. Plasmids were transferred by conjugation or transformation and resistance determinants were investigated by PCR. We showed that at least one plasmid harbouring *qnr*B or *qnr*S determinant was transferred by conjugation in five isolates. *Qep*A determinant was confirmed to be on a non-conjugative plasmid. We found at least one beta-lactamase gene in seven of the eight clinical isolates having plasmid-mediated quinolone resistance, which indicated that these two resistance determinants were mostly on the same conjugative plasmids.

Key words: beta-lactamase, ESBL-producing *Enterobacteriaceae*, plasmid-mediated quinolone resistance

There has been increasing rate of resistance to quinolones and beta-lactam group of agents in *Enterobacteriaceae* members recently. The most frequently seen resistance mechanism against beta-lactam antibiotics is the production of extended spectrum beta-lactamase (ESBL) and the most common ESBLs are the plasmid-borne CTX-M, SHV, and TEM types of enzymes (Pfeifer *et al.*, 2010). Studies have reported that most of the ESBL-producing *Enterobacteriaceae* members are also resistant to fluoroquinolones (Nazik *et al.*, 2011; Pasom *et al.*, 2013; Kim *et al.*, 2014). The role of mutations occurring in the regions encoding DNA gyrase and topoisomerase IV enzymes and overexpression of efflux pumps in the resistance to fluoroquinolones have been known for a long time. The development of plasmid-borne resistance to quinolones in *Enterobacteriaceae* members was first reported in a *Klebsiella pneumoniae* isolate. The Qnr protein, causes a low level quinolone resistance by binding to bacterial DNA gyrase and topoisomerase IV. The *qnr*A gene that had been seen in many *Enterobacteriaceae* members worldwide was followed by *qnr*B, *qnr*C, *qnr*D, *qnr*S and *qnr*VC genes. The other more recent plasmid-borne resistance mechanisms include: *qep*A and *oqx*AB, which are efflux pump encoding genes, and *aac(6')-Ib-cr*, which contributes to the resistance by modifying ciprofloxacin (Pasom *et al.*, 2013; Jacoby *et al.*, 2014). The aim of this study was to explore various plasmid characteristics of ESBL-positive *Escherichia coli* and *K. pneumoniae*, which have been found to have plasmid-mediated quinolone resistance.

The present study included four *E. coli* and four *K. pneumoniae* isolates, which were isolated from various clinical specimens at the Medical Microbiology Department of Ege University Hospital. All the isolates were proved previously to be ESBL-positive by a double disk synergy test and to have at least one plasmid-mediated quinolone resistance (PMQR) determinant (Hoşgör-Limoncu *et al.*, 2012). Minimum inhibitory concentration (MIC) values of ciprofloxacin were determined using the microdilution method as suggested by the Clinical and Laboratory Standards Institute (CLSI). *E. coli* ATCC 25922 was used as the control strain.

Plasmids were isolated from the clinical bacterial isolates and transconjugants by a commercial kit (QIAGEN plasmid maxi kit, Germany) according to the recommendations of the manufacturer. The *qnr*A, *qnr*B, *qnr*S, and *qep*A genes were explored in the isolated plasmids with the PCR method using original primers (Gay *et al.*, 2006; Yamane *et al.*, 2008; Minarini *et al.*, 2008).

* Corresponding author: Mine Hosgor-Limoncu, Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Ege University, Izmir, Turkey; **e-mail: minehosgorlimoncu@yahoo.com.tr**; **mine.hosgor.limoncu@ege.edu.tr**

The positive control strains and plasmids used for *qnr*A, *qnr*B, *qnr*S, and *qep*A were *E. coli* j53 (pMG252), *E. coli* j53 (pMG298), *E. coli* J53 (pMG306) and pSTV28qepA, respectively (Hoşgör-Limoncu *et al.*, 2012). 0.75% and 1.5% agarose were prepared with 1 × TBE for plasmid DNA and PCR products, respectively.

The PMQR gene transfer was performed by the broth culture mating method using *E. coli* J53 Azi^R as a recipient (Jacoby and Han, 1996). From the suspensions of 0.5 McFarland turbidity, first 0.1 ml of each donor strain and then 1 ml of recipient strain (*E. coli* J53 Azi^R) were inoculated into falcon tubes containing 4.5 ml of Luria-Bertani broth (LB-Becton, Dickinson). The tubes were incubated in a 35°C water bath for 18 hours. Transconjugants were selected on LB plates containing sodium azide (100 mg/l) (Sigma-Aldrich, Germany) and nalidixic acid (6 mg/l) (Sigma-Aldrich, Italy). The *qnr*A, *qnr*B, *qnr*S, and *qep*A genes were explored in the plasmids that were isolated from transconjugants with the PCR method using original primers (Gay *et al.*, 2006; Yamane *et al.*, 2008; Minarini *et al.*, 2008).

The transformation of qnr plasmids to *E. coli* DH5α cells was performed by heat shock (Sambrook *et al.*, 1989). Competent *E. coli* DH5α suspension (200 µl) was transferred to sterile microcentrifuge tubes, and plasmid DNA (10 µl– 50 ng) was added to these tubes. The mixtures in the tubes were kept on ice for 30 min by stirring, and then they were kept 90 sec in a water bath adjusted to 42°C. At the end of the incubation they were quickly put on ice and kept there for 1–2 min. Finally, 800 µl of LB medium was added and they were kept for 45 min in a shaker incubator (250 rpm). The transformants were selected on LB agar plates containing ciprofloxacin (0.025–1 µg/ml), and their DNA was isolated to identify the relevant genes by PCR.

The presence of TEM, SHV and CTX-M beta-lactamase genes was explored by PCR in the clinical isolates with identified PMQR determinants and in their transconjugants-transformants (Taşlı and Bahar, 2005, 2010).

Plasmids were isolated from the clinical strains harbouring PMQR determinants. These isolates were found to contain plasmids ranging from approximately 1.5 kbp to 40 kbp. Conjugation experiments were carried out to explore the transferability of plasmids having PMQR determinants. It was observed that ~23 and ~40 kbp plasmids of isolate 130 were transferred. From the three plasmids of isolate 151, only the one of ~23 kbp was transferred; and from the two plasmids of isolate 160, only the one of ~23 kbp was transferred. From the two plasmids of isolates 134 and 146, only those plasmids of ~23 kbp were transferred. The plasmids of ~20 kbp of isolates 13 and 73 and the plasmid of ~23 kbp of isolate 140 could not be transferred to the recipient bacteria by conjugation.

The presence of PMQR determinants in the transconjugants was explored by PCR. It was verified that *qnr*B (in the ~23 kbp plasmid) in isolates 130 and 160, also *qnr*S (in the ~23 kbp plasmid) in isolate 151 were transferred. It was also verified with PCR that *qnr*B was transferred in isolates 134 and 146, but no *qnr*A determinant could be detected in their conjugants (Fig. 1). Therefore, the ~23 kbp conjugative plasmids of isolates 134 and 146 were revealed to carry *qnr*B determinants but not *qnr*A. Transformation experiments were performed with the plasmids of these isolates, but no results could be obtained.

While the transformation trials failed for the plasmids of isolates 13 and 140, it was turned out successful for the plasmid of isolate 73. The presence of *qep*A determinant was verified through the PCR made from the transformant of this strain (Fig. 1). The ciprofloxacin MIC values of the transformant of isolate 73 and recipient strain were 0.2 µg/ml and 0.025 µg/ml, respectively. The ciprofloxacin MIC value was increased 8-fold in the transconjugate.

The presence of TEM, SHV and CTX-M beta-lactamase genes was explored by PCR in the eight clinical isolates harbouring PMQR determinants. TEM, SHV and CTX-M were seen together in three of the iso-

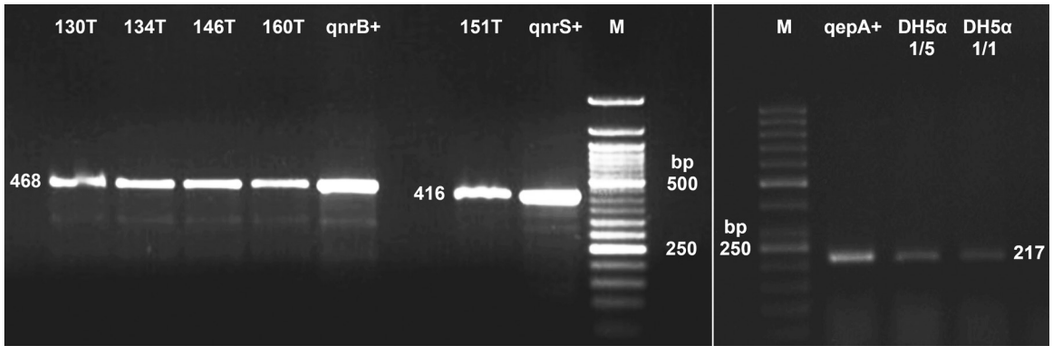

Fig. 1A. PCR results of *qnr*B and *qnr*S from transconjugants of the isolates and Fig. 1B. PCR result of *qep*A from transformant of isolate 73.

Table I
Plasmids and beta-lactamase types of PMQR-positive clinical isolates and transconjugants (*Transformant)

Isolate Number/ Species	PMQR determinant	Plasmid count		Beta-lactamase type	
		Original strain	Transconjugant	Original strain	Transconjugant
13 *K. pneumoniae*	*qnr*S	1	–	TEM SHV	–
73 *E. coli*	*qep*A	1	1*	TEM CTX-M	TEM* CTX-M*
130 *K. pneumoniae*	*qnr*B	2	2	TEM SHV CTX-M	TEM CTX-M
134 *K. pneumoniae*	*qnr*A *qnr*B	2	1	TEM SHV CTX-M	TEM CTX-M
140 *E. coli*	*qnr*B	1	–	TEM CTX-M	–
146 *K. pneumoniae*	*qnr*A *qnr*B	2	1	TEM SHV CTX-M	TEM CTX-M
151 *E. coli*	*qnr*S	3	1	CTX-M	–
160 *E. coli*	*qnr*B	2	1	–	–

lates, TEM and CTX-M in two of them, and TEM and SHV in one. Only CTX-M was present in one isolate and none of these beta-lactamase determinants were encountered in another isolate (Table I). Transferability of beta-lactamase genes by conjugation or transformation was explored by PCR in the isolates (73, 130, 134 and 146). TEM and CTX-M beta-lactamase genes were found in the transformant of isolate 73 and in the transconjugants of isolates 130, 134 and 146. The SHV gene could not be detected in any of the examined transconjugants and transformants (Fig. 2).

Quinolones and beta-lactams are broad-spectrum antibiotics that are frequently used in both community and hospital-acquired infections. Due to plasmids play an important role in the development of resistance to these two antibiotic groups, it is important to investigate these plasmids in detail (Kanamori *et al.*, 2011, Jacoby *et al.*, 2014; Zhao *et al.*, 2015; Piekarska *et al.*, 2015). In our study, we detected plasmids sized about 1.5–40 kbp in clinical isolates with established PMQR determinants. We found that at least one plasmid (~ 23 kbp) was transferred by conjugation in five of the eight clinical isolates included in our study. The presence of *qnr*B and *qnr*S in these conjugative plasmids was verified with PCR (Fig. 1), but no *qnr*A could be detected. Transformation was successful in one of the three isolates whose plasmids could not be transferred through conjugation. The presence of *qep*A in this isolate's transformant was confirmed with PCR (Fig. 1). The ciprofloxacin MIC values of the transformant of isolate 73 and recipient strain were 0.2 µg/ml and 0.025 µg/ml, respectively. The ciprofloxacin MIC value was increased 8-fold in the transconjugate.

In a study conducted in Turkey with ESBL-positive 61 *E. coli* isolates, PMQR was found in only four isolates, and from these only *qnr*A and *qnr*S could be transferred by conjugation but not *qep*A. The ciprofloxacin MIC values have been reported to increase 8 to 62-fold in transconjugates (Nazik *et al.*, 2011). In a study made in Argentina, *qnr*B and *aac(6')-Ib-cr* were transferred through conjugation but the *qep*A determinant could be transferred by electro-transformation (Rincón *et al.*, 2014). While they reported that *qep*A was on a plasmid of around 97 kbp, in our study we found a plasmid of around 20 kbp in the strain which *qep*A was detected. The increase they found in the ciprofloxacin MIC value of the transformant was also the same as found in our study. Very different conjugation rates of plasmids harbouring PMQR determinants may be encountered among studies, such as 17% and 40% (Cai *et al.*, 2011; Pasom *et al.*, 2013). The rate of success in the transfer of plasmids through conjugation is influenced by the

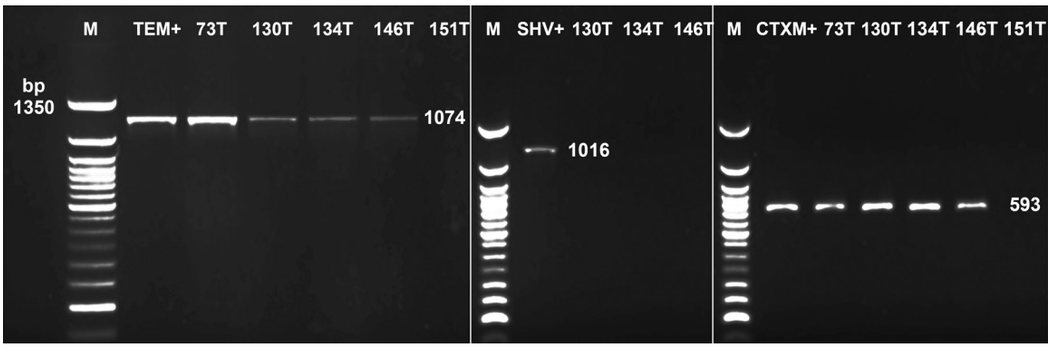

Fig. 2A. Presence of bla_{TEM}, bla_{SHV} (Fig. 2B) and bla_{CTX-M} (Fig. 2C) genes in transconjugants and transformants.

method used (*e.g.* "Filter mating"), recipient strains or antibiotics of varying effectiveness. Having carried out conjugation in five out of the eight clinical isolates in the present study, indicates a high rate of success. Similar to the results of some studies in the literature (Nazik *et al.*, 2011; Rincón *et al.*, 2014), we also found that the plasmid containing *qep*A is non-conjugative. However, Kim *et al.* (2014) stated that the *qep*A determinant could be transferred through conjugation from all of the four isolates containing this determinant.

The presence of TEM, SHV and CTX-M beta-lactamase genes was explored by PCR in the eight clinical isolates harbouring PMQR determinants. TEM, SHV and CTX-M were seen together in three of the isolates. Only CTX-M was present in one isolate and none of these beta-lactamase determinants were encountered in another isolate (Table I). Transferability of beta-lactamase genes by conjugation or transformation was explored by PCR in the isolates (73, 130, 134 and 146). TEM and CTX-M beta-lactamase genes were found in the transformant of isolate 73 and in the transconjugants of isolates 130, 134 and 146. The SHV gene could not be detected in any of the examined transconjugants or transformants (Fig. 2).

As a result, we found at least one beta-lactamase gene in seven out of the eight clinical isolates containing PMQR determinants. We determined that the PMQR and beta-lactamase genes were on the same plasmid in three out of the four isolates harbouring beta-lactamases and conjugative plasmids. The most frequently transferred beta-lactamase genes were bla_{TEM} and $bla_{\text{CTX-M}}$. We also found that the *qep*A determinant was transferred together with bla_{TEM} and $bla_{\text{CTX-M}}$. In our study, *qnr*B was found to be the most frequently transferred PMQR determinant.

Acknowledgements

This study was supported as an Ege University Scientific Research Project (12ECZ012). The authors would like to thank to Prof. George A. Jacoby, Prof. Kunikazu Yamane, Prof. Osman Birol Ozgumus and Prof. Hasan Nazik for kindly providing the positive control strains and plasmids. The authors would like to thank to Prof. Zeki Topcu, Assoc. Prof. Sevil Zencir, PhD Hasan Akbaba and Ege University Faculty of Pharmacy Pharmaceutical Sciences and Research Center (FABAL).

Literature

Cai X., C. Li, J. Huang and Y. Li. 2011. Prevalence of plasmid mediated quinolone resistance *qnr* gene in Central China. *Afr. J. Microbiol. Res.* 5: 975–978.

CLSI. 2013. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement.* CLSI M100-S23. Wayne, PA: Clinical and Laboratory Standards Institute.

Gay K., A. Robicsek, J. Strahilevitz, C.H. Park, G. Jacoby, T.J. Barrett, F. Medalla, T.M. Chiller and D.C. Hooper. 2006. Plasmid-mediated quinolone resistance in non-Typhi serotypes of *Salmonella enterica*. *Clin. Infect. Dis.* 43: 297–304.

Hoşgör-Limoncu M., B. Eraç, A.N. Yurtman and Ş. Aydemir. 2012. Plasmid-mediated quinolone resistance mechanisms in ESBL positive *Escherichia coli* and *Klebsiella pneumoniae* strains at a Tertiary-Care Hospital in Turkey. *J. Chemother.* 24: 144–149.

Jacoby G.A. and P. Han. 1996. Detection of extended-spectrum beta-lactamases in clinical solates of *Klebsiella pneumoniae* and *Escherichia coli*. *J. Clin. Microbiol.* 34: 908–911.

Jacoby G.A., J. Strahilevitz and D.C. Hooper. 2014. Plasmid-mediated quinolone resistance. *Microbiol. Spectr.* 2: 1–42.

Kanamori H., R.B. Navarro, H. Yano, L.T. Sombrero, M.R. Capeding, S.P. Lupisan, R.M. Olveda, K. Arai, H. Kunishima, Y. Hirakata and M. Kaku. 2011. Molecular characteristics of extended-spectrum β-lactamases in clinical isolates of *Enterobacteriaceae* from the Philippines. *Acta. Trop.* 120: 140–145.

Kim E.S., J.Y. Jeonga, S.H. Choia, S.O. Lee, S.H. Kim, M.N. Kim, J.H. Woo and Y.S. Kim. 2009. Plasmid-mediated fluoroquinolone efflux pump gene, *qep*A, in *Escherichia coli* clinical isolates in Korea. *Diagn. Microbiol. Infect. Dis.* 65: 335–338.

Minarini L.A.R., L. Poirel, V. Cattoir, A.L.C. Darini and P. Nordmann. 2008. Plasmid-mediated quinolone resistance determinants among enterobacterial isolates from outpatients in Brazil. *J. Antimicrob. Chemother.* 62: 474–478.

Nazik H., B. Bektöre, B. Öngen, M. İlktaç, M. Özyurt, N. Kuvat, O. Baylan, H. Keküllüoğlu, T. Haznedaroğlu and F.M. Keleşoğlu. 2011. Plasmid-mediated quinolone resistance genes in *Escherichia coli* urinary isolates from two teaching hospitals in Turkey: coexistence of TEM, SHV, CTX-M and VEB-1 type β-lactamases. *Trop. J. Pharm. Res.* 10: 325–336.

Pasom W., A. Chanawong, A. Lulitanond, C. Wilailuckana, S. Kenprom and P. Puang-Ngern. 2013. Plasmid-mediated quinolone resistance genes, *aac(6')-ıb-cr*, *qnr*S, *qnr*B, and *qnr*A, in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae* at a teaching hospital, Thailand. *Jpn. J. Infect. Dis.* 66: 428–432.

Pfeifer Y., A. Cullik and W. Witte. 2010. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *Int. J. Med. Microbiol.* 300: 371–379.

Piekarska K., T. Wołkowicz, K. Zacharczuk, M. Rzeczkowska, A. Chróst, E. Bareja, M. Olak and R. Gierczyński. 2015. Co-existence of plasmid-mediated quinolone resistance determinantsand mutations in gyrA and parC among fluoroquinolone-resistant clinical *Enterobacteriace*ae isolated in a tertiary hospital in Warsaw, Poland. *Int. J. Antimicrob. Agents.* 45: 238–223.

Rincón G., M. Radice, M. Giovanakis, J.A. Di Conza and G. Gutkin. 2014. First report of plasmid-mediated fluoroquinolone efflux pump QepA in *Escherichia coli* clinical isolate ST68, in South America. *Diagn. Microbiol. Infect. Dis.* 79: 70–72.

Sambrook J., E.F. Fritsch and T. Maniatis. 1989. *Molecular Cloning; A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Taşlı H. and I.H. Bahar. 2005. Molecular characterization of TEM- and SHV-derived extended-spectrum beta-lactamases in hospital-based *Enterobacteriaceae* in Turkey. *Jpn. J. Infect. Dis.* 58: 162–167.

Taşlı H. and I.H. Bahar. 2010. Determination of SHV-2, SHV-5, SHV-12 and CTX-M-3 extended-spectrum beta-lactamases in clinical *Enterobacteriaceae* isolates. *Turk. Klin. J. Med. Sci.* 30: 1701–1706.

Yamane K., J. Wachino, S. Suzuki and Y. Arakawa. (2008). Plasmid-mediated *qepA* gene among *Escherichia coli* clinical isolates from Japan. *Antimicrob. Agents Chemother.* 52: 1564–1566.

Zhao L., J. Zhang, B. Zheng, Z. Wei, P. Shen, S. Li, L. Li and Y. Xiao. 2015. Molecular epidemiology and genetic diversity of fluoroquinolone-resistant *Escherichia coli* isolates from patients with community-onset infections in 30 Chinese county hospitals. *J. Clin. Microbiol.* 53: 766–770.