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Original article

# Characterization of plasmid mediated quinolone resistance genes in *Enterobacteriaceae* in a university hospital in Tunisia

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**ABSTRACT**

**Background:** Quinolone-resistant enterobacterial isolates have spread largely across hospitals and community in last years. In Tunisia, numerous studies describe the emergence of plasmid mediated quinolone resistance (PMQR) genes among *Enterobacteriaceae*. **Objective**: Detection of PMQR genes among a collection of enetrobacterial isolates recovered in the Tahar Sfar University Hospital in Tunisia and in the community. **Methods**: In vitro antimicrobial susceptibility testing, extended-spectrum β-lactamases, and PMQR genes were detected using PCR. Clonality of isolates was assessed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). **Results**: In this work, 1710 enterobacterial isolates were recovered in the Tahar Sfar University Hospital in Tunisia between January 2012 and March 2013. Eighty of them were resistant to nalidixic acid: 61 isolates and 19 isolates were isolated from nosocomial and community cases respectively. Detection of PMQR genes leads to the identification of 7 and 8 QnrB producers in nosocomial and community acquired strains respectively. The *qnr*S PMQR gene was less present with 5/61 cases in nosocomial starins and 1/19 case in community one. The most predominant Extended Spectrum Beta Lactamase (ESBL) ESBL was the CTX-M-type one. According to ERIC-PCR profiles, we note a multiclonal dissemination with 12 different profiles in hospital-acquired strains and 9 profiles in community enterobacterial isolates. This result re-emphasize the widely distribution of the QNRB genes and their role in resistance to fluoroquinolones. **Conclusions**: We noticed high prevalence of PMQR genes in human enterobacterial isolates recovered either in clinical and community context.

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## Introduction

*Enterobacteriaceae* are common human pathogens which can cause a broad range of diseases including urinary tract infections, pneumonia and bloodstream infections in both community and hospital settings [1-3].

The increased rate of enterobacterial infections is associated with a rising trends of

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antibiotic use and subsequent resistance in commonly implicated species like *Escherichia coli* and *Klebsiella pneumoniae* [4].

Fluoroquinolones are characterized as broad-spectrum antibacterial drugs active against both gram positive and gram negative bacteria. Besides broad-spectrum activity, the success of fluoroquinolones can be attributed to the fact that they have equivalent oral and intravenous bioavailability , relatively low toxicity and favorable pharmacokinetics [5] .

Fluoroquinolones resistant enterobacterial isolates have been increasingly reported worldwide [6] and in Tunisia [7, 8]. This bacterial resistance to quinolones can be classified into three categories: i- resistance mutation in the target site of quinolone, ii- plasmids carrying quinolone resistance genes, also named plasmid mediated quinolone resistance (PMQR): *qnr* genes, *AAC* (6)-*Ib-cr* and efflux pumps and iii- the down regulation of porins expression [9].

The aim of this retrospective study was to characterize PMQR-producing enterobacterial isolates recovered in Taher Sfar University Hospital in Mahdia, Tunisia, in order to investigate the epidemiology of PMQR genes, and to demonstrate possible commonalities between healthcare-associated and community-acquired clinical enterobacterial isolates.

## MATERIALS AND METHODS

### Bacterial strains

From January 2012 to March 2013, a total of 1710 non-repetitive enterobacterial isolates were consecutively obtained both from clinical specimens of different wards of the Taher Sfar hospital in Mahdia, Tunisia, and from samples recovered from community-acquired infections. Strains resistant to nalidixic acid were selected for this study: nosocomial strains (n= 61) and community-acquired strains (n=19). Isolates were identified by using Api20E system (BioMérieux, Marcy l'Etoile, France).

### Antimicrobial susceptibility testing and extended-spectrum-beta-lactamase (ESBL) screening

Antimicrobial susceptibility was determined by the disk diffusion method on Mueller-Hinton agar plates with β-lactam and non β-lactam antibiotic-containing disks (Bio-Rad, Marnes-la-coquette, France) according to the guidelines of the "Comité de l'Antibiogramme de la Société Française de Microbiologie [10]. *E. coli* ATCC 25922 was used as quality control. Double Disk Synergy Test (DDST) was used to confirm ESBL production.

### Identification of the resistance genes

The 80 nalidixic resistant strains selected for this study were screened by PCR for genes encoding PMQR: *qnrA, qnr*B, *qnr*S, *qnr*C *and qnr*D [11, 12] . Isolates with a positive DDST (n=15) were screened by PCR for genes encoding ESBLs using primers targeting $bla_{\text{CTX-M}}$, $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ genes [13-16].

### Clonality of the isolates

The isolates were genotyped using enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) methods with the primer ERIC-2, as described previously [17].

## Results

### Epidemiological data and selection of strains

Between January 2012 and March 2013, 1710 clinical enterobacterial isolates were recovered in the laboratory of microbiology of the Taher Sfar University Hospital, among which 980 and 730 were isolated from nosocomial and community-acquired infections, respectively. Eighty strains were resistant to nalidixic acid according to the laboratory data and were selected for this study (Table 1).

Among the 1710 enterobacterial isolates, 1267 were identified as *Escherichia coli* (*E.coli*) isolates, 61 between them were nalidixic acid resistant. The second most prevalent enterobacterial isolate was *Klebsiella pneumoniae* (*K. pneumoniae*) (260/1710). *Proteus mirabilis* and *Enterobacter cloacae* were identified in 84 and 43 cases respectively. Species selected for this study are presented in table 1.

The distribution of nalidixic resistant isolates among the different hospital wards and the community acquired origins is presented in Table 2.

### Antimicrobial susceptibility testing and ESBL screening

Nalidixic resistant enterobacterial isolates exhibited various resistance phenotypes, including to β-lactams (except carbapenems), aminoglycosides and trimethoprim. Resistance to ciprofloxacin and ofloxacin was variable.

DDST allowed to characterize 15 ESBL-producing isolates, 9 of which were associated to hospital-acquired infections and 4 to community-acquired infections.

**Characterization of resistance genes**

Table 3 summarizes molecular features of PMQR and ESBL producing isolates.

In hospital-acquired infections, PMQR genes involved in quinolone resistance were *qnr*B and *qnr*S in 7 and 8 cases respectively. However, in community, the *qnr*S gene was detected in only one case (1/8) and *qnr*B was the most prevalent (8/9). So, in total, we have detected 15 *qnr*B gene and 8 *qnr*S gene (23/80) meaning a prevalence of 28.75%. In two strains of *K. pneumoniae*, K2 and K5, we detect the two PMQR genes *qnr*B and *qnr*S in each strain.

No *qnr*C or *qnr*D was detected.

By PCR, we identify 9 ESBL producing isolates in hospital strains (9/12) and 6 in the community one (6/9). Most isolates carried the $bla_{\text{CTX-M}}$ ESBL gene with $bla_{\text{TEM}}$ gene in 11 cases. SHV-enzyme was detected in three nosocomial strains and one community acquired strain.

**Clonality of studied strains**

All strains were subjected to an ERIC-PCR genotyping. We detect a multiclonal dissemination of nalidixic resistant strains in the hospital and community context. ERIC-PCR lead to the identification of 12 ERIC-PCR profiles between the nosocomial strains and 9 profiles between the community one.

**Table 1.** Prevalence of nalidixic acid resistant enterobacterial isolates isolated in Tahar Sfar University Hospital between January 2012 and March 2013

| **Species** | **Number of enterobacterial strains** | **Nalidixic acid resistant strains** |
|---|---|---|
| *E.coli* | 1267 | 61 |
| *Klebsiella pneumonia* | 260 | 11 |
| *Klebsiella oxytoca* | 8 | 1 |
| *Klebsiella spp* | 1 | 0 |
| *Proteus mirabilis* | 84 | 3 |
| *Proteus vulgaris* | 7 | 0 |
| *Proteus spp* | 2 | 0 |
| *Enterobacter cloacae* | 43 | 1 |
| *Enterobacter aerogenes* | 6 | 1 |
| *Enterobacter spp* | 4 | 0 |
| *Citobacter freundii* | 4 | 0 |
| *Citobacter oliveras* | 1 | 0 |
| *Citobacter koseri* | 2 | 0 |
| *Providencia rettgeri* | 2 | 0 |
| *Providencia stuartii* | 3 | 0 |
| *Morganella morganii* | 10 | 1 |
| *Serratia marcescens* | 5 | 0 |
| *Salmonella* | 1 | 1 |
| Total | 1710 | 80 |

**Table 2.** Origin of studied strains resistant to nalidixic acid.

| | **Origin** | **Number (%)** |
|---|---|---|
| **Nosocomial Strains (57,5%)** | Medicine | 20 (25) |
| | Surgery | 8 (10) |
| | Intensive Care Unit | 5 (6.25) |
| | Urology | 3 (3.75) |
| | Pneumology | 2 (2.5) |
| | Nephrology | 2 (2.5) |
| | Pediatrics | 2 (2.5) |
| | Cardiology | 2 (2.5) |
| | Hemodialysis | 1 (1.25) |
| | Gynecology | 1 (1.25) |
| Total Nosocomial Strains | Total Nosocomial Strains | 46 (57.5) |

| | | |
|---|---|---|
| **Community Acquired Strains (42,5%)** | Emergency | 10 (12.5) |
| | Externe consultation | 10 (12.5) |
| | Dispensary | 14 (17.5) |
| Total Community-Acquired Strains | Total Community-Acquired Strains | 34 (42.5) |
| Total Number of strains | Total Number of strains | 80 (100) |

**Table 3.** Characteristics of PMQR- producing enterobacterial isolates

| Isolate | Identification | Date of isolation | Ward | Specimen | Non β-lactams resistance | β-lactamase(s) and PMQR genes |
|---|---|---|---|---|---|---|
| **Nosocomial Strains** | | | | | | |
| **E1** | *Escherichia coli* | **01/27/2012** | Surgery | Urine | CIP/NAL/SXT/OFL | *qnr*S |
| **E2** | *Escherichia coli* | **05/../2012** | Surgery | Pus | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **E3** | *Escherichia coli* | **05/17/2012** | Medicine | Urine | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **E4** | *Escherichia coli* | **02/18/2013** | Medicine | Urine | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **E5** | *Escherichia coli* | **03/02/2013** | Medicine | Urine | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **K1** | *Klebsiella pneumoniae* | **01/24/2012** | Medicine | Urine | CIP/NAL/SXT/OFL | *qnr*S |
| **K2** | *Klebsiella pneumoniae* | **02/02/2012** | Urology | Urine | CIP/NAL/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{SHV}$ , *qnr*S, *qnr*B |
| **K3** | *Klebsiella pneumoniae* | **02/03/2012** | Hemodialysis | Urine | CIP/NAL/TM/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$,*qnr*S |
| **K4** | *Klebsiella pneumoniae* | **05/12/2012** | Nephrology | Urine | CIP/NAL/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{SHV}$, *qnr*S |
| **K5** | *Klebsiella pneumoniae* | **07/14/2012** | Surgery | Urine | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{SHV}$, *qnr*S, *qnr*B |
| **K6** | *Klebsiella pneumoniae* | **02/18/2013** | Medicine | Urine | CIP/NAL/ OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*S |
| **K7** | *Klebsiella pneumoniae* | **02/20/2013** | Medicine | Urine | CIP/NAL/TM/SXT/OFL | *qnr*B |
| ***Community-acquired strains*** | | | | | | |
| **CE1** | *Escherichia coli* | **02/03/2012** | Community | Urine | NAL/TM/ SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **CE2** | *Escherichia coli* | **02/02/2013** | Community | Urine | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **CE3** | *Escherichia coli* | **02/02/2013** | Community | Urine | CIP/NAL/ OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*S |
| **CE4** | *Escherichia coli* | **02/16/2013** | Community | Urine | NAL/TM/ GEN/SXT/OFL | *qnr*B |
| **CE5** | *Escherichia coli* | **02/21/2013** | Community | Urine | CIP/NAL/TM/SXT/OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **CK1** | *Klebsiella pneumoniae* | **02/18/2013** | Community | Epithelial Cell | CIP/NAL/SXT/OFL | *qnr*B |
| **CK2** | *Klebsiella pneumoniae* | **03/06/2013** | Community | Urine | CIP/NAL/TM/GEN/ OFL | *bla*$_{CTX-M}$, *bla*$_{TEM}$, *qnr*B |
| **CEnc1** | *Enterobacter cloacae* | **06/14/2012** | Community | Urine | CIP/NAL/TM/GEN/SXT/OFL | *bla*$_{SHV}$, *qnr*B |
| **CM1** | *Morganella morgannii* | **02/24/2013** | Community | Urine | CIP/NAL/ GEN/ OFL | *qnr*B |

CIP: ciprofloxacin, NAL: Nalidixic acid, SXT: Trimethoprim-sulfamethoxazol, OFL: Ofloxacin, TM: tobramycin, GEN: gentamicin, CIP: ciprofloxacin

**Discussion**

Increasing frequency of drug resistance among *Enterobacteriaceae* in recent years is a major public health concern. These bacteria are at the origin of community-acquired and nosocomial infections and it is common to use quinolones or fluoroquinolones such as nalidixic acid, ciprofloxacin, and norfloxacin for the treatment of this kind of infections.

The spread of quinolone resistance in bacteria is related to different mechanisms such as chromosomal mutations or resistance genes transferred by plasmids. The *qnr* genes have been poorly studied in Tunisia and especially in the Tahar Sfar University Hospital in Mahdia; therefore, this study aimed to investigate the prevalence of nalidixic acid resistance and the presence of *qnr* genes in our structure and in community between January 2012 and March 2013.

In this study 80 of 1710 *enterobacterales* isolates were resistant to nalidixic acid (4,68%); a similar rate is described for nosocomial strains (46/980): 4,7% and community-acquired strains ( 34/730): 4,65%. Occurence of *qnr* genes was in 14 (30.42%) and in 9 (26,47%) nosocomial and community acquired strains respectively.

Neither the *qnr*A, C and D genes were detected in our collection. *qnr*B gene was found in 7/12 hospital-associated isolates and in 8/9 community-associated isolates. This result is in concordance with previous data in Tunisia and in the world relating to the high prevalence of detection of *qnr*B gene. The plasmid-mediated quinolone resistance mechanisms represent a great potential for horizontal spread. They were detected worldwide and few studies explain their prevalence in North Africa and in Tunisia [18].

Fifteen out of 21 of the PMQR producing isolates are also ESBL-producing *Enterobacteriaceae*. In our structure, a large dissemination of $bla_{\text{CTX-M15}}$ was described in a previous study [18]. Here, we confirm the predominance of this enzyme (14/15) in multiresistant *enterobacteriaceae* isolated in Taher Sfar University Hospital.

All *K. pneumoniae* isolates harbored the $bla_{\text{CTX-M1-group}}$ gene, mostly together with the $bla_{\text{TEM-}}$ gene. In addition, the $bla_{\text{SHV-like}}$ genes were rare, and confirm the replacement of SHV *K. pneumoniae* producers with CTX-M-one [20]. A strong association between ESBL production and quinolone resistance has been largely reported in *Enterobacteriaceae* [21-22]. Significant association between the presence of *bla* and *qnr* genes is of serious concern since quinolone resistant ESBL bacteria has limited treatment options. There is a confirmed potential of co-dissemination by horizontal gene transfer of resistance to ß-lactams and quinolones and this finding could be confirmed by further molecular studies and plasmid characterization.

Finally, the diversity of ERIC-PCR profiles in the hospital and the community contexts could be explained by a dissemination of different clones in the hospital in addition to the dissemination of particular one.

This study reports the prevalence of PMQR genes in human enterobacterial isolates recovered either in clinical and community context. The spread of the resistance in our country reinforce the crucial need for antimicrobial surveillance and specially to limitate the indiscriminate use of antibiotic.

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**Competing interests**:

None declared.

**Ethical approval**:

Not required.

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**References**

1- Rodriguez-Martinez JM, Cano ME, Velasco C, Martinez-Martınez L, Pascual A. Plasmid mediated quinolone resistance: an update. J Infect Chemother 2011; 17:149-82.

2- Martirosov D M, Lodise TP. Emerging trends in epidemiology and management of infections caused by carbapenem-resistant Enterobacteriaceae. Diagnostic Microbiology Infectious Disease 2016. 85:266-275.

3- Tischendorf MDJ, Almeida de Avila R, Nasia Safdar MD. 2016. Risk of infection following colonization with carbapenem-resistant *Enterobactericeae*: A systematic review. American J of Infect Control; (44):539-543.

4- Rottier WC, Bamberg YR, Dorigo-Zetsma JW, van der Linden PD, Ammerlaan HS, Bonten MJ. Predictive value of prior colonization and antibiotic use for third-generation cephalosporin-resistant enterobacteriaceae bacteremia in patients with sepsis. Clin Infect Dis. 2015;60(11):1622-30.

5- Naeem A, Badshah SL, Muska M, Ahmad N, Khan K. The current case of quinolones: synthetic approaches and antibacterial activity. Molecules; 2016. 28: 21(4).

6- World Health Organization (2014). Antimicrobial resistance: Global Report on Surveillance 2014. Genova. (06-01-2024)

7- Ferjani S, Saidani M, Quentin C, Slim Ameni F, Boutiba Ben Boubaker I, Dubois V. Prevalence and characterization of uropathogenic Escherichia coli harboring plasmid-mediated quinolone resistance in a Tunisian university hospital. Diagnostic Microbiology and Infectious Disease. 2014.79: 247–251

8- Nasri Yaiche M, Denden Rafraf I, Qinglan G, Mastouri M, Mahjoub A and Minggui W. Type II and type IV topoisomerase mutations in clinical isolates of Morganella morganii harbouring the qnrD gene. Annals of Clinical Microbiology and antimicrobials. 2014. 13:34.

9- Jacoby GA, Strahilevitz J, Hooper DC. Plasmid-mediated quinolone resistance. Microbiol Spectr. 2014 Oct;2(5):10.1128.

10- Comité de l'antibiogramme de la société française de Microbiologie. 2015. www.sfm-microbiologie.fr.

11- Cattoir, V., Poirel, L., Rotimi, V., Soussy, C. J., Nordmann, P. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J Antimicrob Chemother, 2007. 60, 394–397.

12- Robicsek, A., Strahilevitz, J., Sahm, D. F., Jacoby, G. A., Hooper, D. C. qnr prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. Antimicrob Agents Chemother50, 2006. 2872–2874.

13- Robicsek A, Strahilevitz J, Sahm DF, Jacoby GA, and Hooper DC. qnr prevalence in ceftazidime-resistant Enterobacteriaceae isolates from the United States. Antimicrob Agents and Chemother. 2006. 50: 2872–2874

14- Cattoir V, Poirel L, Rotimi V, Soussy C, Nordmann P. Multiplex PCR for detection of plasmid-mediated quinolone resistance qnr genes in ESBL-producing enterobacterial isolates. J Antimicrob Chemother. 2007. 60:394-397.

15- Minghua W, Qinglan G, Xiaogang X, Xiaoying W, Xinyu Y, Shi W, Hooper D, Minggui W. New plasmid-mediated Quinolone resistance gene, qnrC, found in a clinical isolate of Proteus mirabilis. Antimicrob Agents Chemother. 2009. 53: 1892-1897.

16- Park C, Robicsek A, Jacoby GA, Sahm D, Hooper D. Prevalence in the United States of aac(6_)-Ib-cr encoding a Ciprofloxacin-modifying enzyme. Antimicrob Agents Chemother. 2006. 50: 3953-3955.

17- Arpin C, Dubois V, Coulange L, André C, Fischer I, Noury P, et al. Extended-spectrum □-lactamase-producing Enterobacteriaceae in community and private health care centers. Antimicrob Agents Chemother. 2003. 47:3506-14.

18- Bel Haj Khalifa A and Khedher M. Fréquence et résistance aux antibiotiques des bactéries uropathogènes à l'hôpital universiatire Tahar Sfar de Mahdia. Revue Tunisienne d'infectiologie. 2010. 4 (2): 57-61.

19- Mansour W, Grami R, Ben Haj Khalifa A, Dahmen S, Châtre P, Haenni M et al. Dissemination of multidrug-resistant blaCTX-M-15/IncFIIk plasmids in Klebsiella pneumoniae isolates from hospital- and community-acquired human infections in Tunisia. Diagn Microbiol Infect Dis. 2015. 83(3):298-304.
20- Elhani D, Bakir L, Aouni M, Passet V, Arlet G, Brisse S, et al. Molecular epidemiology of extended-spectrum beta-lactamase- producing Klebsiella pneumonia strains in a University Hospital in Tunis,Tunisia, 1999-2005.Clin Microbiol Infect. 2010. 16:157-64.
21- Shibl AM, Al-Agamy MH, Khubnani H, Senok AC, Tawfik AF, Livermore DM. High prevalence of acquired quinolone-resistance genes among Enterobacteriaceae from Saudi Arabia with CTX-M-15 β-lactamase. Diagn Microbiol Infect Dis. 2012. 73(4):350-3.
22- Wu JJ, Ko WC, Tsai SH, Yan JJ. Prevalence of plasmid-mediated quinolone resistance determinants QnrA, QnrB, and QnrS among clinical isolates of Enterobacter cloacae in a Taiwanese hospital. Antimicrobial Agents Chemother. 2007 51(4), 1223-7.

Bahri S, Jarbouii S, El Ahmer I, Bel Haj Khalifa A, Khedher M, Aouni M, Bouallegue O, Mansour W. Characterization of plasmid mediated quinolone resistance genes in Enterobacteriaceae in a university hospital in Tunisia. Microb Infect Dis 2024; 5(4): 1431-1437.