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Research Article

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

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Abstract

Purpose: *To evaluate the occurrence of plasmid-mediated quinolone resistance (PMQR) genes and the prevalence of extended spectrum β-lactamase (ESBL) types in Escherichia coli clinical isolates.*
Methods: *Sixty-one ESBL-producing urinary E. coli isolates were studied. An antibiotic susceptibility test was performed using the disc diffusion method. ESBL production was determined using a double-disc synergy test for all isolates; E-test and Vitek 2 were used for plasmid-mediated quinolone resistance (PMQR)-positive isolates and their transconjugants. The presence of PMQR and β-lactamase genes was determined by polymerase chain reaction (PCR).*
Results: *The strains displayed high rates of resistance to norfloxacin (80 %). The most frequent PMQR gene was aac(6')-Ib-cr (45.9 %). In all, one qnrA1 (1.6 %), one qnrS1 (1.6 %), and two qepA1-positive isolates (5.7 %) were identified. The genes, qnrS1+aac(6')-Ib-cr and qepA1, were co-expressed with $bla_{CTX\text{-}M\text{-}15}$ gene, while qnrA1 occurred with $bla_{TEM\text{-}1}$, bla_{SHV}, and $bla_{VEB\text{-}1}$ genes. The most frequent β-lactamase type was cefotaximase (CTX-M), which generally hydrolyzes cefotaxime (92 %) more than it does ceftazidime; followed by temoneira (TEM, 39 %); sulfhydryl variable (SHV, 5 %), and Vietnamese extended-spectrum beta–lactamase (VEB, 1.6 %).*
Conclusion: *A high prevalence of aac(6')-Ib-cr and CTX-M type β-lactamase was detected in ESBL-producing E. coli strains. This study also identified the co-expression of qnrA1 and $bla_{VEB\text{-}1}$ genes and of qnrS1+aac(6')-Ib-cr in E. coli isolates. The co-existence of PMQR genes with ESBLs may lead to a serious public health problem.*

Keywords: *β-lactamase, Quinolone resistance, aac(6')-Ib-cr, CTX-M-15, VEB-1*

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INTRODUCTION

Although bacterial resistance to quinolones is usually due to chromosomally encoded mechanisms, it can also originate from plasmid-mediated genes. After the identification of *qnr* determinants, which protect target enzymes against quinolone inhibition, two other mechanisms have now been described: the *qepA* gene encodes an efflux pump, which confers reduced susceptibility on hydrophobic fluoroquinolones such as norfloxacin and ciprofloxacin; and the *aac(6')-Ib-cr* gene, which encodes modified aminoglycoside-acetylating enzymes and can inactivate both aminoglycosides and fluoroquinolones [1-3].

Over the last decade, plasmid-mediated quinolone resistance (PMQR), particularly among the various species of *Enterobacteriacae,* has been increasingly reported from many regions of the world. Plasmids carrying genes may contribute to the development of higher levels of fluoroquinolone resistance and may pose a threat by allowing the rapid spread of resistance among organisms. Although these PMQR genes have been associated with low levels of quinolone resistance, it could cause high-level quinolone resistance by facilitating the selection of chromosomal mutations. Several studies have demonstrated that most *qnr*-positive enterobacterial isolates are associated with extended spectrum β-lactamases (ESBLs), including TEM, SHV, VEB, and CTX-M types, which are generally located on plasmids that are highly transferable and may harbor resistance genes to several different groups of antibiotics [4]. Today, many antibiotics, such as β-lactams and fluoroquinolones, which are widely prescribed by clinicians for the treatment of urinary tract *E. coli* infection, are in limited use.

The production of ESBLs and PMQR proteins are a cause for concern. The ciprofloxacin resistance and ESBL rates are high in *E. coli* isolates from Turkey, being 40 - 42% and 28.7 - 32.1 %, respectively [5,6]. However, significantly lower ciprofloxacin resistance and ESBL rates among *E. coli* in North America, Latin America, and Europe were observed - 4.5 - 1.9 %, 7.1 - 9.0 %, and 5.0 - 5.4 %, respectively [7]. Data on the prevalence of ESBL, especially CTX-M and VEB types, are limited in Turkey. Although the prevalence of PMQR genes, *qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr,* and recently *qepA* and associated ESBLs have been reported, the prevalence of *qnrC* and *qnrD* is unknown [8-11].

The aim of this study was to determine the prevalence of PMQR genes and ESBL types in clinical urinary isolates of *E. coli* collected from two large teaching hospitals located in the European and Asian parts of Istanbul in Turkey.

EXPERIMENTAL

Bacterial isolates

A total of 61 consecutive non-repetitive ESBL-producing *E. coli* strains, from the years 2008 and 2009, were collected from the Microbiology Laboratories of two teaching hospitals in Istanbul, Turkey. One of the hospitals, Istanbul Medical Faculty (IMF, 1.750 beds), is located in the European part of Istanbul, while the other, Gulhane Military Medical Academy Haydarpasa Training Hospital (GMMA, 1000 beds), is in the Asian section of Istanbul. Isolates from Istanbul Medical Faculty (n = 26) and from Gulhane Military Medical Academy Haydarpasa Training Hospital (n = 35) were included. The isolates were collected from urine specimens and isolated and identified with the aid of Chromogenic medium and Vitek 2 System (bioMérieux, France).

Antimicrobial susceptibility and synergy testing

Individual strains were tested based on the recommendations of the Clinical and Laboratory Standards Institute (CLSI), using

the Kirby–Bauer disc diffusion method for susceptibility [12]. The double disc synergy test with cefotaxime and ceftazidime was used for screening ESBL production.

The following antibiotic discs (Oxoid, Hampshire, UK) were purchased and used, as instructed by the manufacturer: amoxicillin-clavulanic acid (20/10 µg), imipenem (10 µg), gentamicin (10 µg), norfloxacin (10 µg), co-trimoxazole (1.25/23.75 µg), nitrofurantoin (300 µg), and fosfomycin (200 µg). *E. coli* 25922 was used as control strain. For PMQR-positive isolates and their transconjugants, the minimal inhibitory concentration (MIC) of ampicillin, amoxicillin-clavulanic acid, piperacillin-clavulanic acid, cefazolin, cefuroxime, cefoxitin, ceftazidime, ceftriaxone, cefepime, ertapenem, imipenem, meropenem, amikacin, gentamicin, levofloxacin, tigecycline, and co-trimoxazole were determined by Vitek 2 System. The MICs of ciprofloxacin were determined by E-test method (AB, Biodisk, Solna, Sweden).

Enterobacterial repetitive consensus PCR (ERIC-PCR)

The Enterobacterial Repetitive Intergenic Concensus (ERIC)-PCR with ERIC1 and ERIC2 primers was used to analyze the epidemiological relationship between PMQR-positive *E. coli* isolates. Cycling conditions were as follows: 5 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C; and final extension of 10 min at 72 °C. The PCR products were separated by electrophoresis in 1.5 % agarose gel and visualized on a UV transilluminator, and fingerprints were compared [13].

Transferability of PMQR genes and plasmid analysis

Conjugation experiments with an azide-resistant *E. coli* J53 (AzR) as the recipient were performed in liquid culture media, as described previously [14]. Transconjugants were selected on trypticase soy agar plates containing sodium azide (100 µg/ml) for counter selection and amoxicillin (100 µg/ml), cefotaxime (8 µg/ml), ceftazidime (8 µg/ml), nalidixic acid (16 µg/ml). The High Pure Plasmid Isolation Plasmid DNA Kit (Roche, Mannheim, Germany) was used for the extraction of plasmid DNA. *E. coli* V517 cells harboring plasmids of 54.4, 7.1, 5.6, 5.2, 3.0, 2.7, and 2.1 kb were used as the size marker for the plasmids. The presence of transferred PMQR genes and related ESBLs were confirmed by PCR.

Characterization of ESBL and PMQR genes and sequencing

DNA extraction was performed, as described previously. Briefly, bacterial colonies were suspended in 2 ml centrifuge tubes and then centrifuged at 12,000 *g*. The pellets were washed in 750 µl TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) and then boiled for 10 min in 500 µl TE buffer and centrifuged. The supernatants were stored at -20 °C prior to subsequent DNA amplification [9].

The bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{VEB} genes were investigated by PCR, as previously described [15,16]. A multiplex PCR was performed to detect *qnrA*, *qnrB,* and *qnrS,* as previously described by Cattoir *et al* [17]. PCR amplification of *qnrC*, *qnrD*, *qepA,* and *aac (6')-Ib* was carried out with specific primers and conditions [2,3,18]. The DNA for control for each specific gene region was included with each group of tested strains. After PCR amplifications, the products of *aac(6')-Ib* positives were further analysed by digestion with BtsCI for detection of the –cr variant (New England Biolabs, Ipswich, MA, USA).

The amplification products of PMQR and related β-lactamases were sequenced with an Applied Biosystems sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The nucleotide and amino acid sequences were analyzed and compared by BLAST search (www.ncbi.nlm.nih.gov).

Statistical analysis

Statistical analysis was performed using SPSS for Windows, version 11.5 (SPSS, Inc, Chicago, IL, USA). Rates of resistance were compared using the Chi-square test. A *p*-value of < 0.05 was considered to be statistically significant.

RESULTS

Antibiotic susceptibilities and the prevalence of PMQR determinants/β-lactamase genes

Table 1 shows the prevalence and susceptibility data. The strains displayed highest resistance to norfloxacin (80 %). The most active antibiotics were imipenem (100 %), fosfomycin (100 %) and nitrofurantoin (93.4 %). The rate of resistance to amoxicillin-clavulanic acid for the isolates of IMF was higher than that of the isolates of GMMA (p = 0.022); the opposite was found for the resistance to norfloxacin (p = 0.001). Co-resistance was identified in 96 % of the strains. The highest co-resistance was determined for norfloxacin and the most common two co-resistance phenotypes were amoxicillin-clavulanic acid/norfloxacin/co-trimoxazole (24.5 %) and amoxicillin-clavulanic acid/gentamicin/norfloxacin/co-trimoxazole (15 %).

The prevalence of PMQR genes for *aac(6')-Ib-cr*, *qepA, qnrA, and qnrS* were 45.9, 5.7, 1.6, and 1.6%, respectively. *qnrA1* and *qepA1* were detected alone in strains, but *qnrS1* was co-expressed with *aac(6')-Ib-cr.* All PMQR-positive isolates were resistant to norfloxacin, except the *qnrA1*- positive strain. In addition, norfloxacin resistance in *aac(6')-Ib-cr*-positive isolates (all were resistant) was significantly higher than in the *aac(6')-Ib-cr*-negative ones (p = 0.001). No isolates carrying the *qnrB*, *qnrC,* or *qnrD* genes were detected in this study (Table 1). The most prevalent ESBL type was CTX-M (92 %) (mostly CTX-M group 1 (66 %)), followed by TEM and SHV. Only one isolate harbored the VEB-1 type β-lactamase. Ciprofloxacin-resistant *E. coli* 4 and *E. coli* 6 were isolated from patients with nephrolithiasis, who were operated in the same division of GMMA during the same period, and these were *qepA*1-positive. *E. coli* 4 was isolated 36 days after the operation and the patient was treated with fosfomycin. *E. coli* 6 was isolated from a patient who was admitted with high fever 11 days after the operation and treated with a imipenem-gentamicin combination. Ciprofloxacin-resistant *E. coli*34 (from a kidney transplant patient treated with piperacillin-tazobactam) and ciprofloxacin-susceptible *E. coli* 210 (from a patient who was born with premature rupture of membranes treated with an ampicillin-gentamicin combination) were isolated in different divisions of ITF. *E. coli* 24 harbored both *qnrS1* and *aac(6')-Ib-cr,* while *qnrA1* was detected in *E. coli* 210.

RAPD-PCR typing

E. coli 4 and *E. coli* 6 have similar antibiotic patterns; i.e., they are resistant to amoxicillin-clavulanic acid, cefriaxone, amikacin, gentamicin, norfloxacin, levofloxacin, and co-trimoxazole. RAPD-PCR typing was carried out on the four PMQR-positive isolates. The results showed that the *qepA1*-positive isolates were clonally related (data not shown).

Characteristics of PMQR-positive isolates, transconjugants, and plasmid analysis

PCR assays were used to detect β-lactamase, and identified the $bla_{\text{CTX-M-15}}$ gene in *E. coli* 4, *E. coli* 6, and *E. coli* 34, while $bla_{\text{TEM-1}}$, bla_{SHV}, and $bla_{\text{VEB-1}}$ genes were detected in *E. coli* 210. Despite three separate attempts, conjugative assays failed with the *E. coli* 4 and *E. coli* 6 isolates. However, plasmid analysis demonstrated that both strains harbored multiple plasmids changing 1-7 kb **(**Figure 1**).**

The pattern of susceptibility to the β-lactams of the transconjugants corresponded to the

Table 1: Characteristics of extended-spectrum β-lactamase producing *E. coli* isolates (%)

	qnrA	*qnrB*	*qnrS*	*qnrC*	*qnrD*	*qepA*	*aac(6')-Ib-cr*	*bla*-TEM	*bla*-SHV	*bla*-CTX-M	*bla*-CTX-M grup1	*bla*-VEB	AMC	IPM	GN	NOR	SXT	NIT	FOS
Total (n=61)	1.6	0	1.6	0	0	5.7	45.9	39	5	92	66	1.6	69	0	43	80	70.5	6.6	0
GMMA (n=35)	0	0	0	0	0	3.3	51.4	26	0	91	69	0	57	0	43	94	69	3	0
IMF (n=26)	3.8	0	3.8	0	0	0	38.5	58	11.5	92	61.5	3.8	85	0	42	61.5	73	11.5	0
P	0.242	-	0.242	-	-	0.215	0.315	0.011	0.039	0.901	0.568	0.242	0.022	-	0.966	0.001	0.703	0.176	-

IMF: Istanbul Medical Faculty, GMMA: Gulhane Military Medical Academy Haydarpasa Training Hospital, R: Resistance, AMC: Amoxicillin-clavulanic acid, IPM: Imipenem, GN: Gentamicin, NOR: Norfloxacin, SXT: CO-trimoxazole, NIT: Nitrofurantoin, FOS: Fosfomycin.

expression of clavulanic acid-inhibited ESBLs. Transfer of the *qnrS1+aac(6')-Ib-cr* and $bla_{CTX-M-15}$ of *E. coli* 34 and *qnrA1*, bla_{TEM-1}, bla_{SHV}, and bla_{VEB-1} gene of *E. coli* 210 to the azide-resistant *E. coli* J53 occurred during the conjugation experiments. Tranconjugants of *E. coli* 34 were resistant to amoxicillin-clavulanic acid, cefriaxone, amikacin, gentamicin, levofloxacin, and co-trimoxazole. Transconjugants of *E. coli* 210 were resistant to cefriaxone, gentamicin, and co-trimoxazole.

The MICs of ciprofloxacin and other antibiotics for *E. coli* 34 and *E. coli* 210 parenteral isolates and their transconjugants are presented in Table 2**.** The MICs for ciprofloxacin were increased 63 and 8 times in the transconjugants of *E. coli* 34 and *E. coli* 210, respectively.

DISCUSSION

It is more difficult to treat ESBL-producing *E. coli* because most β-lactams are no longer therapeutic options. In particular, CTX-M type enzymes have emerged worldwide and have rapidly increased in *E. coli* isolated from both community and nosocomial settings [19]. The associated co-resistance of ESBL producers to different groups of antimicrobials, such as quinolones, sulfonamides, and aminoglycosides, is another issue of concern.

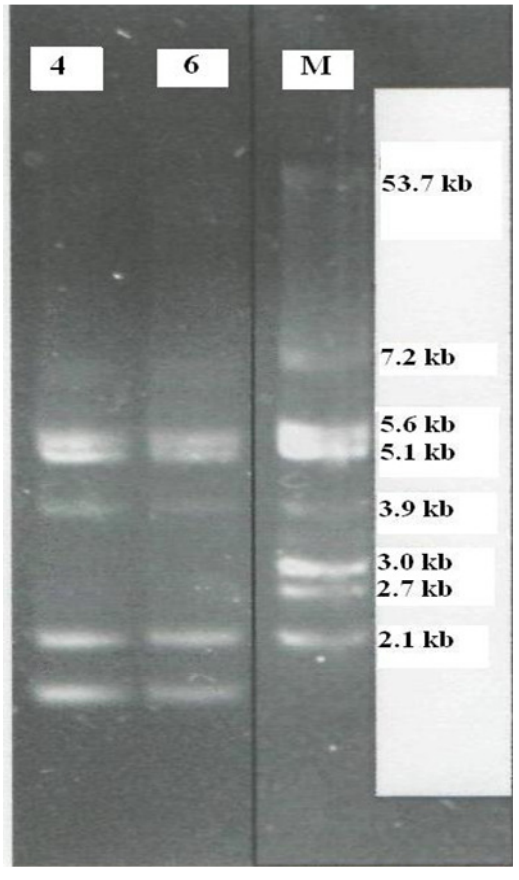

Fig 1: Plasmid DNAs from *E. coli* 4 and *E. coli* 6. Line M, *E. coli* V 517 (used as standard for plasmid size)

Table 2: MICs of β-lactam and non-β-lactam antibiotics*

	MICs (µg/ml) against PMQR-positive isolates and their transconjugants						
Antibiotics	***E. coli* 4 *qepA1*-positive, *bla*$_{CTX-M-15}$**	***E. coli* 6 *qepA1*-positive, *bla*$_{CTX-M-15}$**	***E. coli* 1434 *qnrS1*-positive, *aac(6')-Ib-cr*-positive, *bla*$_{CTX-M-15}$**	***E.coli* J53 (p34) (*qnrS1*-positive, *aac(6')-Ib-cr*-positive *bla*$_{CTX-M-15}$)**	***E. coli* 139210 *qnrA1*-positive bla$_{TEM-1}$, bla$_{SHV}$ *bla*$_{VEB-1}$**	***E. coli* J53 (p210) (*qnrA1*-positive bla$_{TEM-1}$, bla$_{SHV}$ *bla*$_{VEB-1}$)**	***E.coli* J53**
AMP	≥32	≥32	≥32	≥32	≥32	≥32	≤2
AMC	16	16	≥32	≥32	16	4	4
TZP	8	8	≥128	≥128	≤4	≤4	≤4
CZ	≥64	≥64	≥64	≥64	≥64	8	≤4
CXM	≥64	≥64	≥64	≥64	≥64	16	4
FOX	32	32	8	≤4	≤4	≤4	4
CAZ	16	≥64	≥64	16	≥64	4	≤1
CRO	≥64	≥64	≥64	≥64	≥64	≤1	≤1
FEP	8	32	≥64	≥64	≤1	≤1	≤1
ETP	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5
IMP	≤1	≤1	≤1	≤0,5	≤1	≤1	≤1
MEM	≤0,25	≤0,25	≤0,25	≤0,25	≤0,25	≤0,25	≤0,25
AK	4	4	16	≤0,25	16	≤2	≤2
GN	≥16	≥16	≥16	4	≥16	≥16	≤1
CIP	>32	>32	>32	0.38	0.094	0.047	0.006
LEV	≥8	≥8	≥8	1	0,5	0,5	≤0,12
TG	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5	≤0,5
SXT	≥320	≥320	≥320	≥320	≥320	≥320	≤20

**MICs of E.coli 34, E.coli 210 and their transconjugants, E.coli 4 (p4), E.coli 6 (p6) and E.coli J53 were indicated. AMP: Ampicillin, AMC: Amoxicillin–clavulanic acid, TZP: Piperacillin-tazobactam; CZ: Cefazolin, CXM: Cefuroxime, FOX: Cefoxitin, CAZ: Ceftazidime, CRO: Ceftriaxone, FEP: Cefepime, ETP: Ertapenem, IPM: Imipenem, MEM: Meropenem; AK: Amikacin; GN: Gentamicin;; CIP: Ciprofloxacin, LEV: Levofloxacin, TG: Tigecycline, SXT: CO-trimoxazole*

Limited data have been reported on the epidemiology of *E. coli* that produce CTX-M type enzymes in Turkey. Two recent reports from Turkey have shown that the CTX-M enzyme is common among ESBL positive isolates (86.8 %) at our hospital (IMF) in Istanbul [20] and from patients with urinary tract infections (76.5%) [21]. The latter finding of the predominance of CTX-M type enzymes according to TEM and SHV types is reflected in our study, as well. These reports suggest that CTX-M type enzymes are more prevalent than other ESBLs in Turkey. Consistently, a high prevalence of CTX-M type enzymes has been reported in several studies from other countries. In addition, the present study demonstrates that the prevalence of VEB type β-lactamase is low (1.6 %).

Over the past 10 years, PMQR has emerged as an important issue. Different rates of PMQR have been reported depending on the country of origin of the isolates [22]. *E. coli* carrying *qnrA*, *qnrB*, *qnrS,* and *aac(6')-Ib-cr*

genes have been previously reported in Turkey [8-10,23]. In the present study, the most prevalent PMQR determinant was *aac(6')-Ib-cr* (45.9%). A low prevalence was detected for the other PMQR genes (0 - 5.7 %). Many studies have demonstrated an association between TEM-SHV-CTX-M type β-lactamases and PMQR in *Enterobacteriaceae* [4].

In Turkey, as in previous reports, PMQR genes were mostly associated with TEM, SHV, and CTX-M type β-lactamases, such as the *qnrS1+aac(6')-Ib-cr -bla*CTX-M-15 positive *E. coli* 34 strain in this study. The association of *qnrA* with VEB-1 type β-lactamases was first investigated in a single *Enterobacter cloacae* isolate from France and in 11 out of 23 bla_{VEB-1} positive enterobacterial isolates from Thailand, by Poirel *et al* [16]. In addition, a *qnrA*-positive-*Citrobacter freundii* isolate that produces bla_{VEB-1} and bla_{OXA-48} has been reported in Turkey [8]. Here, in addition to the TEM and SHV type, a VEB type β-lactamase was detected, but distinct from that of a *qnrA*-positive *E. coli* isolate from the same hospital. This finding showed that VEB-1 type β-lactamase persists in microorganisms in Turkey. The present study also demonstrated the co-expression of the PMQR genes, similar to previous reports from France, UK, China, and Turkey [11,24-26].

The strain, *qepA*, was first identified in 2007 in two *E. coli* clinical isolates from Japan and Belgium [27], while a new variant (*qepA2*) has already been detected in France [28]. However, recently, a *qepA* producing *E. coli* strain possessing *qnrB2* and *aac(6')-Ib-cr* gene has been reported in Turkey [11]. In the present study, in addition to *qnrA1*, *qnrS1* and *aac(6')-Ib-cr*, *qepA1*-positive *E. coli* isolates that produce CTX-M-15 type β-lactamase were indentified. No *qepA* was found in the isolates screened in ITF, in contrast to other hospitals located in the Asian part of Istanbul. Although these PMQR mechanisms are rare, the association of *qepA1* with multi-drug resistant CTX-M-15 producing *E. coli* can be a cause for concern. There may be a rapid spread of *E. coli*, especially in hospital settings where various antimicrobials are largely used and thus may support the dissemination of these microorganisms.

CONCLUSION

This study documents the high prevalence *aac(6')-Ib-cr* and CTX-M type enzymes in Turkey. In addition to *aac(6')-Ib-cr* and CTX-M type enzymes, the *qepA1* and VEB-1 type enzymes are alarming for Turkey. Our study confirms that CTX-M producing *E. coli* isolates from urine specimens are highly resistant/co-resistant to norfloxacin, co-trimoxazole, amoxicillin-clavulanic acid, and gentamicin. Empiric therapy with these antibiotics may not be adequately effective. However, fosfomycin and nitrofurantoin resistance rates seem low and they may be alternatives for therapy. The emergence of the combination of PMQR and ESBL compromise the usage of valuable antibiotics worldwide. Antibiotic resistance is a public health problem, which requires continuous surveillance, monitoring, and revision of the policy of antibiotic use.

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