Eur J Clin Microbiol Infect Dis (2014) 33:837–843
DOI 10.1007/s10096-013-2022-6

ARTICLE

Fitness cost associated with resistance to fluoroquinolones is diverse across clones of *Klebsiella pneumoniae* and may select for CTX-M-15 type extended-spectrum β-lactamase

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Received: 8 October 2013 / Accepted: 15 November 2013 / Published online: 4 December 2013

Abstract Lowered fitness cost associated with resistance to fluoroquinolones was recently demonstrated to influence the clonal dynamics of methicillin-resistant *Staphylococcus aureus* (MRSA) in the health care setting. We investigated whether or not a similar mechanism impacts *Klebsiella pneumoniae*. The fitness of *K. pneumoniae* isolates from major international hospital clones (ST11, ST15, ST147) already showing high-level resistance to fluoroquinolones and of strains from three minor clones (ST25, ST274, ST1028) in which fluoroquinolone resistance was induced in vitro was tested in a propagation assay. Strains from major clones showed significantly less fitness cost than three of four fluoroquinolone-resistant derivatives of minor clone isolates. In addition, plasmids with CTX-M-15 type extended-spectrum β-lactamase (ESBL) genes were all retained in both major and minor clone isolates, irrespective of the strains' level of fluoroquinolone resistance, while each plasmid harboring SHV-type ESBLs had been lost during the induction of resistance. Major clone *K. pneumoniae* strains harbored more amino acid substitutions in the quinolone resistance determining regions (QRDRs) of the *gyrA* and *parC* genes than minor clone isolates. The presence of an active efflux system could be demonstrated in all fluoroquinolone-resistant derivatives of originally SHV-producing minor clone isolates but not in any CTX-M-15-producing strain. Further investigations are needed to expand and confirm our findings on a larger sample. In addition, a long-term observation of our ciprofloxacin-resistant minor clone isolates is required in order to elucidate whether or not they are capable of restoring their fitness while concomitantly retaining high minimum inhibitory concentration (MIC) values.

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Introduction

Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* turned into a nosocomial pathogen of utmost significance in recent decades. It has not only disseminated extensively in hospitals but also acquired a variety of antibiotic resistance mechanisms, which turned it into a formidable infectious agent [1–3]. With the advent of carbapenemase production, the treatment of infections caused by multidrug-resistant *K. pneumoniae* became a challenge, warranting the use of less than optimal, toxic antibiotics [4–9]. Thus, a better understanding of factors governing the dissemination of *K. pneumoniae* in the health care setting remains a salient issue for infection control.

It is well established that the spread of the ESBL- and carbapenemase-producing *K. pneumoniae* is clonal and some large international clonal complexes of the pathogen are

responsible for the majority of health care-associated infections in many geographical areas [10–23]. Interestingly, among the ESBLs, the production of a particular group—the CTX-M type enzymes—became predominant in *Klebsiella* spp. strains, which largely replaced the earlier SHV-type ESBLs during the last decade [10–12, 24, 25].

In Hungary, *K. pneumoniae* was polyclonal and carried almost exclusively the ESBL genes SHV-2a and SHV-5 prior to 2005 [26–28]. Then, within a few years, the epidemiology of the pathogen changed dramatically: three main clones emerged in adult hospital wards as the primary pathogens (ST11, ST15, ST147), producing the enzyme CTX-M-15 [12, 25]. Remarkably, *K. pneumoniae* isolates in neonatal intensive care units—where fluoroquinolone-type antibiotics were not in use—remained polyclonal and the strains retained the SHV-type ESBL enzymes [28].

Our recent observations suggested that lowered fitness cost associated with resistance to fluoroquinolones bears on the clonal dynamics of methicillin-resistant *Staphylococcus aureus* (MRSA) in the health care setting [29]. Here, we investigate whether or not a similar mechanism impacts *K. pneumoniae*.

Materials and methods

All *K. pneumoniae* strains were isolated by local laboratories in Hungary and sent for typing to the National Reference Laboratory at the National Center for Epidemiology. Strains were isolated from the following samples: blood ($n=6$); bronchoalveolar lavage (BAL) ($n=1$); throat ($n=1$) (Table 1).

The identification of the strains as *K. pneumoniae* was confirmed according to recommendations in the Manual of Clinical Microbiology [30] and by the Micronaut E system (Genzyme Virotech GmbH, Ruesselsheim, Germany).

Minimum inhibitory concentration (MIC) values for ciprofloxacin, ceftazidime, cefotaxime, gentamicin, tobramycin, amikacin, tigecycline, imipenem, meropenem, and ertapenem were determined by the gradient MIC test (Liofilchem, Roseto, Italy) and/or broth microdilution. Broth microdilution tests and evaluation of results were done in line with European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [31].

Multilocus sequence typing (MLST) with seven housekeeping genes was performed as described by Diancourt et al. [32]. Allele sequences and sequence types (STs) were identified as previously described [12].

The typing of plasmids and the detection and identification of ESBLs and carbapenemases was performed as previously described [12, 23].

Ciprofloxacin resistance in strains 5, 6, 7, and 8 was induced by exposing isolates to increasing concentrations of the antibiotic in brain heart infusion broth (BHI) (Oxoid, Basingstoke, UK). The ciprofloxacin-resistant mutants obtained were compared by pulsed-field gel electrophoresis (PFGE) with the original isolates. PFGE was performed in line with the standardized Centers for Disease Control and Prevention (CDC) protocol [33] using *Xba*I restriction enzyme (New England Biolabs, Ipswich, MA, USA). Gels were interpreted with Fingerprinting II Informatix Software (Bio-Rad Laboratories, Hercules, CA, USA). Levels of similarity were calculated with the Dice coefficient, and the unweighted pair group method with arithmetic averages (UPGMA) was used for the cluster analysis of the PFGE patterns.

Relative changes in the fitness of the bacterial strains were determined in propagation assays. In vitro planktonic growth rates were measured for the isogenic ciprofloxacin-sensitive and ciprofloxacin-resistant bacteria in monocultures. Briefly, 200 μL of a suspension of bacterial cultures was diluted to 0.5 McFarland (approx. 10^8 CFU/ml) in BHI (Oxoid, Basingstoke, UK). Cultures were then incubated at 37 °C without shaking in microtiter plates. Bacterial growth was measured after every 45 min by recording the absorbance at 595 nm using a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All strains were tested three times and the results averaged. The area under the curve (AUC) for the comparison of the growth rates was determined using MATLAB software. The assay was also performed in BHI containing 2 mg/L ciprofloxacin.

Strains in which ciprofloxacin resistance was artificially induced or raised (isolates 5/ind., 6/ind., 7/ind., and 8/ind.) were passaged in BHI (Oxoid, Basingstoke, UK) 15 times consecutively (incubation: 24 h; 37 °C). The passaged isolates were regularly plated onto blood agar media and the size of the colonies measured after overnight incubation.

Amino acid substitutions in the gyrase and topoisomerase IV enzymes were investigated by polymerase chain reaction (PCR) and sequencing of quinolone resistance determining regions (QRDRs) of *gyrA*, *gyrB*, *parC*, and *parE* genes. PCR reactions were performed with 1 U Taq DNA polymerase, 0.5 μM of each primer, 0.2 mM dNTP mix, 2.5 mM Buffer with Mg^{2+} (Sigma-Aldrich, St. Louis, MO, USA), and 200 ng DNA prepared from boiled colonies of each strain, in a total volume of 25 μL for PCR reaction. For *gyrA*, the forward primer 5′-CGCTTTTACTCCTTTTCTGTTC-3′ and the reverse primer 5′-CAGCCCTTCAATGCTGATG-3′ and for *parC*, the forward primer 5′-GTATTATCGCGGGTAGTTTT-3′ and the reverse primer 5′-TGTACGATATCCAGCAGTTC-3′ were used. The *gyrA* and *parC* sets of primers were designed using the tools of Eurofins MWG Operon. The thermal profile used was as follows for *gyrA*: initial denaturation 95 °C for 5 min, 30 cycles of 95 °C 1 min, 50 °C 1 min, 72 °C 1 min, and a final elongation at 72 °C for 7 min; for *parC*: initial denaturation 95 °C for 5 min, 35 cycles of 95 °C 1 min, 51 °C 1 min, 72 °C 1 min, and a final elongation at 72 °C for 7 min. For *gyrB* and *parE*, the forward and reverse

Table 1 Minimum inhibitory concentration (MIC) and area under the curve (AUC) values and genetic characterization of *Klebsiella pneumoniae* strains and their derivatives with induced resistance to ciprofloxacin

Strain	Specimen	ST	MIC (mg/L)										ESBL/ carbapanemase genes	PMQR/ efflux	Plasmid size (kb)[b]	Amino acid substitutions in QRDR regions[c]				AUC	
			CIP	CAZ	CTX	GEN	TOB	AMK	TGC	ETP	IMP	MEM				*gyrA*	*parC*	*gyrB*	*parE*	Without antibiotic	2 mg/L CIP
1/2005	blood	11	64	32	>256	64	32	4	0.25	0.064	0.5	0.064	$bla_{\text{CTX-M-15}}$	*aac(6')-lb-cr*	~50*	Ser83Phe; Asp87Ala	Ser80ILe	wt	wt	109.3508	89.4675
2/2009	BAL	11	64	32	64	>256	>256	16	0.5	2	2	0.5	$bla_{\text{CTX-M-15}}$; $bla_{\text{VIM-4}}$	*aac(6')-lb-cr*	~50* (CTX-M-15); ~90 (VIM-4)	Ser83Phe; Asp87Ala	Ser80ILe	wt	wt	100.9185	85.4175
3/2005	blood	15	256	64	>256	32	64	8	4	0.25	0.25	0.064	$bla_{\text{CTX-M-15}}$	*aac(6')-lb-cr*	~90**	Ser83Phe; Asp87Ala	Ser80ILe	wt	wt	113.19	75.93
4/2005	blood	147	64	128	>256	64	64	8	1	0.25	0.25	0.064	$bla_{\text{CTX-M-15}}$	*aac(6')-lb-cr*	~90	Ser83ILe	Ser80ILe	wt	wt	115.5607	85.86
5/1998	throat	25	0.064	8	8	64	8	2	2	0.064	0.5	0.064	$bla_{\text{SHV-2a}}$	–[a]	~90***	wt	wt	wt	wt	117.3083	32.04
5/ind.			16	0.25	0.25	1	1	2	0.25	0.064	0.25	0.032	ESBL-negative	efflux		wt	wt	Ser431Pro	wt	40.9950	48.06
6/2003	blood	1028	0.125	128	16	4	16	8	4	0.25	0.25	0.064	$bla_{\text{SHV-5}}$	–	~90	wt	wt	wt	wt	118.1453	48.7987
6/ind.			4	0.5	0.5	0.5	1	1	2	0.125	0.125	0.032	ESBL-negative	efflux		wt	wt	wt	wt	61.0665	52.8
7/2006	blood	274	2	128	256	128	128	32	1	0.25	0.5	0.064	$bla_{\text{CTX-M-15}}$	*aac(6')-lb-cr*	~90	Ser83Tyr	wt	wt	wt	106.5383	66.2625
7/ind.			64	16	64	128	64	16	1	0.125	0.25	0.064	$bla_{\text{CTX-M-15}}$	*aac(6')-lb-cr*	~90**	Ser83Tyr	wt	wt	wt	40.041	37.5375
8/2008	blood	274	0.5	8	8	128	32	2	1	0.064	0.5	0.064	$bla_{\text{SHV-2a}}$	–	~90***	Ser83Tyr	wt	wt	wt	126.3143	54.585
8/ind.			32	0.5	0.5	128	16	2	1	0.064	0.25	0.032	ESBL-negative	efflux		Ser83Tyr	wt	wt	wt	97.0823	63.015

BAL bronchoalveolar lavage; *ST* sequence type; *CIP* ciprofloxacin; *CAZ* ceftazidime; *CTX* cefotaxime; *GEN* gentamicin; *TOB* tobramycin; *AMK* amikacin; *TGC* tigecycline; *ETP* ertapenem; *IMP* imipenem; *MEM* meropenem; *PMQR* plasmid-mediated quinolone resistance; *AUC* area under the curve; *QRDR* quinolone resistance determining region; *wt* wild-type

[a] Negative for PMQR determinants *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *aac(6')-Ib-cr*, *qepA*, *oqxAB*, and efflux activity

[b] ESBL or carbapenemase gene harboring plasmids published previously [12, 21, 27]; *, **, ***: denoting plasmids identical or differing in a single band after digestion with *PstI* enzyme

[c] Sequences were compared to the full genome sequence of *K. pneumoniae* 1084 strain (accession number CP003785)

primers described by Nam et al. [34] were used with conditions as reported by them.

The presence of plasmid-mediated quinolone resistance determinants was investigated by PCR. Multiplex PCR was carried out for *qnrA*, *qnrB*, and *qnrS* using already published primers and thermal profiles [35, 36]. Separate PCRs were performed for *qnrC* and *qnrD* [37, 38]. The presence of *aac(6')-Ib-cr*, *qepA*, and *oqxAB* determinants was also tested [39–41].

All the isolates were tested for the presence of an active efflux pump conferring resistance to fluoroquinolones. The ciprofloxacin MIC values were determined on all the strains by the broth microdilution method in the absence and presence of 20 μg Phe-Arg-β-naphthylamide (Sigma-Aldrich, St. Louis, MO, USA). Major clone isolates (strains 1, 2, 3, and 4) were incubated in BHI (Oxoid, Basingstoke, UK) with 8 mg/L ciprofloxacin for 24 h prior to testing the efflux activity. Minor clone isolates were tested both prior and subsequent to the induction of resistance to ciprofloxacin.

Results

The derivatives of minor clone *K. pneumoniae* strains—in which resistance to ciprofloxacin was induced in vitro—proved indistinguishable by PFGE from the original isolates (data not shown).

All findings obtained with the original isolates and those obtained with induced resistance to ciprofloxacin are shown in Table 1.

All *K. pneumoniae* strains from major international clones having ab ovo high MIC values to ciprofloxacin (strains 1, 2, 3, and 4) displayed good fitness (Table 1). In contrast, three of the four ciprofloxacin-resistant derivatives of minor clone strains suffered large fitness costs during the induction of fluoroquinolone resistance (strains 5/ind., 6/ind., and 7/ind.), even though in two of the strains (strains 5/ind. and 6/ind.), the ciprofloxacin MIC values attained during induction were significantly lower than those for the major clone isolates (Table 1). The ciprofloxacin-resistant derivative of the SHV-2a-producing minor clone strain (strain 8/ind.), despite developing a high MIC value (64 mg/L), suffered less fitness cost than the derivatives of other minor clone isolates (strains 5/ind., 6/ind., and 7/ind.) (Table 1)

In the propagation assay with 2 mg/L ciprofloxacin, the major clone strains (strains 1, 2, 3, and 4) retained their superior growth rates relative to the induced minor clone isolates (Table 1). Minor clone strain 7 commanding ab ovo an MIC value of 2 mg/L to ciprofloxacin grew significantly faster than its derivative with an MIC of 64 mg/L. Due to elevated bacterial counts in the test medium relative to that used to determine the MIC values, our ciprofloxacin-susceptible original *K. pneumoniae* isolates also showed some growth during the time period of the propagation assay.

Attempts were made to restore fitness lost. All minor clone strains in which ciprofloxacin resistance was artificially induced or the level of resistance raised and suffered fitness cost (strains 5/ind., 6/ind., 7/ind., and 8/ind.) were passaged 15 times in antibiotic-free BHI medium. In strain 6/ind., some restoration of fitness was probable (data not shown); however, the strain displayed strong heterogeneity, thus, the exact characterization of the status of its extant fitness and long-term evolvement needs further investigation. No restoration of fitness in strains 5/ind., 7/ind., and 8/ind. was evident during the testing period.

While $bla_{\text{CTX-M-15}}$ gene-carrying plasmids were retained in all *K. pneumoniae* strains tested irrespective of the MIC values to fluoroquinolones (strains 1, 2, 3, 4, 7, and 7/ind.), each minor clone isolate producing SHV-2a or SHV-5 (strains 5, 6, and 8) lost their plasmids carrying the ESBL genes when their MIC values to ciprofloxacin were raised in vitro (strains 5/ind., 6/ind., and 8/ind.). Among the two ST274 minor clone isolates carrying the SHV-2a and CTX-M-15 types of ESBL genes, respectively (strains 7 and 8), the $bla_{\text{CTX-M-15}}$ gene was retained (strain 7/ind.), while the $bla_{\text{SHV-2a}}$ gene was lost during the course of induction (strain 8/ind.) (Table 1).

All the major clone strains (strains 1, 2, 3, and 4) and both ST274 isolates (strains 7 and 8) showed amino acid substitutions in codon 83 of the *gyrA* gene. In addition, the two ST11 isolates and the ST15 strain each harbored an Asp87Ala *gyrA* amino acid substitution. Furthermore, a Ser80ILe substitution in the *parC* gene could be detected in all of the major clone isolates (strains 1, 2, 3, and 4). In contrast, none of the minor clone strains developed mutations in the *parC* gene during the induction of resistance to ciprofloxacin (strains 5/ind., 6/ind., 7/ind., and 8/ind.) (Table 1). An Ser431Pro substitution was demonstrated in the *gyrB* gene of the ST25 *K. pneumoniae* isolate with induced ciprofloxacin resistance (strain 5/ind.). In general, all major clone isolates developed more amino acid substitutions in the investigated QRDRs than minor clone strains. No amino acid substitution in the *parE* gene could be detected in any of the strains tested (Table 1).

The presence of an active efflux system could be demonstrated in all fluoroquinolone-resistant derivatives of the originally SHV-producing minor clone isolates (strains 5/ind., 6/ind., and 8/ind.), but not in any CTX-M-15-producing strain (stains 1, 2, 3, 4, and 7/ind.) (Table 1). Major clone strains (strains 1, 2, 3, and 4) and strain 7/ind. failed to show efflux activity, even after incubation in BHI with 8 mg/L ciprofloxacin overnight.

Mobile fluoroquinolone resistance determinants *qnrA*, *qnrB*, *qnrS*, *qepA*, and *oqxAB* could not be detected in any of our strains. However, all the major clone strains (strains 1, 2, 3, and 4) and the CTX-M-15-producing ST274 isolate

(strain 7) and its derivative (strain 7/ind.) harbored *aac(6')-Ib-cr* (Table 1).

Discussion

Our investigation suggests that fitness cost associated with resistance to fluoroquinolones is diverse across clones of *K. pneumoniae* in both antibiotic-free medium and in BHI with 2 mg/L ciprofloxacin, and may select for the CTX-M-15 type ESBL. Nevertheless, the number of strains tested by us is small and to expand and confirm our results, the tests should be repeated on a larger sample. In addition, the capacity of induced minor clone strains to regain fitness in the long run and to acquire novel ESBL genes should be investigated.

One of our minor clone strains (strain 8/ind.) suffered much less fitness cost than the other minor clone isolates during the induction of resistance to fluoroquinolones (Table 1), hinting that this isolate may have the potential to develop/acquire characteristics equaling those of major clone strains in the long term.

The ability of minor clone strain 7 having an MIC value for ciprofloxacin just equaling that of the testing medium (2 mg/L) to grow faster than its induced derivative with a much higher MIC for the drug (64 mg/L) (7/ind.) suggests that a strong link between fluoroquinolone resistance and fitness exists in this isolate.

Furthermore, our findings hint that the impact of plasmids carrying various types of ESBL genes may be distinct on the fitness of fluoroquinolone-resistant *K. pneumoniae*. The plasmids carrying $bla_{\text{CTX-M-15}}$ were not (or could not be) eliminated; nevertheless, this did not seem to be associated with great fitness costs in the four major clone *K. pneumoniae* strains tested (strains 1, 2, 3, and 4). Conversely, plasmids harboring SHV-5 and SHV-2a type ESBLs were disposed of during the induction of fluoroquinolone resistance from the three minor clone isolates (strains 5/ind., 6/ind., and 8/ind.), strongly suggesting that these enzymes—or genes associated with them—proved a liability for the strains.

However, our results also indicate that the effect of a particular $bla_{\text{CTX-M-15}}$-carrying plasmid on the fitness of fluoroquinolone-resistant *K. pneumoniae* may be ST specific. The same plasmid carrying the $bla_{\text{CTX-M-15}}$ gene which failed to substantially compromise the fitness of the ST15 major clone strain (strain 3; Table 1) seems to have dramatically impaired the vitality of the fluoroquinolone-resistant derivative of the ST274 CTX-M-15-producing isolate (strain 7/ind.; Table 1), because another fluoroquinolone-resistant isolate from the same ST void of any ESBL-carrying plasmids (strain 8/ind.) showed significantly faster growth rate (Table 1). Though the $bla_{\text{CTX-M-15}}$-carrying plasmid clearly compromised the vitality of strain 7/ind., the isolate—for some reason unknown—proved unable to eliminate it.

It remains to be elucidated whether or not the presence of the plasmid carrying the $bla_{\text{VIM-4}}$ gene was, somehow, responsible for the slightly compromised growth rate of the ST11 metallo-β-lactamase-producing isolate (strain 2) relative to other major clone strains (Table 1) and how the fitness of *K. pneumoniae* strains producing various types of carbapenemases relate to those of isolates from major ESBL-producing clones in general.

The amino acid substitutions detected by us in the QRDRs of the *gyrA* and *parC* genes are all well-known genetic alterations associated with resistance to fluoroquinolones [34, 42–45], while the Ser431Pro substitution in the *gyrB* gene seems to be rare: it has exclusively been observed in a fluoroquinolone-resistant *Coxiella burnetii* strain [46]. Interestingly, the major clone *K. pneumoniae* strains (strains 1, 2, 3, and 4) harbored more amino acid substitutions in the *gyrA* and *parC* genes than the fluoroquinolone-resistant derivatives of minor clone isolates (strains 5/ind., 6/ind., 7/ind., and 8/ind.) (Table 1).

Intriguingly, among our *K. pneumoniae* strains, an active efflux system could be detected exclusively in ciprofloxacin-induced minor clone isolates void of $bla_{\text{CTX-M-15}}$- and *aac(6')-Ib-cr*-carrying plasmids, which raises the prospect of a possible relationship. To our knowledge, there is no report in the literature investigating the prevalence of active efflux in the context of clonal affiliation and/or the type of ESBL produced in *K. pneumoniae*. The sole paper studying a multitude of strains [47] reported a very high frequency of active efflux in *K. pneumoniae* without disclosing clonal classification. However, since the strains investigated were isolated between 1998 and 2002 [47], thus, prior to the advent of CTX-M-15 and the dissemination of the major international *K. pneumoniae* clones, they could have been largely SHV-type ESBL-producing minor clone strains. Consequently, whether or not a relationship between active efflux and $bla_{\text{CTX-M-15}}$- and/or *aac(6')-Ib-cr*-carrying plasmids exists remains to be determined on a larger sample.

These findings are in agreement with the observation of Marcusson et al. [48], who demonstrated that, while genetic alterations enhancing the activity of efflux result in large fitness cost, the combined presence of the three amino acid substitutions observed by us in the *gyrA* and *parC* genes in three of our four major clone isolates (strains 1, 2, and 3) (Table 1) was not associated with any loss of vitality in *Escherichia coli*. In addition, they also showed that the fitness cost conferred by an active efflux can partly be compensated for by the introduction of multiple amino acid substitutions in the *gyrA* and *parC* genes in genetically engineered *E. coli* strains [48].

The genetic background accounting for the diverse capacity of our *K. pneumoniae* clones to develop mutations in the *gyrA* and *parC* genes remains to be elucidated and would require the whole genome sequencing of multiple strains.

Acknowledgment We thank Alexander Friedrich for his helpful comments and useful discussion.

Conflict of interest The authors declare that they have no conflict of interest.

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