IN VIVO EVOLUTION AND EMERGENCE OF A NEW CTX-M β-LACTAMASE WITH «CEFTAZIDIMASE» ACTIVITY IN A HYPERMUTABLE CLINICAL STRAIN

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INTRODUCTION AND OBJECTIVES

The family of CTX-M β-lactamases is rapidly growing and currently includes at least 50 enzymes. CTX-Ms generally have higher activity against cefotaxime (CTX) than against ceftazidime (CAZ). Recently, twelve CTX-M variants have been reported to contain point mutations at positions 167 or 240 associated with increased CAZ hydrolyzing activity. These include six enzymes of the CTX-M-1 cluster: CTX-M-15, -23, -28, -29, -32, -33; two enzymes of the CTX-M-2 cluster: CTX-M-35, -43; three enzymes of the CTX-M-9 cluster: CTX-M-16, -19, -27; and CTX-M-25. The CTX-Ms containing mutations at position 167 have been shown to confer higher levels of resistance to CAZ than to CTX, and thus could be defined as "ceftazidimases". [4, 5, 6].

During a survey of ESBL producers, an *E. coli* clinical isolate (Irk2320) was identified that produced a single CTX-M β-lactamase and expressed an unusual resistance phenotype being moderately resistant to cefotaxime (MIC 8 mg/L) but highly resistant to ceftazidime (MIC 128 mg/L). Notably, this isolate was obtained from a patient who had been treated with CAZ. The second *E. coli* isolate (Irk2322) with identical phenotype of resistance was isolated from another patient in the same ward one month later.

In this study we aimed to investigate whether resistance to CAZ arose in vivo as a result of mutation(s) in a CTX-M β-lactamase.

METHODS

Bacterial strains. Isolates Irk2320 and Irk2322 were included in this study along with 19 nosocomial *E. coli* strains that were earlier isolated in the same ward and were found to produce a single CTX-M ESBL conferring low-level resistance to CAZ.

Susceptibility testing. MICs of ampicillin (AMP), amoxicillin-clavulanate (AM-C, 2:1), CTX, CAZ, ceftriaxone (CTR), cefepime (FEP), cefotaxime-clavulanate (CTX-C, 4 mg/L fixed clavulanate concentration) and ceftazidime-clavulanate (CAZ-C, 4 mg/L fixed clavulanate concentration) were determined using agar dilution method according to the NCCLS (2005) guidelines.

Determination of spontaneous mutation rate. The rates of spontaneous mutations to rifampin resistance were determined as described elsewhere [3].

Resistance transfer by conjugation. The Irk2320 was mated in broth with *E. coli* AB1456 (Rif^R) to determine transferability of CTX-M-coding plasmid. The transconjugants were selected on plates containing rifampin (100 mg/L) and cefotaxime (1 mg/L).

Typing by arbitrarily primed PCR (AP-PCR). The clonality between Irk2320, Irk2322 and 19 CTX-M producing strains with low-level CAZ resistance was assessed by AP-PCR with primers ERIC1, AP7, OPA4, OPB17 and M13 as described previously [1, 2]. Cluster analysis of AP-PCR profiles was done with the GelCompar software v.4.1 (Applied Maths, Sint-Martens-Latem, Belgium) using the Pearson correlation coefficient and unweighted pair group method using arithmetic averages (UPGMA) algorithm.

Amplification, cloning and sequencing of bla_{CTX-M} genes. DNA fragments containing the entire bla_{CTX-M} gene and the upstream part of ISEcp1 including the putative promoter region were amplified by PCR with primers 5'-TGTCTGGTATAATAAGAATATCATC-3' and 5'-CTATTACAAACCGTCGGTGAC-3' from strains Irk2320 and Irk1224, ligated into pCC1 vector and cloned in *E. coli* EPI300 (EPICENTRE, Madison, WI).

Plasmids isolated from the respective transformants were used as templates in sequencing reactions with M13 and bla_{CTX-M}-internal primers. Sequencing was performed using an ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Stafford, TX).

RESULTS AND DISCUSSION

In mating experiments, the resistance to CAZ was transferred from Irk2320 to the recipient *E. coli* strain at a frequency of 7E-03. The transconjugants carried a CTX-M ESBL and resembled the original strain in resistance pattern to β-lactams (data not shown). Direct sequencing of the internal bla_{CTX-M} gene fragment amplified by PCR from the transconjugant and clinical isolates Irk2320 and Irk2322 revealed the presence of a single point mutation leading to a P167T change in the deduced aa sequence.

Noteworthy, the Irk2320 had elevated rate of spontaneous mutations to rifampin resistance (~1E-5) and was therefore suggested as a hypermutable strain.

Based on these initial findings, we hypothesized that the Irk2320 could have acquired de novo the P167T mutation in its bla_{CTX-M} gene. In order to verify this hypothesis and to identify the possible progenitor strain of Irk2320 lacking the mutation in bla_{CTX-M} gene, we determined clonality between the Irk2320, Irk2322 and 19 CTX-M producing *E. coli* strains with low-level CAZ resistance that were isolated in the same ICU up to eight months before the isolation of Irk2320. According to the results of cluster analysis of AP-PCR profiles (Fig. 1), the strain Irk1224 was identified as a most likely progenitor of Irk2320 and Irk2322 that shared identical banding patterns.

The sequence of the cloned 1225bp DNA fragment from Irk1224 was completely identical to the previously published sequence of $bla_{CTX-M-3}$ and the 5' adjacent part of ISEcp1 (GenBank Acc. No. AF550415) while the same fragment from Irk2320 contained a single nucleotide substitution (deposited in GenBank under Acc. No. DQ061159). The respective β-lactamase differing from CTX-M-3 at position 167 was designated CTX-M-42 (Fig. 2).

When expressed in the isogenic background in *E. coli* EPI300, the CTX-M-3 and CTX-M-42 displayed the "cefotaximase" and "ceftazidimase" phenotypes, respectively (Tab. 1), thus clearly supporting the involvement of T167 in CAZ hydrolysis.The P167T substitution was previously detected in CTX-M-23 which is derived from CTX-M-1 [5]. Our study for the first time provides an example of convergent evolution of CTX-M-3 towards the acquisition of CAZ-hydrolyzing activity. The in vivo acquisition of P167T mutation in CTX-M-42 is strongly supported by identification of the clonally and epidemiologically related strains producing the CTX-M-3 and CTX-M-42. It seems likely, although not proven, that this mutation arose in a hypermutable *E. coli* strain and was selected by CAZ treatment.

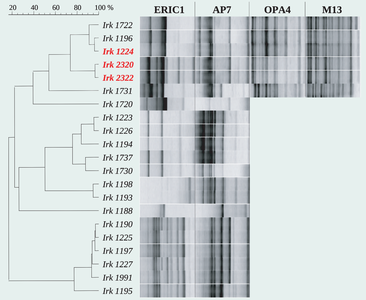

Figure 1. UPGMA clustering of combined AP-PCR profiles of CTX-M-producing *E. coli* strains.

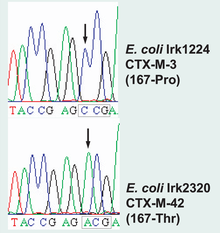

Figure 2. Chromatogram showing nucleotide changes at codon 167 (Ambler's numbering) of bla_{CTX-M}

Table 1. Characteristics of *E. coli* clinical isolates and transformants.

Strain	Date of isolation	MICs of β-lactams, mg/L								β-lactamase
		AMP	AM-C	CTX	CTX-C	CAZ	CAZ-C	CTR	FEP	
E. coli Irk1224	March, 16 - 2003	≥256	32	≥256	2	32	4	≥256	32	CTX-M-3
E. coli Irk2320	May, 23 - 2003	≥256	16	8	0.125	128	2	8	1	CTX-M-42
E. coli Irk2322	June, 3 - 2003	≥256	16	8	0.125	128	2	8	1	CTX-M-42
E. coli EPI300 pCC1-1224		≥256	8	4	0.06	0.5	0.25	4	0.5	CTX-M-3
E. coli EPI300 pCC1-2320		≥256	8	0.5	0.06	32	1	1	0.25	CTX-M-42
E. coli EPI300 pCC1		2	2	0.06	0.06	0.25	0.25	0.06	0.06	none

CONCLUSIONS

A new CTX-M variant, CTX-M-42, was described that differs from CTX-M-3 by a single point mutation (P167T) and confers higher level of resistance to CAZ than to CTX.

An in vivo evolution of a CTX-M β-lactamase towards the acquisition of a "ceftazidimase" activity was demonstrated.

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