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Identification of the Naturally Occurring Variant Genes *bla*$_{TEM-1d}$ and *bla*$_{TEM-70}$ Encoding Broad-Spectrum TEM-Type β-lactamases

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BACKGROUND AND OBJECTIVES

In recent years, there have been numerous reports describing the sequences of the genes for extended-spectrum and inhibitor resistant β-lactamases which belong to the TEM-family. At the same time genetic diversity of the naturally occurring parental genes encoding TEM-type penicillinases was studied to a lesser extent. Sequences of the genes *bla*$_{TEM-1a}$, *bla*$_{TEM-1b}$ and *bla*$_{TEM-2}$ carried by transposons Tn*3*, Tn*2* and Tn*1* were determined in the middle 1980-s, and since then it has been commonly accepted that other members of the TEM-group evolved from three distinct genetic lineages. More recently, a TEM-55 penicillinase with an altered isoelectric point (pI 5.2) was found in a single clinical isolate of *E.coli* using the isoelectric focusing (IEF) method, and in 1999 the third allelic variant encoding TEM-1 β-lactamase was described that received a designation *bla*$_{TEM-1c}$.

We have used a recently developed Polymerase Chain Reaction - Single Strand Conformational Polymorphism (PCR-SSCP) method to screen for mutational events which account for the sequence diversity of parental *bla*$_{TEM}$ genes in unrelated uropathogenic strains of *E.coli*. We have also determined the sequences of the variant genes and characterised the properties of mutant β-lactamases.

METHODS

Selection of bacterial strains: The *E.coli* strains used in this study were collected from outpatients with UTI in four medical centers located in 3 different cities of Russia: Moscow (2 centers), Novosibirsk (1 center) and Smolensk (1 center) in 1998. Forty five ampicillin-resistant isolates equally representing each of the 4 participating medical centers were analysed by ERIC-PCR. For 30 strains representing the distinct molecular types the presence of a TEM β-lactamase was determined by isoelectric focusing and confirmed by PCR-amplification of the respective *bla*$_{TEM}$ gene. The amplified sequences were screened for the presence of mutations using the PCR-SSCP analysis. Mutations contributing to an unusual SSCP profile or an altered pI value of a β-lactamase were studied by direct sequencing of a PCR product.

Susceptibility testing: Susceptibility of *E.coli* isolates to ampicillin, gentamicin, nitrofurantoin, trimethoprim/sulphamethoxazole, nalidixic acid and ciprofloxacin was determined by standard agar dilution method on Mueller-Hinton agar (Becton Dickinson, USA) and interpreted according to the current NCCLS standards. The MICs of ticarcillin, piperacillin, cefuroxime, cefoperazone, ceftazidime, cefotaxime, cefoxitin, aztreonam and combinations of penicillins with clavulanic acid and tazobactam were additionally determined for the clinical strains and their transconjugants producing β-lactamase TEM-70. *E.coli* strains ATCC25922 and ATCC35218 were used for quality control.

Typing by ERIC-PCR: Template DNA was extracted from 3-4 colonies of each strain grown overnight on MacConkey agar using the InstaGene matrix (BioRad, USA). Reaction mixes were set up by adding 2μl of 25μM primer ERIC1 (5'-GTGAATCCCCAGGAGCTTACAT-3'), 2μl of template DNA and 21μl of autoclaved Milli-Q water to the 0.5ml tubes containing premixed and predispensed PCR reagents (Ready-To-Go PCR Beads; Amersham Pharmacia Biotech (APB), USA). The PCR was carried out in a PTC-200 thermocycler (MJ Research, USA) under the following conditions: initial denaturation at 94°C for 3 min followed by 35 cycles of annealing at 47°C for 1 min, elongation at 72°C for 1 min and denaturation at 94°C for 30 sec, with a final elongation step extended to 4 min. After electrophoresis in 1.3% agarose gel DNA fragments were stained with ethidium bromide and documented using a PhotoDoc-IT Link Gel Documentation System (UVP, USA). Cluster analysis of ERIC-PCR fingerprints was done using a GelCompar software (Applied Maths BVBA, Belgium) by the UPGMA method applied to densitometric tracks.

Isoelectric focusing: Crude sonic extracts containing β-lactamases were examined using a PhastSystem apparatus and precast IEF 5-8 pI gels (APB, USA) as previously described, and β-lactamases with known pIs (TEM-37 (pI 5.2), TEM-1 (pI 5.4), TEM-2 (pI 5.6) and TEM-3 (pI 6.3)) were used as standards.

Amplification of *bla*$_{TEM}$ genes and SSCP-analysis: A pair of primers (A: 5'-ATAAAATTCTTGAAGACGAAA-3' and B: 5'-GACAGTTACCAATGCTTAATCA-3') was used to amplify a 1080-bp fragment that covers the entire *bla*$_{TEM}$ gene sequence. The PCR mixture contained: 12.5mM Tris-HCl (pH 8.3), 62.5mM KCl, 2mM $MgCl_2$, 200μM of each dNTP, 0.25μM of each primer, 1.25U AmpliTaq DNA polymerase (Perkin-Elmer, USA) and 20μl of template DNA in a total volume of 50μl. The PCR was carried out in a PTC-200 thermocycler (MJ Research, USA) under the following protocol: initial denaturation at 94°C for 2 min followed by 35 cycles of annealing at 54°C for 10 sec, elongation at 72°C for 45 sec and denaturation at 94°C for 10 sec, with a final elongation step at 72°C for 3 min. 20μl of amplified DNA was digested with either 1U *Taq* I and 10U *Pst* I or 1U *Taq* I and 4U *Ava* II restriction endonucleases (APB, USA) for 2 h at 37°C and 1 h at 65°C. Restriction fragments were then denatured to single-stranded (ssDNA) form by by mixing 2μl of digest with double volume of SSCP buffer (98% formamide, 2% glycerol, 0.05% bromphenol blue, 10mM EDTA) and heating the mixture at 98°C for 10 min with subsequent cooling at 0°C in a thermocycler. ssDNA fragments were separated on a PhastSystem (APB, Sweden) using a PhastGel Homogeneous 12.5 system with PhastGel Native Buffer Strips. The program had three steps as follows: 1) Pre-run step at 400V, 5mA, 2W, 15°C for 70Vh; 2) Sample loading step at 400V, 1mA, 2W, 15°C for 2Vh; 3) Separation step at 400V, 5mA, 2W, 15°C for 350Vh in the case of *Taq* I - *Pst* I digests or for 230Vh in the case of *Taq* I - *Ava* II digests. The gels were stained with a PhastGel DNA Silver Staining Kit (Pharmacia Biotech, Sweden) as recommended by manufacturer.

DNA sequencing: Sequencing of the mutant *bla*$_{TEM}$ genes was performed on 1080-bp PCR-products using a dideoxy-chain termination method with a Cycle Reader DNA Sequencing Kit (Fermentas, Latvia), with primers A, B and additional internal primers (5'-ATTCTCAGAATGACTTGGTTGAG-3' and 5'-TTACTGTCATGCCATCCGTAAG-3').

Kinetic Analysis of b-lactamases: The genes coding for β-lactamase TEM-70 in clinical isolates NS13 and SM91 were transferred by conjugation and expressed in *E.coli* C600. Kinetic parameters of TEM-70 were determined by using partially purified extracts obtained from transconjugants and compared with those of TEM-1 (*E.coli* C600, *bla*$_{TEM-1b}$). The complete time course of hydrolysis of benzylpenicillin, ampicillin, ticarcillin, cephalothin and cefoperazone was recorded with an Ultrospec 3000 spectrophotometer (APB, Sweden) with two different starting concentrations of each substrate. The K_m and V_{max} values were calculated using the results 3 independent measurements fitted to the Hanes plot.

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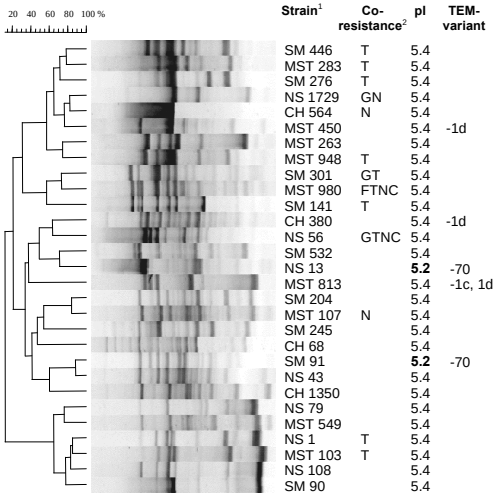
RESULTS



Strain[1]	Co-resistance[2]	pI	TEM-variant
SM 446	T	5.4	
MST 283	T	5.4	
SM 276	T	5.4	
NS 1729	GN	5.4	
CH 564	N	5.4	
MST 450		5.4	-1d
MST 263		5.4	
MST 948	T	5.4	
SM 301	GT	5.4	
MST 980	FTNC	5.4	
SM 141	T	5.4	
CH 380		5.4	-1d
NS 56	GTNC	5.4	
SM 532		5.4	
NS 13		**5.2**	-70
MST 813		5.4	-1c, 1d
SM 204		5.4	
MST 107	N	5.4	
SM 245		5.4	
CH 68		5.4	
SM 91		**5.2**	-70
NS 43		5.4	
CH 1350		5.4	
NS 79		5.4	
MST 549		5.4	
NS 1	T	5.4	
MST 103	T	5.4	
NS 108		5.4	
SM 90		5.4	

Figure 1: UPGMA clustering of ERIC-PCR fingerprints showing the distribution of mutant *bla*$_{TEM}$ genes in unrelated *E.coli* strains.
[1] Letters are used to encode the medical center from which the isolate was obtained.
[2] Co-resistance is defined as an MIC in the resistant range by NCCLS criteria.
G - gentamycin, F - nitrofurantoin, T - trimethoprim/sulphamethoxazole, N - nalidixic acid, C - ciprofloxacin.

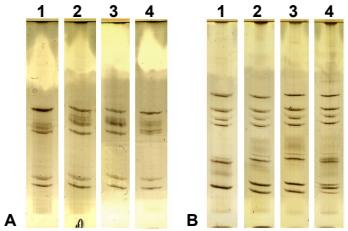


Figure 2: Selected SSCP profiles of *bla*$_{TEM}$ genes obtained with the combinations of restriction endonucleases *Taq* I - *Pst* I (**A**) and *Taq* I - *Ava* II (**B**).
Lane 1: Standard profile of *bla*$_{TEM-1a}$; Lanes 2-4: SSCP profiles corresponding to the clinical isolates *E.coli* MST450, *E.coli* CH380 and *E.coli* MST813, respectively.

According to IEF, 28 isolates produced a single β-lactamase with pI 5.4, corresponding to a TEM-1 enzyme. Two strains (SM91 and NS13) isolated in different cities expressed a β-lactamase with pI 5.2 (Fig.1). DNA sequencing revealed that the *bla*$_{TEM}$ genes of the last-named isolates were identical and differed from *bla*$_{TEM-1b}$ by a single G_{813}→A transition leading to a substitution Arg_{204}→Gln in the deduced amino acid sequence. Until now this mutation has not been described in TEM β-lactamases and the new enzyme containing a glutamine residue at position 204 received a designation TEM-70 (Tab.1). Comparative susceptibility testing of *E.coli* strains producing β-lactamases TEM-1 and TEM-70 has demonstrated that the MICs of different β-lactams, including ceftazidime, cefotaxime and inhibitor-protected penicillins, were not affected by mutation, although, the MIC of cefoperazone for *E.coli* C600 encoding TEM-70 was slightly high (2 μg/ml) in comparison with that of the same strain producing TEM-1 (0.5 μg/ml). The K_m and relative V_{max} values were very similar for both enzymes except for the K_m value of cefoperazone (143 for TEM-70 and 205 for TEM-1).

Another variant gene was identified by PCR-SSCP analysis in 3 unrelated strains (CH380, MST450 and MST813) producing a β-lactamase with pI 5.4 (Fig. 1, 2). As determined by sequencing, the gene was associated with P3 promotor and resembled *bla*$_{TEM-1a}$ at nucleotides 175, 226, 317 but contained 4 silent mutations at positions 346, 436, 682 and 925 which were previously found in *bla*$_{TEM-2}$ (Tab. 1). The new gene, therefore, encodes a TEM-1 penicillinase and could be designated *bla*$_{TEM-1d}$. It may be proposed, that *bla*$_{TEM-1d}$ could result from recombination of *bla*$_{TEM-1a}$ and *bla*$_{TEM-2}$ between positions 317 and 346. Interestingly, the SSCP profiles and sequencing data suggested that MST813 strain carried two genes (*bla*$_{TEM-1c}$ and *bla*$_{TEM-1d}$) encoding TEM-1 penicillinase.

The nucleotide sequence data for *bla*$_{TEM-70}$ and *bla*$_{TEM-1d}$ have been submitted to the GenBank under accession no. AF188199 and AF188200, respectively.

Table 1: Substitutions in *bla*$_{TEM}$ genes and derived penicillinases.

Nucleotide no.[1]	32	175	226	317	346	436	604	682	813	925	pI
Amino acid substitution[2]				Q_{39}-K					R_{204}-Q		
bla$_{TEM-1a}$	C	A	C	C	A	C	G	T	G	G	5.4
bla$_{TEM-1b}$		G	T			T	T				5.4
bla$_{TEM-1c}$						T					5.4
bla$_{TEM-2}$	T			A	G	T		C		A	5.6
bla$_{TEM-1d}$[3]					G	T		C		A	5.4
bla$_{TEM-70}$[3]		G	T			T	T		A		5.2

[1] **Numbering is according to Sutcliffe (1978).**
[2] **Numbering is according to Ambler et al. (1991). Q - glutamine; K - lysine; R - arginine.**
[3] **Genes identified in this study**.
pI - isoelectric point of a corresponding β-lactamase.

CONCLUSIONS

Although, the sequences coding for TEM penicillinases were generally conserved among the *E.coli* strains used in this study, new mutant genes have been identified and even more variants may be expected in future if the appropriate genetic methods, like PSR-SSCP, will be used to screen for mutations. The identification of new parental *bla*$_{TEM}$ genes may provide further insight into the understanding of heterogeneity and evolution of TEM-type β-lactamases.