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A MALDI TOF MS-based minisequencing method for rapid detection of TEM-type extended-spectrum beta-lactamases in clinical strains of Enterobacteriaceae

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ABSTRACT

A minisequencing method based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) was developed for rapid identification of single nucleotide polymorphisms at bla_{TEM} gene codons 104, 164 and 238 associated with extended-spectrum activity on TEM-type beta-lactamases. The method was validated by testing the *Escherichia coli* and *Klebsiella pneumoniae* strains possessing the known bla_{TEM} gene sequences.

1. Introduction

Gram-negative bacteria of the Enterobacteriaceae family are important causes of urinary tract infections, bloodstream infections, hospital- and healthcare-associated pneumonias, and various intra-abdominal infections. They have become increasingly resistant to antibiotics over the past 2 decades due to selective pressure imposed by the extensive use of antibiotics in the hospitals and community (Gootz, 2006). One of the main resistance mechanisms identified so far is a production of beta-lactamases, bacterial enzymes capable of hydrolysing beta-lactam antibiotics such as penicillins, cephalosporins and carbapenems. Extended-spectrum beta-lactamases (ESBLs) are among the most clinically significant beta-lactamases since they confer resistance to all penicillins, cephalosporins, including oxyimino-compounds, and aztreonam. The widespread use of modern (3rd- and 4th-generation) cephalosporins is believed to be a major contributor to the emergence and global dissemination of ESBL-producing organisms (Paterson, 2006; Babic et al., 2006).

ESBLs are found in many species of the family Enterobacteriaceae. Most ESBLs belong to the three major genetic groups of molecular class A beta-lactamases: CTX-M, SHV and TEM. The TEM-type ESBLs are derived from parental penicillinases (TEM-1, TEM-2 or related variants) and differ from the latter by one to several amino acid substitutions (Bradford, 2001; http://www.lahey.org/Studies/). In TEM enzymes, the most important for an extension of the substrate spectrum residues are situated at Ambler's positions 104, 164 and 238–240. Substitutions of Ser, His or Cys for Arg164 and Ser for Gly238 result in an expansion of the substrate binding site, whereas Lys for Glu changes at positions 104 and 240 enhance the interaction with oxyimino side-chains of ceftazidim and aztreonam (Gniadkowski, 2001). Since infections with ESBL-producing organisms are registered with increased prevalence, revealing and subtyping of such enzymes, particularly TEM ESBL, take on special, epidemiological significance.

The approach presented in this paper uses the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) based minisequencing reaction for the rapid, sensitive and specific detection of nucleotide polymorphisms, which are responsible for the extension of substrate specificity in TEM beta-lactamases towards the oxyimino-cephalosporins. Minisequencing methodology of SNP detection is derived from classic Sanger DNA sequencing and uses dideoxynucleotides for termination of the growing DNA strand from a primer with its 3′-end, immediately nearby of a polymorphic site (Blondal et al., 2003; Haff and Smirnov, 1997). Recently, minisequencing method was successfully applied for genotyping of the hepatitis C virus (Ilina et al., 2005), detection of drug resistance related mutations in *N. gonorrhoeae* (Vereshchagin et al., 2005) and *M. tuberculosis* (Ikryannikova et al., 2007).

In our work, a MALDI TOF MS-based minisequencing approach was adapted for the analysis of polymorphisms at codons 104, 164 and 238 of bla_{TEM} gene.

2. Materials and methods

2.1. Sources of template DNA

Twenty two strains of *Escherichia coli* and one strain of *Klebsiella pneumoniae* carrying natural plasmids encoding various TEM-type enzymes were used in this study (Table 1). Beta-lactamases produced

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Table 1
Control strains carrying different *bla*$_{TEM}$ genes and artificial oligonucleotide templates

No.	Host strain	TEM variant	Enzyme type	Amino acid substitutions (codon changes)				
				104	164	237	238	240
1	*E. coli* L-862	TEM-1	Penicillinase	No mutations in codons 104, 164 and 238				
2	*E. coli* 197	TEM-1b	Penicillinase					
3	*E. coli* 450MST	TEM-1f	Penicillinase					
4	*E. coli* 813MST	TEM-1f+TEM-1c	Penicillinase					
5	*E. coli* 200	TEM-2	Penicillinase					
6	*E. coli* j62	TEM-2	Penicillinase					
7	*E. coli* 199	TEM-37	Penicillinase					
8	*E. coli* 189	TEM-39 (IRT-10)	Penicillinase					
9	*E. coli* 196	TEM-40 (IRT-167)	Penicillinase					
10	*E. coli* SM91	TEM-70	Penicillinase					
11	*E. coli* j62	TEM-117	Penicillinase					
12	*E. coli* j53.2	TEM-3	ESBL	Glu104Lys (GAG-AAG)			Gly238Ser (GGT-AGT)	
13	*E. coli* CF604	TEM-3	ESBL	Glu104Lys (GAG-AAG)			Gly238Ser (GGT-AGT)	
14	*E. coli* j53.2 pUD16	TEM-4	ESBL	Glu104Lys (GAG-AAG)			Gly238Ser (GGT-AGT)	
15	*E. coli* C1A	TEM-7	ESBL		Arg164Ser (CGT-AGT)			
16	*E. coli* 190	TEM-9	ESBL	Glu104Lys (GAG-AAG)	Arg164Ser (CGT-AGT)			
17	*E. coli* j53 RHH-1	TEM-9	ESBL	Glu104Lys (GAG-AAG)	Arg164Ser (CGT-AGT)			
18	*E. coli* j53	TEM-9	ESBL	Glu104Lys (GAG-AAG)	Arg164Ser (CGT-AGT)			
19	*E. coli* j53	TEM-10	ESBL		Arg164Ser (CGT-AGT)			Glu240Lys (GAG-AAG)
20	*E. coli* j53	TEM-11	ESBL		Arg164His (CGT-CAT)			
21	*E. coli* DH5α	TEM-12	ESBL		Arg164Ser (CGT-AGT)			
22	*E. coli* j53	TEM-26	ESBL	Glu104Lys (GAG-AAG)	Arg164Ser (CGT-AGT)			
23	*K. pneumoniae* 3151	TEM-68	ESBL				Gly238Ser (GGT-AGT)	Glu240Lys (GAG-AAG)
24	TEM164C	Artificial oligonucleotide templates			Arg164Cys (CGT-TGT)			
25	TEM237T					Ala237Thr (GCC-ACC)		
26	TEM240K					Ala237Thr (GCC-ACC)		Glu240Lys (GAG-AAG)

by these strains were previously characterized by partial or complete sequencing of the coding genes and analysis of their phenotypic expression (Jacoby and Sutton, 1991; Fiett et al., 2000; Leflon-Guibout et al., 2000; Edelstein et al., 2000). Bacterial DNA was isolated by rapid boiling of suspensions of 1–3 colonies of each strain in TE buffer. Centrifuged lysates were used as sources of template DNA in PCR.

Additionally, three synthetic oligonucleotides (TEM164C, TEM237T and TEM240K) were used to simulate the rarely occurring *bla*$_{TEM}$ gene sequences with single mutations at codons 164, 237 or 240 (Table 1). These oligonucleotides served as templates for the primer extension reactions (see below).

2.2. PCR of *bla*$_{TEM}$ genes

A 1080-bp fragment containing the entire *bla*$_{TEM}$ gene sequence was amplified with TEM-Fwd and TEM-Rew (Mabilat and Goussard, 1993) oligonucleotide primers (Table 2). The PCR was carried out in 25 μL of reaction mixture containing 66 mM of Tris–HCl (pH 9.0), 16.6 mM of $(NH_4)_2SO_4$, 2.5 mM of $MgCl_2$, 250 μM of each dNTP, 1 U of *Taq* DNA polymerase (Lytech LTD, Moscow, Russia) and 10 pmol of each primer. A TETRAD DNA ENGINE thermocycler (MJ Research, Inc.) was used. Initial heating step was at 94 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, 55 °C for 30 s and 72 °C for 45 s. The results of amplification were confirmed by agarose gel electrophoresis.

All PCR products were treated with 0.5 U of shrimp alkaline phosphatase (SAP) and 2.5 U of *E. coli* Exonuclease I (Fermentas, Vilnius, Lithuania) at 37 °C for 20 min, to dephosphorylate the 5′-end phosphate groups of deoxynucleoside triphosphates, and to remove residual primers. After that, the enzymes were deactivated at 85 °C for 10 min.

2.3. Whole-length sequencing of *bla*$_{TEM}$ genes

The entire coding regions of *bla*$_{TEM}$ genes were sequenced for all the strains used in this study to confirm the presence of key mutations

Table 2
Oligonucleotides involved into described experiments

Name	Sequence 5′–3′	Function	T_m, °C[a]	T_w, °C[b]
TEM164C	GGGGGATCATGTAACTCGCCTTGATTGTT GGGAACCGGAGCTGAATGAAGCC	Artificial template DNA		
TEM237T	TGGCTGGTTTATTGCTGATAAATCTGGAA CCGGTGAGCGTGGGTCTCGCGGTATCAT			
TEM240K	TGGCTGGTTTATTGCTGATAAATCTGGAA CCGGTAAGCGTGGGTCTCGCGGTATCAT			
TEM-Fwd	ATAAAATTCTTGAAGACGAAA	Primers for amplification and whole-length *bla*$_{TEM}$	55	55
TEM-Rew	GACAGTTACCAATGCTTAATCA	gene sequencing	60	
TEM1f	AGAGAATTATGCAGTGC	Primers for whole-length *bla*$_{TEM}$ gene sequencing	48	48
TEM4r	CTCGTCGTTTGGTATGGC		56	56
TEM5r	TTACTGTCATGCCATCC		50	50
TEM104f	CTCAGAATGACTTGGTT	Primer for extension at codon 104	48	48
TEM164f	ATCATGTAACCCGCCTTGAT	Primers for extension at codon 164	58	
TEM164r	TCAGCTCCGGTTCCCAA		54	
TEM237f1	ATTGCTGATAAATCTGGA	Primers for extension at codons 237, 238 and 240	41	41
TEM238f2	TGCTGATAAATCTGGAGCC		49	41
TEM238f3	CTGATAAATCTGGAACC		42	
TEM240r1	GATACCGCGAGATCCACGCT		56	

[a]T_m — calculated melting temperature; [b]T_w — actual working temperature.