Evolution of TEM β-lactamase genes identified by PCR with newly designed primers in Korean clinical isolates

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Among Gram-negative pathogens in Korea, the incidence of resistance to extended-spectrum β-lactam antibiotics is becoming an ever-increasing problem in nosocomial infections [1]. The single most prevalent mechanism responsible for resistance to β-lactam antibiotics among clinical isolates of the family Enterobacteriaceae is the production of extended-spectrum β-lactamases (ESBLs). We designed 16 primer pairs from most β-lactamase genes belonging to four molecular classes [2] (classes A, B, C and D) of ESBL to differentiate the genes encoding ESBLs and to detect a new ESBL gene.

The BLASTN program at the National Center for Biotechnology Information was used for database searches of ESBL genes, and the Clustal W program was used to align multiple nucleotide sequences. Nucleotide sequence accession numbers taken from GenBank, EMBL or DDBJ databases, and reference reports, are available on our website: http://www.inticity.com/ulg07100. The primers (Table 1) were designed by selecting identical sequences in multiple nucleotide alignments of ESBL genes and using the Primer Calculator program (Williamstone Enterprises, Waltham, MA, USA). The electrophoretic analysis of polymerase chain reaction (PCR) products of reference genes ($bla_{\text{TEM-1a}}$, $bla_{\text{SHV-12}}$, $bla_{\text{OXA-2}}$, $bla_{\text{MOX-1}}$, $bla_{\text{CMY-2}}$, $bla_{\text{MIR-1}}$, $bla_{\text{IMP-1}}$, $bla_{\text{IMI-1}}$, and $bla_{\text{Toho-1}}$) showed that the fragment sizes of PCR products were identical to those indicated in Table 1.

Using the criteria for the reduced susceptibility or resistance to oxyimino-cephalosporins (cefotaxime and ceftazidime) and monobactams (aztreonam) and confirmation by double disk test, we detected five *Escherichia coli* and two *Klebsiella pneumoniae* clinical isolates producing ESBLs from January 1999 to March 1999 in Kosin Medical Center, South Korea. The clinical isolates were recovered from urine (four strains), exudates (two strains), or sputum samples (one strain). Genotypes of ESBLs determined by PCR using plasmid DNA as a template with 16 primer pairs included seven TEM-derived and seven SHV-derived ESBLs. The seven clinical isolates yielded positive results with TEM- and SHV-specific primers. Nucleotide sequence analysis (automatic sequencer 373A; Applied Biosystems, Weiterstadt, Germany) of PCR products revealed that $bla_{\text{TEM-1b}}$ and $bla_{\text{SHV-12}}$ were the dominant types of β-lactamase gene. In addition, we also identified $bla_{\text{TEM-52}}$, $bla_{\text{SHV-5}}$, and a new ESBL gene. The $bla_{\text{TEM-17}}$ gene was only identified in *Capnocytophaga ochracea* CIP 105321, a capnophilic gram-negative fusiform rod with gliding motility [4]. Because a new ESBL gene first detected in *E. coli* K992740-1 is different from $bla_{\text{TEM-17}}$ at seven positions (silent point mutations in the coding region: $C_{226} \rightarrow T$, $C_{436} \rightarrow T$, $A_{469} \rightarrow T$, $G_{604} \rightarrow T$, $C_{682} \rightarrow T$, $T_{863} \rightarrow C$, and $T_{985} \rightarrow C$), we named it $bla_{\text{TEM-17b}}$. The nucleotide sequence of $bla_{\text{TEM-17b}}$ has been submitted to GenBank and assigned accession number AF264753. PCR of $bla_{\text{TEM-17b}}$ containing promoter and whole coding regions was carried out with T1′ primer (5′-CAATAACCCTGGTAAATGCT-3′) and T2′ primer (5′-TTACCAATGTTTGGTAAGGGA-3′). Nucleotide sequence analysis of the PCR product showed that four mutations at positions (according to the numbering scheme of Sutcliffe [5]) 175, 226, 436 and 604 were the same as those of $bla_{\text{TEM-1b}}$ (Tn-2 type) [6]. The results suggest that $bla_{\text{TEM-17b}}$ is harbored in a Tn-2 type transposon. The strain containing $bla_{\text{TEM-17b}}$ was resistant to ceftazidime (MIC 32 mg/L), aztreonam (MIC 64 mg/L), and amoxycillin (MIC 512 mg/L). The strain had intermediate resistance to cefotaxime (MIC 16 mg/L) and was susceptible to cefotetan (MIC 1 mg/L), cefoxitin (MIC 16 mg/L), and imipenem (MIC 0.25 mg/L). The MIC profile of *E. coli* K992740-1 was similar to that of *C. ochracea* CIP 105321 [4]. The $bla_{\text{TEM-17b}}$ gene presents the single Glu104 → Lys amino acid substitution. The $bla_{\text{TEM-52}}$ gene presents Glu104 → Lys, Met182 → Thr and Gly238 → Ser amino acid substitutions. Therefore, the detection of $bla_{\text{TEM-17b}}$ suggests that the evolution of $bla_{\text{TEM-52}}$ from those genes identified from Korean clinical isolates would seem to be

Table 1 Nucleotide sequences of the oligonucleotides used for amplification

Gene and primer name[a]	Sequence[b]	Nucleotide position[c]	Expected size (bp) of PCR product (primer pair)
bla$_{TEM}$			
T1	5′-AGAGTATGAGTATTCAACATT-3′	204	
T2	5′-ATCTCAGCGATCTGTCTAT-3′	1041	837 (T1 and T2)
T3	5′-AGTCACAGAAAAGCATCTTA-3′	523	
T4	5′-ATAGTTTGCGCAACGTTGT-3′	782	259 (T3 and T4)
bla$_{SHV}$			
S1	5′-GGGTTATTCTTATTTGTCGCT-3′	58	
S2	5′-TAGCGTTGCCAGTGCTCG-3′	987	929 (S1 and S2)
S3	5′-AGATCCACTATCGCCAGCA-3′	393	
S4	5′-TCATTCAGTTCCGTTTCCCA-3′	624	231 (S3 and S4)
bla$_{OXA}$			
O(10)1	5′-GTTCAATTACAGAAAATACGT-3′	180	
O(10)2	5′-TTAGCCACCAATGATGCC-3′	912	732 (O(10)1 and O(10)2)
O(2)1	5′-GTTTTCCGATGGGACGG-3′	980	
O(2)2	5′-ACCCATCCTACCCACCA-3′	1361	381 (O(2)1 and O(2)2)
bla$_{CMY-1}$, *bla*$_{FOX}$, and *bla*$_{MOX}$			
C1	5′-GAGCAGACCCTGTTCGAGAT-3′	570	
C2	5′-GATTGGCCAGCATGACGATG-3′	1416	846 (C1 and C2)
C3	5′-TACTCCAACCCCAGCATAGG-3′	852	
C4	5′-CCACATAGGCGCCAAAGCC-3′	1371	519 (C3 and C4)
bla$_{CMY}$, *bla*$_{AmpC[C]}$, *bla*$_{LAT}$, and *bla*$_{BIL}$			
CA1	5′-TGCTGCTGACAGCCTCTTTC-3′	71	
CA2	5′-TTTCAAGAATGCGCCAGGCC-3′	1177	1106 (CA1 and CA2)
CA3	5′-GCGATCCGGTCACGAAATAC-3′	359	
CA4	5′-ATAACGCTGGATTTCACGCCA-3′	785	426 (CA3 and CA4)
bla$_{MIR}$, *bla*$_{AmpC[E]}$, and *bla*$_{ACT}$			
M1	5′-CTATAAGTAAAACCTTCACCGG-3′	1178	
M2	5′-TATGCCGCCTCAACGCGTG-3′	2048	870 (M1 and M2)
M3	5′-TGCGCTTTTATCAAAACTGGCA-3′	1382	
M4	5′-GCCACGTAGCTGCCAAACC-3′	1967	585 (M3 and M4)
bla$_{IMP}$			
I1	5′-CTACCGCAGCAGAGTCTTTG-3′	494	
I2	5′-ACAACCAGTTTTGCCTTACCAT-3′	1082	588 (I1 and I2)
bla$_{IMI}$, *bla*$_{NMC}$, and *bla*$_{Sme}$			
IN1	5′-ATGTCATTAGGTGATATGGCT-3′	492	
IN2	5′-GCATAATCATTTGCCGTACC-3′	889	397 (IN1 and IN2)
bla$_{CTX-M}$ and *bla*$_{Toho}$			
CT1	5′-ATCTGACGCTGGGTAAAGC-3′	646	
CT2	5′-ATATCGTTGGTGGTGCCATA-3′	808	162 (CT1 and CT2)
bla$_{PER}$			
P1	5′-GATTTGTTATTTGAACTGGT-3′	729	
P2	5′-TGAACCTAATAACTGCATAA-3′	1326	597 (P1 and P2)

[a]Primers are consensus sequences of the *bla* genes taken from GenBank, EMBL, DDBJ database, or reference report (in the text). [b]PrimersT1,T3, S1, S3, O(10)1, O(2)1, C1, C3, CA1, CA3, M1, M3, I1, IN1, CT1 and P1 are identical to the leading strand; primersT2, T4, S2, S4, O(10)2, O(2)2, C2, C4, CA2, CA4, M2, M4, I2, IN2, CT2 and P2 are identical to the lagging strand. [c]Numbers correspond to the position of the first 5′ base of each oligonucleotide according to the numbering of the nucleotide sequences of V01119 (*bla*$_{TEM-1a}$) forT1, T2, T3 and T4; M59181 (*bla*$_{SHV-1}$) for S1, S2, S3 and S4; U37105 (*bla*$_{OXA-10}$) for O(10)1 and O(10)2; X07260 (*bla*$_{OXA-2}$) for O(2)1 and O(2)2; X92508 (*bla*$_{CMY-1}$) for C1, C2, C3 and C4; *bla*$_{AmpC[C]}$ of *Citrobacter freundii* [3] for CA1, CA2, CA3 and CA4; M37839 (*bla*$_{MIR-1}$) for M1, M2, M3 and M4; S71932 (*bla*$_{IMP-1}$) for I1 and I2; U50278 (*bla*$_{IMI-1}$) for IN1 and IN2; X92506 (*bla*$_{CTX-M-1}$) for CT1 and CT2; Z21957 (*bla*$_{PER-1}$) for P1 and P2.

bla$_{TEM-1b}$ → *bla*$_{TEM-17b}$ → a novel β-lactamase gene (Glu104 → Lys plus Met182 → Thr or Met182 → Thr plus Gly238 → Ser) → *bla*$_{TEM-52}$, although the novel β-lactamase gene has not been identified (http://www.lahey.org/studies/webt.htm). Furthermore, *bla*$_{TEM-1b}$ (*E. coli* K992806-2), *bla*$_{TEM-17b}$ (*E. coli* K992740-1) and *bla*$_{TEM-52}$ (*K. pneumoniae* K992806-3) were detected in the different samples of one patient (1-year-old baby). *E. coli* K992806-2 and *E. coli*

K992740-1 had intermediate resistance to cefotaxime (MIC 16 mg/L), whereas *K. pneumoniae* K992806-3 was resistant to cefotaxime (MIC 64 mg/L). Three strains were resistant to ceftazidime and aztreonam, but the MICs were higher in *E. coli* K992806-2, *E. coli* K992740-1, and *K. pneumoniae* K992806-3, in that order. Sequence analysis revealed that $bla_{\text{TEM-17b}}$ was more similar to $bla_{\text{TEM-1b}}$ than to $bla_{\text{TEM-1a}}$, and four of seven silent mutations of $bla_{\text{TEM-17b}}$ were the same as those of $bla_{\text{TEM-52}}$ (at positions 469, 682, 863 and 985). Our findings from the same patient appear to represent the in vivo evolution of β-lactamase genes (from $bla_{\text{TEM-1b}}$ to $bla_{\text{TEM-17b}}$ and from $bla_{\text{TEM-17b}}$ to $bla_{\text{TEM-52}}$) under the selective pressure of antimicrobial therapy (especially cefotaxime), as was the case with $bla_{\text{SHV-8}}$ [7].

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Rhodotorula septicemia: case report and minireview

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Rhodotorula, a yeast member of the family Cryptococcaceae, was only recently recognized as a human pathogen especially affecting immunocompromised patients [1]. To our knowledge, over the last 40 years, no more than 40 cases of rhodotorula infections have been reported in patients with central indwelling vascular catheters. We isolated *Rhodotorula rubra* from both the catheter and the peripheral venous blood of a patient on azole prophylaxis. On this occasion, we reviewed the literature and the limited experience with rhodotorula hospital infections in Greece.

A 21-year-old female patient, with non-Hodgkin's lymphoma in second remission, underwent autologous peripheral blood progenitor cell transplantation. A large peripheral double lumen catheter was placed in the subclavian vein for the collection and reinfusion of stem cells. The conditioning regimen was given on days −5 to −1. The patient received prophylactic oral ciprofloxacin 500 mg twice daily and fluconazole 200 mg/day. Because of severe vomiting and gastrointestinal mucositis, total parenteral nutrition was mandatory. One day after stem cell reinfusion (day 1), the patient developed grade 4 neutropenia on the WHO toxicity scale (PMN count $<100/\mu L$), and 3 days later (9 days after insertion of the catheter) she became febrile. Blood cultures were obtained from both the catheter sites as well as from the antecubital vein. Ciprofloxacin was discontinued, and intravenous ceftazidime, vancomycin and amikacin were started. Microscopic examination of all blood cultures revealed budding yeasts.

Blood cultures were performed with the continuously monitored non-invasive system BACTEC 9240 (Becton Dickinson Medical Systems, Rutherford, NJ, USA). Blood specimens were inoculated in BACTEC plus F aerobic and anaerobic bottles (Becton Dickinson Microbiology Systems, Sporks, Maryland, USA) and were incubated for a standard 7-day