IMPROVED PCR DETECTION AND SUBTYPING OF CTX-M-β-LACTAMASE-ENCODING GENES

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ABSTRACT

Backround: Rapid detection and differentiation of CTX-M β-lactamases is of need in many laboratories as this family of ESBLs is rapidly disseminating. PCR has been used widely to detect bla_{CTX-M} genes but detection of all the known variants usually required multiple reactions with specific primers for different CTX-M subtypes. Consensus primers have also been described but the recent emergence of new CTX-M variants (e.g. CTX-M-25, -26) made them no longer versatile. In this study we describe a novel real-time PCR technique for detection of all CTX-Ms and a single-enzyme post-PCR restriction analysis for differentiation between the 5 CTX-M genetic clusters.

Methods: A pair of primers matching conserved sequences at positions 205 to 227 and 724 to 706 with respect to the CTX-M translational starting point was designed to amplify a 520-bp fragment of all the known bla_{CTX-M} genes using a real-time approach with SYBR Green product detection. A computer analysis was used to identify BseDI as a restriction endonuclease capable of distinguishing the subtypes of bla_{CTX-M} genes. PCR products were digested with BseDI and digests analyzed by agarose gel electrophoresis. Bacterial strains producing known β-lactamases (CTX-M-2, -3, -4, -5, -9, -15; TEM-1; SHV-1; KluA) were used for quality control and assessment of specificity of PCR and discriminative power of the restriction analysis.

Results: A single DNA fragment of the expected size was amplified in all CTX-M-positive control strains. Melting-curve peaks were detected at 84.5, 85.5 and 87.5°C for CTX-M-2, -3 and CTX-M-9 genetic subtypes, respectively. No increase in fluorescence was detected for CTX-M-negative isolates. Further restriction analysis produced the predicted restriction patterns and, therefore, allowed to distinguish different CTX-M subtypes.

Conclusions: The proposed real-time PCR is a versatile and specific tool for rapid detection of CTX-M β-lactamases. When needed, the post-PCR restriction analysis may be used to confirm differentiation of CTX-M genetic subtypes.

INTRODUCTION

In the past several years the CTX-M β-lactamases have become the most widespread ESBLs. The global expansion of these enzymes has provoked the need for their effective detection and subtyping. In response to this need several PCR-based tests have been developed which imply the use of either universal [1, 4] or cluster-specific CTX-M primers [2, 3].

In this work we have redesigned our previously reported [1] universal CTX-M primers based on comprehensive analysis of the sequences of new CTX-M variants, including members of the growing CTX-M-8 cluster (CTX-M-40 and CTX-M-41) as well as of the novel cluster comprised by the CTX-M-25 and CTX-M-26. We have also developed a single-tube real-time PCR assay which uses the newly designed primers and a SYBR Green I chemistry for the rapid detection and differentiation of the CTX-Ms that belong to different clusters.

MATERIALS AND METHODS

Analysis of CTX-M sequences and design of primers. The BLAST tool (http://www.ncbi.nlm.nih.gov/blast/) was used to retrieve sequences of the CTX-M-coding genes from the GenBank database. For this purpose five independent searches using the full length nucleotide sequences of $bla_{CTX-M-1}$ (GB Accession: X92506), $bla_{CTX-M-2}$ (GB Accession: X92507), $bla_{CTX-M-9}$ (GB Accession: AF174129), $bla_{CTX-M-8}$ (GB Accession: AF189721) and $bla_{CTX-M-25}$ (GB Accession: AF518567) were performed. A total of 127 sequences of bla_{CTX-M} genes and 18 genes for species-specific β-lactamases of *Kluyvera* spp. were retrieved. The sequences were aligned using the ClustalW algorithm in BioEdit Sequence Alignment Editor v7.0.1 (Tom Hall, Isis Pharmaceuticals, Inc). The primers were selected "manually" to anneal to the most conserved regions of the bla_{CTX-M} genes and were verified for the absence of secondary structures and self-complementarity using the GeneRunner v3.05 software (Hastings Software, Inc). Thermodynamic parameters of the primer-templatebinding were predicted using the MeltCalc program (E. Schutz & N. von Ahsen). Primers were selected to allow minimum number of mismatches and minimum Tm differences for all the CTX-M subtypes.

Bacterial strains. The following strains producing the known β-lactamases were used: *Salmonella typhimurium* CAS5 (CTX-M-2), *Citrobacter freundii* 2525 (CTX-M-3), *S. typhimurium* SP-893 (CTX-M-4), *Escherichia coli* KZ-Ma12 (CTX-M-5), *E. coli* (CTX-M-9), *E.coli* BH3223/2 (CTX-M-15), *Kluyvera ascorbata* T861 (KluA), *Klebsiella oxytoca* (OXY-2), *E.coli* J53 (TEM-3), *E.coli* J53 (SHV-2).

Real-time PCR and melting-curve analysis. The amplification was initially attempted and compared with degenerate (CTX-M/Fwob: 5'-TTTSCVATGTGCAGYACCAGTAA-3' and CTX-M/Rwob: 5'- CDCCGCTGCCGGTYTTATC-3') and nondegenerate (CTX-M/F': 5'-TTTGCGATGTGCAGTACCAGTAA-3' and CTX-M/R1: 5'- CTCCGCTGCCGGTTTTATC-3') primers. The latter primers matching conserved sequences at positions 205 to 227 and 706 to 724 with respect to the CTX-M translational starting point were selected for further testing. The 25-µl PCR mixtures contained: 0.5µM of each primer, 200µM of each dNTP, 15mM $MgCl_2$, 67mM Tris-HCl (pH=8.3), 17mM $(NH_4)_2SO_4$, 0.1% Tween-20, 0.12mg/ml BSA, 8% Glycerol, 10% DMSO, 2.5U of DiaTaq polymerase (AmpliSens, Russia), 1µl of SYBR Green I (1:1000 dilution, BioGene, UK) and 2.5µl of template DNA prepared by rapid boiling of 2-3 bacterial colonies in 1mM TE buffer. Amplification was performed in a Rotor-Gene 2000 Real-Time Cycler (Corbett Research, Australia) under the following conditions: 95°C for 2 min, followed by 27 cycles of 95°C for 20 sec, 58°C for 30 sec, 72°C for 30 sec. The final elongation step was at 72°C for 3 min. Melting of amplification products was performed by increasing the temperature from 72 to 94°C by 1°C every 10 sec. Fluorescence of SYBR Green I was acquired on the "SYBR" channel at the end of each amplification cycle and during the melting analysis.

Restriction analysis and electrophoresis of PCR-products. A *Bse*DI restriction endonuclease was identified by computer analysis as the enzyme capable of distinguishing the PCR fragments of bla_{CTX-M} genes which belong to different clusters. Amplification products (5 µl) were digested with *Bse*DI (Fermentas, Lithuania) in a Yellow Tango™ Buffer (33mM Tris-acetate (pH 7.9), 10mM magnesium acetate, 66mM potassium acetate, 0.1mg/ml BSA) and electrophoresed in a 3% TBE agarose gel.

RESULTS AND DISCUSSION

Real-time PCR amplification of blaCTX-M genes. The positions of the primers selected for universal amplification of bla_{CTX-M} genes are shown in figure 1. Comparison of the degenerate and non-degenerate primers matching the same sites has demonstrated more consistent amplification of bla_{CTX-M} genes that belong to different clusters with the non-degenerate oligonucleotides (data not shown). The CTX-M/F' and CTX-M/R1 primers have 100% identity to all the sequences of the CTX-M-2 cluster but have 1 or 2 mismatches for the members of the CTX-M-1, CTX-M-8, CTX-M-9 and CTX-M-25 clusters. Nevertheless, the expected PCR product was obtained with selected primers from all the strains producing CTX-M enzymes of 3 distinct clusters. The efficiencies of amplification of $bla_{CTX-M-2}$ and $bla_{CTX-M-3}$ were nearly identical and that of $bla_{CTX-M-9}$ was only slightly lower, as follows from the comparison of respective threshold cycle values (Ct = 11.9; 12.1 and 18.0) (Figure 2A).

Control strains carrying the genes of the $bla_{CTX-M-8}$ and $bla_{CTX-M-25}$ groups were not available, but the structure of primer binding sites in these genes is similar to that of the $bla_{CTX-M-1}$ group. It is therefore expected that PCR products from $bla_{CTX-M-8}$ and $bla_{CTX-M-25}$-related genes should be readily obtained with selected primers. The specificity of PCR with selected primers was verified by testing a panel of strains producing various class A β-lactamases (TEM, SHV, OXY, Klu). As expected, only the *K.ascorbata* strain carrying a chromosomal *kluA* gene which is 100% identical to the CTX-M-5 gave stable amplification, and the *K.oxytoca* strain carrying the chromosomal bla_{oxy-2} gene which is partially homologous to bla_{CTX-M}s gave weak amplification.

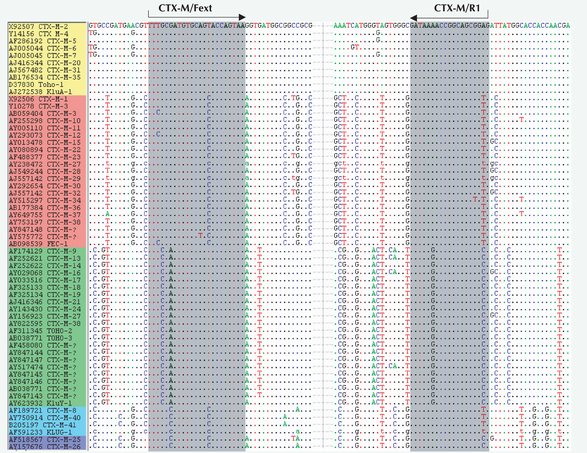

Figure 1. Primer binding sites (shown in grey) in the aligned bla_{CTX-M} sequences.

No PCR products were obtained from strains, producing the TEM- and SHV-type ESBLs (data not shown).

Melting-curve analysis of PCR products. Figure 2B shows the results of melting-curve analysis of PCR-products. The PCR products corresponding to CTX-Ms of 3 different clusters were characterised, respectively, by three distinct dissociation profiles with well-separated Tm peaks. The Tm values for the members of the same cluster were identical (84.5°C for CTX-M-2, CTX-M-4, CTX-M-5 and KluA; 85.5°C for CTX-M-3, CTX-M-15; 87.5°C for CTX-M-9) and were highly reproducible in repetitive experiments. It may be also expected that within each cluster all members should have the same melting temperatures as they differ only by a few point mutations. Therefore, melting-curve analysis may possibly be used for discrimination between the CTX-M clusters. Further studies with larger panel of CTX-M variants (particularly those of the CTX-M-8 and CTX-M-25 groups) are needed to confirm this possibility.

Electrophoresis and restriction analysis of real-time amplification products. The specificity of amplification was confirmed by electrophoretic analysis of real-time PCR products. A single band of expected size (520 bp) was amplified from all the CTX-M-producing strains (Figure 3A). When digested with a single *Bse*DI endunuclease which is capable of distinguishing between the CTX-M clusters (Table 1), all the samples yielded predicted patterns of bands (Figure 3B).

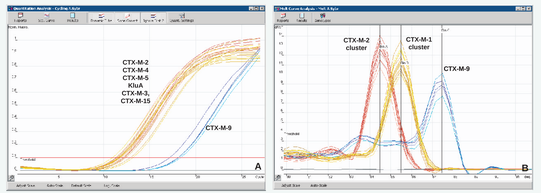

Figure 2. Real-time amplification (A) and melting-curve analysis (B) of bla_{CTX-M}s.

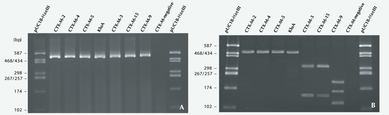

Figure 3. Electrophoresis of real-time PCR products (A) and their *Bse*DI-restriction fragments (B).

TABLE 1. Predicted *Bse*DI restriction patterns of PCR products

CTX-M cluster	Expected length of restriction fragments, bp
CTX-M-1	32, 6, 314, 136, 32
CTX-M-2	35, 485
CTX-M-9	35, 202, 115, 168
CTX-M-8	176, 148, 196
CTX-M-40, -41	35, 66, 75, 148, 196
CTX-M-25, -26	324, 74, 1, 121

CONCLUSIONS

The developed real-time PCR method allows rapid and specific detection of CTX-M β-lactamases which belong to different genetic clusters. The simplicity and reduced risk of PCR-contamination make this method particularly suitable for the epidemiological studies.

A preliminary differentiation of the CTX-M clusters may be achieved using a postamplification melting-curve analysis. When needed a restriction analysis of PCR products with a single *Bse*DI endunuclease may be used to unambiguously confirm the results of typing.

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