A SINGLE-TUBE PCR WITH MGB ECLIPSE PROBES FOR DETECTION OF SHV-TYPE EXTENDED-SPECTRUM β-LACTAMASES (ESBLs)

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ABSTRACT

Objectives: ESBLs of the SHV-type are one of the most common and clinically significant beta-lactamases. The number of SHV variants is continuously growing however ESBL activity of SHV enzymes has been associated with mutations at relatively few amino-acid positions (aa-s) as compared to the TEM enzymes. Here we propose a simple and rapid method that allows detection of all the known SHV ESBLs in a single real-time PCR reaction.

Methods: The proposed method is based on amplification of bla_{SHV} genes in the presence of short (13-14nt) fluorogenic probes capable of hybridization-triggered fluorescence. These probes commercially known as MGB Eclipse probes contain a dark quencher with a conjugated minor groove binder at the 5'-end and a fluorescent dye at the 3'-end. This structure allows detection and differentiation of nucleotide polymorphisms at targeted sites by post-PCR melting curve analysis. Four probes were designed to perfectly match the wild-type (WT) sequences at mutation sites corresponding to aa-s 146, 149, 156, 179 and 238. Thus, mutations conferring ESBL activity were expected to specifically lower the melting temperatures (Tm-s) of the probe-template duplexes. Each probe was labeled with a unique dye permitting analysis of mutations at multiple sites in a single reaction.

Results: The method was validated using laboratory strains producing the SHV-1 (WT, non-ESBL control), SHV-2, 3, 4, 5 (G238S), SHV-18 (G238A), SHV-6 (D179A), SHV-8 (D179N) and strains carrying cloned bla_{SHV} fragments to which the naturally-occurring mutations D179G, G156D, T149S and A146V were introduced by site-directed mutagenesis. Following careful design of the probes and optimization of PCR conditions, all the above mutations were successfully detected and discriminated from the WT sequence and each other according to specific Tm-s. The detection was precise and highly reproducible in repeated experiments. Furthermore, when applied to the analysis of ten clinical isolates of *Klebsiella pneumoniae* expressing ESBL phenotype, the method was able to detect multiple SHV alleles (WT and G238S or D179A) in the same isolates. This observation is particularly important considering the high frequency of co-production of the SHV-1 and ESBLs in klebsiellae.

Conclusions: A PCR with MGB Eclipse probes has a great potential for studying the epidemiology of SHV ESBLs and possibly for analysis of other antimicrobial resistance mechanisms associated with mutations at defined loci.

INTRODUCTION

SHV β-lactamases are very common among *Enterobacteriaceae*. SHV-1, the first member of the SHV β-lactamase family, is currently considered a species-specific enzyme of *Klebsiella pneumoniae* (J.Chaves, 2001) and confers resistance to penicillins and early-generation cephalosporins. A plasmid-mediated SHV-1 is also commonly found in klebsiellae and *Escherichia coli* and is seen in many other species as well. Extended-spectrum β-lactamases (ESBLs) of SHV-type are of particular therapeutic concern as they confer resistance to newer cephalosporins. Though various ESBL derivatives differ from SHV-1 at multiple amino acid (aa) residues, there are five crucial aa positions: 146, 149, 156, 179, and 238. A substitution in at least one of them is found in all the currently described SHV ESBLs.

The existing molecular tests for detection of SHV ESBLs include PCR-RFLP (M.T. Nuesch-Inderbinen, 1996), PCR-SSCP (F. M'Zali, 1996), LCR (C. Niederhauser, 2000), Restriction Site Insertion-PCR (A. Chanawong, 2001), real-time PCR with FRET probes (C. Randegger, 2001), and minisequencing (C. Howard, 2002), however none of these tests allows the identification of all polymorphisms at the above-mentioned positions. We aimed to develop a single-tube real-time PCR assay for detection of all the naturally found SHV ESBLs.

MATERIALS AND METHODS

Bacterial strains. The reference strains used in this study are listed in the table 1. These strains carried either natural plasmids encoding previously characterised SHV variants or recombinant vectors to which mutations conferring ESBL activity were introduced by site-directed mutagenesis. In addition, fifty clinical isolates of *K.pneumoniae* and *E. coli* in which the presence of SHV ESBLs was confirmed by PCR-NheI test (M.T.Nuesch-Inderbinen, 1996) and ten bla_{SHV}-gene-negative strains of various species were tested to assess sensitivity and specificity of the real-time PCR assay.

Preparation of template DNA. A 1 µl loopful of bacteria harvested from an agar plate was suspended in 100 µl of autoclaved MilliQ-water and heated at 99ºC for 20 min. Heat-treated samples were centrifuged at 10000 g for 3 min. Five microliters of supernatant was used in PCR.

Real-time PCR and melting curve analysis. Four MGB Eclipse probes (Epoch Biosciences, USA) were designed to perfectly match the wild-type (WT) bla_{SHV}-sequences at mutation sites corresponding to aa-s: 146-149, 156, 179 and 238. The probes were labelled with different dyes (FAM, TET, Cy3.5 and Cy5) to allow multiplexing. Three pairs of primers complementary to the highly conserved bla_{SHV} regions were additionally designed to amplify the fragments of the gene containing mutations of interest (Fig. 1). The ratio of primers and probes was optimised to achieve the maximum fluorescent signal from each probe hybridised to its target. Each primer pair was tested both in monoplex PCR with the respective probe and in multiplex reaction with the whole set of primers and probes. Finally, amplification was performed using the asymmetric (1:8) ratio of primers complementary to the same strand as the respective probes and opposite strand primers. A 50-cycle PCR was followed by melting curve analysis on a Rotor-Gene 2000 real-time PCR system (Corbett Research, Australia). Fluorescence detection was conducted on the four standard channels: FAM (410/510 nm), JOE (530/555 nm), ROX (585/610 nm) and Cy5 (625/660 nm).

Table 1. Control strains.

Mutation	Enzyme	Host strain	Plasmid	Reference
WT	SHV-1	*E.coli* J53	R1010[n]	G. A. Jacoby, 1991
	SHV-1	*E.coli* DH5a	pCTA-1[r]	C. C. Randegger, 2000
238S	SHV-2	*E.coli* J53	pMG229[n]	G. A. Jacoby, 1991
	SHV-2	*E.coli* DH5a	pCTA-2[r]	C. C. Randegger, 2000
	SHV-3	*E.coli* J53	pUD18[n]	G. A. Jacoby, 1991
	SHV-4	*E.coli* J53	pUD21[n]	G. A. Jacoby, 1991
	SHV-5	*E.coli* DH5a	pCTA-5[r]	C. C. Randegger, 2000
	SHV-5	*E.coli* J53	pAFF2[n]	G. A. Jacoby, 1991
238A	SHV-18	*K.pneumoniae* K6	[n]	J. K. Rasheed, 2000
179A	SHV-6	*E.coli* J53	[n]	G. Arlet, 1997
		E.coli TOP10	pGEM-179A[r]	this study
179N	SHV-8	*E.coli* DH5a	pCTA-8[r]	C. C. Randegger, 2000
179G		*E.coli* TOP10	pGEM-179G[r]	this study
156D		*E.coli* TOP10	pGEM-156D[r]	this study
149S		*E.coli* TOP10	pGEM-149S[r]	this study
146V		*E.coli* TOP10	pGEM-146V[r]	this study

n – native; **r** – recombinant plasmid

RESULTS

The discrimination of mutations at positions 146, 149, 156, 179 and 238 is shown on figure 2. Due to the perfect complementarity of the probes to the wild-type (non-mutated) sequences, they produced the most stable duplexes, as demonstrated by high melting temperatures (Tm-s). The mismatches resulting from mutations at the probe binding sites lowered Tm values by 5.5-14ºC, thus allowing unambiguous detection of SHV ESBL genotypes. Furthermore, all mutations, except 149S and 146V, were successfully distinguished from each other according to specific Tm-s. In repetitive experiments, positions of the melting peaks were highly reproducible.

When applied to the analysis of clinical enterobacterial isolates, the assay correctly detected all SHV ESBL producers. In *K. pneumoniae* isolates, it was possible to detect the presence of multiple SHV alleles (e.g., WT (238G) and 238S) appeared as dual peaks on melting curve of the FAM-labelled probe (Fig. 3). No unspecific interaction of the probes with DNA from SHV-β-lactamase-negative strains was found.

The developed method offers two major advantages over the earlier described real-time PCR with FRET probes (C.Randegger, 2001). Firstly, it permits detection of a broader range of mutations. Secondly, the use of short MGB-containing probes that span only 12-13 nt surrounding each mutation site eliminate the effect of additional nucleotide polymorphisms at adjacent sequences.

CONCLUSIONS

To our knowledge, this is the first report of a real-time PCR assay that allows detection of 8 nucleotide polymorphisms at 5 codons in a single tube. We believe that a PCR with MGB Eclipse probes has a great potential for studying the epidemiology of SHV ESBLs due to its high accuracy, simplicity and speed.

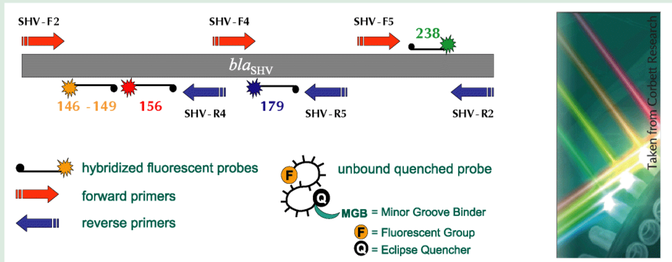

Figure 1. Primers and probes mapped to the bla_{SHV} gene internal sequence

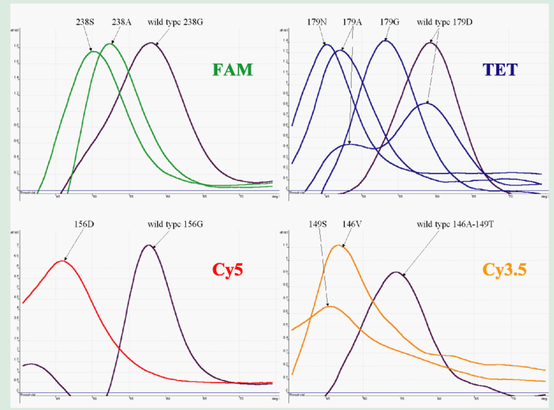

Figure 2. Detection and discrimination of the "key" mutations responsible for SHV ESBL activity by melting curve analysis with four MGB Eclipse probes

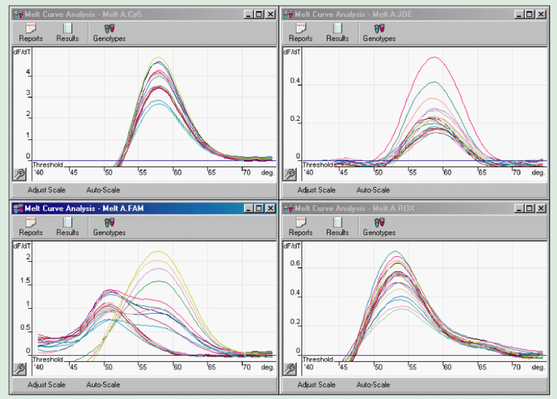

Figure 3. Detection of the wild-type and mutant (238S) bla_{SHV} alleles in clinical isolates

ACKNOWLEDGMENTS We are grateful to Prof. H. Hächler (University of Berne, Switzerland), Prof. G.A. Jacoby (Lahey Clinic, Burlington, MA, USA) and Dr. T. Muratani (University of Occupational and Environmental Health, Kitakyushu, Japan) for donation of the reference SHV β-lactamase-producing strains.