Discrimination of SHV β -Lactamase Genes by Restriction Site Insertion-PCR

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Restriction site insertion-PCR (RSI-PCR) is a simple, rapid technique for detection of point mutations. This technique exploits primers with one to three base mismatches near the 3' end to modulate a restriction site. We have developed this technique to identify described mutations of the *bla*_{SHV} genes for differentiation of SHV variants that cannot be distinguished easily by other techniques. To validate this method, eight standard strains were used, each producing a different SHV β -lactamase: SHV-1, SHV-2, SHV-3, SHV-4, SHV-5, SHV-6, SHV-8, and SHV-18. Mismatch primers were designed to detect mutations affecting amino acids at positions 8 (*SspI*), 179 (*HinII*), 205 (*PstI*), 238 (Gly→Ala) (*BsrI*), and 240 (*NruI*) of *bla*_{SHV} genes. All amplimers of the *bla*_{SHV} genes used in this study yielded the predicted restriction endonuclease digestion products. In addition, this study also makes theoretical identification of *bla*_{SHV-6}, *bla*_{SHV-8}, and 12 novel *bla*_{SHV} variants using the PCR-restriction fragment length polymorphism (RFLP) technique possible. By using a combination of PCR-RFLP and RSI-PCR techniques, up to 27 SHV variants can now be distinguished rapidly and reliably. These simple techniques are readily applied to epidemiological studies of the SHV β -lactamases and may be extended to the characterisation of other resistance determinants.

SHV extended-spectrum β -lactamases are spread worldwide in members of the family *Enterobacteriaceae* (8, 9). Methods used to characterize these enzymes, including isoelectric focusing and nucleotide sequence analysis, are time-consuming, expensive, or both. PCR–single-strand conformational polymorphism (PCR–SSCP) analysis is useful for characterization of the genes encoding SHV β -lactamases, particularly for detection of new SHV variants, or where a single strain harbors different *bla*_{SHV} genes (13). Mutations leading to the production of some SHV variants, however, yield PCR–SSCP patterns that are difficult to differentiate, and the technique relies upon relatively expensive instrumentation that is not available routinely to diagnostic laboratories. Recently, the ligase chain reaction has been developed and has been successful for typing known SHV variants (11). That technique, however, needs special reagents or a detection kit, which may be expensive, making routine application impractical. Hence, methods that are convenient for the rapid and reliable molecular epidemiological analysis of these resistance determinants are still required.

We previously applied PCR–restriction fragment length polymorphism (PCR–RFLP) analysis to the differentiation of variant *bla*_{SHV} genes (3), extending the molecular characterization of SHV β -lactamases using PCR–SSCP analysis. However, PCR–SSCP analysis has a number of drawbacks as described above. The PCR–RFLP technique is a simple and rapid alternative but cannot identify all known mutations, such as those affecting amino acids at positions 8, 238 (altering glycine to alanine), or 240, according to Ambler's numbering scheme

(1). Thus, *bla*_{SHV-3}, *bla*_{SHV-4}, *bla*_{SHV-7}, and *bla*_{SHV-13} cannot be identified unambiguously by PCR–RFLP analysis, unless the PCR–SSCP analysis is also applied (3, 13). In the previous study, neither PCR–SSCP nor PCR–RFLP analysis could differentiate the *bla*_{SHV-1}, *bla*_{SHV-6}, and *bla*_{SHV-8} genes (3). In addition, no commercial supplies are available for restriction endonuclease *BceII*, used to demonstrate mutations affecting the amino acid at position 205. Furthermore, 12 novel *bla*_{SHV} variants have also been reported recently: *bla*_{SHV-14} (17), *bla*_{SHV-16} (C. Arpin, R. Labia, F. Tessier, and C. Quentin, GenBank accession no. AF072684), *bla*_{SHV-18} (15), *bla*_{SHV-19} to *bla*_{SHV-22} variants (5), *bla*_{SHV-23} (S. Y. Essack, L. M. C. Hall, and D. M. Livermore, GenBank accession no. AF117747), *bla*_{SHV-25} and *bla*_{SHV-26} (L. K. Siu, F. Y. Chang, and M. H. Huang, GenBank accession no. AF208796 and AF227204, respectively), *bla*_{SHV-27} (J. E. Corkill, C. A. Hart, L. Cuevas, and J. Greensill, GenBank accession no. AF293345), and *bla*_{SHV-28} (Y. Yu, W. Zhou, and Y. Chen, GenBank accession no. AF299299). This further complicates the characterization of the genes of the *bla*_{SHV} family, and we have included them in our study.

Restriction site insertion-PCR (RSI-PCR) was first developed to detect point mutations between closely related DNA sequences (4, 7, 12). Primers with one to three base mismatches near the 3' end are used to modulate target restriction sites. In this study, RSI-PCR has been applied to the detection of the mutations of *bla*_{SHV} genes that cannot be identified unambiguously by PCR–RFLP. In addition, PCR–RFLP can theoretically be applied to differentiate *bla*_{SHV-6}, *bla*_{SHV-8}, *bla*_{SHV-14}, *bla*_{SHV-18} to *bla*_{SHV-23}, and *bla*_{SHV-25} to *bla*_{SHV-27}, thus allowing almost all the described *bla*_{SHV} variants to be differentiated easily and reliably.

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FIG. 1. Mutations of *bla*_{SHV} genes demonstrated by RSI-PCR analysis with various restriction endonucleases. (a) Position 8 with *Ssp*I digestion; (b) position 179 with *Hinf*I digestion; (c) position 238 with *Bsr*I digestion. Lanes A to H, amplimers of *bla*_{SHV} genes *bla*_{SHV-1} (A), *bla*_{SHV-2} (B), *bla*_{SHV-3} (C), *bla*_{SHV-4} (D), *bla*_{SHV-5} (E), *bla*_{SHV-6} (F), *bla*_{SHV-8} (G), and *bla*_{SHV-18} (H) after digestion; lane I, amplimer of *bla*_{SHV-1} gene before digestion; lane M, 100-bp ladder.

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MATERIALS AND METHODS

Bacterial strains. Eight standard strains were used in this study, including *Escherichia coli* C600(R1010), encoding *bla*_{SHV-1}; *E. coli* C600(pMG229), encoding *bla*_{SHV-2}; *E. coli* J53-2(pUD18), encoding *bla*_{SHV-3}; *Klebsiella pneumoniae* K25, encoding *bla*_{SHV-4}; *E. coli* HB101(pAFF611), encoding *bla*_{SHV-5}; *E. coli* C1A(pSLH06), encoding *bla*_{SHV-6}; *E. coli* strain 2-75, encoding *bla*_{SHV-8}; and *K. pneumoniae* ATCC 700603, encoding *bla*_{SHV-18} (2, 10, 15, 16).

Primers. Mismatch primers comprising at least 20 nucleotides were designed with modification of one or two nucleotides near the 3' end based on the nucleotide sequence of the *bla*_{SHV-1} flanking the primers; thus, a restriction site is created on an amplimer of the *bla*_{SHV-1} gene (Table 1). These included the primers that detect mutations affecting amino acids at positions 8 (*Ssp*I), 179 (*Hinf*I), 238 (*Bsr*I), and 240 (*Nru*I). A further mismatch primer pair was designed to remove the *Pst*I restriction site affecting the amino acid at positions 208 to 209. This permits identification of mutants that carry a *Pst*I recognition site affecting the amino acid at position 205. The mismatch primers were designed using primer design software: Primer3 from the PCR Jump Station. Pairs of primers that yielded amplimer products not longer than 350 bp were chosen (4).

PCR amplification. PCR amplification was performed in a final volume of 25 μ l as described previously (3) except that dimethyl sulfoxide was excluded and

0.5 to 1 U of SuperTaq DNA polymerase was used. In addition, 1.25 and 1.125 mM concentrations of magnesium chloride were used for PCR amplification that detected mutations affecting amino acids at position 238 and 240, respectively. PCR amplification was carried out using predenaturation at 95°C for 3 min; followed by 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; and with a final elongation at 72°C for 5 min. The PCR products were digested with restriction endonucleases according to the manufacturer's instructions (*Bsr*I, *Nru*I, and *Ssp*I were purchased from New England Biolabs, *Pst*I and *Hinf*I were supplied from Promega and GibcoBRL, respectively). After digestion, the products were analysed by gel electrophoresis using 3% low-melting-point agarose (Metaphore; FMC Bio-Products, Flowgen, Staffordshire, United Kingdom). A 100-bp ladder was used as a DNA size marker.

RESULTS AND DISCUSSION

In this study, mismatch primers were designed to identify mutations affecting the amino acids at positions 8, 179, 205, 238, and 240. All *bla*_{SHV} variants described to date are derived from *bla*_{SHV-1} with one to seven amino acid substitutions. Thus, the primers were designed to create a restriction site specific to the *bla*_{SHV-1}, except the primer detecting mutations affecting the amino acid at position 205. The latter primer was designed to delete a *Pst*I restriction site found just downstream at the nucleotides encoding amino acids 208 and 209. This site is present in all *bla*_{SHV} genes. The mismatch primer pair will thus yield an amplimer that will only be digested by the *Pst*I restriction endonuclease if it is generated from genes that carry

FIG. 2. Mutations of *bla*_{SHV} genes demonstrated by RSI-PCR analysis with various restriction endonucleases. (a) Position 240 with *Nru*I digestion; (b) position 205 with *Pst*I digestion. Lanes A to E, amplimers of *bla*_{SHV} genes *bla*_{SHV-1} (A), *bla*_{SHV-2} (B), *bla*_{SHV-3} (C), *bla*_{SHV-4} (D), and *bla*_{SHV-5} (E) after digestion; lane F, amplimer of *bla*_{SHV-1} gene before digestion; lane M, 100-bp ladder.

TABLE 1. Primers used for the RSI-PCR technique

Amino acid affected	Primer	Oligonucleotide sequence (5'→3') ^a	Restriction recognition site (enzyme) ^b	PCR product (bp)	Size of digested PCR product (bp)	Variants carrying mutations at various positions
8	F8- <i>SspI</i> R8	ATGTATTGTGGTTATGCGGAAT TGCTGGCGATAGTGGATCTT	AAT↓ ATT (<i>SspI</i>)	300	278, 22	<i>bla</i> _{SHV-7} , <i>bla</i> _{SHV-14} , <i>bla</i> _{SHV-18}
179	S-4 R179- <i>HinfI</i>	TCAGCGAAAAACACCTTGC ATGCTGGCCGGGGTAGTGGAG	G↓AN TC (<i>HinfI</i>)	233	214, 19	<i>bla</i> _{SHV-6} , <i>bla</i> _{SHV-8} , <i>bla</i> _{SHV-24}
205	S-4 R209- <i>PstI</i>	TCAGCGAAAAACACCTTGC CGATCGTCCACCATCCAGTG	CTG CA↓G (<i>PstI</i>) ^c	322	293, 29	<i>bla</i> _{SHV-3} , <i>bla</i> _{SHV-4}
238	F238- <i>BsrI</i> S-8	ATCGCCGATAAGACCGGAAGTGC AGTCATATCGCCCGGCAC	ACTG GN↓ (<i>BsrI</i>)	248→175, 73 ^d	151, 73, 24	<i>bla</i> _{SHV-13} , <i>bla</i> _{SHV-18}
240	F240- <i>NruI</i> S-8	CCGATAAGACCGGAGTTCGC AGTCATATCGCCCGGCAC	TCG↓C GA (<i>NruI</i>)	248	225, 23	<i>bla</i> _{SHV-4} , <i>bla</i> _{SHV-5} , <i>bla</i> _{SHV-7} , <i>bla</i> _{SHV-9} , <i>bla</i> _{SHV-10} , <i>bla</i> _{SHV-12} , <i>bla</i> _{SHV-15} , <i>bla</i> _{SHV-17} , <i>bla</i> _{SHV-18} , <i>bla</i> _{SHV-22} , <i>bla</i> _{SHV-23}

^a Underlining indicates the nucleotides involved in the restriction site. Boldface type indicates mismatch nucleotides that create or delete the restriction site.

^b The down arrow (↓) indicates the position at which the chosen restriction endonuclease cleaves DNA. The line (|) indicates the position of the 3' terminus of the mismatch primer. Boldface type indicates mismatch nucleotides that create or delete the restriction site. Underlining indicates nucleotides found in *bla*_{SHV-1}, where mutant forms of the gene have differing bases.

^c The *PstI* restriction recognition site affecting amino acid positions 208 and 209 of all *bla*_{SHV} genes was removed by the mismatch primer, which changed CTGCAG to CTGCAC.

^d There is another *BsrI* restriction site within the 248-bp amplicon; thus, wild-type amplicon is digested to yield three fragments, whereas the mutant amplicon yields two fragments.

TABLE 2. Restriction patterns of PCR products showing mutations affecting amino acids at various positions compared with *bla*_{SHV-1}

Amino acid position ^a in <i>bla</i> _{SHV} (codons in <i>bla</i> _{SHV-1} ^b and mutations in other <i>bla</i> _{SHV} genes)	Endonuclease ^c	Size(s) (bp) of DNA fragment(s) in <i>bla</i> _{SHV-1}	Size(s) (bp) of DNA fragment(s) generated by mutation	Genes carrying mutations affecting amino acids at various positions
35 (CTA→CAA)	<i>DdeI</i>	492, 198, 137	629, 198	<i>bla</i> _{SHV-2a} , <i>bla</i> _{SHV-11} , <i>bla</i> _{SHV-12} , <i>bla</i> _{SHV-13} , <i>bla</i> _{SHV-15} , <i>bla</i> _{SHV-25}
43 (CGC→AGC)	<i>BglI</i>	333, 237, 165, 92	498, 237, 92	<i>bla</i> _{SHV-7} , <i>bla</i> _{SHV-14} , <i>bla</i> _{SHV-18}
54 (GGC→deletion)	<i>EagI</i> , <i>EclXI</i> , <i>Eco52I</i> , <i>NotI</i> , <i>XmaIII</i>	634, 193	824 ^d	<i>bla</i> _{SHV-9} , <i>bla</i> _{SHV-10}
80 (GTG→ATG)	<i>BstI</i>	389, 276, 162	665, 162	<i>bla</i> _{SHV-15}
122 (CTC→TTC)	<i>EcoRI</i> , <i>RsrI</i>	827	433, 394	<i>bla</i> _{SHV-21} , <i>bla</i> _{SHV-23}
129 (ATG→GTG)	<i>Hsp92II</i> , <i>NlaIII</i>	236, 171, 170, 147, 66, 36	236, 207, 170, 147, 66	<i>bla</i> _{SHV-25}
130 (AGC→GGC)	<i>BstXI</i> , <i>DsaI</i> , <i>EcoT14I</i> , <i>NcoI</i> , <i>StyI</i>	827	414, 410 ^d	<i>bla</i> _{SHV-10}
140 (GCC→CGG)	<i>AvaII</i> , <i>Eco47I</i> , <i>RsrII</i>	686, 141	450, 233, 141 ^d	<i>bla</i> _{SHV-9} , <i>bla</i> _{SHV-10}
140 ^e (GCC→ACN) or 141 ^e (ACC→GCN)	<i>BalI</i> , <i>MscI</i>	375, 264, 188	636, 188 ^d	<i>bla</i> _{SHV-1} , <i>bla</i> _{SHV-2}
156 (GGC→GAC)	<i>BglI</i> <i>TaqI</i>	333, 237, 165, 92 827	425, 237, 165 498, 329	<i>bla</i> _{SHV-27}
158 (AAC→AAG)	<i>MaeII</i> , <i>HpyCH4IV</i> <i>BstEII</i> , <i>BstPI</i> , <i>Eco91I</i> , <i>Eco065I</i>	506, 321 827	827 506, 321	<i>bla</i> _{SHV-22}
173 (CTT→TTT)	<i>Bsp143II</i> , <i>BstH2I</i> , <i>HaeII</i>	434, 192, 116, 85	434, 201, 192	<i>bla</i> _{SHV-19} , <i>bla</i> _{SHV-20} , <i>bla</i> _{SHV-21}
179 (GAC→AAC)	<i>BstUI</i>	287, 164, 161, 68, 64, 54, 29	316, 164, 161, 68, 64, 54	<i>bla</i> _{SHV-8}
179 (GAC→GGC)	<i>Cfr42I</i> , <i>KspI</i> , <i>Sfr303I</i> , <i>SacII</i> , <i>SstII</i>	827	567, 260	<i>bla</i> _{SHV-24}
187 (GCC→ACC)	<i>BglI</i>	333, 237, 165, 92	333, 329, 165	<i>bla</i> _{SHV-26}
188 (GCG→GGG)	<i>AvaII</i> , <i>Eco47I</i> , <i>SinI</i> <i>Eco0109I</i>	686, 141 827	596, 141, 90 596, 231	<i>bla</i> _{SHV-23}
192 (AAG→AAC)	<i>MaeII</i>	506, 321	504, 218, 102 ^d	<i>bla</i> _{SHV-9} , <i>bla</i> _{SHV-10}
193 (CTG→GTG)	<i>AluI</i>	306, 153, 151, 91, 84, 42	348, 151, 150, 91, 84 ^d	
238 (GGC→AGC)	<i>MaeI</i> , <i>NheI</i>	971 ^f	744, 227	<i>bla</i> _{SHV-2} to <i>bla</i> _{SHV-5} , <i>bla</i> _{SHV-2a} , <i>bla</i> _{SHV-7} , <i>bla</i> _{SHV-9} , <i>bla</i> _{SHV-10} , <i>bla</i> _{SHV-12} , <i>bla</i> _{SHV-15} , <i>bla</i> _{SHV-20} to <i>bla</i> _{SHV-23}

^a Amino acid position numbering according to the scheme of Ambler et al. (1).

^b *K. pneumoniae bla*_{SHV-1} gene, strain KPZU-8, GenBank accession number X98100 (14). Mutations are highlighted by boldface type.

^c Alternative restriction endonucleases are included.

^d Mutations affecting amino acids at positions 130, 140, 192, and 193 are found in *bla*_{SHV-9} (except position 130) and *bla*_{SHV-10} genes that also have a deletion of amino acid at position 54; thus, both genes yield an amplicon of 824 bp.

^e *bla*_{SHV-1} and *bla*_{SHV-2} genes determined by Barthelemy et al. (cited in reference 6) have threonine (ACN) and alanine (GCN) at amino acid positions 140 and 141, respectively.

^f Using primer S-8 (this study), as a reverse primer for PCR amplification, instead of that described previously (3).

TABLE 3. Identification of genes encoding SHV β -lactamases by a combination of PCR-RFLP and RSI-PCR analysis

Mutations detected by PCR-RFLP analysis or RSI-PCR with ^a :									<i>bla</i> _{SHV} variants yielding amplimers with various RFLP and RSI profiles ^c
<i>SspI</i> * (8 ^b)	<i>DdeI</i> (35)	<i>BglI</i>			<i>HinPI</i> * (179)	<i>PstI</i> * (205)	<i>NheI</i> (238)	<i>NruI</i> * (240)	
43	156	187							
—	—	—	—	—	—	—	—	—	[<i>bla</i> _{SHV-11}], <i>bla</i> _{SHV-16} , ^d <i>bla</i> _{SHV-19} (173/ <i>HaeII</i>), <i>bla</i> _{SHV-28} ^e
+	—	+	—	—	—	—	—	—	<i>bla</i> _{SHV-14}
+	—	+	—	—	—	—	—	+	<i>bla</i> _{SHV-18}
—	—	—	+	—	—	—	—	—	<i>bla</i> _{SHV-27}
—	—	—	—	+	—	—	—	—	<i>bla</i> _{SHV-26}
—	—	—	—	—	+	—	—	—	<i>bla</i> _{SHV-6} , <i>bla</i> _{SHV-8} (179/ <i>BstUI</i>), <i>bla</i> _{SHV-24} (179/ <i>SacII</i>)
—	+	—	—	—	—	—	—	—	[<i>bla</i> _{SHV-11}], <i>bla</i> _{SHV-13} (238/ <i>BsrI</i> *), <i>bla</i> _{SHV-25} (129/ <i>NlaIII</i>)
—	+	—	—	—	—	—	+	—	[<i>bla</i> _{SHV-2a}]
—	+	—	—	—	—	—	+	+	[<i>bla</i> _{SHV-12}], <i>bla</i> _{SHV-15} (80/ <i>BtsI</i>)
—	—	—	—	—	—	—	+	—	[<i>bla</i> _{SHV-2}], <i>bla</i> _{SHV-20} (173/ <i>HaeII</i>), <i>bla</i> _{SHV-21} (173/ <i>HaeII</i> and 122/ <i>EcoRI</i>)
—	—	—	—	—	—	+	+	—	[<i>bla</i> _{SHV-3}]
—	—	—	—	—	—	+	+	+	[<i>bla</i> _{SHV-4}]
—	—	+	—	—	—	—	+	+	<i>bla</i> _{SHV} ^f
+	—	+	—	—	—	—	+	+	<i>bla</i> _{SHV-7}
—	—	—	—	—	—	—	+	+	[<i>bla</i> _{SHV-5}], <i>bla</i> _{SHV-9} (54/ <i>NotI</i>), <i>bla</i> _{SHV-10} (54/ <i>NotI</i> and 130/ <i>StyI</i>), <i>bla</i> _{SHV-22} (158/ <i>MaeII</i>), <i>bla</i> _{SHV-23} (122/ <i>EcoRI</i> and 188/ <i>AvaII</i>)

^a Mutations (amino acid positions are shown in parentheses or as subordinate headings) are detected by either PCR-RFLP analysis or RSI-PCR (indicated by asterisks). Symbols —, yielding amplimers with the same PCR-RFLP or RSI-PCR pattern as that of *bla*_{SHV-1}; +, yielding amplimers with PCR-RFLP or RSI-PCR pattern generated by mutation.

^b Amino acid position numbering according to Ambler et al. (1).

^c Brackets indicate the *bla*_{SHV} genes that are commonly found and can be differentiated by detecting four mutations (*DdeI*, position 35; *PstI*, position 205; *NheI*, position 238, and *NruI*, position 240) (see text).

^d Parentheses enclose mutation position and restriction endonuclease for PCR-RFLP or RSI-PCR (indicated by asterisk) analysis that can be applied for further identification of the gene. The gene encoding SHV-16 (Arpin et al., GenBank accession no. AF072684) can be differentiated from that encoding SHV-1 by comparing sizes of their amplimers using a pair of primers identifying the mutation at position 179; the amplimer generated from *bla*_{SHV-16} will yield a fragment of 250 bp, whereas that of *bla*_{SHV-1} will give a 235-bp fragment.

^e The gene encoding SHV-28 (Yu et al., GenBank accession no. AF299299) differs from that encoding SHV-1 by an amino acid substitution at position 7 from tyrosine in SHV-1 to phenylalanine in SHV-28. No restriction endonucleases detecting this mutation are available.

^f This gene was described by Winokur et al. and appears to be identical to SHV-17, which has been withdrawn (see text).

a mutation that creates a *PstI* site, such as the mutations that cause alterations in the amino acid at position 205. In this study, target restriction endonucleases were chosen based on a recognition site at least 5 bp in length, for their cost-effectiveness and for their commercial availability.

When amplifying *bla*_{SHV-1}, the primers, with the exception of the primer pair that detects mutations affecting the amino acid at position 205, all generated the expected novel restriction sites that yield fragments of the predicted sizes when digested by their specific restriction endonucleases (Fig. 1 and 2). Amplimers from *bla*_{SHV} genes carrying mutations remained undigested by these endonucleases. Since there is a *BsrI* restriction site within the 248-bp product amplified by a pair of primers identifying the mutation that alters the amino acid at position 238 (Table 1) and the mismatch primer creates a second *BsrI* restriction site, after digestion with *BsrI* amplimers from the wild type and the mutant yielded fragments as predicted (Table 1 and Fig. 1). For the primer pair designed to detect mutations affecting the amino acid at position 205, the *bla*_{SHV-1} amplimer remained undigested, whereas the mutant *bla*_{SHV} amplimers were digested to yield fragments of the appropriate sizes (Fig. 2).

As of 10 January 2001, (last date accessed by us) 29 variants of the *bla*_{SHV} gene have been deposited in GenBank. This study also makes theoretical considerations for the identification of *bla*_{SHV-6}, *bla*_{SHV-8}, *bla*_{SHV-14}, *bla*_{SHV-18} to *bla*_{SHV-23}, and *bla*_{SHV-25} to *bla*_{SHV-27} using the PCR-RFLP technique. DNASIS as described previously (2) and Webcutter 2.0 were used to identify restriction endonucleases capable of distinguishing the point mutations of these *bla*_{SHV} genes. All muta-

tions of the *bla*_{SHV} genes that can be detected by PCR-RFLP analysis from a previous study (3) and this study are summarized in Table 2. Although these studies make a theoretical PCR-RFLP analysis for the differentiation of genes encoding SHV β -lactamases, the technique relies on the high specificity of restriction endonucleases for their restriction sites. Thus, amplimers of SHV mutant genes would yield predicted PCR-RFLP patterns if mutations are present in their nucleotide sequences as described. This technique has been proved to identify *bla*_{SHV} variants successfully as predicted (3)—in this case, a novel gene, *bla*_{SHV-27}. The mutation affecting the amino acid at position 156 of the *bla*_{SHV-27} gene can be detected easily using PCR-RFLP analysis with either *BglI* or *TaqI* (data not shown).

The RSI-PCR technique has been developed to extend the identification of SHV β -lactamases by PCR-RFLP analysis as described previously (3). By a combination of both techniques, genes encoding the 27 SHV variants *bla*_{SHV-2} to *bla*_{SHV-27} may be differentiated as proposed in Table 3. Common *bla*_{SHV} variants such as *bla*_{SHV-1} to *bla*_{SHV-5}, *bla*_{SHV-2a}, *bla*_{SHV-11}, and *bla*_{SHV-12} (8, 9) may be differentiated by detecting four mutations affecting the amino acids at positions 35, 205, 238 (Gly→Ser), and 240 (Table 3). However, this study suggests that other *bla*_{SHV} variants that are uncommon may be underestimated due to the lack of methods suitable for screening these genes in instances when many isolates are to be characterized. The approach in Table 3 may be useful for the differentiation of all known SHV β -lactamase genes described to date. Although the nucleotide sequence of the gene previously designated *bla*_{SHV-17} has recently been withdrawn from Gen-

Bank, a novel SHV-type extended spectrum β -lactamase also designated SHV-17 by Winokur et al. (P. L. Winokur, D. L. Desalvo, R. N. Jones, and M. A. Pfaller, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother., abstr. 2045, 1999) has three amino acid substitutions at positions 43 (Arg→Ser), 238 (Gly→Ser), and 240 (Gln→Lys) identical to those described in the sequence withdrawn from GenBank. Thus, this SHV variant is included in the identification scheme. In the present study, the PCR-RFLP technique cannot differentiate the *bla*_{SHV-28} gene from the *bla*_{SHV-1} gene since there are no restriction endonucleases that can detect a mutation affecting the amino acid at position 7 of the *bla*_{SHV-28} gene. However, RSI-PCR could be applied to identify this mutation. In addition, the gene encoding SHV-16 may be distinguished from that encoding SHV-1 by the sizes of their amplimers using a pair of primers identifying mutation at position 179. The amplimer generated from *bla*_{SHV-16} will yield a fragment of 250 bp, whereas that of *bla*_{SHV-1} will give a 235-bp fragment since the product of the *bla*_{SHV-16} gene has an extra five amino acids, starting at position 167.

The PCR-RFLP and RSI-PCR techniques can be used as screening methods for groups of strains when many isolates are to be characterized or when it is not possible to apply nucleotide sequence determination. Either PCR-RFLP or RSI-PCR analysis can also be applied to confirm new mutations demonstrated by nucleotide sequence analysis, allowing new SHV variants to be differentiated. The flexibility of RSI-PCR, with the ability to create or remove restriction sites, makes this the method of choice for characterizing newly described variants of *bla*_{SHV}. A limitation of this technique is that it can only detect mutations at sites where the current range of primers create restriction sites. In new epidemiological studies, there may be *bla*_{SHV} variants with mutations in previously undescribed positions. Nucleotide sequence analysis is thus still required to confirm absolutely the nature of any *bla*_{SHV} encountered in such studies.

PCR-RFLP and RSI-PCR techniques are simple and rapid and require only basic molecular biology equipment, namely, a thermocycler and simple electrophoresis apparatus. These techniques are readily applied to epidemiological studies of the genes encoding variant SHV β -lactamases and may easily be extended to the discrimination of other polymorphic resistance determinants.

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