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 \( \text{bla}_{\text{SHV}} \) 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 (HindII), 205 (PstI), 238 (Gly→Ala) (BsrI), and 240 (NruI) of \( \text{bla}_{\text{SHV}} \) genes. All amplimers of the \( \text{bla}_{\text{SHV}} \) genes used in this study yielded the predicted restriction endonuclease digestion products. In addition, this study also makes theoretical identification of 8 special 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. Recent applications of RSI-PCR to differentiate the \( \text{bla}_{\text{SHV}} \) genes have been reported recently: \( \text{bla}_{\text{SHV}}\) (C. Arpin, R. Labia, F. Tessier, and C. Quentin, GenBank accession no. AF072684), \( \text{bla}_{\text{SHV}}\) (D. M. Livermore, GenBank accession no. AF117747), \( \text{bla}_{\text{SHV}}\) to \( \text{bla}_{\text{SHV}}\) variants (5), \( \text{bla}_{\text{SHV}}\) (S. Y. Essack, L. M. C. Hall, and D. M. Livermore, GenBank accession no. AF117747), \( \text{bla}_{\text{SHV}}\) and \( \text{bla}_{\text{SHV}}\) 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 \( \text{bla}_{\text{SHV}} \) variants have also been reported recently: \( \text{bla}_{\text{SHV}}\) (J. E. Corkill, C. A. Hart, L. Cuevas, and J. Greensill, GenBank accession no. AF208796 and AF227204, respectively), \( \text{bla}_{\text{SHV}}\) (J. E. Corkill, C. A. Hart, L. Cuevas, and J. Greensill, GenBank accession no. AF293345), and \( \text{bla}_{\text{SHV}}\) (Y. Yu, W. Zhou, and Y. Chen, GenBank accession no. AF299299). This further complicates the characterization of the genes of the \( \text{bla}_{\text{SHV}} \) family, and we have included them in our study.

Restiction 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 \( \text{bla}_{\text{SHV}} \) genes that cannot be identified unambiguously by PCR-RFLP analysis. Thus, \( \text{bla}_{\text{SHV}}\), \( \text{bla}_{\text{SHV}}\), \( \text{bla}_{\text{SHV}}\), and \( \text{bla}_{\text{SHV}}\) 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 \( \text{bla}_{\text{SHV}}\) 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 \( \text{bla}_{\text{SHV}} \) variants have also been reported recently: \( \text{bla}_{\text{SHV}}\) (C. Arpin, R. Labia, F. Tessier, and C. Quentin, GenBank accession no. AF072684), \( \text{bla}_{\text{SHV}}\) (D. M. Livermore, GenBank accession no. AF117747), \( \text{bla}_{\text{SHV}}\) and \( \text{bla}_{\text{SHV}}\) genes (3). In addition, no commercial supplies are available for restriction endonuclease BceII, used to demonstrate mutations affecting the amino acid at position 205.
MATERIALS AND METHODS

Bacterial strains. Eight standard strains were used in this study, including Escherichia coli C600(R1010), encoding \textit{bla}SHV-1; E. coli C600(pMG229), encoding \textit{bla}SHV-2; E. coli J53-2(pUD18), encoding \textit{bla}SHV-3; Klebsiella pneumoniae K25, encoding \textit{bla}SHV-4; E. coli HB101(pAFF611), encoding \textit{bla}SHV-5; E. coli C1A(pSLH06), encoding \textit{bla}SHV-6; E. coli strain 2-75, encoding \textit{bla}SHV-8; and K. pneumoniae ATCC 700603, encoding \textit{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 \textit{bla}SHV-1 flanking the primers; thus, a restriction site is created on an amplimer of the \textit{bla}SHV-1 gene (Table 1). These included the primers that detect mutations affecting amino acids at positions 8 (SspI), 179 (HinfI), 238 (BsrI), and 240 (NruI). A further mismatch primer pair was designed to remove the PstI restriction site affecting the amino acid at positions 205. The mismatch primer pair was thus yield an amplimer that will only be digested by the PstI restriction endonuclease if it is generated from genes that carry

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 \textit{bla}SHV variants described to date are derived from \textit{bla}SHV-1 with one to seven amino acid substitutions. Thus, the primers were designed to create a restriction site specific to the \textit{bla}SHV-1, except the primer detecting mutations affecting the amino acid at position 205. The latter primer was designed to delete a PstI restriction site found just downstream at the nucleotides encoding amino acids 208 and 209. This site is present in all \textit{bla}SHV genes. The mismatch primer pair will thus yield an amplimer that will only be digested by the PstI restriction endonuclease if it is generated from genes that carry
Amino acid affected | Primer | Oligonucleotide sequence (5′−3′) | Restriction recognition site (enzyme) | PCR product (bp) | Size of digested PCR product (bp) | Variants carrying mutations at various positions
--- | --- | --- | --- | --- | --- | ---
8 | F8-SpI R8 | ATGTATTTGGTATTGCGGAGTT | AAT1 | 278, 22 | blaSHV-5, blaSHV-14, blaSHV-15
179 | S-4 R179-HinfI | TCACTGGGAAAAACCTTTGC | G | 233 | blaSHV-6, blaSHV-8, blaSHV-24
205 | S-4 R209-PstI | TCACTGGGAAAAACCTTTGC | CTG/C/A | 322 | blaSHV-3, blaSHV-4
238 | F238-BsrI S-8 | ATCGCCGATAAGACCAGGA | ACTG/C/G | 498 | blaSHV-10, blaSHV-15, blaSHV-16
240 | F240-NruI S-8 | AGTCTAAGACCGGAGTT | TCG/C/G(A)/NruI | 248 | blaSHV-4, blaSHV-6, blaSHV-8, blaSHV-9, blaSHV-12

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 blaSHV-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 SHV genes was removed by the mismatch primer, which changed CTGCCAG to CTGCAC.

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

| Amino acid position in blaSHV (codons in blaSHV, and mutations in other blaSHV genes) | Endonuclease | Size(s) (bp) of DNA fragment(s) generated by mutation | Genes carrying mutations affecting amino acids at various positions
--- | --- | --- | ---
35 (CTA→CAA) | BstI | 492, 198, 137 | blaSHV-7, blaSHV-10, blaSHV-12, blaSHV-13
43 (GC→AGC) | BglI | 333, 237, 165, 92 | 498, 237, 92 | blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-25
54 (GC→deletion) | EagI, EclXI, Eco521, NorI, XmaIII | 634, 193 | 824<sup>a</sup> | blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-25
80 (GTG→ATG) | BstI | 389, 276, 162 | 665, 162 | blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-25
122 (CTC→TTC) | EcoRI, BglI | 236, 171, 170, 147, 66, 36 | 236, 207, 170, 147, 66 | blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-25
129 (ATG→GTG) | Hpy922, Bsu36I | 827 | 433, 394 | blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-25
130 (AGC→GGC) | BstXI, DasI, EcoT14I, NcoI, SylI | 827 | 414, 410<sup>a</sup> | blaSHV-10
140 (GCC→CGG) | AvrII, Eco471, RsaII | 868, 141 | 450, 233, 141<sup>a</sup> | blaSHV-10
140<sup>b</sup> (GCC→ACN) or 141<sup>c</sup> (ACC→GCN) | BclI, MscI | 375, 264, 188 | 636, 188<sup>b</sup> | blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-25
156 (GGC→GAC) | BglI | 333, 237, 165, 92 | 425, 237, 165 | blaSHV-27
158 (AAC→AAG) | MreII, HpyCH4IIV | 506, 321 | 827 | blaSHV-22
173 (CTT→TTT) | Bsp134II, BstHII, HaeII | 434, 192, 116, 85 | 434, 201, 192 | blaSHV-19, blaSHV-26, blaSHV-21
179 (GAC→AAC) | BstUI | 287, 164, 161, 68, 64, 54 | 316, 164, 161, 68, 64, 54 | blaSHV-19, blaSHV-26, blaSHV-21
179 (GAC→GGC) | CfoI, KelI, Stf301, SacII, SrrI | 827 | 506, 321 | blaSHV-24
188 (GGC→GGG) | AvrII, Eco47I, SstI | 668, 141 | 596, 141, 90 | blaSHV-23
192 (AAG→AAC) | MreII | 506, 321 | 504, 218, 102<sup>a</sup> | blaSHV-19, blaSHV-26, blaSHV-10
193 (CAG→GTG) | AatI | 306, 153, 151, 91, 84, 42 | 348, 151, 150, 91, 84<sup>a</sup> | blaSHV-19, blaSHV-26, blaSHV-10
238 (GGC→AGC) | MreI, NheI | 971<sup>a</sup> | 744, 227 | blaSHV-2, blaSHV-5, blaSHV-26, blaSHV-7, blaSHV-9, blaSHV-10, blaSHV-12, blaSHV-15, blaSHV-16, blaSHV-22, blaSHV-23

<sup>a</sup>Amino acid position numbering according to the scheme of Ambler et al. (1).<br><sup>b</sup>K. pneumoniae blaSHV-1, gene, strain KPZU-8, GenBank accession number X08100 (14). Mutations are highlighted by boldface type.<br><sup>c</sup>Alternative restriction endonucleases are included.<br><sup>d</sup>Mutations affecting amino acids at positions 130, 140, 192, and 193 are found in blaSHV-9 (except position 130) and blaSHV-10 genes that also have a deletion of amino acid at position 54; thus, both genes yield an amplimer of 824 bp.<br><sup>e</sup>blaSHV-5 and blaSHV-6 genes determined by Barthélémy et al. (cited in reference 6) have threonine (ACN) and alanine (GCN) at amino acid positions 140 and 141, respectively.<br><sup>f</sup>Using primer S-8 (this study), as a reverse primer for PCR amplification, instead of that described previously (3).
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<sub>SHV-1</sub>, 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<sub>SHV</sub> 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<sub>SHV-1</sub> primer remained undigested, whereas the mutant bla<sub>SHV</sub> 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<sub>SHV</sub> gene have been deposited in GenBank. This study also makes theoretical considerations for the identification of bla<sub>SHV-6</sub>, bla<sub>SHV-14</sub>, bla<sub>SHV-18</sub> to bla<sub>SHV-23</sub>, and bla<sub>SHV-25</sub> to bla<sub>SHV-27</sub> 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<sub>SHV</sub> genes. All mutations of the bla<sub>SHV</sub> 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<sub>SHV</sub> variants successfully as predicted (3)—in this case, a novel gene, bla<sub>SHV-27</sub>. The mutation affecting the amino acid at position 156 of the bla<sub>SHV-1</sub> gene has been withdrawn from GenBank. This gene was described by Winokur et al. and appears to be identical to SHV-17, which has been withdrawn (see text).

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

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**Notes:**
- Mutations (amino acid positions are shown in parentheses or as subheadings) 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<sub>SHV-1</sub>; +, yielding amplimers with PCR-RFLP or RSI-PCR pattern generated by mutation.
- Amino acid position numbering according to Ambler et al. (1).
- Brackets indicate the cases where mutations can be used to identify restriction endonucleases capable of distinguishing the point mutations of these bla<sub>SHV</sub> genes.
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 \( bla_{SHV-26} \) 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 \( bla_{SHV-26} \) 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 amplifiers using a pair of primers identifying mutation at position 79. The amplimer generated from \( bla_{SHV-16} \) will yield a fragment of 250 bp, whereas that of \( bla_{SHV-1} \) will generate a 235-bp fragment since the product of the \( bls_{SHV-16} \) 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 \( bls_{SHV} \) variants with mutations in previously undescribed positions. Nucleotide sequence analysis is thus still required to confirm absolutely the nature of any \( bls_{SHV} \) 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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REFERENCES