Brief reports

Detection of mutations conferring extended-spectrum activity on SHV β-lactamases using polymerase chain reaction single strand conformational polymorphism (PCR-SSCP)

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Single strand conformational polymorphism (SSCP) is a recently developed technique used to detect single base mutations in short PCR-generated amplimers. The method has been adapted and applied to differentiation of β-lactamase genes. Each of the five standard SHV strains used produced a unique SSCP pattern, allowing the possibility of rapid identification of the SHV genes of other isolates. A clinical isolate that phenotypically produced SHV-5 yielded a pattern of major bands indistinguishable from that of the SHV-5 standard strain, illustrating the applicability of this technique. We therefore report a reliable and reproducible technique that can be applied to the characterisation of the SHV β-lactamases.

Introduction

Soon after the introduction of extended-spectrum cephalosporins into widespread clinical use, strains resistant to these agents emerged (Philippon, Labia & Jacoby, 1989). The first SHV-type extended-spectrum β-lactamase was reported by Knothe et al. (1983) who described a transferable determinant in clinical isolates of Klebsiella pneumoniae and Serratia marcescens conferring resistance to cefotaxime. The basis of this resistance was a β-lactamase that was closely related to the SHV-1 β-lactamase, and this enzyme was later designated SHV-2 (Kleibe et al., 1985). Other groups have reported the occurrence of extended-spectrum β-lactamases belonging to the SHV family (Barthelemy et al., 1988; Bure et al., 1988; Jarlier et al., 1988; Gutmann et al., 1989). The evolutionary relationship of the SHV family of β-lactamases is summarised in Figure 1. This small family of well-defined β-lactamases provides an opportunity to develop a rapid and reliable method of characterising and identifying mutations that are responsible for the extended-spectrum cephalosporinase phenotype demonstrated by representatives of this group.

Until now, a complete characterisation of the genes encoding these enzymes has required determination of the nucleotide sequence of the resistance determinant. Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP),
first described by Orita et al. (1989) for the detection of mutations in human genes, was chosen as a simpler method of characterising these genes.

PCR-SSCP relies upon the observation that single nucleotide mutations affect the electrophoretic mobility of very short sequences of single-stranded DNA. Point mutations cause these short sequences to migrate at a different and characteristic rate from the parental DNA. This allows identification of strains with known mutations by comparison of the electrophoretic mobility of a short amplimer from the test strain with that of amplimers generated using known standard genes as the PCR template. Generation of an amplimer with a different electrophoretic mobility from the pattern obtained from standard strains permits the presumptive identification of a novel mutation in the gene family. Thus PCR-SSCP allows for a rapid screening of strains to identify known mutations and to indicate novel mutants for confirmation by a full nucleotide sequence determination.

Materials and methods

Bacterial strains

The five strains, each producing one of the five recognised SHV β-lactamases, were: Escherichia coli C600 (R1010) encoding SHV-1; E. coli C600 (pMG229) encoding SHV-2 donated by Prof. G. A. Jacoby, Massachusetts General Hospital, Boston, USA; E. coli strain J53-2(pUD18) encoding SHV-3; Klebsiella pneumoniae K25 expressing SHV-4, both donated by Prof. M. H. Nicolas, Ambroise Paré Hospital, Boulogne, France; and E. coli HB101(pAFF611) encoding SHV5 and provided by Prof. E. Collatz, University of Paris, Paris, France.

Klebsiella pneumoniae strain 99866P, isolated from a wound swab from a patient at the Leeds General Infirmary, and producing a β-lactamase with an iso-electric point
equivalent to a pI value of 8.2, and with a substrate profile consistent with the SHV-family was also used in this study.

Iso-electric focusing

Analytical iso-electric focusing was carried out as described by Heritage et al. (1992), except that the enzyme was extracted from cells grown in Brain Heart Infusion (Oxoid, Basingstoke, UK) using the sonication method described by Seetulsingh, Hall and Livermore (1991).

PCR amplification

A pair of primers was used to amplify a 475 bp sequence of the SHV gene. These have the following nucleotide sequences: 5'-TCAGCGAAAAACACCTTG-3' and 5'-TCCCGCAGATAATCAACCA-3'. For amplification, 30 pmoles of each primer were mixed in a sterile 500 μL microcentrifuge tube with 10 μL of magnesium chloride, 5 μL of Taq DNA polymerase reaction buffer (Promega Ltd, Southampton, UK) and 4 μL of a 2.5 mM dNTP pool, made up from the Ultrapure dNTP set (Pharmacia, Milton Keynes, UK). The amplification mixture was made up to 45 μL with sterile distilled water, and then 2 U of SuperTaq DNA polymerase (HT Biotechnologies, Cambridge, UK) were added. Finally, 5 μL of DNA template were added to the reaction mixture. The template was prepared by picking off three or four colonies of the strain to be tested from an overnight culture on IsoSensitest Agar (Oxoid, Basingstoke, UK), into 50 μL distilled water and heating the cells at 95°C for 5 min. The 50 μL amplification mixture was overlayed with approximately 20 μL of mineral oil.

The 475 bp fragment of the SHV gene was amplified in an Omnigene thermocycler (Hybaid, Teddington, UK) using 30 amplification cycles. Each cycle consisted of a denaturation step at 94°C for 15 sec, followed by an annealing step at 60°C for 30 sec and an extension step at 72°C for 1 min. The final elongation period was for 5 min at 72°C.

Polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis

The 475 bp SHV amplimer generated using PCR was digested with 5 U Pst I restriction endonuclease (Boehringer Mannheim, Lewes, UK) according to the manufacturer's instructions for 1.5 h at 37°C to yield one 300 bp fragment and one 175 bp fragment. The digested amplimer was then denatured to yield single-stranded DNA fragments by mixing 2 μL of the digestion product with an equal volume of denaturation solution comprising 97% deionised formamide, 4.6 M urea, 0.3% bromophenol blue, 0.3% xylene cyanol and 10 mM EDTA. The mixture was then heated in a thermocycler at 95°C for 5 min and separated on a PhastSystem (Pharmacia, Milton Keynes, UK), and using a PhastGel homogeneous 20 system with PhastGel native buffer strips. The program had three separation steps as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Voltage (V)</th>
<th>Current (mA)</th>
<th>Power (W)</th>
<th>Temperature (°C)</th>
<th>Time (Vh)</th>
</tr>
</thead>
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<tr>
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<td>10</td>
<td>2.5</td>
<td>15</td>
<td>100</td>
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<tr>
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<td>400</td>
<td>1</td>
<td>2.5</td>
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<tr>
<td>3</td>
<td>400</td>
<td>10</td>
<td>2.5</td>
<td>15</td>
<td>500</td>
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</tbody>
</table>
Results and discussion

All strains used in this study carried SHV-encoding DNA sequences that were amplified using the described primers and PCR conditions. Upon PCR-SSCP analysis, each of the five representative SHV β-lactamases yielded a unique profile (Figure 2). To test the applicability of this technique for identification of unknown SHV β-lactamases, a clinical isolate was also included in this study. Phenotypically, this strain produced the SHV-5 enzyme, as shown by its resistance profile and its iso-electric point of 8.2.
PCR-SSCP detection of SHV \( \beta \)-lactamase mutants

PCR-SSCP analysis of this strain showed a pattern of major bands indistinguishable from the SHV-5 standard strain. This technique has been repeated on 17 occasions using the five standard strains, and has been used to characterise nine clinical isolates. On every occasion a consistent and reproducible PCR-SSCP pattern has been obtained, confirming biochemical and iso-electric focusing data.

PCR-SSCP was first developed by Orita et al. (1989) to examine mutations in the human genome. The technique makes possible the detection of both known and unknown single point mutations, and the method has been applied to detect genetic alterations in a number of human diseases. For the successful detection of point mutations, it is recommended that the PCR amplimer to be examined should be no more than 200 base-pairs long. The SHV primers used in this study generate an amplimer 475 bp in length, but this amplimer carries a unique \textit{Pst} I site, and digestion with this restriction endonuclease generates two fragments, one of 300 bp and one of 175 bp. The Pharmacia PhastSystem permits a highly standardised method of running analytical gels and thus provides the reproducibility required for the successful implementation of the PCR-SSCP technique.

With PCR-amplified DNA the fidelity of base incorporation has occasionally been shown to be inaccurate, but the reproducibility of the method for the identification of SHV \( \beta \)-lactamases described here was not affected. This was probably because short amplimers were required, and to produce a detectable spurious result, the mis-substitution would have to occur within the first two or three cycles of the PCR reaction. Thereafter, such infidelity of replication would yield only a small number of molecules within the amplimer population, and these would become increasingly difficult to detect.

Until recently, the characterisation and identification of \( \beta \)-lactamases have relied upon the determination of their substrate profile, biochemical kinetic analysis and isolectric focusing properties. These methods are poorly reproducible, and can be technically demanding. The alternative approach is to undertake a full nucleotide sequence determination. This can also be technically demanding, and is time-consuming and expensive, though reliable. Oligonucleotide probing of regions of the TEM \( \beta \)-lactamase gene carrying known mutations has been applied to identification of mutants, but this method can detect only previously described mutants. PCR-SSCP affords the opportunity to screen a large array of strains for both known and unexpected mutations occurring within the amplimer. The technique enabled differentiation of all the known SHV \( \beta \)-lactamases. The genes for SHV-1 and SHV-3 yielded patterns that were very similar, but that could be distinguished with careful examination. This technique was also applied successfully to the identification of an SHV \( \beta \)-lactamase from a clinical strain.

The SHV family of extended-spectrum \( \beta \)-lactamases is small and well defined, and has proved to be a good model for the development of PCR-SSCP technology as applied to the characterisation of antibiotic resistance genes in bacteria. We aim to extend this technique to characterise the mutations that have given rise to the much larger family of TEM-derived extended spectrum \( \beta \)-lactamases.

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Prof. E. Collatz for providing strains that produce the standard SHV β-lactamases. Part of this work has been presented at the 35th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, 1995.

References


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