A rapid immunoassay method for the direct
detection of PCR products: application to detection
of TEM β-lactamase genes

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A rapid immunoassay for the detection of specific PCR products is described in which a positive PCR amplification result is detected, usually in less than 5 min, by applying a few drops of the diluted PCR end-product to a small immunoassay sample device. The method was evaluated in comparison with conventional susceptibility tests and isoelectric focusing (IEF) for the detection of TEM-family β-lactamase genes in 477 Escherichia coli isolates from urine samples. Of 187 isolates identified as presumptive TEM β-lactamase producers by conventional methods, 185 generated a positive signal in the PCR immunoassay system. Two further signal-positive isolates were recognised when the PCR was repeated. In addition, one of the 276 ampicillin-susceptible isolates gave a positive signal in repeated PCR-immunoassay experiments despite being ampicillin susceptible and failing to give a TEM-type enzyme band in iso-electric focusing experiments.

PCR techniques have been used in many laboratories for the direct detection of specific bacteria and viruses in clinical specimens – including blood, CSF and tissues – and have also been used in a few studies for the direct detection of bacterial antibiotic resistance genes in sputum samples and CSF [1-3]. Although the PCR itself is usually complete within 2-3 h, detection of specific PCR products requires a time-consuming electrophoresis step on agarose or polyacrylamide gels. This requirement limits the number of assays that can be completed within a working day and complicates the introduction of PCR tests into routine laboratories. This paper describes a new, simple and user-friendly immunoassay for the rapid detection of PCR products. With this system, a positive amplification result is detected, usually in less than 5 min, by applying a few drops of the diluted PCR end-product to a small immunoassay sample device.

To validate the system, 477 consecutive clinical isolates of Escherichia coli were examined by PCR for the presence of TEM family β-lactamase genes, and the results obtained were compared with those from conventional methods. All isolates were tested for ampicillin susceptibility by multipoint inoculation of c.10^4 cfu on to IsoSensitest Agar (Unipath) containing doubling dilutions of ampicillin (Smith-Kline Beecham, Brockham Park, Surrey). Where appropriate, cultures that were insusceptible to ampicillin 8 mg/L were tested for β-lactamase production by preparing crude extracts as described previously [4] and mixing these in microtitration plates in equal volumes with the chromogenic cephalosporin nitrocefin (Unipath) 1 mg/ml. β-Lactamases were characterised further by analytical iso-electric focusing (IEF) on cellulose acetate membranes [5], initially over a broad pH range of 2-11, followed by a narrow pH range of 4-6, as described previously [4].

Total DNA for PCR amplification was extracted from each isolate by emulsifying a small loopful of overnight growth from an agar plate in 100 μl of sterile H2O in a microfuge tube, overlaying with sterile mineral oil (Sigma), heating to 98°C for 15 min in a DB2A Dri Block (Techne, Cambridge), followed by cooling and diluting into 900 μl of sterile H2O. The resulting extract was centrifuged for a few seconds at 12000 g in a microcentrifuge. The super-
nate was then used directly in multiplex PCR reactions in which two sets of custom-synthesised (Oswel DNA Service, Southampton, Hants) labelled primers were present simultaneously. These primers (Table 1) were for TEM family β-lactamase genes and a conserved sequence coding for 16S rRNA. The specificity of these primer sets for their targets has been demonstrated previously [3, 6] and, in so far as data are available, all members of the TEM family are detected by the primer set used. After initial trial experiments, PCR mixes were amplified with the following set of conditions: 4 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 67°C and 1 min at 72°C, followed by 2 min at 72°C. Each PCR mix contained, in a final volume of 25 μl: 2.5 pmol of each of the four primers; 0.2 mM dNTPs (Boehringer Mannheim); 1.5 mM MgCl₂; 0.6 U Taq polymerase (Boehringer Mannheim); and 4 μl of bacterial DNA extract, prepared as above. The reaction end-product was diluted 1 μl in 99 μl of Tris-borate buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) and then applied to an immunoassay detection device (Unipath). This consisted of a small plastic device (Fig. 1) enclosing a membrane and a sample application pad containing blue latex beads labelled with an anti-dinitrophenol (DNP) antibody. The membrane was coated with lines of anti-biotin antibody (in the ‘test’ window) and antidigoxigenin (DIG) antibody (in the ‘control’ window). The labelled primers listed in Table 1 were designed to generate a DNP and biotin-labelled product from TEM-family β-lactamase genes, and a DNP and DIG-labelled product from the conserved sequence coding for 16S rRNA. Diluted PCR product (100 μl) was applied to the sample application pad. In the presence of a specific dual-labelled (DNP and biotin, or DNP and DIG) PCR end-product, the blue beads localised

<table>
<thead>
<tr>
<th>Primer</th>
<th>Label</th>
<th>Sequence</th>
<th>Target</th>
<th>Product</th>
<th>Reference no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>5'-triple-DNP</td>
<td>5'-GGAATTCAA/GAATTGAGGGGCGG</td>
<td>Conserved 16S rRNA sequence</td>
<td>479 bp</td>
<td>6</td>
</tr>
<tr>
<td>Y</td>
<td>5'-DIG</td>
<td>5'-CGGATCCAGGCCCAGGAACGTATTCAC</td>
<td>TEM β-lactamase genes</td>
<td>526 bp</td>
<td>3</td>
</tr>
<tr>
<td>Amp1</td>
<td>5'-triple-DNP</td>
<td>5'-TGGGTGACAGTGAGGTTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amp2</td>
<td>5'-biotin</td>
<td>5'-TTATCCGCTCCATCCAGTC</td>
<td></td>
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</tr>
</tbody>
</table>

DIG, digoxigenin; DNP, dinitrophenol.

Fig. 1. Examples of immunoassay detection devices showing positive and negative results after PCR amplifications with the labelled primers. a, no DNA template in PCR mix and no signal in either ‘test’ (lower) or ‘control’ (upper) window; b, ampicillin-susceptible isolate with a signal in the ‘control’ window only; c, ampicillin-resistant TEM+ isolate with signals in both the ‘test’ and ‘control’ windows.
to the appropriate antibody line on the membrane (i.e., in either the ‘test’ or ‘control’ window) to yield an easy-to-read blue line as the end-point. The ‘control’ line confirmed a negative ‘test’ result by indicating that sufficient DNA was present and that PCR amplification of the conserved control DNA sequence had occurred successfully. Results were usually obtained within 5 min, without washes or any additional manipulations.

In initial trial experiments, a range of primer concentrations and ratios (1–25 pmol of each primer/reaction mix) and annealing temperatures (50°–70°C) were tested, based on those used previously for these sets of primers [3, 6]. Addition of > 5 pmol of primers to reaction mixes, or the use of annealing temperatures at the lower end of the range tested, yielded false positive results, presumably because of non-specific amplification products or primer dimers. Specific and reproducible results were obtained with 2.5 pmol of each of the four primers and an annealing temperature of 67°C.

When tested with these conditions, all 477 isolates generated a positive result in the ‘control’ window, indicating that successful amplification of the conserved sequence coding for 16S rRNA had occurred. The signal in the ‘control’ window was sometimes rather faint if a TEM+ ‘test’ result was also obtained, but a strong ‘control’ signal was always obtained if the ‘test’ window was negative. Of 201 ampicillin-insusceptible isolates (MIC of > 8 mg/L), 187 were identified as presumptive TEM β-lactamase producers by conventional methods, and 185 of these gave a positive signal in the PCR-immunoassay system, indicating that PCR amplification of a TEM gene had occurred. The two isolates that appeared TEM-positive by conventional tests, but were initially negative by PCR, were repeatedly positive when the PCR was repeated. One of the 276 ampicillin-susceptible isolates generated a positive signal for TEM-family β-lactamase genes in repeated PCR-immunoassay experiments. As non-specific binding of the primer set has not been reported to occur [3], this isolate may have produced a defective or non-functional TEM enzyme or have contained a ‘silent’ TEM-family β-lactamase gene that was not being expressed.

The experiments described were designed to test the utility of the PCR-immunoassay system by detecting β-lactamase genes belonging to the TEM family in clinical isolates of E. coli. The system potentially could be adapted to detect any antibiotic resistance gene, or indeed any PCR amplification product, providing appropriate labelled primers are available. It is important that the PCR itself should be optimised to reduce the likelihood of non-specific amplification products. This was clearly not a problem in the detection of TEM β-lactamase genes with the conditions described in this paper, but multiplex PCR systems are particularly sensitive to varying primer concentrations and ratios. Further experiments will also be required to optimise the DNA template preparation and PCR amplification steps to ensure that reproducible results can be obtained from direct tests on clinical samples.

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References