Elimination of Bacterial DNA from Taq DNA Polymerases by Restriction Endonuclease Digestion

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The incidence of false positives due to the presence of bacterial DNA in Taq DNA polymerase is an obstacle to the use of PCR in the diagnosis of infection. We describe a method that uses a restriction enzyme to destroy the ability of contaminating sequences to act as templates for a nested PCR which uses primers based on the 16S rRNA genes. The method was used prior to a PCR that amplified 10 fg of bacterial DNA. This method can be readily adapted to suit other sensitive PCRs required for clinical applications.

Due to its ability to exponentially amplify regions of DNA, the PCR has the potential to be used as a diagnostic tool. It has been used in the detection and identification of a wide range of bacterial species using oligonucleotide primers based on the conserved regions of rRNA sequences. The detection of organisms which are difficult to cultivate has been improved by the use of this technique (1, 5, 8), as it relies on the presence of DNA and not the viability of the organism. The disadvantage of primers with broad specificity is the concomitant amplification of contaminating DNA, which gives rise to false-positive results. The elimination of false positives is an essential prerequisite to the development of PCR protocols for use in a clinical setting. This objective has been partly achieved by rendering PCR amplicons unsuitable for reamplification and also by the elimination of contaminating template DNA in PCR reagents. The presence of bacterial DNA in preparations of Taq DNA polymerase is well established (3, 4). Several methods have been used to eradicate this contamination (6, 7, 9), but none were found to be 100% effective. Diagnosis by culture is especially difficult with clinical samples when a limited volume is available. A set of nested primers was developed for the detection of bacteria which were capable of detecting 10 fg of bacterial DNA (unpublished observation). However, these primers were found to be of limited use, as every reagent control which was subjected to two rounds of amplification gave rise to a product of the size expected for a bacterial control which was subjected to two rounds of amplification. These primers were found to be of limited use, as every reagent control which was subjected to two rounds of amplification gave rise to a product of the size expected for a bacterial control which was subjected to two rounds of amplification. These primers were found to be of limited use, as every reagent control which was subjected to two rounds of amplification gave rise to a product of the size expected for a bacterial control which was subjected to two rounds of amplification.

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The water, buffer, MgCl₂ and Taq DNA polymerase components were mixed and incubated for 30 min at 37°C with 1.0 U of Sau3AI (Boehringer Mannheim) per U of Taq DNA polymerase. The restriction enzyme was inactivated by incubation at 95°C for 2 min, following which the deoxynucleoside triphosphates, primers, and template DNA were added and PCR amplification commenced. Taq DNA polymerase for the second round of amplification was used as supplied. Details of the two primer pairs used in this study appear in Table 1. PCR mixtures contained 60 μM each deoxynucleoside triphosphate (Pharmacia), 0.3 μM each primer, 3.0 mM Mg₂⁺, and 1 U of various Taq DNA polymerases in 25 μl. First-round amplifications were conducted with 2.5 pmol each of primers 16SF and 16SR, with an initial denaturation at 95°C (3 min) and cycling as follows: 95°C for 10 s, 54.2°C for 10 s, and 72°C for 15 s for 30 cycles (Genius Thermal Cycler; TECHNE). The second-round amplification was carried out as was the first, except that the Mg²⁺ concentration was 2.5 mM and 5 pmol of each primer was used. One microliter of the product from the first-round amplification was amplified with primers NF and NR as follows: denaturation at 95°C (3 min) and cycling at 95°C for 7 s, 60°C for 7 s, and 72°C for 10 s (30 cycles). Reagent controls from the first round were always subjected to a second round of amplification to control for contamination.

Amplicons were resolved on a 1% agarose–Tris-acetate-EDTA gel, visualized by using ethidium bromide under UV illumination, and recorded by using the UVP gel documentation system (UVP Ltd.). Samples for Southern analysis were purified by using Qiaquick PCR purification kits (Qiagen) and slot blotted onto Hybond N⁺ (Amersham). Probes for Southern hybridizations were 3' labelled and detected by using the ECL 3' labelling kit (Amersham).

Contaminating sequences in Taq DNA polymerase are readily amplified by using universal bacterial primers based on ribosomal genes. All assays to check for DNA in Taq DNA polymerase were carried out by using fresh reagents to rule out the possibility of exogenous contamination. The level of contamination was insufficient to give a detectable product after one round of PCR but was easily detected in all preparations after two rounds of amplification (Fig. 1). Amplitaq LD

TABLE 1. Names, sequences, and positions of oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Position on E. coli rRNA gene sequence</th>
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<tbody>
<tr>
<td>16SF</td>
<td>5' TTGGAGAGTTTGATCGTGCTTC 3'</td>
<td>4–25</td>
</tr>
<tr>
<td>16SR</td>
<td>5' ACCTCACTCCCACCTCCCTTC 3'</td>
<td>1174–1194</td>
</tr>
<tr>
<td>NF</td>
<td>5' GCGCGAAGCTCTAAYACATGCAAGT 3'</td>
<td>42–66</td>
</tr>
<tr>
<td>NR</td>
<td>5' GACGACAGGATGCACCCCTGCATG 3'</td>
<td>1084–1067</td>
</tr>
</tbody>
</table>
AmpliTaq LD prior to the second round of amplification was used for nested PCR amplification, Amplitaq LD was treated with Sau3AI as already described. It can be seen from Fig. 2 that use of very high levels of Sau3AI (4 U/U of Taq DNA polymerase) as a pretreatment resulted in reduced product formation from 20 ng of template DNA. Lower levels of Sau3AI achieved the aim of avoiding false positives while maintaining an acceptable level of sensitivity after two rounds of PCR amplification. Treatment of the AmpliTaq LD prior to the second round of amplification was not performed, as the subsequent sensitivity of detection was compromised (data not shown). Southern hybridization of the test sample DNA. Lower levels of Sau3AI achieved the aim of avoiding false positives while maintaining an acceptable level of sensitivity after two rounds of PCR amplification. Treatment of the AmpliTaq LD prior to the second round of amplification was not performed, as the subsequent sensitivity of detection was compromised (data not shown). Southern hybridization of the negative reaction mixtures from round 1 that had been pretreated with Sau3AI. Lane 9 contained the 1-kb size marker (Promega). Lanes 10 to 17 contained the products of the amplification of 1 µl of each of the first-round samples with primers NF and NR. Lane 18 contains a reagent control for the second round of amplification.

FIG. 2. Effect on product amplification of treating Taq DNA polymerase with decreasing amounts of Sau3AI. Positive reactions contained 20 ng of Escherichia coli NTCC 11151 DNA. Negative reaction mixtures contained no added template. (A) First round of amplification with primers 16F and 16R. Lanes 1, 3, 5, 7, 9, 11, and 13 were positive reaction mixtures treated with 1 µl of the negative reaction mixtures from round 1 that had been treated with 4, 2, 1, 0.5, 0.25, 0.13, and 0 µl of Sau3AI, respectively. Lane 15 contained the molecular size marker (GIBCO, Paisley, Scotland). (B) Second round of amplification with primers NF and NR. Lanes 1 to 7 contained 1 µl of the negative reaction mixtures from round 1 that had been treated with 4, 2, 1, 0.5, 0.25, 0.13, and 0 µl of Sau3AI, respectively. Lane 8 contained the positive control for the amplification, and lane 9 contained the reagent control. Lane 10 contained the molecular size marker (GIBCO).

FIG. 3. Sensitivity of nested PCR amplification of E. coli NTCC 11151 DNA using primer pairs 16F plus 16R and NF plus NR. The template was E. coli NTCC 11151 DNA purified as described in the text. Lanes 1 to 8 contained the products of the amplification of 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, and 10 fg of DNA or no DNA using primers 16F and 16R and AmpliTaq LD that had been pretreated with Sau3AI. Lane 9 contained the 1-kb size marker (Promega). Lanes 10 to 17 contain the products of the amplification of 1 µl of each of the first-round samples with primers NF and NR. Lane 18 contains a reagent control for the second round of amplification.

REFERENCES
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