Optimization of randomly amplified polymorphic DNA-polymerase chain reaction for molecular typing of Salmonella enterica serovar Typhi

Optimização da reação de amplificação aleatória do DNA polimórfico – reação em cadeia da polimerase para tipagem molecular de Salmonella enterica serovar Typhi

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ABSTRACT
Optimization of the RAPD reaction for characterizing Salmonella enterica serovar Typhi strains was studied in order to ensure the reproducibility and the discriminatory power of this technique. Eight Salmonella serovar Typhi strains isolated from various regions in Brazil were examined for the fragment patterns produced using different concentrations of DNA template, primer, MgCl2, and Taq DNA polymerase. Using two different low stringency thermal cycle profiles, the RAPD fingerprints obtained were compared. A set of sixteen primers was evaluated for their ability to produce a high number of distinct fragments. We found that variations associated to all of the tested parameters modified the fingerprinting patterns. For the strains of Salmonella enterica serovar Typhi used in this experiment, we have defined a set of conditions for RAPD-PCR reaction, which result in a simple, fast and reproducible typing method.

Key-words: Optimization. RAPD – PCR. Salmonella enterica serovar Typhi. Brazil.

RESUMO
A otimização da reação de RAPD para a caracterização de cepas de Salmonella enterica serovar Typhi foi estudada com o objetivo de assegurar a reprodutibilidade e o poder discriminatório desta técnica. Oito cepas de Salmonella serovar Typhi isoladas de algumas regiões do Brasil foram usadas para examinar os padrões de fragmentação produzidos quando foram empregadas concentrações diferentes do DNA molde, do iniciador, do MgCl2, e da enzima Taq DNA polimerase. Com a utilização de dois diferentes perfis de ciclos termais de baixa estrinência, foram comparados os padrões de bandas obtidos. Um conjunto de dezenove iniciadores foi avaliado quanto à capacidade de produzir número elevado de fragmentos distintos. Observou-se que variações associadas a todos os parâmetros testados modificaram os padrões de bandeamento. Para as amostras de Salmonella enterica serovar Typhi utilizadas neste experimento, definiu-se um conjunto de condições para a reação de RAPD-PCR que resultou num método de tipagem simples, rápido e reprodutível.


For the study of the epidemiology of typhoid fever, new molecular typing methods have been developed and improved to complement Vi phagetyping, the most useful technique to distinguish one S. enterica serovar Typhi strain from another. One of them, RAPD-PCR (Random Amplified Polymorphic DNA), can generate simple and reproducible fingerprints of genomic DNA in a PCR reaction by using single primers chosen irrespective of the genome sequence to be fingerprinted20 22. Thus, RAPD-PCR requires no prior knowledge of the molecular biology of the organisms to be investigated. The amplification occurs at low stringency, allowing the primers to anneal to several locations on the two strands of the DNA. These primers detect polymorphisms in the absence of specific sequence information and the polymorphisms function as genetic markers.

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As described previously\(^4\), it has been demonstrated that RAPD-PCR reaction has the potential to provide a discriminatory, reproducible and easy to interpret method to type S. enterica serovar Typhi strains. However, in order to use RAPD-PCR for differentiation between bacterial strains, the optimization of the reaction is imperative to eliminate most of the variations that are sometimes observed in duplicate DNA profiles\(^4,5\). The standardization of some parameters such as the MgCl\(_2\) and Taq DNA polymerase enzyme concentrations, the annealing temperature and the thermal cycling profile\(^6,7\) may lead to a more robust and reliable reaction capable of recognizing related strains and discriminating between unrelated strains\(^8\).

In the present study we define the conditions for the optimization of RAPD-PCR to S. enterica serovar Typhi DNA using 10 bp primer and demonstrate the effects in the fingerprint pattern caused by varying the target DNA, MgCl\(_2\) and Taq DNA polymerase enzyme concentrations and the thermal cycling profile. We also evaluate a total DNA extraction methodology, observing its time consumption and the stability of the resulting genetic material.

**MATERIAL AND METHODS**

The strains of S. enterica serovar Typhi used in the present study are listed in Table 1. These strains isolated from humans had been maintained on nutrient agar slopes in the culture collection of the National Reference Center for Cholera and Enteric Diseases, Department of Bacteriology, Oswaldo Cruz Institute/IOCRUZ, Rio de Janeiro, Brazil. The cultures for DNA extraction were grown in 5ml Brain Heart Infusion broth (Difco) for 18 to 24h at 37°C.

Total DNA was extracted as described by Sambrook et al\(^15\), using 1ml of each culture. The DNA was quantified, after electrophoresis in a 1% agarose gel, by comparison with known amounts of Hind III digested by bacteriophage \(\lambda\) (lambda) DNA. Using S. enterica serovar Typhi genomic DNA as template in the optimization steps, we first evaluated the DNA extraction method. An amount of 100 to 200ng/ml was produced and this was adequate to perform the amplification reactions. The genetic material remained stable for about 4 weeks, when stored at -20°C.

Preliminary assays with one, randomly chosen, S. enterica serovar Typhi strain (501 - Oswaldo Cruz Institute Collection) were carried out with sixteen 10-mer oligonucleotides primers, commercially synthesized, and aimed to test their ability to produce discriminatory RAPD profiles in S. enterica serovar Typhi. The primers were synthesized at the Escola Paulista de Medicina, São Paulo, Brazil (Table 2).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>GC (%)</th>
<th>Tm (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>784</td>
<td>5'-GGG GAA AFA G 3'</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>785</td>
<td>5'-GGG CAG CCA A 3'</td>
<td>70</td>
<td>34</td>
</tr>
<tr>
<td>786</td>
<td>5'-GGG ATC ACC A 3'</td>
<td>70</td>
<td>34</td>
</tr>
<tr>
<td>787</td>
<td>5'-GGG CCA A 3'</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>788</td>
<td>5'-GGG ACC A 3'</td>
<td>60</td>
<td>32</td>
</tr>
<tr>
<td>789</td>
<td>5'-GGG CCA TTT C 3'</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>790</td>
<td>5'-GGG CCA GAA C 3'</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>791</td>
<td>5'-GGG AAT ACC C 3'</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>792</td>
<td>5'-GGG ACT ACC C 3'</td>
<td>70</td>
<td>34</td>
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<td>793</td>
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<td>794</td>
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<td>798</td>
<td>5'-GGG AAT ACC C 3'</td>
<td>80</td>
<td>36</td>
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<tr>
<td>799</td>
<td>5'-GGG AAT ACC C 3'</td>
<td>80</td>
<td>36</td>
</tr>
</tbody>
</table>

We conducted this step under non-standardized conditions: the reaction was prepared in a total volume of 25μl per tube, containing 20ng DNA of the strain 501, 3U Taq DNA polymerase (CENBIOT/RS), 10mM Tris HCl, 50mM KCl, 1.5mM MgCl\(_2\), 200mM of each dNTP and 20 pM/ml of primer.

Considering the amplification products fingerprint, we selected one primer and first evaluated the effects of varying the Taq DNA polymerase enzyme concentration (1U/25μl, 2 U/25μl, 3 U/25μl, 4 U/25μl and 5 U/25μl) using strain number 501. Furthermore, the assays were carried out at different concentrations of MgCl\(_2\) (2mM, 3mM and 4mM), DNA (20ng and 40ng) and primer (20pM/ml and 40pM/ml) at thermal cycle A, programmed for 30 cycles composed of one step of denaturation for 1 min at 94°C, one annealing step for 1 min at 36°C followed by a final synthesis for 2 min at 72°C.

To evaluate the influence of thermal cycle profile, six strains were submitted to thermal cycle A and thermal cycle B, programmed for five cycles, composed of one initial step of denaturation for 1 min at 94°C, one step of low stringency temperature of annealing for 1 min at 36°C and one step of synthesis or amplification for 2 min at 72°C. This was followed by 25 cycles of high-stringency temperature of annealing, consisting of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, finishing with an amplification step for 7 min at 72°C.

The reaction tubes were covered with 60μl of sterilized mineral oil and the amplification took place in a DNA thermal cycler (Perkin Elmer 480). Amplification products were submitted to electrophoresis in 1.5% agarose gel, Tris-borate buffer and a constant voltage of 100V, followed by staining with ethidium bromide and visualization in a UV transilluminator. A negative control was included in each PCR run with no target DNA.
The λ phage DNA cleaved by Hind III restriction enzyme (Sigma) and the synthetic DNA, ladder 100 (Pharmacia) were employed as patterns for band molecules weight.

RESULTS

Submitting 20ng/µl DNA template to 20 pM/µl primer, 1.5 mM MgCl₂, 1 U/25µl Taq DNA polymerase at thermal cycle A, the primer 784 (sequence 5’GCG GAA ATA3’; 50% GC content and Tm = 30°C) was chosen due to its ability to produce a higher number of bands and a better fingerprinting pattern (Figure 1). Varying the Taq DNA polymerase concentrations markedly affected the amplification profiles obtained with primer 784 (Figure 2). Thus, reactions performed with increasing concentrations of the enzyme showed the best performance when using a concentration of 5 U/25µl. The loss of fragments in the profiles observed using 1 U/25µl and 2 U/25µl highlights the influence of this parameter in the optimization of RAPD reaction.

The number and the intensity of fragments produced varied in function of the thermal cycling profile, the MgCl₂, DNA template, primer and Taq DNA polymerase enzyme concentrations. Maintaining the DNA and primer 784 concentrations at 20ng/µl and 20pM/µl, respectively, and varying the MgCl₂ concentration, we observed that the best fingerprinting pattern was obtained using 2.0mM MgCl₂ at thermal cycle A. Higher MgCl₂ concentrations (3.0 and 4.0 mM) had significant effects upon the RAPD profiles produced, showing fewer and indistinguishable bands.

When we increased the DNA template concentration from 20 to 40ng/µl and the primer concentration from 20 to 40 pM/µl, only a few or no amplification fragments were observed.

The analysis of two different thermal cycle profiles applied to 6 strains of S. enterica serovar Typhi showed that 30 low stringency cycles, named cycle A was sufficient to promote primer/template interactions and generate discriminatory amplification fragments. However, a lack of bands and sometimes no amplification was observed when applying cycle B (Figures 3a and 3b).

DISCUSSION

Various molecular biological techniques such as ribotyping¹ and pulsed-field gel electrophoresis (PFGE) have recently been applied to Salmonella enterica serovar Typhi for epidemiological purposes. Nevertheless, there is still no rapid, reliable and sufficiently discriminative method for laboratory investigation of the epidemiology of typhoid fever. Thus, the use of random amplification of polymorphic DNA fingerprinting technique (RAPD), a modification of the polymerase chain
reaction (PCR) in which arbitrary oligonucleotides primers are used to promote DNA synthesis at low stringency conditions in order to determine genomic diversity, may be considered a promising alternative typing method capable of discriminating between S. enterica serovar Typhi strains of the same phage type14. Besides differentiating isolates of serovar Typhi and other Salmonella isolates, RAPD-PCR proved capable of discriminating between S. enterica serovar Typhi strains 16. RAPD-PCR assays are simpler, faster, more convenient and easier to perform than most other molecular typing methods. However, only when used under well defined and optimized conditions RAPD is capable of reproducing the amplification of random fragments of DNA and generating high degrees of polymorphism6 17. The DNA extraction method reported here was technically easy to perform and not time consuming. The total material obtained was maintained stable when stored at -20°C for about four weeks, reproducing the same profiles without lack of bands or clearness. However, whole cell reaction products if analyzed after 24h of amplification may produce smeared profiles probably due to the presence of degradative enzymes that are stable during the cycling reaction and active during storage6.

The standardization of DNA template concentration is important to avoid artifacts on the band patterns. Using 20ng/µl DNA template, RAPD produced well resolved profiles, but many bands were lost when using 40ng/µl. As reported before, an extraction kit to extract DNA from the cells worked well with RAPD analysis and although it did not quantify the amount of DNA, the template concentration over a wide range had no significant effect on the RAPD profiles produced16 23. The primer concentration influence was also evaluated. Increasing the primer concentration from 20pM/µl to 40pM/µl produced similar fingerprint profiles.

We found that magnesium ion concentration was a critical element in determining the performance of amplification reaction and significantly varied the profiles produced. Low magnesium ion concentrations may result in poor reaction efficiency and high concentrations may result in poor reaction specificity13. Because we were working with nonspecific primer-template interaction we expected that increasing the concentration of magnesium ion had the net effect of decreasing the stringency of primer binding. Surprisingly, a notorious lack of bands was noticed when using 3.0mM MgCl₂ and the best performance occurred at a lower concentration, 2.0mM MgCl₂. The same results were observed for Yersinia enterocolitica strains, which have the best performance for RAPD reaction at 2.0mM. In contrast, for S. enterica serovar Enteritidis, no difference in the RAPD fingerprint profiles was obtained varying MgCl₂ concentration from 2.0mM to 4.0mM.

Reaction buffer pH was maintained at 8.0 in all experiments although a variation in pH as small as 0.4 can make the difference between a discriminatory array of fragments and no amplification1.

Because RAPD variations associated to thermal cycle profile (Figures 3a and 3b) may occur, this parameter should be evaluated when standardizing RAPD reaction. The Taq DNA polymerase preparations can be classified as a major source of variations (Figure 2). Besides the concentration, we also found variations when changing the production lot of Taq DNA polymerase of the same brand, showing the importance of acquiring an appropriate quantity of this enzyme to complete the research. As performed previously, the use of different brands of Taq DNA polymerase revealed major variations between the patterns obtained10.

If RAPD is to be useful as a typing method, a sufficient discriminatory number of amplified fragments must be produced, which essentially depends on the primer-template interaction1. Each fingerprint reflects the successful primer-direct targeting of a set of sites in the genome2. For this reason, the choice of an appropriate set of 10-mer oligonucleotides is imperative or the discriminatory power of RAPD analysis may decrease. Thus, each primer gave a different fingerprint pattern although each had the potential of detecting polymorphisms between strains, thereby allowing the differentiation of even
closely related strains. From 16 arbitrary primers examined for suitability, primer 784 was found to be specially useful for optimization steps and generated the best fingerprint patterns. In addition, due to the arbitrary selection of primers, RAPD reaction may produce many amplification fragments or no fragments to any extent (Figure 1). The use ofERIC (Enterobacterial Repetitive Intergenic Consensus) primer in the RAPD reaction of S. enterica serovar Typhi resulted in indistinguishable fingerprint patterns that were unable to discriminate between strains from different geographical origins.

RAPD-PCR fingerprint strategy should be applicable to bacterial typing for its rapidness, simplicity, low cost and potential to generate polymorphisms. Our results suggest that there is a considerable possibility for increasing the efficiency of the RAPD-PCR reaction if a precise standardization protocol is determined. We have proposed a model that can be used as a support for typing strains of S. enterica serovar Typhi.

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REFERENCES