

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

Otimização da reação de amplificação aleatória do DNA polimórfico – reação em cadeia da polimerase para tipagem molecular de *Salmonella enterica* sorovar 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 sorovar Typhi foi estudada com o objetivo de assegurar a reprodutibilidade e o poder discriminatório desta técnica. Oito cepas de Salmonella sorovar 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 stringência, foram comparados os padrões de bandamento obtidos. Um conjunto de dezesseis iniciadores foi avaliado quanto à capacidade de produzir elevado número de fragmentos distintos. Observou-se que variações associadas a todos os parâmetros testados modificaram os padrões de bandamento. Para as amostras de Salmonella enterica sorovar 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.

Palavras-chaves: Otimização. RAPD – PCR. Salmonella enterica sorovar Typhi. Brasil.

For the study of the epidemiology of typhoid fever, new molecular typing methods have been developed and improved to complement Vi phagotyping, 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 fingerprinted^{20,22}. 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 previously¹⁴. 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 profiles²⁴. The standardization of some parameters such as the MgCl₂ and *Taq* DNA polymerase enzyme concentrations, the annealing temperature and the thermal cycling profile^{10 21} may lead to a more robust and reliable reaction capable of recognizing related strains and discriminating between unrelated strains⁵.

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, MgCl₂ 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/FIOCRUZ, 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 al¹⁵, 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 λ (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,

Table 1 - Strains of *Salmonella enterica* serovar Typhi, area, isolation source and year of isolation.

Strain n°	Area	Isolation source	Year
492	north (Acre)	blood	1995
496	north (Acre)	blood	1995
501	north (Acre)	blood	1995
30	mid west (Distrito Federal)	blood	1972
655	mid west (Distrito Federal)	blood	1995
656	mid west (Distrito Federal)	blood	1995
T68	northeast (Pernambuco)	blood	1937
T54	northeast (Pernambuco)	blood	1938

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 2 - Characteristics of the primers used for the RAPD-PCR reaction with *S. enterica* serovar Typhi.

Primer	Sequence	GC (%)	Tm (°C)
784	5'GCG GAA ATA G 3'	50	30
785	5'CCG CAG CCA A 3'	70	34
786	5'GCG ATC CCC A 3'	70	34
787	5'AAC GCG CAA C 3'	60	32
788	5'GTG GAT GCG A 3'	60	32
789	5'AGC CAG TTT C 3'	50	30
790	5'GTC AAC GAA G 3'	50	30
791	5'GAG ACT CCC C 3'	50	30
792	5'GGT ACT CCC C 3'	70	34
793	5'GAC CGA CCC A 3'	70	34
794	5'ACT GAA CGC C 3'	60	32
795	5'GAC ACG CAC A 3'	60	32
796	5'ACC TCA GCT C 3'	60	32
797	5'AGC GTC ACT C 3'	60	32
798	5'TGA CCC GCC G 3'	80	36
799	5'GGC TTG GCC G 3'	80	36

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 MgCl₂, 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 MgCl₂ (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/ μ l DNA template to 20 pM/ μ l primer, 1.5 mM MgCl₂, 1 U/25 μ l *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/25 μ l. The loss of fragments in the profiles observed using 1 U/25 μ l and 2 U/25 μ l highlights the influence of this parameter in the optimization of RAPD reaction.

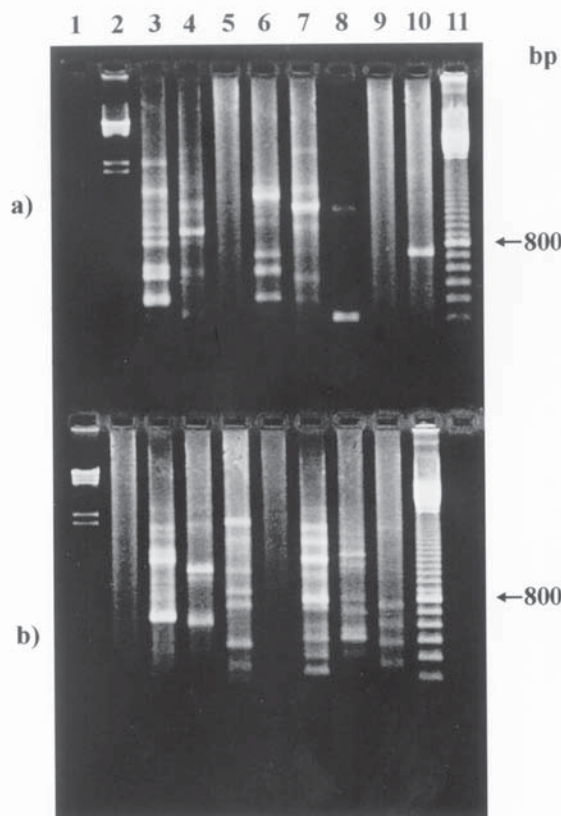


Figure 1 - RAPD-PCR fingerprinting of *S. enterica* serovar Typhi strain 501 DNA using sixteen arbitrary primers for selection. a) Lanes: 1, negative control; 2, phage λ digested with *Hind* III; 3 to 10, primers 784 to 791; 11, ladder 100 DNA. b) Lanes: 1, phage λ digested with *Hind* III; 2 to 9, primers 792 to 799; 10, ladder 100; 11, negative control.

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/ μ l and 20pM/ μ l, respectively, and varying the MgCl₂ concentration, we observed that the best fingerprinting pattern was obtained using 2.0mM MgCl₂ at

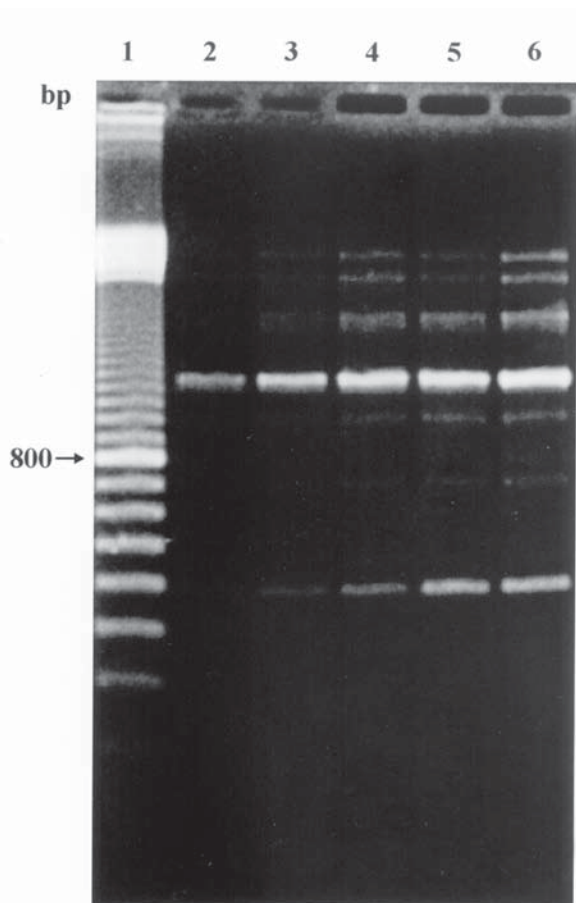


Figure 2 - RAPD patterns variations associated to *Taq* DNA polymerase enzyme concentration obtained from *S. enterica* serovar Typhi strain 501 DNA using primer 784. Lanes: 1, ladder 100 DNA; 2, 1 U/25 μ l; 3, 2 U/25 μ l; 4, 3 U/25 μ l; 5, 4 U/25 μ l; 6, 5 U/25 μ l.

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/ μ l and the primer concentration from 20 to 40 pM/ μ l, 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)^{11 12 18 19} 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 fever⁸. Thus, the use of random amplification of polymorphic DNA fingerprinting technique (RAPD), a modification of the polymerase chain

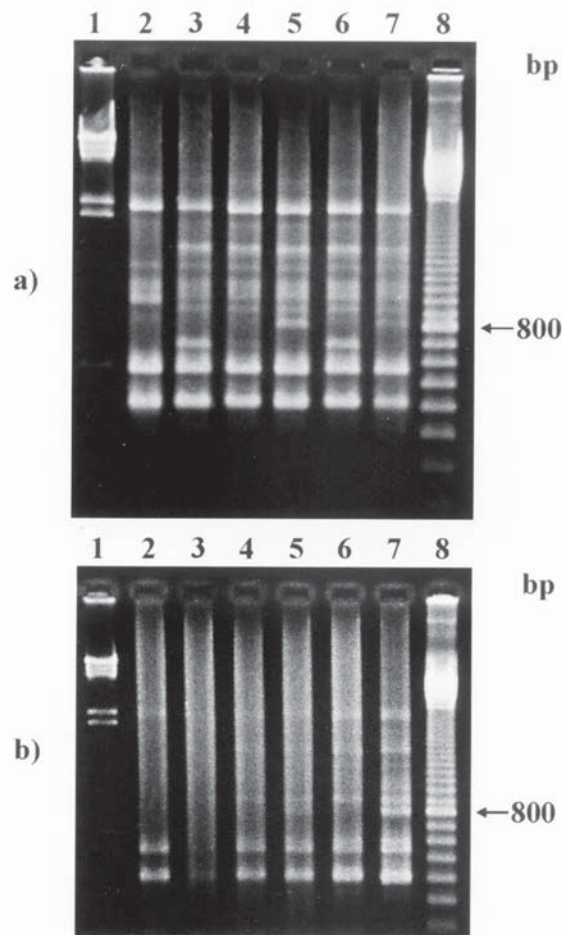


Figure 3 - RAPD patterns variations associated to the thermal cycle profile obtained from six strains of *S. enterica* serovar Typhi using primer 784. a) cycle A (30 cycles at 94°C, 1 min; 36°C, 1 min; 72°C, 2 min); b) cycle B (5 cycles at 94°C, 1 min; 36°C, 1 min; 72°C, 2 min and 25 cycles at 94°C, 1 min, 50°C, 1 min; 72°C, 2 min; 72°C, 7 min). Lanes: 1, phage λ digested with Hind III; 2, strain 492 DNA; 3, strain 30 DNA; 4, strain 655 DNA; 5, strain 656 DNA; 6, strain T68 DNA; 7, strain T54 DNA; 8, ladder 100 DNA.

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 strains¹⁶. 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 polymorphism^{6,17}.

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/ μ l DNA template, RAPD produced well resolved profiles, but many bands were lost when using 40ng/ μ l. 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 produced^{16,23}. The primer concentration influence was also evaluated. Increasing the primer concentration from 20pM/ μ l to 40pM/ μ l 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 2.0mM⁹. In contrast, for *S. enterica* serovar Enteritidis, no difference in the RAPD fingerprint profiles was obtained varying MgCl₂ concentration from 2.0mM to 4.0mM⁷.

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 amplification⁶.

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 interaction⁶. 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 of ERIC (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 origins³⁴.

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

1. Altwegg M, Hickman-Brenner FW, Farmer III JJ. Ribosomal RNA gene restriction patterns provide increased sensitivity for typing *Salmonella typhi* strains. *The Journal of Infectious Diseases* 160: 145-149, 1989.
2. Caetano-Anollés G, Bassam BJ, Gresshoff P. Primer-template interactions during DNA amplification fingerprinting with single arbitrary oligonucleotides. *Molecular Genomic Genetics* 235: 157-165, 1992.
3. Gruner E, Flepp M, Gabathuler U, Thong K, Altwegg M. Outbreak of typhoid fever in a non-endemic area: comparison of three molecular typing methods. *Journal of Microbiologic Methods* 28: 179-185, 1987.
4. Hermans PWM, Saha SK, Van Leeuwen WJ, Verbrugh HA, Van Belkum A, Goessens WHE. Molecular typing of *Salmonella typhi* strains from Dhaka (Bangladesh) and development of DNA probes identifying plasmid-encoded multidrug-resistant isolates. *Journal of Clinical Microbiology* 34: 1373-1379, 1996.
5. Hilton AC, Banks JG, Penn CW. Random amplification of polymorphic DNA (RAPD) of *Salmonella*: strain differentiation and characterization of amplified sequences. *Journal of Applied Bacteriology* 81: 575-584, 1996.
6. Hilton AC, Banks JG, Penn CW. Optimization of RAPD fingerprinting *Salmonella*. *Journal of Applied Bacteriology* 24: 243-2483, 1997.
7. Lin AW, Usera MA, Barrett TJ, Goldsby RA. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella enteritidis*. *Journal of Clinical Microbiology* 34: 870-876, 1996.
8. Ling JM, Loo NW, Ho YM, Kam KM, Hoa NT, Phi LT, Cheng AF. Molecular methods for the epidemiological typing of *Salmonella enterica* serotype Typhi from Hong Kong and Vietnam. *Journal of Clinical Microbiology* 38: 292-300, 2000.
9. Makino S-I, Okada Y, Maruyama T, Kaneko S, Sasakawa C. PCR-based random polymorphic DNA fingerprinting of *Yersinia pseudotuberculosis* and its practical applications. *Journal of Clinical Microbiology* 32: 65-69, 1994.
10. Meunier JR, Grimont PAD. Factors affecting reproducibility of random polymorphic DNA fingerprinting. *Research Microbiology* 144: 373-379, 1993.
11. Nair S, Poh CL, Lim YS, Tay L, Goh KT. Genome fingerprinting of *Salmonella typhi* by pulsed-field gel electrophoresis for subtyping common phage types. *Epidemiological Infectious* 113: 391-402, 1994.
12. Navarro E, Lovet T, Echeita MA, Coll P, Aladueña A, Usera MA, Prats G. Molecular typing of *Salmonella enterica* serovar Typhi. *Journal of Clinical Microbiology* 34: 2831-2834, 1996.
13. Persing DH. Target selection and optimization of amplification reactions. In: Persing DH, Smith TF, Tenover FC, White TJ (ed) *Diagnostic Molecular Microbiology: Principles and Applications*, Washington, p. 88-103, 1993.
14. Quintaes BR, Leal NC, Reis EME, Fonseca EL, Hofer E. Conventional and molecular typing of *Salmonella* Typhi strains from Brazil. *Revista do Instituto de Medicina Tropical de São Paulo* 44: 315-319, 2002.
15. Sambrook J, Fritsch EE, Maniatis T. *Molecular Cloning, A Laboratory Manual*. 2nd edition. Cold Spring Harbour Laboratory Press, New York, p. 1.25-1.28, 1989.
16. Shangkuan Y-H, Lin H-C. Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella typhi* and other *Salmonella* species. *Journal of Applied Microbiology* 85: 693-702, 1998.
17. Soto SM, Guerra B, Gozález-Hevia MA, Mendoza MC. Potential of three-way randomly amplified polymorphic DNA analysis as a typing method for twelve *Salmonella* serotypes. *Applied Environmental Microbiology* 65: 4830-4836, 1999.
18. Thong KL, Cheong YM, Puthuchery S, Koh CL, Pang T. Epidemiologic analysis of sporadic *Salmonella typhi* isolates and those from outbreaks by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* 32: 1135-1141, 1994.
19. Thong KL, Puthuchery S, Yassin RM, Sudarmono MP, Soewandojo E, Handojo I, Sarasombath S, Pang T. Analysis of *Salmonella typhi* isolates from Southeast Asia by pulsed-field gel electrophoresis. *Journal of Clinical Microbiology* 33: 1938-1941, 1995.
20. Welsh J, McClelland M. Genomic fingerprinting using arbitrarily primed PCR and a matrix of pairwise combinations of primers. *Nucleic Acids Research* 19: 5275-5279, 1991.
21. Welsh J, Petersen C, McClelland M. Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. *Nucleic Acids Research* 19: 303-306, 1991.
22. Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* 18: 6531-6535, 1990.
23. Wong NACS, Linton CJ, Jalal H, Millar MR 1994. Randomly amplified polymorphic DNA typing: a useful tool for rapid epidemiological typing of *Klebsiella pneumoniae*. *Epidemiological Infectious* 113: 445-454, 1994.
24. Yu K, Pauls KP. Optimization of the PCR program for RAPD analysis. *Nucleic Acids Research* 20: 2606, 1992.