



Performance of the tetra-primer PCR technique compared to PCR-RFLP in the search for rs12979860 (C/T) and rs8099917 (T/G) single nucleotide polymorphisms (SNPs) in the *IFNL4* gene

Desempenho da técnica tetra-*primer* PCR em relação a PCR-RFLP na pesquisa de polimorfismos de nucleotídeos únicos (SNPs) rs12979860 (C/T) e rs8099917 (T/G) no gene *IFNL4*

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ABSTRACT

Single nucleotide polymorphisms (SNPs, rs12979860 e rs8099917) in the Interferon Lambda 4 gene (*IFNL4*, formerly *IFNL3* and/or *IL28B*) has been associated with failure in the innate immune response, sustained virological response in hepatitis C, and HTLV-1-associated myelopathy (HAM) development. To search for these polymorphisms several methodologies can be employed, such as sequencing, real-time or quantitative polymerase chain reaction (qPCR), restriction fragment length polymorphism analysis in PCR products (PCR-RFLP), and tetra-primer PCR. The present study compared the performance of the tetra-primer PCR in relation to the PCR-RFLP, both optimized in the Research HTLV Laboratory of the Center of Immunology of Instituto Adolfo Lutz in São Paulo. One hundred DNA samples obtained from patients of STD/Aids Reference Centre in São Paulo, previously analyzed for *IL28B* SNPs by PCR-RFLP were selected for analysis, after confirming that they represent all *IL28B* SNPs patterns described in the literature. The results obtained showed concordance between the PCR-RFLP and the tetra-primer PCR SNPs results, and because of the low cost, easy to perform, and minor employment of biological specimen and reagents, the tetra-primer PCR is of choice to be used in routine.

Keywords. Interferon Lambda 4 (IFNL4), Interleukin 28B (IL28B), Single Nucleotide Polymorphism (SNP), Polymerase Chain Reaction (PCR), Amplified Fragment Length Polymorphism Analysis, Cost Analysis.

RESUMO

Polimorfismos de nucleotídeos únicos (*single nucleotide polymorphisms*, SNPs rs12979860 e rs8099917) no gene que codifica o Interferon Lambda 4 (*IFNL4*, antigamente *IFNL3* e/ou *IL28B*) têm sido associados às falhas na resposta imune inata e resposta virológica sustentada na hepatite C, e a mielopatia associada ao HTLV-1 (*HTLV-1-associated myelopathy*, HAM). A pesquisa destes polimorfismos pode empregar diversas metodologias: sequenciamento, reação em cadeia da polimerase em tempo real ou quantitativa (*quantitative polymerase chain reaction*, qPCR), análise de fragmentos de restrição enzimática em produtos de PCR (*restriction fragment length polymorphism in PCR products*, PCR-RFLP) e a tetra-*primer* PCR. Este estudo comparou o desempenho da tetra-*primer* PCR em relação a PCR-RFLP, ambas otimizadas no Laboratório de Pesquisa em HTLV do Centro de Imunologia do Instituto Adolfo Lutz de São Paulo. Foram selecionadas 100 amostras de DNA obtidas de pacientes do Centro de Referência e Treinamento em DST/Aids de São Paulo cujos SNPs na *IL28B* foram anteriormente determinados por PCR-RFLP e representaram todos os perfis descritos em literatura. Os resultados obtidos mostraram concordância entre elas, e pelo fato da tetra-*primer* PCR ter menor custo, ser de fácil execução, empregar menos tempo, insumos e material biológico, é a técnica de escolha para uso em rotina.

Palavras-chave. Interferon Lambda 4 (IFNL4), Interleucina 28B (IL28B), Polimorfismo de Nucleotídeo Único (SNP), Reação em Cadeia da Polimerase (PCR), Polimorfismos de Fragmentos de Restrição (RFLP), Análise de Custo.

INTRODUCTION

The interferon lambda 4 gene (*IFNL4*) is one of the most recent human genes discovered and the last added to the interferon lambda family¹. The official symbols for the interferon lambda family genes are *IFNL1* (formerly *IL29*), *IFNL2* (formerly *IL28A*), *IFNL3* (formerly *IL28B*) and *IFNL4*. These genes are located on the long arm of chromosome 19, region q13+13^{1,2}. The *IFNL3* (*IL28B*) gene sequence had been described to have similarity of 96% and 81% with *IFNL2* (*IL-28A*) and *IFNL1* (*IL-29*) genes, respectively²⁻⁴.

The proteins encoded by interferon lambda family genes bind to a shared co-receptor complex, leading to activation of the JAK-STAT signaling pathway and upregulating of numerous interferon-stimulated genes¹. These proteins are involved in immune mechanisms, from cell stimulation/activation to cytokines self-production in response to an antigen, as occurs in inflammatory and degenerative processes (pro-inflammatory and anti-inflammatory cytokines).

Commins et al⁴ pointed the antiviral activity of the Interleukin 28B (*IL28B*) mainly in viral infections caused by hepatitis B virus (HBV) and hepatitis C virus (HCV), and other researchers in HIV and in human T-cell lymphotropic virus type 1 (HTLV-1) infections⁵⁻⁸.

Due to the antiviral activity of *IFNL3/IL28B*, IFN- α (the first drug to demonstrate activity against HCV) was developed by using biosynthetic processes. Later, in order to improve the IFN- α pharmacokinetics, an addition of bis-mono methoxy polyethylene glycol (PEG) in its chemical structure, originated PEG-IFN- α ⁹. Even with this drug change, the treatment was not operative because the adverse effects and poor adherence. In addition, low frequency (around 55%) of sustained virological response (SVR), which means undetectable plasmatic HCV RNA after 12-24 weeks of therapy, was detected in patients undergoing treatment¹⁰. Currently, direct acting antiviral agents (DAAs) that target specific HCV proteins involved in viral replication and assembly are available for treatment, but until 2013, the universal therapeutic scheme was based on PEG-IFN- α with or without ribavirin (RBV); the same scheme recommended by the Brazilian Ministry of Health¹¹.

During follow-up of patients with HCV infection, different response profiles were observed; good responders denoting patients who had spontaneous viral load clearance or SVR after treatment, and poor responders in cases of therapeutic failure. Such different responses have been linked to a combination of viral and host factors^{12,13}. Viral factors refer mainly to the HCV genotype and viral load (HCV RNA levels), and host factors to ethnicity, sex, age, body mass index (BMI), and single nucleotide polymorphisms (SNPs); the last one pointed out as early markers of treatment success and SVR^{12,13}.

In fact, genome-wide association studies (GWASs) of *IL28B* with response to PEG-IFN- α treatment identified the SNPs rs12979860 and rs8099917 as useful pharmacogenetics tool for prediction of hepatitis C treatment response (HCV clearance and SVR) when applied to populations from different geographic regions¹⁴⁻¹⁶. These SNPs were firstly located in a genomic region of 24 Kb between the *IFNL2* and *IFNL3* genes, more precisely, at the 3 Kb (rs12979860) and 8 Kb (rs8099917) positions that precede *IFNL3*¹⁷, but after the discovery of *IFNL4* they were repositioned within intron 1 of *IFNL4* (rs12979860), and in an intergenic region near to and upstream of *IFNL4* (rs8099917)¹. The polymorphism at the rs12979860 position represented by the CC, TT or CT genotypes, disclosed the C allele as associated with a better response to HCV infection treatment^{14,18}, while the polymorphism at position rs8099917 represented by TT, GG or TG genotypes, associates the G allele with a higher risk for hepatitis C disease chronicity^{15,16,19}.

As the treatment based on PEG-IFN- α with or without association with RBV was expensive and had many adverse effects causing patients abandon of treatment, it was recommended to research the SNPs rs12979860 and rs8099917 before starting therapy. Regardless of others important and unusual polymorphisms that control the generation of IFNL4 protein have been detected recently, like the *IFNL4*- $\Delta G/TT$ (rs368234815) variant within exon 1, the rs12979860 and rs8099917 SNPs whose are in linkage disequilibrium with *IFNL4*- $\Delta G/TT$ continue to be important predicted markers associated with HCV clearance¹.

The determination of such polymorphisms can be performed by many methods; genetic sequencing, real-time or quantitative polymerase chain reaction (qPCR), restriction fragment length polymorphism analysis in PCR products (RFLP-PCR), and amplification-refractory mutation system-polymerase chain reaction / tetra-primer polymerase chain reaction (ARMS-PCR/tetra-primer PCR). The choice of the method to be used for SNPs searching will depend on the facilities and equipment available in the laboratory. Furthermore, it should take into account the quantity of inputs and biological material, as well as the training of its technical staff. Therefore, in regions and countries with a lot of financial resources, sequencing, and qPCR techniques (commercially available) can be used without problems, but in conditions of low financial resources, PCR-RFLP and tetra-primer PCR techniques seem to be a great alternative.

Briefly, the PCR-RFLP technique consists of PCR amplification of a DNA region of interest and subsequent enzymatic restriction. For PCR, a pair of specific primers is used to amplify the target region. After confirming the amplification of the specific product by agarose gel electrophoresis, these amplicons are incubated with restriction endonucleases that recognize and cut specific sites (usually four to six base pairs) in a DNA sequence, generating fragments that can be separated on a gel mesh (agarose or polyacrylamide) after electrophoresis according to its size²⁰. The genotype profiles are analyzed after the second electrophoresis and are discriminated according to the fragments presented. Polymorphisms in homozygosis (wild-type and mutant) have distinct fragments profiles, while heterozygosis for the same SNP combines the profile of both.

The tetra-primer PCR differs from the conventional reaction by using four primers in a single step. Two outer primers and two inner primers, both with forward and reverse. The outer primers amplify a larger fragment that comprises the region where the SNPs are to be identified and function as an amplification control. While the forward and reverse combination between the inner and outer primer [(inner-forward and outer-reverse) or (outer-forward and inner-reverse)] amplify smaller products corresponding to the specific alleles of the studied polymorphism^{21,22}. The high specificity of this technique depends on an error in the second nucleotide of the 3' end of the inner primers (allele-specific). This mistake destabilizes the sharing of the polymorphic base between the primers and the target region, increasing the specificity of the reaction and minimizing false-positive results²¹. Identification of genotypes occurs by analysis of PCR products, separated in the agarose gel after electrophoresis. The interpretation of products according to size in base pairs (bp) is specific for the control and each allele. In cases of homozygous genotypes, two bands are seen, one referring to the control (larger segment) and the other to the specific allele. Samples with heterozygosis present in addition to the control band, two bands indicative of the alleles^{23,24}.

Based on the above descriptions, the HTLV Research Laboratory of the Center of Immunology of Institute Adolfo Lutz standardized in 2015 the PCR-RFLP technique for searching *IL28B* SNPs rs12979860 and rs8099917 according to the protocols from Moreira et al²⁵. However, due to the high cost of restriction

endonucleases and the scarcity of biological material from patients, this Laboratory has been concerned with standardizing another technique that use less DNA inputs and reagents. Thus, the present study aimed to optimize the tetra-primer PCR and compare its performance in relation to the PCR-RFLP technique.

MATERIAL AND METHODS

Study design and samples

The samples came from a cross-sectional study that investigated the prevalence of HTLV-1/2 in patients attended at the Reference and Training Center for STD/Aids (CRT DST/Aids) in São Paulo. 1,608 patients agreed to participate, and signed the Informed Consent which included the search for *IL28B* SNPs rs12979860 and rs8099917. The project was approved by the Ethics Committees for Research of Instituto Adolfo Lutz under protocols number CTC # 106D/2012 and by the Brazilian Ministry of Health CAAE # 11302512.0.0000.0059. DNA samples from the blood of 100 patients (50 HIV single-infected and 50 HIV/HCV co-infected) whose SNPs profiles (rs12979860 and rs8099917) were previously determined by PCR-RFLP and showed all genotypes described in literature were employed in the present study (project approved by the Ethics Committees for Research of Instituto Adolfo Lutz under protocols number CTC # 62H/2015 and the Brazilian Ministry of Health CAAE # 52493316.1.0000.0059). No data that could identify the patients were presented. All procedures were performed following the principles established in the Declaration of Helsinki of 1975, as revised in 1983, 1989, 1996, and 2000.

Methods

DNA extraction

DNA extractions were performed from peripheral blood leukocytes, using the Roche robotic system which consists of purification by magnetic beads (MagNA Pure LC 2.0 extractor) and/or the MagNA Pure LC DNA isolation kit I (Roche Diagnostics GmbH – Roche Applied Science-Mannheim, Germany), following the manufacturers' instructions.

PCR-RFLP

Briefly, in the PCR reaction, primers IL28B-860F - 5' AGC AGG ACA GAT TGG CAA AG 3' and IL28B-860R - 5' CAC AAT TCC CAC CAC GAG AC 3' were used for SNP rs12979860, and primers IL28B-917F - 5' CTG GAA CAA ATC GTC CCA AT 3' and IL28B-917R - 5' TTC CTT TAG GCC TGT GGA TG 3' for SNP rs8099917; they amplify products of 694 bp and 496 bp, respectively. The PCR reactions employed protocols adapted from Moreira et al²⁵. Briefly, 50-500 ng of DNA template, 1U of *Taq* DNA polymerase, 200 µM of each dNTPs, 1.5 mM of MgCl₂, PCR buffer, 10 pmol of each primer were added to a final volume of 50 µL of reaction. For rs12979860 5% DMSO was added. The primer annealing temperature was 59 °C for rs12979860 and 57.5 °C for rs8099917. Cycling was performed in a PTC 100TM MJ Research device, using temperatures of 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 59 or 57.7 °C for 30 s and 72 °C for 1 min. The final extension took place at 72 °C for 7 min. The restriction enzymes used for RFLP were *Hpy166II* (37 °C for 2 h) and *BsrDI* (65 °C for 2 h), both from New England Biolabs Inc. The reaction products were visualized in 2% and 3% agarose gels, respectively (**Figure 1**).

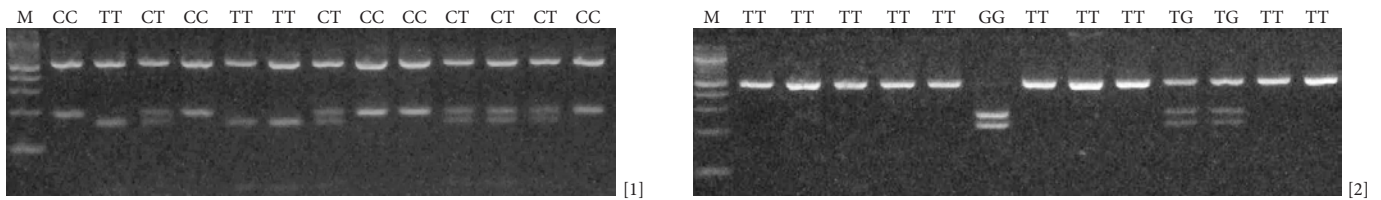


Figure 1. PCR-RFLP electrophoretic patterns related to *IFNL4* (*IL28-B*) SNP rs12979860 [1] and rs8099917 [2] after digestion by *Hpy*166II, and *Bsr*DI, respectively

Legend: [1] SNP rs12979860; M, molecular size marker (100 bp Ladder); CC genotype products of 509 bp and 185 bp; CT genotype products of 509 bp, 185 bp, and 155 bp; TT genotype products of 509bp and 155 bp; [2] SNP rs8099917; M, molecular size marker (100 pb Ladder); TT genotype products of 496 bp; TG genotype products of 496 bp, 272 bp, and 224 bp; GG genotype products of 272 bp and 224 bp

Source: Laboratório de Pesquisa em HTLV

Tetra-primer PCR

The technique described by Delvaux et al²³ was employed and adapted for the reagents and conditions of the HTLV Research Laboratory of the Center of Immunology, Instituto Adolfo Lutz.

For rs12979860, primers ARMS 860F1 (outer forward) CCA GGG CCC CTA ACC TCT GCA CAG TCT G, ARMS 860R1 (outer reverse) CTA TGT CAG CGC CCA CAA TTC CCA CCA C, ARMS 860F2T (inner forward) ACT GAA CCA GGG ACG TCC CCG AAG GAG T, and ARMS 860R2C (inner reverse) CGG AGT GCA ATT CAA CCC TGG TGC G were used, and for rs8099917, primers ARMS 917F1 (outer forward) CAT CAC CTA TAA CTT CAC CAT CCT CCT C, ARMS 917R1 (outer reverse) GGT ATC AAC CCC ACC TCA AAT TAT CCT A, ARMS 917F2C (inner forward) CTT TTG TTT TCC TTT CTG TGA GCA GTG, and ARMS 917R2T (inner reverse) TAT ACA GCA TGG TTC CAA TTT GGG TAA A. The PCR reactions were conducted with 3 μ L of DNA, 0.6 μ L (inner reverse) primers, 0.8 μ L (inner forward) primers, and 0.2 μ L (outer reverse e outer forward) primers, 10 μ L of Go *Taq* Colorless master mix (Promega Corporation, USA), $MgCl_2$, and ultrapure water, in a total volume of 20 μ L.

The temperature for the annealing of both sets of primers was defined at 64 $^{\circ}C$ for rs12979860 and 54.5 $^{\circ}C$ for rs8099917. The best results were obtained with the initial denaturation at 94 $^{\circ}C$ for 5 min; followed by 35 cycles, including denaturation at 94 $^{\circ}C$ for 30 s; annealing for 30 s; extension at 72 $^{\circ}C$ for 1 min, and a final extension step at 72 $^{\circ}C$ for 10 min. The DNA tetra-primer PCR products were visualized on 3% agarose gels with SyberSafe[®] (Invitrogen). For SNPs rs12979860 products of 277 pb (control); 198 bp (T allele) and 132 bp (C allele) were detected, and for rs8099917 products of 437 bp (control); 295 bp (T allele), and 197 bp (G allele).

RESULTS

For the tetra-primer PCRs optimization, several primers temperatures for the annealing were tested; 56 $^{\circ}C$ to 76 $^{\circ}C$ for rs12979860, and 53 $^{\circ}C$ to 57 $^{\circ}C$ for rs8099917. The same was conducted for $MgCl_2$ concentrations (1.5 mM to 3.0 mM), inner primers (0.1 pmol to 0.4 pmol) and DNA input (2 μ L and 3 μ L).

The best results were obtained using 3.0 mM of $MgCl_2$; temperature of primers annealing of 64 $^{\circ}C$ and 54.5 $^{\circ}C$ (rs12979860 and rs8099917, respectively); concentration of primers: 0.3 pmol of each inner primers, except for inner forward rs12979860 (0.4 pmol), and 3 μ L of DNA input (**Figures 2 and 3**).

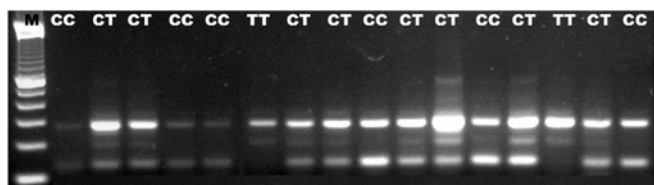


Figure 2. *IFNL4* (*IL28B*) genotypes profiles for SNPs rs12979860.

Legend: M, molecular size marker (100 bp DNA Ladder); CC genotype (products of 277 bp and 132 bp); CT genotype (products of 277 bp, 198 bp, and 132 bp); TT genotype (products of 277 bp and 198 bp)

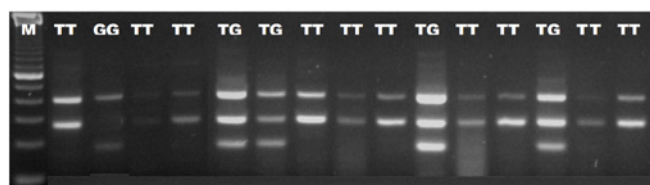


Figure 3. *IFNL4* (*IL28B*) genotypes profiles for SNPs rs8099917

Legend: M, molecular size marker (100 bp DNA Ladder); TT genotype (products of 437 bp and 295 bp); TG genotype (products of 437 bp, 295 bp, and 197 bp); GG genotype (products of 437 bp and 197 bp)

After tetra-primer PCR optimization, 100 DNA samples whose SNPs rs12979860 e rs8099917 patterns had been determined by PCR-RFLP were now analyzed by tetra-primer PCR, and the results obtained were 100% concordant.

It is important emphasize that although the low number of samples analyzed, all genotypes for both SNPs were correctly identified. The frequencies detected were: 35% (CC), 46% (CT), and 19% (TT) for rs12979860, and 72% (TT), 24% (TG), and 4% (GG) for rs8099917. In relation to the allelic distribution, the majority of wild alleles were detected: C (58%) and T (84%) for rs12979860 and rs8099917, respectively.

In order to choose the best technique to be employed as routine, an analysis of cost was conducted. **Table** shows the parameters analyzed for comparing the PCR-RFLP and tetra-primer PCR. The tetra-primer PCR disclosed a reduction of 45.5% in the cost of SNPs genotyping taking into account only the value of reagents and technical handle, without computing the required equipment.

Table. Comparative analysis of the PCR-RFLP and tetra-primer PCR techniques applied for *IFNL4* (formerly *IFNL3* and/or *IL28B*) genotyping SNPs rs12979860 and rs8099917

| Parameters | PCR-RFLP | tetra-primer PCR |
|--|----------------------------|----------------------------|
| | (rs12979860 and rs8099917) | (rs12979860 and rs8099917) |
| PCR (number of reactions) | 2 | 2 |
| Enzymatic digestion (number of reaction) | 2 | – |
| Electrophoresis (number of reactions) | 2 | 1 |
| DNA input (µL) | 10 | 6 |
| Time of reactions (hours) | 10 | 5.5 |
| Cost of PCR (R\$)* | 3.20 | 6.17 |
| Cost of RFLP (R\$)* | 5.29 | – |
| Cost of electrophoresis (R\$)* | 82.80 | 41.40 |
| Cost of technical work [R\$ (R\$ 40.00/hour)]* | 400.0 | 220.0 |
| Total cost [R\$* (2 SNPs)] | 491.29 | 267.57 |

Legend: N, number; R\$, real; * values in real in April 2023

DISCUSSION AND CONCLUSION

Currently, the determination of SNPs has a wide arsenal of molecular methods. The choice of the best technique to be applied in routine laboratory depends on the advantages and disadvantages the technique present, allowing adaptation to the particularities of each laboratory.

The gold standard of molecular techniques is genetic sequencing, especially when there is a need to know or discriminate the nucleotide present in a specific position (mutation), as is the case with polymorphisms. However, it is a laborious methodology, requires specialized technicians to analyze the results, in addition to being quite expensive²²⁻²⁵.

Since analysis of single nucleotide mutations on a large scale (routine laboratory) is difficult by sequencing, other techniques have been standardized. The qPCR showed high sensitivity, specificity and 100% agreement with the sequencing, indicating that it is the best choice, aiming at practicality, as it has the shortest execution time (about 130 minutes) and low risk of contamination^{23,26,27}. In addition, it is a closed system with ease of automation, eliminating post-amplification manipulation and allowing the reading of results with the software in real time²⁸. However, commercial kits and even in-house reactions are expensive, due to the fluorescent chemistries that allow the interpretation of the results, in addition to the maintenance and calibration of thermal cyclers coupled with a fluorescence reader, specific for real-time PCR²³.

In an attempt to reduce costs and make SNP research more accessible, some PCR reactions followed by enzyme restriction to identify genotypes were standardized, as they used only conventional thermocyclers and electrophoresis equipment. Comparing the PCR-RFLP results with those obtained by sequencing, 100% of agreement were found, which demonstrated the good performance of the technique^{17,23,29,30}. Unlike qPCR, in this reaction, there is manipulation of amplified material (electrophoresis and preparation of mix for digestion), which increases the risk of contamination. Also, a lot of time is used for the reaction, due to the various steps and the incubation with the enzyme, taking around 10 hours until the final result. Another disadvantage relates to the endonucleases that differentiate the mutation in each SNPs, they are usually very expensive and depend on importation most of the time²³. In particular, the protocol previously used in the laboratory (adapted from Moreira et al)²⁵, still presented as a limiting factor, the required volume of DNA sample [10µL for the two polymorphisms (rs12979860 and rs8099917)].

Because it is a simple, fast, economical technique that does not require elaborate equipment, tetra-primer PCR has been increasingly used in polymorphism research. Studies that compared its performance in relation to the gold standard genotyping methodology of rs12979860 and rs8099917 found 98% to 100% agreement of results^{22-24,30}. The relatively short execution time (approximately 5 hours) is another of its advantages, being superior only to the qPCR performance time. In addition, it is a very safe reaction, as it manipulates amplicons only once during application on the gel for electrophoresis and is economical in terms of DNA input (3 µL per test)²³.

In the present study, comparative analysis of cost-effectiveness between PCR-RFLP and tetra-primer PCR methodologies that considered some operational characteristics, reagent costs and technical fees in the genotyping of SNPs (rs12979860 and rs8099917), disclosed the tetra-primer PCR the best one. It was cheaper, had a shorter execution time, and 100% agreement with PCR-RFLP results.

Delvaux et al²³ evaluated the cost-effectiveness of four molecular techniques [Sanger sequencing (US\$ 202.80); qPCR (US\$ 22.90); PCR-RFLP (US\$ 27.80) and tetra-primer PCR (US\$ 19.40)] for genotyping rs12997860 and rs8099917. They also found the tetra-primer to be the best cost/benefit methodology, highlighting the ease of its implementation in laboratories with little routine or with scarce financial resources, common in developing countries. The same was observed by Fateh et al³⁰ who evaluated three methods in relation to cost and turn-around time: direct sequencing, PCR-RFLP and ARMS-PCR (tetra-primer PCR). The authors excluded the value of sample extraction, as it is common in all three methods, and estimated the costs of US\$ 4.70 for PCR-RFLP, US\$ 3.10 for ARMS-PCR, and US\$ 16.20 for sequencing, and the turn-around time of 6 hours and 45 minutes for PCR-RFLP, 2 hours and 40 minutes for ARMS-PCR, and 2 days for sequencing. Thus, they concluded that the ARMS-PCR was the most efficient and reliable method to determine polymorphisms in routine clinical practice.

Regarding the different costs of each methodology disclosed here, we have to consider that the technical fees, equipment, inputs and the value of imported products (US\$) differ depending on the country, but despite these differences, all studies point to tetra-primer PCR as the best in terms of cost-benefit.

Due to the simplicity, safety and low cost of tetra-primer PCR, it is a good choice for routine use in Public Health Laboratories, where human and financial resources, as well as sample volumes are limited.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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AUTHORS' CONTRIBUTIONS

All authors have contributed significantly to this study: ACA contributed to the study conception and design, and wrote the manuscript; EHSK and KRC performed the lab experiments, analyzed the data, and wrote the monography. All authors approved the final content.

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PRESENTATION NOTE

This manuscript is based on the Research Technical Training Monograph by Ellen Hochleitner de Souza Kindermann, entitled: Técnicas para pesquisa de polimorfismos de nucleotídeos únicos (SNPs) próximos ao gene que codifica o interferon lambda III (*IFNL3*) e comparação do desempenho da técnica tetra-*primer* PCR em relação a PCR-RFLP. Instituto Adolfo Lutz, Centro de Imunologia, Laboratório de Pesquisa em HTLV. São Paulo, 2018. 38p. Advisor: Adele Caterino-de-Araujo.

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