An Optimized Nested Polymerase Chain Reaction (PCR) Approach Allows Detection and Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) *env* and *gag* Genes From Clinical Samples

Dayse Locateli,¹ Patricia H. Stoco,² Carlos R. Zanetti,¹ Aguinaldo R. Pinto,^{1*} and Edmundo C. Grisard²

¹Laboratório de Imunologia Aplicada, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

²Laboratórios de Protozoologia e de Bioinformática, Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, Florianópolis, SC, Brazil

The needs for development and/or improvement of molecular approaches for microorganism detection and characterization such as polymerase chain reaction (PCR) are of high interest due their sensitivity and specificity when compared to traditional microbiological techniques. Considering the worldwide importance of human immunodeficiency virus type 1 (HIV-1) infection, it is essential that such approaches consider the genetic variability of the virus, the heterogeneous nature of the clinical samples, the existence of contaminants and inhibitors, and the consequent needs for standardization in order to guarantee the reproducibility of the methods. In this work we describe a nested PCR assay targeting HIV-1 virus *gag* and *env* genes, allowing specific and sensitive

diagnosis and further direct characterization of clinical samples. The method described herein was tested on clinical samples and allowed the detection of HIV-1 presence in all samples tested for the *gag* gene and 90.9% for the *env* gene, revealing sensitivities of 1 fg and 100 fg, respectively. Also, no cross-reactions were observed with DNA from infected and noninfected patients and the method allowed detection of the *env* and *gag* genes on an excess of 10^8 and 10^4 of human deoxyribonucleic acid (DNA), respectively. Furthermore, it was possible to direct sequence all amplified products, which allowed the sub typing of the virus in clinical samples. J. Clin. Lab. Anal. 22:106–113, 2008. © 2008 Wiley-Liss, Inc.

Key words: diagnosis; standardization; HIV-1; nested PCR

INTRODUCTION

The search for new or improved methods allowing a fast, sensitive, and specific detection of the human immunodeficiency virus type 1 (HIV-1) infection is of outmost importance. The use of polymerase chain reaction (PCR) has been of particular importance (1–3), being the method of choice for diagnosis of HIV-1 infection when anti-HIV-1 antibodies are present in low levels or may be passively acquired (4,5).

In this context, nested PCR provides a good strategy for detection of HIV in blood samples due to its enhanced sensitivity. However, carryover contamination (6), the presence of PCR inhibitors, the variability of the

biological samples, as well as the presence of retrovirus-like sequences in normal human cells (7) may lead to false-positive or inconclusive diagnosis results and have influenced the reliability of such a method (3,8). Also, several studies reported a low efficiency or failure of

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*Correspondence to: Aguinaldo R. Pinto, MIP/CCB, Universidade Federal de Santa Catarina, Campus Trindade, 88040-900, Florianópolis, SC, Brazil. E-mail: pintoar@ccb.ufsc.br

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PCR-based diagnosis to detect HIV-1 genes in clinical samples (9–11), reinforcing the needs for standardization and/or optimization of this technique for diagnosis and/or characterization of the virus (12,13).

Nowadays, HIV-1 subtyping by nested PCR is usually directed to the *env* and/or *gag* genes due to their high genetic variability and importance on treatment and vaccine development. However, characterization based on the protease and reverse transcriptase genes have been observed (14–16). Thus, considering the socioeconomic and epidemiologic importance of a precise HIV-1 diagnosis and characterization, the aim of this work was to standardize a nested PCR assay for specific and sensitive detection of HIV-1 *env* and *gag* genes, which allows direct subtyping of the virus from clinical samples.

MATERIALS AND METHODS

Clinical Samples

Deoxyribonucleic acid (DNA) samples were obtained by standard phenol/chloroform extraction (17) from peripheral blood cells of 10 noninfected individuals and from 88 HIV-seropositive individuals attended at the HIV/ acquired immunodeficiency syndrome (AIDS) ambulatory care unit at the Dr. Homero de Miranda Gomes Hospital (São José, SC, Brazil). HIV-1 infection was assessed by enzyme-linked immunosorbent assay (ELISA) and confirmed by Western blotting at the Laboratório Central de Saúde Pública (Lacen-Santa Catarina State Health Authority). Briefly, all individuals were bled using vacuum-ethylene diamine tetraacetic acid (EDTA) tubes and 6 mL of venous blood was immediately processed for separation of peripheral blood mononuclear cells (PBMCs) by centrifugation on Ficoll-Hypaque gradient (Sigma-Aldrich, St. Louis, MO), following proteinase K (200 mg/mL) digestion in a lysis buffer (sodium dodecyl sulfate [SDS] buffer 1%, EDTA 25 mM, 10 mM NaCl, 10 mM Tris, pH 7.5). The study was approved by the Universidade Federal de Santa Catarina (UFSC) Ethics Committee and informed consent was obtained from all participating individuals.

Control Samples

Plasmids pCR2 and pNL4-3 (Heteroduplex Mobility Assay [HMA] Kit; National Institutes of Health [NIH] AIDS Research and Reference Reagent Program, Bethesda, MD), containing the HIV *env* and *gag* gene sequences, respectively, were used as positive controls in each reaction set.

Nested PCR Conditions

Nested PCR for the *env* gene was performed using 10 pmol of primers ED5 (5'-ATG GGA TCA AAG

CCT AAA GCC ATG TG-3') and ED12 (5'-AGT GCT TCC TGC TGC TCC CAA GAA CCC AAG-3'), followed by a second amplification round using primers ES7 (5'-TGT AAA ACG ACG GCC AGT CTG TTA AAT GGC AGT CTA GC-3') and ES8 (5'-CAG GAA ACA GCT ATG ACC CAC TTC TCC AAT TGT CCC TCA-3'), allowing the amplification of 1,250 bp and 700 bp fragments (18), respectively. Both amplifications were carried out in a Mastercycler Gradient® thermal cycler (Eppendorf, Hamburg, Germany), consisting of an initial step at 94°C for 2 min, three cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, following 32 cycles of 94°C for 15 sec, 55°C for 45 sec, 72°C for 1 min, and a final extension step at 72°C for 5 min.

DNA samples revealing negative results using primers ES7/ES8 were further tested by PCR using primers ED3 (5'-TTA GGC ATC TCC TAT GGC AGG AAG AAG CGG-3') and ED14 (5'-TCT TGC CTG GAG CTG TTT GAT GCC CCA GAC-3') for the first PCR and ED31 (5'-CCT CAG CCA TTA CAC AGG CCT GTC CAA AG-3') and ED33 (5'-TTA CAG TAG AAA AAT TCC CCT C-3') for the following nested PCR, resulting in amplicons of 2 kb and 640 bp, respectively (19).

For *gag* gene amplification, 5 pmol of primers H1G822 (5'-GCT TTC AGC CCA GAA GTA ATA CC-3') and H1GHMA1317 (5'-CCA AAT TCT CCC TAA AAA ATT AGC CT-3') for the first PCR and H1Gag1584 (5'-AAA GAT GGA TAA TCC TGG G-3') and g17 (5'-TCC ACA TTT CCA ACA GCC CTT TTT-3') for the nested PCR, resulted in amplicons of 830 bp and 460 bp (20), respectively. These amplifications were performed with the following thermal profile: 94°C for 2 min, 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min, and a final extension step of 7 min at 72°C.

The proper annealing temperatures for each primer pair were determined by gradient temperature assays performed with pure control plasmids DNA or mixtures of control DNA with excesses of human DNA as templates and annealing temperatures from 45 to 60°C. The temperature at which a single and strong band was observed in agarose gel electrophoresis was determined as the optimal annealing temperature. A schematic representation of each primer annealing site, as well as their relative positions on the HIV-1 HXB2 genome (GenBank accession number AF033819), is presented in Fig. 1.

Both PCR and nested PCR reactions were initially standardized using 1 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 mM of deoxyribonucleotide triphosphate (dNTP) (Invitrogen, Carlsbad, CA), 30 ng of template DNA, and distinct buffers varying in pH (8.4, 8.8, or 9.2), MgCl₂ (15 or 35 mM)

Fig. 1. Schematic representation of the HIV-1 *env* and *gag* genes showing the annealing positions of the primers used in this study as well as their relative positions on the HIV-1 HXB2 virus genome (numbers). Adapted from the HIV-1 *env* HMA Subtyping Kit and HIV-1 Group M *gag* HMA Subtyping Kit Manual (NIH AIDS Research and Reference Reagent Program). Scale bar represents ~300 bp.

and KCl (250 or 750 mM) concentrations. Along with the pCR2 and pNL4-3 plasmid DNA used in three dilutions (1:10, 1:100, and 1:1,000) as positive PCR controls, negative controls consisted of: 1) the absence of template DNA; and 2) noninfected human DNA extracted as described in Clinical Samples, above, also in three dilutions (1:10, 1:100, and 1:1,000). Nested PCR reactions used as template 1 μ L of the initial PCR reactions but due the concern for possible carryover contamination, all pre- and post-PCR manipulations were performed in distinct areas not sharing reagents or equipment. Also, negative controls on the nested PCR reactions were performed by adding an aliquot (1 μ L) from the previous PCR round negative controls. After amplification, all products were resolved in 1% agarose gels, stained with ethidium bromide (0.01% vol/vol), and digitally recorded.

Determination of Nested PCR Sensitivity and Specificity

Sensitivity tests were carried out using 10-fold dilutions (10 ng to 1 fg) of the pCR2 and pNL4-3 reference plasmids DNA and the nested PCR conditions described above. Specificity assays were performed using reconstitution tests mixing 10-fold dilutions of noninfected human DNA (1 ng–1,000 ng) with 1 pg of each reference plasmid DNA (pCR2 or pNL4-3). In both cases, endpoint detection of the nested PCR amplification products was determined through agarose gel electrophoresis as described above in Nested PCR Conditions.

Detection of HIV-1 *gag* and *env* Genes in Clinical Samples by Nested PCR

After DNA extraction, three dilutions (1:10, 1:100, and 1:1,000) of all clinical samples were tested in duplicate for the presence of HIV-1 using the herein described nested PCR approach. After amplification, products were resolved in 1% agarose gel electrophoresis as described in Nested PCR Conditions, above, and digitally recorded. Also, 2 μ L of each positive sample was used as a template for direct sequencing reactions in order to verify their usefulness for HIV-1 typing. Thus, sequencing was performed in an automated MegaBace 1000[®] DNA Analysis System using the DYEnamic[®] ET Dye Terminator kit (GE/Amersham Biosciences, Uppsala, Sweden) as formerly described (21). Briefly, after isopropanol (70%) precipitation of the PCR products, sequencing was carried out using primers ES7 or ED31 and H1Gag1584. Sequencing reaction was set up using 50–200 ng of purified nested-PCR products and 5 pmol of each primer, using the following thermal profile: denaturation for 25 sec at 95°C, following 35 cycles of 95°C for 15 sec, 55°C for 30 sec, and a final extension step of 120 sec at 60°C. After a new purification with isopropanol 70%, samples were injected at 2 kV for 120 sec and electrophoresed at 9 kV for 1–2 hr. After quality evaluation and clustering by the Phred/Phrap/Consed package (22,23), high quality sequences (Phred $\geq$ 20) with at least 150 bp had their identity checked using the BLAST program (24) using the GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>) and

Fig. 2. Agarose gel electrophoresis stained with ethidium bromide, revealing the amplification products of the sensitivity assays for detection of the HIV-1 *env* (A) and *gag* (B) genes for the first and for the nested PCR. MW, molecular weight marker (*Lambda* phage DNA digested with *Pst*I); NC, negative control of the reaction (without DNA).

at the Los Alamos (http://hiv-web.lanl.gov/content/hiv-db/BASIC_BLAST/basic_blast.html) databases.

RESULTS

Based on the herein described standardized PCR/nested PCR reaction and using 10-fold dilutions of plasmid pCR2, a clear and specific amplification of the HIV-1 *env* gene was obtained at concentrations as low as 0.1 pg and 1 fg, respectively (Fig. 2A). For the *gag* gene, the sensitivity of the first PCR and the following nested PCR was of 1 pg and 0.1 pg, respectively (Fig. 2B) and no cross-reaction was observed with noninfected patients DNA in any assay (Fig. 3).

Reconstitution tests carried out using artificial mixtures of control plasmids and human DNA as described in Determination of Nested PCR Sensitivity and Specificity, above, allowed the detection of the HIV-1 *env* and *gag* genes by nested PCR in an excess of 10^8 and

10^4 times of human DNA, respectively. Since similar results were obtained for both primer pairs, a representation of the obtained results for the *env* gene is shown in Fig. 3.

Based on these results and considering the possibility of the presence of inhibitors in the reaction, we have used three dilutions of each testing sample, which proved to be effective since several clinical samples revealed positive in a single dilution (Fig. 4B). No carryover contamination of any nested PCR reactions was observed during the study, confirming that proper separation of pre- and post-PCR procedures are effective in avoiding such problem.

The use of the nested PCR approach for HIV-1 proviral DNA detection through amplification of *env* and *gag* genes from clinical samples revealed for both genes a single and clear amplification product of the expected size (Fig. 4). During the testing of 12 distinct buffers varying in pH, and MgCl_2 and KCl

Fig. 3. Agarose gel electrophoresis stained with ethidium bromide, revealing the sensitivity and specificity of the detection of the HIV-1 *env* gene for the first and nested PCR assays using reconstitution tests. MW, molecular weight marker (*Lambda* phage DNA digested with *Pst*II); PC, positive control (pCR2 plasmid); NC, negative control (without DNA).

Fig. 4. Agarose gel electrophoresis stained with ethidium bromide, revealing the first PCR (A) and the nested PCR (B) for detection of HIV-1 *env* and *gag* genes using DNA from two clinical samples (S1 and S2) in three dilutions (1:10; 1:100; and 1:1,000). NS, negative sample (DNA from noninfected patients); PC1 and PC2, positive controls (1 fg and 1 pg of pCR2 or pNL4-3 plasmids); MW, molecular weight marker (*Lambda* phage DNA digested with *Pst*II); NC, negative control (without DNA).

concentration for PCR and nested PCR reactions, several revealed the expected amplification products. Among these, a buffer containing 100 mM Tris-HCl (pH 9.2), 15 mM MgCl₂, and 250 mM KCl revealed the strongest amplicons for the *gag* gene and for the *env* gene the best amplification patterns were obtained with a buffer containing 100 mM Tris HCl (pH 8.4), 15 mM MgCl₂, and 750 mM KCl. All positive clinical samples tested for the *gag* gene by the above described nested-PCR assay were positive. On the other hand, only 75% of the positive clinical samples were confirmed by the nested PCR approach for the *env* gene.

Considering that in our conditions the PCR/nested PCR with primers ES7/ES8 and ED5/ED12 was not able to amplify the *env* gene in 22 clinical samples (25%), these same samples were further tested with an alternative primer set (ED3/ED14 and ED31/ED33) directed to the V3 region of the *env* gene. Among these samples, 14 out of 22 revealed positive, reducing to around 60% the number of false-negative results. False-positive results were not observed for any primer set used in detection of both genes, revealing the specificity of the method. Figure 4 shows the detection of HIV-1 *gag* and *env* genes in two clinical samples representing

the profile observed for all analyzed samples. It is clear that the use of the herein described nested PCR approach enhance the amplification of the target genes and that dilutions may enhance the overall method sensitivity by reducing any inhibitory effect (Fig. 4B).

All amplification products obtained by the nested PCR detection of the *gag* ($n = 88$) and *env* ($n = 80$) genes were purified and directly sequenced as described in Detection of HIV-1 *gag* and *env* Genes in Clinical Samples by Nested PCR, above, resulting in high quality (Phred ≥ 20) sequences of 300–700 bp, which allowed confirmation of the fragments' identity. Furthermore, these results allowed a sequence-based HIV-1 subtyping using the GenBank and Los Alamos databases, which were the subject of another publication (21).

DISCUSSION

Diagnosis of early, postexposure HIV infection prior to seroconversion can be achieved by the detection of proviral DNA in mononuclear cells by nested PCR. This technique allows also the study of mutations, recombination, drug resistance, and the identification of HIV subtypes. Other techniques as cloning and HMA also allow HIV-1 subtyping; however, they involve distinct technical difficulties. The aim of this study was to standardize a nested PCR technique in order to verify the reaction efficiency in detecting HIV-1 infection, thus reducing unspecific amplification and the occurrence of false-negative results. The proposed method was revealed to be highly sensitive and specific in detecting HIV-1 infection by PCR amplification of *gag* and *env* genes in 100% and 90.9% of the clinical samples tested, respectively.

All positive clinical samples tested for the *gag* gene by the proposed method were positive; however, only 75% of the positive clinical samples were confirmed by the nested PCR approach directed to the *env* gene. This result is in agreement with previous reports indicating that detection of the *gag* gene is more efficient than the *env* gene (25). However, using a different primer set, Brigido et al. (26) were able to amplify 66% of samples tested for the *gag* gene and 84% for the *env* gene. Using distinct combinations of several primers, Castro et al. (27) proposed an approach by which PCR detection of the *env* gene was obtained for 71% of the tested samples. Such variable results and the difference observed in the present study may be due the variability of the virus genome, leading to an occurrence of a particular HIV subtype, as recently reported by Powell et al. (28). In this case, the commonly used primers may be unable to hybridize to the template sequence and lead to false-negative results, which reinforces the importance of generating sequences of such target genes for

comparative analysis. Furthermore, reports indicate that PCR amplification of protease and reverse transcriptase genes is even more difficult to achieve, being successful in 42–70% of the attempts for both genes (10,11).

The standardization of the buffer composition was demonstrated to be more efficient and less costly in increasing the reaction sensitivity (29) and in reduction of unspecific bands and false-negative results than just testing multiple annealing temperatures (30). Thus, using temperature gradients and a variety of distinct buffers, we have minimized the occurrence of unspecific bands during the standardization procedures, which enabled us to have a single, specific band in all assays that can be directly applied to sequencing reactions.

Despite the use of standard and widely used methods for blood sampling, Ficoll density-gradient collection of PBMC and DNA extraction (17) in the present study, variable recovery of target cells and DNA was obtained. Considering that such variation can often lead to false-negative PCR results due the presence of contaminants, inhibitors, or even low amounts of target sequences, we herein propose the sampling of 6 mL of blood from each patient and the use of three dilutions for each DNA sample in all PCR assays. This protocol proved to be effective in increasing the sensitivity since several samples revealed positive in a single dilution (Fig. 4B), probably due to reduction of inhibitors/contaminants and/or by changing the stoichiometry of the reaction favoring the amplification.

Primer choices are also a determinant factor when aiming for specific and sensitive molecular diagnosis. In this work, primer selection considered oligonucleotides commonly used for amplification of both HIV-1 *env* and *gag* genes in the literature, including the NIH AIDS Research and Reference Reagent Program. Thus, based on regular DNA extraction and common primers, the nested PCR standardization procedures carried out in this study resulted in strong and specific amplification of the target genes, not revealing unspecific amplification products even in the excess of human DNA (Figs. 3 and 4).

Since nested PCR reactions use PCR-amplified products as template, there is a major concern of carryover contamination, especially when working on diagnosis of infectious diseases (31). Despite the existence of several approaches to avoid such contamination, the use of strict separation of reagents and equipment and the inclusion of negative controls on the nested PCR reactions revealed no contamination.

The presence of inhibitors is also a major concern in PCR-based methods and is often a problem for standardizing a technique or method (32). Such inhibitors may be carried from the biological sample

or may be introduced during the separation and/or extraction processes, varying among distinct samples, sampling, or extraction methods, reagents, or even laboratories. Thus, due the difficulty of having a standard protocol, the use of dilutions proposed in this study proved to be efficient in increasing the test's sensitivity, as observed in *gag*- or *env*-based HIV-1 detection on clinical samples (Fig. 4).

Addressing the *gag* gene, we have obtained a complete concordance with standard diagnostic methods used in clinical routine (ELISA and Western blotting), obtaining the amplification of the gene from 100% of the 88 clinical samples tested. However, for the *env* gene the standardization procedures have shown higher sensitivity values than those described in the literature (26,27), obtaining amplification products of the gene for 90.9% of the clinical samples. This result revealed the higher sensitivity of the proposed methodology when compared to previous results (9–11). The difference in the efficacy of amplification of *env* and *gag* genes is usually related to the high variability of the HIV-1 *env* gene (16) and, thus, primer design aiming diagnosis methods targeting the *env* gene present variable results and must be constantly standardized, which is a problem for routine diagnosis.

The nested PCR is a highly reproducible and specific technique for HIV-1 detection in clinical samples, allowing further subtyping of the infecting virus, as reported herein. Although this work points out alternatives to increase the efficacy of the amplification of both HIV-1 *gag* and *env* genes by nested PCR, we must emphasize that the reproducibility in this study was far more dependent on buffer than on primer annealing temperature. The optimal buffers revealed no additional bands on lower annealing temperatures when compared to others tested, even in the presence of excess of human DNA. Thus, we may conclude that buffer testing is a highly effective and lower cost standardization procedure for PCR and nested PCR for the detection of HIV-1 *gag* and *env* genes, increasing the specificity of the detection and avoiding nonspecific interactions with human DNA.

Attempting to offer a feasible alternative for HIV-1 diagnosis and subtyping, the possibility of direct sequencing of the nested PCR products was tested in the present study. This strategy proved to be effective and lower in cost by avoiding cloning procedures, resulting in high-quality sequences that allowed precise identification of the PCR fragments and subtyping of the clinical samples by direct sequencing. The results of the subtyping of such samples was addressed in another study (21).

In conclusion, the specificity and sensitivity values of a variety of PCR and nested PCR methods described in

the literature for HIV-1 diagnosis are dependent on several factors, including the virus' genetic variability due to mutation or recombination events and the geographical origin of the sample. Thus, our results indicate that the existing tools can be used worldwide depending on proper standardization, as suggested in the present work.

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