Available online at www.sciencedirect.com
ScienceDirect
Molecular and Cellular Probes 21 (2007) 368–378
Molecular and Cellular Probes
www.elsevier.com/locate/ymcpr

Fast and sensitive quantitative detection of HIV DNA in whole blood leucocytes by SYBR green I real-time PCR assay

Anna Casabianca[a,*], Caterina Gori[b], Chiara Orlandi[a], Federica Forbici[b], Carlo Federico Perno[b], Mauro Magnani[a]

[a]*Institute of Biological Chemistry "Giorgio Fornaini", University of Urbino, via Saffi 2, 61029 Urbino (PU), Italy*
[b]*National Institute for Infectious Diseases IRCCS "Lazzaro Spallanzani", via Portuense 292, 00149 Rome, Italy*

Received 31 January 2007; accepted 1 May 2007
Available online 21 May 2007

Abstract

The aim of this study was the development of a real-time PCR for HIV DNA quantification in whole blood leucocytes providing an alternative assay to those already described, almost based on the *gag* gene detection. The technique (*pbs*-rtPCR assay) is more rapid (the whole assay required less than 5 h), easy to perform, omitting both PBMC purification step and data normalization to a housekeeping gene, when compared to previously published assays. Our method is able to detect all group M HIV-1 subtypes in the highly conserved primer-binding site (PBS) region and to avoid the interference by retroviral endogenous sequences in HIV DNA quantification. The sensitivity was 100% for 2 copies even in the presence of high amounts of genomic DNA (1 μg). To monitor the HIV DNA level in all possible clinical conditions, the assay has been validated and compared with a previously developed *gag*-PCR assay on 73 HIV-1 HAART-treated patients with a plasma HIV-1 RNA range from <50 to $>500\,000$ copies/ml. The 50% of the samples with a viremia below the limit of detection (50 copies/ml) was found to contain HIV DNA between 2 and 10 copies/μg DNA. The *pbs*-rtPCR offers a significant improvement in the percentage of quantitatively detectable sample (99%) compared with the *gag*-PCR (42%) suggesting caution in the choice of HIV DNA assay.

Keywords: HIV DNA quantification; Human endogenous retroviruses; Primer-binding site; SYBR Green I real-time PCR; Whole blood leucocytes

1. Introduction

In the HAART (highly active antiretroviral therapy) era, HIV testing has gained immense therapeutic relevance: initiating HAART on time may improve the quality of life and indeed prolong life considerably. Although the introduction of HAART results in undetectable levels of plasma virus (50 copies/ml) in the majority of patients, it fails to completely eradicate the virus. Indeed, despite a powerful long-term viral suppression which can involve up to 9 years of uninterrupted suppressive therapy, there is evidence of ongoing viral replication, and of HIV DNA persistence in cellular reservoir (CD4+ T lymphocytes and cells of the macrophage lineage) and anatomical sanctuary sites (brain and possibly testis). These viral reservoirs are one of the major obstacles in HIV-1 eradication [1–5].

At present, the plasma HIV-1 RNA assay which has a sensitivity of approximately 50 copies/ml, is the most widely used and most sensitive commercially available method to monitor the effectiveness of current therapeutic regimens [5].

However, other virological and immunological markers appear to be relevant. Recent studies suggest that peripheral blood mononuclear cells (PBMC) HIV DNA may be a marker for HIV-1 disease progression [6,7]. The quantification of HIV DNA, in blood cells may be, along other associated parameters (i.e. plasma HIV RNA or CD4 count), an additional tool in clinical practice and may be of assistance to define the best time to initiate and prescribe treatment for each patient [7–10]. Moreover, Yerly and colleagues founded that the level of HIV DNA in PBMC have a predictive value in term of the viral set point after

*Corresponding author. Tel.: +39 0722 305211; fax: +39 0722 305324.
E-mail address: a.casabianca@uniurb.it (A. Casabianca).

0890-8508/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.mcp.2007.05.005

discontinuation of HAART [11]. Recently, Shiramizu et al. demonstrated that high HIV DNA levels in PBMC correlate with HIV-1 associate dementia in HAART-experienced individuals even after controlling for plasma HIV-1 RNA loads and with HAART-naïve patients. The study provides evidence that HIV DNA may be an important factor in HIV-1 neuropathogenesis [12,13]. The study of intracellular HIV DNA dynamics could be of particular interest in that 30% of HAART-treated patients show discordant viro-immunological responses. As reported in [10], the high HIV DNA content founded in naïve and memory CD4+ T cells was strictly associated to deficiency of T-cell reconstitution in immunological-non-responders.

Cellular HIV DNA content will give more detailed information, thus contributing to the development of new therapeutic strategies involving drug design that targets the virus during various phases of its life cycle such as entry and fusion, integration, virus assembly and maturation [14,15].

Another important application of HIV DNA detection is in the early diagnosis of pediatric patients born from seropositive mothers. Pediatric HIV infection, can be diagnosed using HIV DNA PCR within the first 10 days of life (http://www.hivguidelines.org/Content.aspx?pageID=258).

In adults, the delay between infection and HIV seroconversion (window period), which may be several months long, represents one possible cause of false ELISA negativity. Moreover, HIV-1 negative specimens can give non-specific-reactivity on Western Blot [16,17]. Quantitative HIV DNA detection is able to identify HIV infection prior to evaluation of HIV antibodies while providing valuable information through the quantitative results obtained. It may also prevent ambiguous results given by antibody tests. In the past few years PCR-based assays to quantify cell-associated HIV DNA have been developed; many of them are based on conventional end-point PCR [9,18–21] and others on the more sensitive real-time PCR [22–26]. Recently, real-time PCR detection of HIV DNA in dried spots, for the early diagnosis in infants, has been developed [27,28]. Since, to our knowledge, an HIV DNA assay that could be employed in all the applications mentioned above does not yet exist, we attempted to create an alternative SYBR Green I real-time PCR assay (*pbs*-rtPCR assay) based on HIV DNA quantification in the whole blood cells of patients. This method detects all forms of intracellular HIV DNA that is, unintegrated and integrated linear DNA, as well 1-long terminal repeat (LTR) and 2-LTR circles [9,23]. Subsequently, a group of HIV-1 positive patients under HAART and having a broad range of plasma viremia (HIV RNA between <50 and $>500\,000$ copies/ml) was selected to validate the PCR assay in determining the exact HIV DNA copy number normalized per μg of DNA or per ml of blood and to compare the quantitative results obtained by *pbs*-rtPCR with those of a previously optimized quantitative *gag*-PCR assay [10].

2. Materials and methods

2.1. Reference strains and patients

Seventy-three HIV-1 positive patients treated with HAART at the National Institute for Infectious Diseases "Lazzaro Spallanzani", Rome, Italy were enrolled in the study, after informed consent following the Helsinki declaration, selected on the basis of their plasma viremia (range from <50 to $>500\,000$ copies/ml). Following with clinical assessment and routine laboratory monitoring, 1 ml of whole blood was collected from patients. Each DNA sample was assayed in SYBR Green I real-time PCR assay to quantify HIV DNA levels in infected leukocytes . The level of HIV-1 RNA in plasma has been quantified directly with use of a solid-phase nucleic acid hybridization assay based on branched DNA (bDNA) signal amplification technology (Versant HIV-1 RNA 3.0 assay, Bayer Diagnostics, Emeryville, CA, USA). This test is FDA approved for in vitro diagnostic use and has a linear dynamic range from 50 to 500 000 copies/ml.

Lymphocyte surface phenotypes and CD4+ lymphocyte counts were determined using flow cytometry analysis by Immunotech-Beckman Coulter (Marseille, France).

The sequence analysis showed that 65 of 73 samples (89%) were B-subtype and 8 (11%) were non-B (two subtypes F, two subtypes C and four circulating recombinant forms: two 01_AE and two 02_AG). Three molecular cDNA clones of HIV-1 and one of HIV-2 from the National Institute for Biological Standards and Control (NIBSC, UK) were also analyzed: Ugandan strains (HIV-1 subtype A, NIBSC repository reference ARP2014.1 and HIV-1 subtype D, NIBSC repository reference ARP2014.4), Central African Republic strain (HIV-1 subtype H, NIBSC repository reference ARP2014.10) [29] and complete proviral sequence of HIV-2 ROD (HIV-2, NIBSC repository reference EVA232.1). Additionally, 100 HIV-1 seronegative blood donors were used to test specificity of the assay in HIV-1 DNA detection.

2.2. DNA isolation

For the isolation of DNA from blood cells, two different protocols were first settled in Urbino at the Institute of Biological Chemistry "G. Fornaini", and then validated in laboratories at the National Institute for Infectious Diseases "L. Spallanzani". The first protocol (phenol method), previously described [30], was used with some modifications. Briefly, 1 or 0.5 ml of frozen blood was washed twice with 1-10 volumes of physiological solution containing 0.1% NP-40 and 1 volume of SDS 5% was added to the cell pellet. After an incubation of 30 min at 37 °C, an equal volume of lysis buffer (8 M Urea, 0.3 M NaCl, 10 mM Tris–HCl pH 7.5, 10 mM EDTA pH 8.0) was added, followed by a further incubation for 30 min at 37 °C. The DNAs were then purified by phase extraction with organic solvent followed by ethanol precipitation and