A rapid RT-PCR assay for the detection of HIV-1 in human plasma specimens

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A R T I C L E   I N F O
Article history:
Received 13 June 2014
Accepted 16 June 2014
Available online 16 June 2014

Keywords:
HIV-1
RT-PCR
AIDS
Molecular diagnostics

A B S T R A C T

Introduction: The CDC estimates that there are currently over 1 million people living with human immunodeficiency virus (HIV-1) in the United States, with new cases increasing by approximately 50,000 each year. HIV-1 consists of four distinct groups: the major M group, and the rare N, O, and P groups, each comprising of various subtypes. Without proper care, HIV-1 can lead to cardiovascular, kidney, and liver diseases, cancer, and rapid progression into acquired immune deficiency syndrome (AIDS). Here, we describe a novel, rapid, and highly sensitive assay for the detection of HIV-1 using intercalating dye based RT-PCR and melt curve analysis.

Materials and methods: We designed an RT-PCR assay for the detection of the major M subtypes in addition to the rare (O, N, and P) HIV-1 groups, as well as an extraction/RT-PCR control, using melt curve analysis. Viral RNA was extracted using the automated QiaGen EZ1 robotic system (QiaGen, Valencia, CA). To establish the limit of detection (LOD) for this assay, we diluted the AcroMetrix HIV-1 panel (LifeTechnologies, Grand Island, NY) to concentrations ranging from 25 to 500 copies/ml. Armored RNA® BCR/ABL b3/a2 (Asuragen, Austin, Texas) was used as our extraction and RT-PCR control. Specificity and accuracy were assessed by testing plasma specimens from 48 anonymized patients negative for HIV-1.

Results: This assay has a turnaround time of less than 2.5 h and has a limit of detection of 50 copies/ml of plasma. Our assay also demonstrated 100% concordance with 53 previously quantified plasma patient specimens, including 48 negative samples and 5 positive samples. HIV-1 and our extraction/RT-PCR control were consistently identified at 79 °C and 82.5 °C, respectively.

Conclusions: We developed a comprehensive, easy to use assay for the detection of HIV-1 in human plasma. Our assay combines a rapid and cost-effective method for molecular diagnostics with the versatility necessary for widespread laboratory use. These performance characteristics make this HIV-1 detection assay highly suitable for use in a clinical laboratory.

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Introduction

The CDC estimates that there are currently over 1 million people living with human immunodeficiency virus (HIV-1) in the United States and for the past decade, the number of persons infected with the virus has increased at an alarming rate of almost 50,000 new cases per year. Of these persons, approximately 1 in 5 are unaware of the infection and thus do not seek treatment (http://www.cdc.gov/hiv/statistics/basics/ataglance.html). Without proper care, HIV-1 can lead to cardiovascular, kidney, and liver diseases, cancer, and rapid progression into acquired immune deficiency syndrome (AIDS) (http://www.cdc.gov/hiv/topics/basic/index.html; Zeng et al., 2014). AIDS is the latter stage of HIV-1 infection, resulting in a severely damaged immune system incapable of adequately fighting diseases and certain cancers (http://www.cdc.gov/hiv/topics/basic/index.html). Current combinations of medications can greatly slow the progression of HIV-1 to AIDS, as well as reduce the risk of transmission of the virus (http://www.cdc.gov/hiv/topics/basic/index.html). Early detection of the virus is therefore critical for patient care and management.

In addition, there are four distinct groups of HIV-1: the major M group, and the rare N, O, and P groups. Group M comprises 90% of HIV-1 infections and contains at least 9 genetically unique subtypes (A, B, C, D, F, G, H, J, and K) and 15 circulating recombinant forms (CRFs) (Arien et al., 2005) (Fig. 1). CRFs are formed when two viruses of different subtypes combine genetic material in the cell of an infected person, resulting in a hybrid virus (Burke, 1997). Group O is typically found only in west-central Africa, while group N – a strain only identified...
dye based RT-PCR and melt curve analysis. Intercalating dye, such as SYBR Green, based real-time RT-PCR with melt curve analysis, is an example of such a platform. This has been shown as a reliable method for the detection of nucleic acid targets with varying sequences, across a broad range of optical thermocyclers (Varga and Delano, 2006). It is therefore highly beneficial to develop assays that incorporate this technique, due to the commonality of such instruments in clinical laboratories.

In this study, we describe a novel, rapid, and highly sensitive assay for initial detection as well as patient monitoring of HIV-1 during acute and chronic stages of infection, respectively, using intercalating dye based RT-PCR and melt curve analysis.

**Materials and methods**

**Samples**

In this study, we used the plasma AcroMetrix HIV-1 panel (LifeTechnologies, Grand Island, NY) and 48 clinical samples that had been previously tested and quantified using the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 (Roche Molecular Diagnostics, Branchburg, NJ). For the limit of detection study, AcroMetrix samples were diluted with negative plasma to concentrations ranging from 25 to 500 copies/ml. Armored RNA® BCR/ABL b3/a2 (Asuragen, Austin, Texas) was used as an extraction and RT-PCR control.

**Extraction**

We used 400 μl of plasma for each patient sample and made a separate internal control mixture containing 52.2 μl Buffer AVE, 3.6 μl Carrier RNA, and 4.2 μl Armored RNA® BCR/ABL b3/a2. This internal control mixture was incorporated into the plasma sample during the automated extraction. Viral RNA was extracted with the QiaGen EZ1 robotic system using the Virus v2.0 card and the EZ1 Virus Mini extraction kit/cartridges (QiaGen, Valencia, CA). Viral RNA was eluted in 60 μl elution buffer and subsequently concentrated using the RNasey MinElute Cleanup Kit (QiaGen, Valencia, CA) with a final elution volume of 14 μl. We used 11.2 μl of the eluted viral RNA per RT-PCR reaction.

**RT-PCR and fluorescence melting curve analysis**

RT-PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA). Each 25 μl reaction mixture contained 12.5 μl of 2 × iTaqtM Universal SYBR® Green One-Step Kit (Bio-Rad, Hercules, CA), 0.3 μl of iScript Reverse Transcriptase (Bio-Rad, Hercules, CA), 0.1 μM of HIV Forward Primer (5′-GCT TTA TTA CAG GGA CAG CAG AGA-3′), 0.1 μM of HIV Reverse Primer (5′-ACT CCT GCC CCT TCA CTT TGC CA-3′), 0.085 μM of AR Forward Primer (5′-GACT GGA TTT AAC CAG AGT TC-3′), 0.085 μM of AR Reverse Primer (5′-TTC GGA GTT CCA AGC AGC-3′), 0.06 μl of RNase OUT, and 11.2 μl of eluted viral RNA. The RT-PCR amplification profile was as follows: cDNA synthesis for 10 min at 50 °C, followed by...
by reverse transcriptase inactivation for 5 min at 95 °C, then 45 cycles of denaturation at 95 °C for 10 s and anneal/extension at 57.5 °C for 30 s. Once amplification was complete, melting curves were obtained by performing a temperature gradient from 65 to 90 °C at 0.1 °C/s. Fluorescence was continually measured during the heating period and the melting peaks were visualized by plotting the fluorescence versus the temperature.

**Results**

In this assay, we designed specific primers targeting the pol integrase genomic region for one step RT-PCR detection of HIV-1. This region is highly conserved in the major M subtypes in addition to the rare (O, N, and P) HIV-1 groups. Armored RNA (AR) is integrated into each plasma sample during extraction and is therefore used as an extraction and RT-PCR control (Fig. 2). Both HIV-1 and AR primers were designed to amplify specific targets with characteristic melting temperatures of ~79 °C and ~82.5 °C, respectively.

**Analytical sensitivity (limit of detection)**

To establish the limit of detection (LOD), we diluted the plasma AcroMetrix HIV-1 to concentrations of 25 copies/ml, 50 copies/ml, 75 copies/ml, 100 copies/ml, and 500 copies/ml. We extracted viral RNA from each plasma sample and spiked it into our RT-PCR reactions. Our assay was able to easily resolve 50 copies/ml, 75 copies/ml, 100 copies/ml, and 500 copies/ml across both tests (Fig. 3). Our AR extraction and RT-PCR control were resolved in each sample, showing that extraction and RT-PCR worked as expected. This demonstrates a high sensitivity for the detection of HIV in plasma sample types.

**Specificity and accuracy**

To assess the specificity of this assay we tested 53 previously quantified plasma patient specimens, including 48 negative samples and 5 positive samples with concentrations ranging from 100 to 200,000 copies/ml. We chose patients who had been found negative in the most recent previous screening on the COBAS AmpliPrep/COBAS TaqMan HIV-1 Test, v2.0 because HIV viral loads can fluctuate while receiving treatment. This test quantifies HIV-1 group M and N subtypes with a limit of detection of ≤50 cp/ml, ranging from <15 to 46 cp/ml in group M (http://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/PremarketApprovalsPMAs/ucm092879.pdf; Schumacher et al., 2007). Our assay identified all 48 clinical specimens as negative for HIV (Fig. 4). Both the RNA extraction and amplification processes were intact as shown by the detection of the AR control peak. Our assay also identified all 5 positive clinical specimens as positive for HIV infection. These results were 100% concordant with what was expected and demonstrate a high level of accuracy and specificity for our assay.

**Turnaround time**

The total turnaround time for this assay is less than 2.5 h. The assay begins with a 45 min automated extraction, followed by a 15 min viral RNA cleanup and concentration. RT-PCR amplification takes approximately 1 h and 20 min to set up and run (Fig. 4). The results of the
melt-curve are available within minutes of completion of the RT-PCR and easy to interpret.

Discussion

In this study, we report a new method for initial detection and monitoring of HIV from plasma specimens. This RT-PCR based assay uses an intercalating dye and melt-curve analysis to determine the presence of HIV. Such an assay is affordable, sensitive, and can be implemented on real-time PCR platforms frequent to most molecular diagnostics laboratories. Based on our in silico primer design, this assay should provide universality, suggesting that it is capable of detecting a wide range of HIV-1 groups and/or subtypes. We have, however, only tested the AcroMetrix HIV-1 panel, which is made solely from HIV-1 Subtype B source material.

Intercalating dyes and melt curve analysis are much less expensive to use than fluorescently labeled probes, yet maintain high specificity and sensitivity. This lowers the cost of our RT-PCR without sacrificing the lower limit of detection. Our assay also uses an automated viral RNA extraction system, which greatly reduces the hands-on time, making it fast and easy to perform. This minimizes the risk of contamination as well as accidental exposure to the active virus. After automated extraction we are able to concentrate the viral RNA, which greatly improves the sensitivity of our assay.

Our results exhibit this assay as sensitive, specific, and accurate. The LOD for this assay was 50 copies/ml, which is comparable to other commercial kits whose LODs range from 20 to 50 copies/ml (Church et al., 2011; Kaur et al., 2014; Pas et al., 2010). The U.S. Department of Health and Human Services recommends an LOD of 200 copies/ml in order to determine virologic success as opposed to failure (http://www.aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf; Kaur et al., 2014). However, some studies suggest that this threshold is too high for patients with quantifiable and detectable viremia (Gandhi and Deeks, 2012). The target of highly active antiretroviral therapy is suppression of plasma HIV-1 RNA levels to <50 copies/ml, since this cutoff has been shown to slow virus evolution and T-cell activation (Hammer et al., 2006). This has been shown to allow for immune system reestablishment, slow disease progression, and improve survival rates (Hammer et al., 2006; Wood et al., 2000). While we use an automated extraction method in order to lower the variability of our assay, the plasma input is limited to 400 μl. Some manual extractions increase the amount of plasma input to 1 ml, which improves the overall LOD but adds risk and variability. At 50 copies/ml of plasma, our RT-PCR reaction is receiving an input of only ~20 copies of viral RNA.

Our results were also 100% specific with what was expected for the 48 previously tested negative patient plasma specimens. The use of an extraction and RT-PCR control is vital for HIV-1 detection as inhibition rates can reach as high as 3.7% in large-scale validations, resulting in a false-negative (Drosten et al., 2006). Should the control fail to amplify, all of the samples and controls from the initial run must be retested, starting with extraction.

HIV-1 demonstrates a vast genetic diversity with many different genotypes of HIV-1 in existence today. The primers we designed for this assay cover all the subtypes of HIV-1 detailed in the WHO Reference Panel (http://www.nibsc.org/documents/ifu/12-224.pdf), including subtype group P. This gives our assay the versatility to detect a wide range of HIV-1 types, which makes for a very comprehensive detection method. Further testing should be done across all known groups and subtypes to verify the universality of HIV-1 detection in this assay.

In conclusion, we developed a comprehensive, easy to use assay that can be used to detect acute and chronic HIV-1 infections in human plasma. This assay combines a rapid and cost-effective method for molecular diagnostics with the versatility necessary for use across various laboratory settings. These performance characteristics make this HIV detection assay highly suitable for use in a clinical laboratory.

Funding

This work was supported by the Bill and Melinda Gates Foundation [Global Health Grant Number OPP1028794].

References


