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Low cost HIV-1 quantitative RT-PCR assay in resource-limited settings: Improvement and implementation

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A B S T R A C T

Monitoring of HIV viral load in low and middle income settings is limited by high cost of the commercial assays. Therefore, we developed a novel RT-PCR quantitative assay was developed. This assay targets the HIV-1 *pol integrase* gene (INT). Subsequently, the performance of the INT assay, described previously as a Long Terminal Repeat (LTR) assay and a combined INT/LTR dual target RT-PCR assay was compared. The LTR-assay was found to be sensitive and cost-effective (50–70% cheaper than commercial assays) with the lowest coefficient of variation (%CV). Introduction of an internal standard further improved assay reliability. Therefore, this LTR assay was implemented in West Java, Indonesia. Linearity and precision of the LTR assay were good: %CV ranged from 1.0% to 10.4%. The limit of quantitation was 616 copies/ml. Performance was comparable with the commercial assay (Abbott assay) ($r^2 = 0.01$), although on average the viral loads were 0.39 $\log_{10}$ copies/ml lower. In clinical practice, it had excellent capability for monitoring treatment failure, the positive predictive value was 99% and the negative predictive value was 93%. In conclusion, the implementation of the improved HIV-1 viral load LTR-assay for routine diagnosis in resource poor settings can be a good alternative when commercial assays are unaffordable.

1. Introduction

UNAIDS estimates that at the end of 2009, 33.3 million people were living with HIV, 83.6% of them live in low- and middle-income countries (UNAIDS, 2010). The monitoring of HIV viral load of patients treated with anti retroviral treatment (ART) is an effective and common practice in high-income countries. However, the high cost of the commercial HIV viral load tests limits their implementation in low- and middle-income settings.

Although affordable alternative tests are available for measuring HIV RNA, such as the heat-associated HIV-1 p24 antigen enzyme-linked immunosorbent assay (Bonard et al., 2003; Ribas et al., 2003; Mwapasa et al., 2010) and the reverse transcriptase activity kit assay from Cavide Exa Vir (Braun et al., 2003; Malmsten et al., 2003; Huang et al., 2010; Stewart et al., 2010), these have not been implemented widely in low income settings because they are labor-intensive and involve complex techniques. "in-house" real-time quantitative RT-PCR assays have the advantage that they are cheap, sensitive and easy to perform. One of these assays targets the HIV-1 Long Terminal Repeat (LTR), which is moderately conserved among HIV-1 subtypes B and non subtype B. This assay has been evaluated by several research groups and found to have limit of detection varying from 92.4 copies/ml to 300 copies/ml (Candotti et al., 2004; Drosten et al., 2006; Rouet et al., 2005, 2007; Edelmann et al., 2010; Vessière et al., 2010). Given the large variation of HIV-1 subtypes found in some developing countries, instead of using single target assay, it could be more advantageous to use dual target assays such as the COBAS Taqman HIV-1 v2.0 (Sizmann et al., 2010; Paba et al., 2011; Sire et al., 2011).

Most of the "in-house" assays were developed in high-income countries that have adequate laboratory capacity and high skilled technicians, but there are few reports of their implementation in low- or middle-income countries. The difference in capacity between those two settings may affect the technical and clinical performance of the assay. Therefore, it is crucial to evaluate the

Abbreviations: RNA, ribonucleic acid; LTR, Long Terminal Repeat; INT, integrase; CV, coefficient variation; NPV, negative predictive value; PPV, positive predictive value; UNAIDS, Joint United Nations Program on HIV/AIDS; WHO, World Health Organization; RT-PCR, real time-polymerase chain reaction; LOD, limit of detection; LOQ, limit of quantitation; Ct, cycle threshold; NIBSC, National Institute for Biological Standards and Control; PDV, Pochine distempter virus; ART, anti retroviral.

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total performance of the assay in the laboratory in resource-limited countries. The assays internal control is important when monitoring the efficiency of extraction and amplification of an individual sample. It is essential to select internal controls that can be mixed in one extraction tube without interfering with the target PCR outcome.

This research was conducted at Erasmus MC, the Netherlands and at Hasan Sadikin hospital, Indonesia. The technical performance of three "in-house" assays, two single (LTR and INT) target assays and one dual target (combination of LTR and INT) assay, in combination with three different nucleic acid extraction procedures were examined. The most optimal configuration assay was chosen and its implementation in West Java, Indonesia, was evaluated. The ability of the assay to predict therapy failure, and its running cost compare with a commercial HIV-1 viral load assay were assessed. The internal control of the assay was determined and its application with the HIV-1 "in-house" assay was observed.

This study provides information which could be useful for monitoring HIV viral load as a part of a routine service for HIV/AIDS patients under therapy in countries facing financial and capacity constraint.

2. Materials and methods

2.1. Test and clinical samples

2.1.1. Test samples

The WHO HIV-1 RNA standard (HIV-1 RNA, 2nd International Standard, 97/650; NIBSC) containing 5.56 $\log_{10}$ IU of HIV-1, subtype B, per ml was diluted in HIV negative plasma and used to determine the limit of detection (LOD) and the limit of quantitation (LOQ) of each assay. Twenty replicates at each concentration (725, 325, 180, 90 and 45 copies/ml) were extracted using QIAamp RNA viral mini kit using two sample input volumes, 140 μl and 560 μl.

To evaluate the performance of three nucleic acid systems, a HIV-1 culture supernatant was diluted in HIV negative plasma. Ten replicates at concentration 3.29 $\log_{10}$ copies/ml were run per assay per purification system.

To test the efficiency of the assay on different types of the HIV strains, the WHO subtype reference plasma panel (NIBSC code: 08/358, UK) including HIV-1 subtypes A, B, C, D, AE, F, G, H and N was assessed.

To determine the intra- and inter-assay variation, 10 aliquot of HIV-1 IIIB purified virus at concentrations between 7.43 and 2.83 $\log_{10}$ copies/ml were tested. In the assessment of the intra-assay variation, five assays were run independently, while in the inter-assay variation, ten assays were run independently.

A HIV-1 IIIB electron microscopy counted virus stock (2.3×10^{11} vp/ml, tebu-bio, France) was calibrated relative to the WHO HIV-1 RNA standard diluted in QIAamp RNA viral mini kit lysis buffer and used as secondary standard and for further experiments.

2.1.2. Clinical samples

To determine the acceptable detection limit of the developed HIV-1 "in-house" assays in resource-limited settings, a total of 2121 clinical samples from 915 patients were tested using the Abbott RealTime HIV-1 assay over a period of four years.

To assess the agreement between "in-house" assay and the Abbott RealTime HIV-1 assay, a total of 210 clinical samples from patients treated with antiretroviral therapy (ART) at Hasan Sadikin Hospital, West Java, Indonesia were selected at random after obtaining informed consent. Those selected samples were also used to define the treatment predictive value of the "in-house" assay. The samples were tested with the Abbott RealTime HIV-1 viral load assay and stored at −80 °C until tested by the "in-house" assays. The Bland–Altman analysis, the positive predictive value (PPV) and the negative predictive value (NPV) of the "in-house" assay relatively to the commercial assay were calculated.

2.2. RNA isolation

HIV-RNA was extracted with three purification systems: MagnaPure LC (Roche Diagnostics, GmBH), Abbot HIV-1 preparation kit (Abbott Molecular, USA) and QIAamp RNA viral mini kit (Qiagen, GmBH). For the MagnaPure LC system, the input volume was 200 μl and the output volume was 100 μl, for the Abbott HIV-1 system, the input volume was 200 μl and the output volume was 89 μl, for the QIAamp RNA system, the input volume was 140 μl and the output volume was 80 μl. The extraction procedures followed the manufacturer's instructions.

2.3. Assay design

Primers and probes for the LTR assay were obtained from Drosten et al. (2006). This assay targets Long Terminal Repeat region of HIV-1. The primers and probe for INT assay were designed targeting conserved HIV-1 *pol* integrase gene (nt 4835–4972) using Primer Express software. To assess the presence of primer/probe mismatches in those two assays, alignments were made using all complete sequences of Los Alamos database. An alignment of the LTR region was built from 257 and of the INT region was built from 1456 different HIV-1 subtype sequences. The INT fragment was amplified using the forward primer 5′-cataatagcaacagacatacaaactaaaga-3′, reverse primer 5′-gccccttcacctttcca-3′ and probe 5′-aaaattttcgggtttattacagggacagca-3′. Probes of the LTR and INT assay were labeled with FAM fluorescent dye at the 5′ end and non-fluorescent quencher at the 3′ end. For the dual target assay (MIX), primers and probes from the LTR and INT assay were combined in a single RT-PCR reaction.

2.4. Real-time quantitative RT-PCR

RNA was amplified using the TaqMan® Gold RT-PCR amplification kit (Roche Molecular System, New Jersey, USA). Briefly, 20 μl of RNA was added into 30 μl amplification-mix containing 20 pmol of each primer and 5 pmol probe. Thermal cycling program was as follows: 48 °C for 30 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Cycling was performed in ABI 7500 sequence detection system (Applied Biosystems, Nieuwkerk a/d Ijssel, the Netherlands). RNAse free water (Baxter, the Netherlands) was used as negative template control (NTC) and low (10^3 copies/ml), mid (10^4 copies/ml) and high (10^6 copies/ml) concentrations of the EM counted virus stock were used as positive external quantitation controls. An obtained Cycle threshold (Ct)-value was valid if the NTC was undetectable and if Ct values of the positive controls were in range of mean ± two times standard deviation (STD).

2.5. Internal control

Pochine distemper virus (PDV) was used as a universal internal control (GenBank accession: AF479274). The PDV internal control (IC) was added to each sample upon HIV-1 RNA isolation. PDV RNA was detected in a separate RT-PCR system described by Clancy et al. (2008) and contained forward primer 5′-cgggtgccttttacaagaac-3′ and reverse primer 5′-ttctttcctcaacctcgtcc-3′ which were modified to generate an 83 base pair amplicon. The probe 5′-atgcaagggccaattcttccaagtt-3′ was labeled with a 5′-Dragonfly1 dye and matching 3′ quencher BHQ-2. An obtained Ct-value of a sample