Establishment of In-House Quantitative Real-Time RT-PCR Assay for HIV-1 Viral Load Measurement: Application to Evaluate Efficacy of ART in Ghanaian Patients in an Urban Setting

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Abstract

The aim of this study was to establish and apply a real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) for human immunodeficiency virus (HIV) RNA quantification in patients on antiretroviral treatment (ART) in Ghana, where recombinant strains including CRF02_AG are prevalent. The primers and TaqMan probe concentrations as well as reaction temperatures were optimized to establish an efficient in-house quantitative assay system for HIV RNA, a tool for HIV viral load measurement in patients. Then an already established HIV-specific PCR amplicon (HIV-1 gag) was used as an external standard to estimate the linearity, amplification efficiency, analytical sensitivity and reproducibility of the in-house real time quantitative assay. Finally, the assay was applied to quantify the viral load in clinical samples of HIV patients on ART. The real time quantitative assay was shown to have good linearity (R²=1.0), high amplification efficiency (E=1.91), high sensitivity (180 copies/ml), and high reproducibility (variation coefficient range, from 1.25% to 3.58%). Analytical specificity and sensitivity of the assay in clinical samples was 96.7% and 95.0%, respectively. The established tool is reliable and covers all relevant genotypes including rare and recombinant forms that circulate in the sub-region. It could therefore allow general monitoring of antiretroviral therapy in patients living in resource-limited settings due to its simplicity, rapidity and less-labour intensiveness.

Keywords: ART; Evaluation; Ghana; HIV; qRT-PCR; Viral load

Abbreviations: HIV: Human Immunodeficiency Virus; ART: Antiretroviral treatment; PMTCT: Prevention of mother-to-child transmission; qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; NASBA: Nucleic acid sequence-based amplification; LTR: Long terminal repeat

Introduction

Antiretroviral therapy (ART) can effectively manage human immunodeficiency virus type 1 (HIV-1) infection. Lower ART costs make treatment programs possible in non affluent countries, where 95% of HIV infections occur [1,2]. However, patients receiving antiretroviral therapy (ART) frequently develop treatment resistance. A key determinant of treatment failure is an increase in viral load, tests for which generally are not available in developing countries. As such, the WHO developed guidelines for ART failure that are based upon CD4 counts and clinical criteria, as these measures are generally available [3]. However, objective evaluation of these criteria has shown that there is always poor correlation between these parameters for clinical evaluation of patients. The quantification in plasma of human immunodeficiency virus type 1 (HIV-1) RNA is therefore a necessary useful test for predicting clinical outcome, evaluating disease progression, and monitoring the effect of ART on HIV-1-infected patients [4,5].

Programs to scale-up ART in resource-limited countries have lately received a lot of attention in the last 7 years [6,7]. Currently, almost 1.5 million people in these countries are receiving ART and the number continues to grow. However, due to limited resources and inadequate laboratory capacity, many programs have minimized the laboratory monitoring of patients on treatment, in an effort to accelerate the widespread availability of the antiretroviral drugs [8]. There are, however, several reasons why equal efforts should be made to implement HIV-1 RNA viral load monitoring in these regions. The inability to detect early virological failure could lead to an accumulation of drug-resistant mutations and the selection of viruses with broad cross resistance to antiretroviral drugs. An increasing number of patients carrying drug-resistant virus will inevitably lead to a spread of these resistant strains in the population.

Hence, ART should as a matter of compulsion include accurate monitoring of plasma virus concentrations (viral load) [2,9-12]. There is therefore an urgent need for low-cost, simple, and accurate HIV-1 viral load monitoring technologies in resource-limited settings, particularly at the time of the scaling-up of first- and second-line ARTs. Earlier, for the prevention of mother-to-child transmission (PMTCT), virus detection is required for testing newborns and mothers milk. However, in current practice viral load in mothers milk is used as a correlate to plasma viral load, and therefore plasma viral load suppression considerably reduces the risk of transmission through breastfeeding. Testing for HIV-1 in the context of ART and PMTCT is best done
by molecular methods, e.g., reverse transcription-PCR (RT-PCR), nucleic acid sequence–based amplification (NASBA), or branched DNA (bDNA) assays [10,11,13–16]. Because of the high diversity of HIV-1, assay design is extraordinarily demanding. Therefore, viral load testing relies almost exclusively on expensive commercial tests. With decreasing prices for drugs in many countries, therapy monitoring has become more expensive than the treatment itself [17,18], a situation that leads to insufficient therapy monitoring, suboptimal patient management, and increased risk for emergence of drug-resistant virus strains. In addition, because they have been optimized for strains prevalent in the Northern Hemisphere, many commercial tests are not accurate for testing “exotic” HIV-1 subtypes found mainly in developing countries [19]. Moreover, some commercial assays are based on the gag gene, which is too variable for detection of outlier strains [20], and hence consequently in using such assays, certain HIV-1 subtypes and recombinants may not be detected [21,22].

There is therefore an urgent need for low-cost, simple, and accurate HIV-1 viral load monitoring technologies in resource-limited settings, particularly at the time of the scaling-up of first and second-line highly active antiretroviral therapies [23,24]. Technological improvements, from automated sample isolation to real time amplification technology, have given the ability to develop and introduce systems for most virulent of clinical interest, and to obtain clinically relevant information needed for optimal ART options [25,26]. On the basis of this established experience [20], we have developed an inexpensive real-time quantitative RT-PCR viral-load assay that at least equals to commercial tests with regard to technical features and performance. Instead of the gag gene, it targets the highly conserved long terminal repeat (LTR) region, thereby providing a spectrum of detectable and quantifiable genotypes beyond that of current assays. Without doubt, the fluorescence-based real-time RT-PCR is widely used for the quantification of steady-state mRNA levels and is a critical tool for basic research, molecular medicine and biotechnology. Assays are easy to perform, capable of high throughput, and can combine high sensitivity with reliable specificity.

Material and Methods

Gold standard for monitoring antiretroviral treatment success or failure, HIV patients and clinical samples

According to the guide line and criteria for management of HIV patients on antiretroviral treatment advanced and approved by the Ministry of Health of Ghana, plasma viral load measurement (gold standard test) augmented with the dynamics of CD4 and CD8 counts and other clinical parameters serve as index for treatment success or failure. There were a total of 405 HIV patients on ART recruited for cross-sectional monitoring at the Koforidua Government Hospital during the application of the in-house quantitative real-time RT-PCR (qRT-PCR) evaluation of efficacy. From the 405 patients, veinomous blood from patients was collected in vacutainer tubes containing EDTA (Japan). The HIV

AAG CTT TA-3'. Primers were chemically synthesized by Invitrogen (Gene Diagnosis Inc., Japan) on ABI Prism 7300 (Applied Biosystems, Foster City, USA). The HIV

GCC TGT AC-3', and the reverse primer was 5'-AGC ACT CAA GGC

GTG CCC GTC T-Quencher-MGB-3'; set 3-fwd, 5'- CAG GCA GCA

ACC TAC CAG ATG TTA A-3'; set 1-fwd, 5'- GCC TAC ATA AAG GTT GCC TTG A-3'; set 1-rv, 5'- GCC GCC ACT GCT AGA GAT TTT-3'; set 1-probe, 5'-FAM-AAG TAC TCG GTG CGC GTC T-Quencher-MGB-3'; set 2-fwd, 5'-GTC AAG TAA CGG TAT GCC TCC TGT TG-3'; set 2-rv, 5'-TAC GCT AAT TTT GGT CAT-3'; set 2-probe, 5'-FAM-AAG TAC TCG GTG CGC GTC T-Quencher-MGB-3'; set 3-fwd, 5'-CAG GCA GCA ATG CAT AGT TTA A-3'; set 3-rv, 5'- AGT AGT TCC TGT CGC ACT TCC C-3'; set 3-probe, 5'-FAM-CAG TAC ATG CAG GGC CTA TTC CAC CAG TAMRA-3'; set 4-fwd, 5'-CAA GCA GCC ATG CAA

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ATG TTA A-3'; set 4-rv, 5'-AGT AGT TCC TGC TAT GTC ACT TCC C-3'; set 4-probe, 5'-FAM-CAG TGC AGG CTA TGG CAC CAG TAMRA-3'; set 5-fwd, 5'-GTA GTG TGT GCC CGT CTG TTG-3'; set 5-rv, 5'-CAAG GAC GAC TGC TGC GT-3'; set 5-probe, 5'-FAM-TGG CGC CCG AAC AGG GACT T-TAMRA-3'.

For preparation of viruses, HEK 293T cells in 6-well-plates were transfected with 2 µg of the plasmid encoding NL4-3 or 97GH-AG2 using 6 µl of FuGENE 6 (Roche), according to the manufacturers’ protocol. Culture medium was changed after 12 h of incubation, and culture supernatant was collected 24 h after changing culture medium. The concentration of p24 in the collected culture supernatants was measured with the HIV-1 p24 CLEIA assay. Virus-containing medium was diluted to make the concentration of NL4-3 p24 to be 100 ng per 140 µl and that of 97GH-AG2 p24 to be 100 pg per 140 µl. RNA was extracted from 140 µl of the diluted virus-containing medium by using viral RNA purification kit (QIAGEN). Purified RNA was subjected to quantification by real-time RT-PCR assay with 5 primer-probe sets. Cycle threshold value (CT value) was compared to identify the primer-probe set with the highest sensitivity.

Plasma HIV-1 RNA quantification by real-time RT-PCR assay

Briefly, RNA was extracted from 200 µl of stored (-70°C) plasma by using the QIAGEN procedure. The TaqMan PCR target was a conserved consensus region in the long terminal repeat (LTR) region of the HIV-1 major group. The primer/probe set 1 was used in this study. All runs were performed in a 50-µl volume containing RNA extract (20 µl), primers (a 500 nM concentration of each), probe (200 nM), 1× PCR buffer, and 1× RT RNase Inhibitor Mix (Taqman One-Step RT-PCR Master Mix; Applied Biosystems).

Statistical analysis

Linear regression was used to analyse the data obtained from the standard curve of qRT-PCR in this research. The assessing parameters including amplification efficiency, specificity, sensitivity and reproducibility were analysed by SPSS for windows version 13.0 software (SPSS Inc, Chicago, IL USA). The correlation between results of three replicate runs by 2 independent operators at 3 different occasions for our in-house HIV RNA viral load assays were analyzed using Pearson’s correlation coefficient (r). Variability within-run and between-run was determined by calculating the coefficient of variation (CV) [30]. The analytical sensitivity of operator 1 and operator 2 were measured with the HIV-1 p24 CLEIA assay. Virus-containing medium was diluted to make the concentration of NL4-3 p24 to be 100 ng per 140 µl and that of 97GH-AG2 p24 to be 100 pg per 140 µl. RNA was extracted from 140 µl of the diluted virus-containing medium by using viral RNA purification kit (QIAGEN). Purified RNA was subjected to quantification by real-time RT-PCR assay with 5 primer-probe sets. Cycle threshold value (CT value) was compared to identify the primer-probe set with the highest sensitivity.

Results

HIV specific PCR amplicon standard

An HIV specific PCR amplicon standard was prepared in the laboratory. The HIV amplicon was made by conventional PCR. In the electrophoresis gel, the HIV amplicon of a proper size (503 bp) was acquired as shown in Figure 1B. Then the amplicon produced by many PCR reactions was purified and quantified. The purified HIV PCR amplicon was measured in triplicate on ABI Prism 7300. The average concentration was 2.75×10^9 copies/µl (2.70−2.80×10^9 copies/µl). The above-quantified HIV stock amplicon solution and a dilution of 2.75×10^9 copies/ml used throughout the study as an external standard were then both kept at −20°C.

Linearity, amplification efficiency and detection limit of the TaqMan real-time quantitative RT-PCR assay

First, the sequences of the primers and TaqMan probes, their concentrations, and reaction temperatures were optimized to establish an efficient qRT-PCR assay for HIV RNA. Five sets of primers and a probe targeting HIV-1 LTR and gag were designed based on the sequence of NL4-3 laboratory strain and 97GH-AG2 Ghanaian CRF02_AG strain (Figure 1A). We analyzed sensitivity of these primer-probe sets using diluted viruses produced from 293T cells transfected with pNL4-3 or p97GH-AG2. Sensitivity analysis revealed that the primer-probe set 1 and 3 efficiently detect the Ghanaian CRF02-AG strain (97GH-AG2) (Figure 1C), while the primer-probe set 1 and 4 detect the NL4-3 strain (Figure 1D). Our primer-probe set 1 could sensitively quantify both strains. Figure 1A shows primer construction and selection within tile profile of tile LTR and Gag regions of HIV genome. Figure 1B shows the amplicon standards was electrophoresed in 1.5 % agarose gel containing M, 1kb ladder; lane 1, 97GH-AG2 (Ghanaian isolate) expected size of 503 bp respectively; lane 3, distilled water (negative control). The AG strains are currently driving the infections in Ghana.
The TaqMan quantitative assay was carried out on a series of ten-fold dilutions from 1×10⁷ to 1×10¹ of HIV amplicon standard and Ghanaian CRF02_AG, a major strain driving HIV infection in Ghana. The standard curve (Figure 2) was generated automatically. The standard curve showed the linear relationship between the log of the concentration of target RNA and the cycle threshold (Ct) value. The correlation coefficient of the standard curve was 1.0, indicating a precise log-linear relationship between the concentration of target RNA and the Ct value. The regression equation was as Equation 1: y = -3.3206x + 47.245 [1]. Based on conventional quantitative RT-PCR theory [31,32], the regression coefficient and the PCR amplification efficiency are related according to Equation 2: b = -(1/logE) [2] where E is efficiency of PCR reaction, b is regression coefficient (slope of the standard curve). According to the equation, the PCR amplification efficiency calculated was 1.91. The detection limit of the assay was determined by performing a series of dilutions of HIV amplicon standard for five repeats. The detection limit of the quantitative RT-PCR assay was 180 copies/mL (Table 1).

**Reproducibility of HIV-1 viral RNA quantification assay**

Variability of viral RNA quantification within-run and between-run was determined by calculating the CV [30]. The CV of within-run by operator-1 ranged from 0.34% to 4.12%, and the CV of between-run values ranged from 2.10% to 6.41%. The CV of the within-run operator-2 activity ranged from 1.00% to 3.80%, and the CV of between-run by operator-2 ranged from 1.12% to 5.20%. The variation of within-run and between-run analyses was very low, as indicated in Table 2.

**Application of the real-time quantitative RT-PCR assay on clinical samples**

The real-time quantitative RT-PCR assay was applied to detect HIV RNA in plasma specimens collected from 405 study subjects. The quantification of HIV RNA showed that there was suppression of virus expression in 302 patients on ART and 99 patients showed varying degrees of viral load while four patients exhibited undetermined values which might probably mean the patients have no RNA detectable in their plasma due to ART (Table 3). Among the 30 plasma specimens collected from healthy persons in the control group, one was found falsely positive. Therefore the analytical specificity and sensitivity of the in-house HIV RNA quantitative RT-PCR assay were 96.7% and 95.0%, respectively as indicated by the gold standard (Table 4). It was probable that the plasma specimens from the one patient sample which was negative could be due to failure of the inhibition of the RT-PCR inhibitors. The inhibitors of the RT-PCR negatively affect PCR amplification efficiency during template

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**Table 2: Reproducibility of in-house HIV RNA viral load**

| Run 1 | Run 2 | Run 3 | Mean | SD | CV (%) |
|-------|-------|-------|------|----|--------|
| 48 335.57 | 5318.71 | 785.81 | 6.41 |
| 49 430.62 | 650 5 62 | 268 266.87 | 3.12 |
| 50 298.75 | 218 278.96 | 8039 0.00 | 2.12 |
| 51 89 527.03 | 2750.25 | 5.20 |
| 52 98 289 | 1069.86 | 4.02 |
| 53 83 88 90.00 | 94.70 | 1.12 |

| HIV RNA Assay Operators | Sample Tested | With-in Variation (Copies/ML) | Between- run Variation (Copies/ML) |
|-------------------------|---------------|-------------------------------|-----------------------------------|
| Operator 1              |               |                               |                                   |
| 1                       | 49 317 ± 165  | 51 881 ± 1321 | 45 640 ± 261 | 48 933.57 | 3137.71 | 6.41 |
| 2                       | 25 121 ± 656  | 25 177 ± 1037 | 24 266 ± 867 | 25 187.96 | 785.81 | 3.12 |
| 3                       | 80 92 ± 162   | 78 31 ± 125   | 81 94 ± 139  | 80 390.00 | 168 31 | 2.12 |
| Operator 2              |               |                               |                                   |
| 1                       | 50 227 ± 712  | 53 718 ± 604  | 55 706 ± 552 | 53 217 00 | 2750.25 | 5.20 |
| 2                       | 25 821 ± 892  | 25 970 ± 986  | 27 980 ± 1012 | 26 590 00 | 1069.86 | 4.02 |
| 3                       | 82 35 ± 205   | 85 21 ± 161   | 84 08 ± 198  | 83 880.00 | 94.70 | 1.12 |

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**Table 1: Detection limit of the TaqMan HIV quantitative assay using a series of dilutions of HIV Amplicon Standard.**

| HIV RNA Assay (Copies/ML) | Number of positive samples/ (Number of tested samples) | Positive (%) |
|---------------------------|--------------------------------------------------------|--------------|
| 540                       | 5/5                                                     | 100%         |
| 420                       | 5/5                                                     | 100%         |
| 300                       | 5/5                                                     | 100%         |
| 180                       | 4/5                                                     | 80%          |
| 60                        | 0/5                                                     | 0%           |

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**Note:**
- All samples were tested in triplicate for each run. Every run was operated by different operator on 3 independent occasions.
- Between-assay SDs were calculated using the modified SD formula: ([SD of means]² - mean with-assay SD) / 3²
- The SD achieved was used to calculate the CV by using the formula CV = (SD / mean) * 100
few years ago with the increasing availability of ART in resource-limited settings was only supplemented with [34]. In Western countries, the combined cost of a CD4 cell count and a viral load test is higher than the cost of 2 months ART therapy [36]. The combined cost of a CD4 cell count and a viral load test is higher than the cost of 2 months ART therapy [36].

In developing the assay, we took into account that for resource-limited countries, special attention should be paid to the high genotypic diversity of HIV in these regions. Subtyping was therefore performed on a subset of the samples used in this study and revealed the presence of at least five subtypes and a number of unique recombinations. CRF02_AG is the major driver of HIV infection in Ghana and others in minor proportion of circulation such as CRF06_cpx, sub-subtype A3, CRF09_cpx and subtypes A, G and D had been identified. Studies have shown that RT activity is independent of the HIV-subtype [40-45]. This is in agreement with other studies that evaluated an in-house real-time RT-PCR assay that targeted the same Long Terminal Repeat (LTR) region probing for viral RNA. The LTR region is one of the more conserved regions of the HIV-1 genome and therefore perfectly suitable for the development of assays with broad subtype specificity [46-49].

Real time PCR was carried out in a closed system with PCR post treatment (no gel analysis is needed), and so the risk of contamination was substantially reduced. The qRT-PCR assay out performs previous clinical diagnostics not only because of its speed, lower contamination risk, and ability for quantification but also because of its higher sensitivity and specificity for the RNA determination of clinical specimens [50]. In this study, primers and TaqMan probes were designed, and PCR conditions were defined for efficient amplification and quantitation of HIV RNA. An HIV_c3 NL4-3 amplicon standard of a 503-bp highly conserved sequence from LTR gene was made by conventional PCR to estimate analytically the quantitative RT-PCR assay. The qRT-PCR assay established in this study was shown to have a good linearity ($R^2=1.0$), a high PCR amplification efficiency (1.91), a high sensitivity (180 copies/ml), and a good reproducibility (variation coefficient range from 1.25% to 3.58%). In this study, we used a relatively stable HIV amplicon DNA as an external standard because handling of DNA is easier and safer than RNA. Compared with the gold standard Real-Time HIV-1 assay (Abbott), the specificity and sensitivity of the assay for detection of the HIV RNA in clinical samples were 96.7% and 95.0%, respectively, which were close to those of the quantitative RT-PCR for detection of other RNA virus in clinical specimens [50]. Hence, the 74.6% viral suppression in HIV patients as detected by the application of the in-house assay system gives indication that management of HIV patients on ART in Ghana is efficient. However, varying levels of viral load detected in 25.4% of patients is worrisome and therefore calls for a pragmatic program of longitudinal study to follow-up on this group of patients for effective clinical management to prevent them from developing resistant mutations. On the basis of the overall results obtained in the study, we conclude that the assay could be useful in poor-resource settings to measure HIV viral load in clinical samples for the management of patients on ART. This study confirmed that in general TaqMan qRT-PCR is one of the most appealing techniques in the field of basic research and molecular laboratory diagnosis for quantification of nucleic acids [51] adoptable as an in-house for resource limited setting especially in south Saharan African countries like Ghana.

**Conclusions**

We have developed in-house quantitative real-time RT-PCR assay method for HIV-1 viral load measurement which is rapid, simple, and cost-efficient therefore very suitable for resource-limiting settings. The validity of this assay has been confirmed by its application to evaluate the efficacy of ART in Ghanaian patients.
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