Evaluation of HIV-1 Viral Load Detection by modified a Commercial Real-Time PCR Reagent used for Light Cycler 1.2 Instrument

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Objective: Commercial TaqMan real-time PCR reagent was modified and applied on Light Cycler 1.2 for quantifying HIV-1 RNA in plasma and compared with the reference method; COBAS AmpliPrep/COBAS Amplicor HIV-1 monitor test version 1.5.

Material and Method: Three hundred and eight frozen and fresh plasma samples were used for evaluation. Sequential specimens were also tested for follow-up cases.

Results: The correlation between HIV-1 RNA values obtained by reference and modified method with automated and manual sample preparation were significant with r = 0.916 and 0.908 (p < 0.001, p < 0.001) respectively with similar agreement log of mean bias (0.5 versus 0.48). High degree of correlation and agreement were observed between the assays in blind fresh plasma, r = 0.953 (p < 0.001) with 0.15 log difference in HIV-1 RNA level. Among follow-up samples, both methods gave 100% concordant results.

Conclusion: This modified protocol provided evidence for using modified commercial real-time PCR reagent for HIV-1 RNA quantitative detection as a monitoring tool for HIV/AIDS patients in Thailand.

Keywords: HIV-1 viral load, ARV monitoring: real-time PCR, Light Cycler

Since the World Health Organization launched the program of “3 by 5” initiative in 2005 it has stimulated many countries to accelerate and strengthen mechanisms for providing antiretroviral therapy (ART) to the people living with HIV. The Ministry of Public Health (MOPH) Thailand launched a program called National Access to Antiretroviral Programs for People who have HIV/AIDS (PHA) or NAPHA that has offered free antiretroviral drugs to all PHA starting from 2002(1). This “NAPHA” program has improved the morbidity and the mortality rate among HIV patients. However, the increased usage of ART has been accompanied by the need of monitoring tests such as CD4 and viral load (VL) testing. The “NAPHA” program covered CD4 count but not for HIV-1 viral load testing.

As the quantification of HIV-1 viral load is a biological marker for assessing disease prognosis and outcome of ART(2). Several commercial kits are available for quantification of HIV-1 viral load in Thailand. The technologies based on either target or signal amplification such as the COBAS Amplicor HIV-1 Monitor test, the HIV-1 RNA Quantitative assay and Quantiplex HIV RNA assay(3-5). The cost of viral load testing is very expensive but the AIDS patients treated with ART can have viral load testing once a year according to National Health Security Office of Thailand. However, many HIV-1 infected patients are not able to access for viral load test, thus a low-cost alternative test is urgently needed. Several real-time PCR testing could represent low-cost HIV VL monitoring technology(6,7). Global Fund for AIDS, TB and Malaria (GFATM) gave funding for 11 real-time PCR machines (Light Cycler 1.2), but there is no reagent perfect match or compatible with the instrument for the quantification
of HIV-1 RNA in plasma.

The purposes of the present study were to evaluate the performance of real-time PCR for HIV-1 quantitative assays and provide this test for HIV-1 infected cases in ARV clinic. The authors would like to transfer this low-cost technique to other laboratories that have Light Cycler (LC) machine from the Global Fund. This easy and cost effective reagent was modified and used with the LC 1.2 machine. The automated machine, COBAS AmpliPrep, was used to extract the plasma sample for VL testing in routine service at Bamrasnaradura Infectious Disease Institute. The option of manual sample extraction was evaluated in the present study. The viral load detection by COBAS AmpliPrep/COBAS AMPLICOR HIV-1 MONITOR test, version 1.5 (CAP/CA v1.5) was compared with real-time TaqMan HIV-1 test using LC instrument.

Material and Method

Samples

EDTA blood samples from HIV-1 infected patients who attended Anti-retroviral (ARV) clinic at Bamrasnaradura Infectious Disease Institute at various stages of infection were used in the present study. Three hundred and eight plasma specimens were divided into three groups for evaluation. The first and second groups of frozen plasma were composed of 135 plasma samples and 36 sequential samples among nine patients of ARV monitoring cases. The last group of 137 blind of fresh plasma was evaluated parallel to the routine testing. Of the 135 samples, 104 used for sample extraction by automated COBAS AmpliPrep machine with Total Nucleic Acid Isolation reagent (CAP-TNAI) and 31 samples for manual preparation with Amplicor HIV-1 monitor v1.5 extraction kits.

The plasma specimens were separated from EDTA blood after centrifugation at 1,600 rpm for 20 min, and aliquot into three tubes within 6 hours. The first tube was tested for HIV-1 viral load quantization. The other two left over plasma samples were kept in -70°C freezer for frozen specimen back up.

Standard curve and in-house independent run control (IRC) preparation

Seven frozen plasma samples were commercially available as OptiQuant HIV-1 RNA panel (Acrometrix Inc, USA). The panel contains seven vials, of which, 6 points of positive members for standard curve construction and one of negative control sample. For HIV-1 viral load quantization of established real-time PCR protocol, the standard curve was generated with a range from 50, 500, 5,000, 50,000, 500,000, 50,000,000, and 5,000,000 copies/mL with both manual and automated sample preparation, each point was run in duplicate. The IRC was prepared from pooled HIV-1 infected plasma samples and viral load level was tested by COBAS AmpliPrep/COBAS AMPLICOR HIV-1 Monitor test version 1.5; CAP/CA v1.5 (Roche Molecular Systems, USA.) then it was diluted with normal human plasma, which created homogenous mix and was checked for viral load again. After it was mixed well, IRC was aliquoted and kept at -70°C until performing RNA extraction. HIV-1 RNA in IRC was tested by both CAP/CA and real-time LC. The IRC must be included for every run of real-time PCR in order to calculate the HIV-1 viral load with standard curve.

COBAS AmpliPrep/COBAS AMPLICOR HIV-1 MONITOR test, version 1.5 (CAP/CA, v1.5: RT-PCR)

This CAP/CA v1.5 (Roche Molecular Systems, USA) was used as a reference method in the present study and performed according to the manufacturer’s instruction. The principle is based on end-point RT-PCR. The process of viral load detection started from sample extraction by COBAS AmpliPrep instrument then amplification and detection by COBAS Amplicor. The reported upper and lower limits of quantitation were 1,000,000 (6.0 log_{10}) RNA copies/ml and 50 (1.7 log_{10}), respectively. Low positive, high positive, and negative controls were included in each run. HIV-1 quantization standard (QS) was used to calculate HIV-1 RNA result.

Extraction of HIV-1 RNA by COBAS total nucleic acid isolation kit (TNAI) on COBAS AmpliPrep instrument

This CAP/TNAI (Roche Molecular Systems, USA) was used as automated sample preparation method for evaluation and performed according to the manufacturer’s instruction, except QS reagent, which was not added or loaded to the machine. The protocol for extraction was TNAI500. Extraction volume was done with 500 μL of plasma sample and an elution volume of 100 μL.

Extraction of HIV-1 RNA by Amplicor HIV-1 monitor kit version 1.5 (manual sample preparation)

The volume of 500 μL plasma sample was ultracentrifuged at 13,000 rpm for 1 hour. Extraction reagent was used without HIV-1 QS added due to the limitation of LC filter detection. The processes were performed by following the manufacturer’s instruction.
Briefly, after centrifugation, supernatant was discarded and HIV-1 RNA pellet was lysed by GuSCN. Then, RNA was isolated by isopropanol precipitation and washed with 70% ethanol. This RNA was again suspended in 100 μL of HIV-1 diluent.

**HIV-1 viral load detection by modified real-time TaqMan HIV-1 reagent using Light Cycler 1.2 instrument**

The principle of test was the combination of real time RT-PCR system and TaqMan probe technology, which amplification and detection were processed on LC1.2 instrument with software version 2.0 (Roche Applied Sciences, Germany). The master mix reagent was prepared following the modified protocol with optimized ratio Master Mix and Manganese 7.33 to 1. The volume ratio of reagent and extracted RNA were 2 to 1. The limitation of total volume in glass capillary is 20 μL but it could be added up to 30 μL to increase the sensitivity. The ratio of 20 μL and 10 μL was used for amplification and detection on LC 1.2 instrument. The PCR protocol consisted of RT and amplification steps; 3 pre-cycles at 50°C for 2 min, 60°C for 20 min and 95°C for 2 min followed by amplification steps; at 95°C for 5 sec and 55°C for 10 sec and 60°C for 20 sec, amplification for 60 cycles. The measurement of amplification signal was set at 55°C in amplification cycle. The log copy number for each sample was analyzed by comparison of the threshold cycle (Ct) value of each sample with a standard curve calibrated by known copy number HIV-1 standard from OptiQuant, Acrometrix Inc., USA. There were six levels of standard and at least one point would be included in every run for test calculation. However, IRC can be comparably used in the present study due to the cost of commercial standard.

**Statistical analysis**

The statistical calculations were performed using SPSS/PC+ software (version 10.0, SPSS Inc., Chicago III) and the Microsoft Excel function statistic category. HIV-1 RNA quantization in plasma of each method was based on the log copy number of HIV-1 RNA (log copies/mL) in plasma. Log of mean ± coefficient of variation was used to describe the average. Linear regression and Pearson’s correlation coefficient were used to analyze the relationship between real time systems and the reference method, CAP/CA Monitor v1.5. The agreement between two methods was analyzed by using Bland-Altman plot analysis. The log difference between respective quantization was plotted against the log mean of the two assays. The 95% confidence intervals (95% CI) for slope and mean log difference were calculated. A p-value of less than 0.05 was considered significant.

**Ethical considerations**

The present research was conducted on the project: Strengthening laboratory for HIV diagnosis and ARV monitoring at Bamrasnaradura Infectious Disease Institute (BIDI) under the main project: National Center for HIV/AIDS Care and Training in BIDI (Project code: BI01) during the year 2004-2006. The research proposal was submitted to the institute ethical committee for using left over plasma samples after routine testing.

**Results**

**Independent run control result**

HIV-1 viral load from IRC samples were detected by CAP/CA v1.5 (RT-PCR) and established real-time PCR/LC 1.2 with automated and manual sample preparation. HIV-1 level detected by RT-PCR was log 5.2 copies/mL whereas the average log from automated and manual sample preparation was log 5.53 and 5.5 copies/mL, respectively. Log of mean ± coefficient variation of HIV-1 RNA detection from automated and manual sample extraction were log 5.53 ± 0.13 (n = 10) and log 5.5 ± 0.49 (n = 8) copies/mL, respectively. The IRC samples were tested in duplicate for amplification and detection.

**Evaluation of HIV-1 viral load detection by modified real-time TaqMan HIV-1 reagent using Light Cycler 1.2 instrument among three groups of specimens**

(i) **Frozen plasma with automated and manual sample preparation**

One hundred and thirty five frozen plasma samples were used for the method evaluation, 104 samples used for extraction with automated CAP-TNAI and 31 samples used manual preparation with Amplicor HIV-1 monitor extraction kit. These samples were tested in duplicate for amplification and detection. Regression analysis of HIV-1 viral load detection by CAP/CA v1.5 (RT-PCR) and these two extraction methods of real-time PCR are shown in Fig. 1 A and 1B. Linear regression of log-transformed HIV-1RNA level between RT-PCR and real-time PCR; automated and manual revealed slope 1.016 with Pearson’s correlation (r) of 0.916 and slope 1.015 with correlation (r) of 0.908; p < 0.001 and p < 0.001 respectively. The mean log copy number of 104 samples detected by RT-PCR was 3.45 ± 1.507 and real-time with automated preparation was 3.95 ± 1.67.
whereas mean log copy of 31 samples by RT-PCR and manual preparation was 3.12 ± 1.37 and 3.59 ± 1.53, respectively. The agreement between RT-PCR and real-time PCR result with two extraction methods were analyzed by using Bland-Altman plot analysis (Fig. 2 A and 2B). High levels of agreement were observed with the mean bias between these two methods. Mean bias and limit of agreement were 0.5 and -0.84 to 1.84 (n = 104) versus 0.48 and -0.81 to 1.77, respectively. In addition, regression analysis and Bland-Altman plot of HIV-1 viral load detected by modified real-time PCR with automated and manual sample preparation were compared among 31 pairs plasma samples (Fig. 3A and 3B). Linear regression analysis of log HIV-1 RNA levels from these two methods revealed a slope of 0.927, with correlation (r) of 0.924. The mean bias and limit of
agreement were -0.12 and -1.35 to 1.09. When the first evaluation samples were tested in duplicate, an excellent correlation was obtained with automated sample preparation. Thus, all subsequent specimens were quantified just once.

(ii) Fresh plasma samples with automated sample preparation
The HIV-1 viral load detection by CAP/CA v1.5 and modified commercial real-time TaqMan PCR using LC1.2 instrument with automated sample preparation were compared among 137 clinical samples. These tests were performed in parallel without freeze-thawing of samples. The comparison between RT-PCR and real-time PCR demonstrated that the log transformed of RNA level was significantly correlated \( (r = 0.953, p < 0.01) \) (Fig. 4A). These values were calculated by the formula \( y = \text{slope}(x) + \text{intercept} \) for the curve fit with equation \( y = 1.225x - 0.309 \). Bland-Altman plot of HIV-1 viral load between these two methods showed excellent agreement with mean bias and limit of agreement as 0.15 and -0.69 to 0.99 (Fig. 4B).

(iii) Sequential of frozen plasma samples
Mean log copy number of 36 sequential plasma samples with four time-points among nine patients were tested for the follow up case. The result of HIV-1 viral load detected by RT-PCR and real-time PCR showed 100% of concordance result. The viral load detection of these two principles was shown in Fig. 5 as mean difference between these two principles among ARV monitoring cases.

Discussion
MOPH, Thailand launched “NAPHA” program in the country so the need of low cost viral load testing among HIV-infected patients became very urgent. From the GFATM, Light Cycler 1.2 was supported and distributed to the regional laboratories throughout Thailand, not at BIDI. As no in vitro diagnostic reagents were designed for LC machine, there were many challenges such as modification, evaluation or validating the performance of other real-time PCR reagent. In the present study, the authors applied the commercial real-time TaqMan reagent in order to use this real-time PCR instrument for HIV-1 viral load monitoring. Another important point is about its cost. Many HIV-1 infected patients especially in developing countries are poor and cannot afford the payment. It is still difficult to access the viral load test if the price is similar to other reagents in the market. The limitation of LC 1.2 is the volume for capillary tube but it could be a major advantage in case of good optimization between reagent and extracted sample. This modified method used less than half volume of the normal reagent, which was 20 µl versus 50 µl. About the standard curve, plasma samples

**Fig. 3** HIV-1 viral load detected by modified real-time TaqManHIV-1/LightCycler among 31 pair samples with automated and manual sample preparation were compared using linear, regression analysis (A) and Bland-Altman test; mean bias and limit of agreement were shown as -0.12 and -1.35 to 1.09 (B)
Fig. 4  HIV-1 viral load detected by CAP/CA v1.5 (RT-PCR) and modified real-time TaqManHIV-1/LightCycler among 137 blind fresh plasma with automated sample preparation were compared using Regression analysis (A) and Bland-Altman Regression analysis; mean bias and limit of agreement were shown as 0.15 and -0.69 to 0.99 (B).

Fig. 5  Mean change of HIV-1 viral load detection by CAP/CA v1.5 (RT-PCR) and establish modified real-time TaqManHIV-1/LightCycler were shown as 36 sequential plasma samples from ARV monitoring cases with 4 time points.

are commercially available and are a necessary cost. However, IRC could be prepared and used as one standard in the experiment. The stability and homogeneity of IRC are the most critical points for a consistent result. Therefore, standard curve can be used as a master file for each lot of reagent.

The commercial TaqMan real-time PCR reagent is designed to be used with COBAS TaqMan.
machine with Quantization Standard (QS) used for result calculation. This reagent was modified and applied to LC 1.2 with optimized PCR protocol. The ratio of reagent and extracted RNA was used as 13:7, 10:10, and 20:10. The best result was found with ratio of 2 to 1 (20:10). This modified real-time PCR protocol could cover the dynamic range of viral load detection from 50 to 5,000,000 copies/ml. In the established process, manual extraction protocol with QS added gave interference of Ct signal in the real-time PCR reaction (data not shown). This problem was solved by omitting the QS addition for specimen extraction.

Real-time PCR systems have been developed to quantify HIV-1 RNA, which can reduce the time and cost of the test and could be of great benefit, particularly to developing countries, by the comparison of reagent performance among real-time reagents[7,9]. The correlation and agreement data indicated that FDA approved reagent such as CAP/CA v 1.5 had similar performances for the quantization of HIV-1 RNA in the samples tested in the present study. As shown in Fig. 1A, 1B, 2A, 2B, automated sample preparation gave slightly better correlation than manual sample preparation ($r = 0.916$ versus 0.908) but similar agreement of mean log average (0.5 versus 0.48). However, this manual sample extraction could be used as an alternative way in case the automatic machine was not available in the laboratory with comparable result (Fig. 3A and 3B). In case of multi-site evaluation, increasing more samples and avoidance of frozen samples are very crucial points. The evaluation of blind plasma with automated sample preparation ($n = 137$), as shown in Fig. 4A, gave an excellent correlation of viral load between real-time PCR test results and those obtained from reference method of routine service ($r = 0.953$). Its agreement was also excellent with a mean bias of 0.15 log VL between the two methods (Fig. 4B). Among the 137 cases, there were 112 cases of undetectable HIV-1 viral load and both methods gave similar results. In addition, amongst the ARV follow up cases, both RT-PCR and real-time PCR gave 100% concordance result between mean changes of HIV-1 viral load (Fig. 5).

In conclusion, the authors showed that modified commercial real-time PCR reagent for HIV-1 RNA quantitative detection could be used for monitoring ARV effectiveness at Bamrasnaradura Infectious Disease Institute. After the evaluation, this test was able to provide viral load testing for almost 420 HIV-1 infected patients that had never had access to the test because of budget constraint. Furthermore, these results have provided evidence to support and employ LC 1.2 real-time PCR instrument for national ARV program in Thailand and other developing countries.

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References

การตรวจหาปริมาณไวรัสเอชไอวีด้วยน้ำยาสำเร็จรูป real-time PCR ที่ปรับใช้กับเครื่อง light cycler รุ่น 1.2

สุมนมาสภ ดุษณภศ, ศิริรัตน์ ลิกานนท์สกุล, รวงผึ้ง สุทเธนทร์, อัจฉรา เชาวะวณิช

วัตถุประสงค์: เพื่อดัดแปลงน้ำยาสำเร็จรูป real-time PCR และนำมาใช้กับเครื่อง light cycler รุ่น 1.2 ในการตรวจหาปริมาณไวรัส โดยเปรียบเทียบกับน้ำยามาตรฐาน (COBAS AmpliPrep/COBAS Amplicor HIV-1 monitor test version 1.5)

วัสดุและวิธีการ: ทำการตรวจหาปริมาณไวรัสในพลาสมาทั้งหมด 308 ตัวอย่างในตัวอย่างสดและตัวอย่างแช่แข็ง รวมทั้งกลุ่มตัวอย่างที่มีผลการตรวจย้อนหลัง ซึ่งเปรียบเทียบน้ำยา real-time PCR โดยใช้เครื่อง light cycler รุ่น 1.2 กับน้ำยามาตรฐาน

ผลการศึกษา: ปริมาณไวรัสที่ตรวจพบในตัวอย่างแข็งแช่แข็งของสองวิธี ไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ทั้งจากการสกัดด้วยเครื่องอัตโนมัติและสกัดด้วยมือ มีค่า $r = 0.916$ และ $0.908$ ($p < 0.001, p < 0.001$) และความแตกต่างเฉลี่ยของปริมาณไวรัสที่ตรวจโดยสองวิธีมีค่า $log 0.5$ และ $0.48$ ตามลำดับ ซึ่งปริมาณไวรัสที่ตรวจโดยมีค่า $r = 0.953$ ($p < 0.001$) และความแตกต่างเฉลี่ยของปริมาณไวรัสเป็น $log 0.15$ ทั้งในตัวอย่างที่มีผลการตรวจย้อนหลังพบว่าไม่แตกต่างกันบ่งบันระดับ 100

สรุป: วิธีการดัดแปลงน้ำยาสำเร็จรูป real-time PCR เพื่อนำมาใช้ตรวจหาปริมาณไวรัสด้วยเครื่อง light cycler รุ่น 1.2 สามารถนำมาใช้ในการตรวจสอบความสามารถเร็วในประเทศไทย

References:

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