Quantification of HIV-1 RNA Load by One-Tube-One-Step RT PCR and Real Time PCR Assay with TaqMan Probe

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Objectives: *To develop a less expensive assay to calculate HIV-1 viral load for use in resource-limited countries.*

Material and Method: *An In-house One-tube-one-step Viral Load Assay (IOVA) was developed by using real-time PCR-based with TaqMan probe. Primers and probe were designed from the conserved region of sequences from all HIV subtypes. A standard curve was generated from reference virus in various dilutions. IOVA was applied on 105 HIV-positive and 25 HIV-negative samples and compared with the results from ROCHE AMPLICLOR.*

Results: *IOVA measured HIV RNA in the samples ranging from 125 to 2 x 10^6 copies/mL. The coefficient of variation of intra- and inter-assay ranged from 0.68% to 7.89%. The sensitivity, specificity, positive and negative predictive values were 92%, 100%, 100% and 79.5% respectively. The parallel quantitative analysis showed high correlation (r=0.95) between IOVA and AMPLICOR.*

Conclusion: *A new HIV-1 viral load assay was developed and validated. It was reliable and less expensive.*

Keywords: *HIV, Viral load, Real time PCR, TaqMan*

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HIV monitoring during antiretroviral therapy (ART) includes both clinical and laboratory aspects. CD4 count and HIV viral load are the two common tests to determine whether there is laboratory failure for ART during follow up. The HIV-1 RNA level in patient's circulation is the parameter has the highest predictive value with regard to disease progression[1]. The assay to measure viral burdens in plasma is also critical for drug development, clinical medicine and pathogenesis studies in humans[2].

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Many commercial assays were developed for measuring HIV viral load with the cost ranged from 50-100 US$ per test which was higher than the monthly cost of ART in Thailand. This expense is not affordable by most of the HIV infected patients in developing countries. Several techniques were modified in order to create an in-house assay with a less expensive price[3,4]. To be used in a clinic, the new test should be validated; have a wide dynamic range, good intra- and inter-assay reproducibility, good sensitivity and specificity, be easy to perform and be high throughput instrument[5].

Thailand is working on the campaign 'Access for All'. All HIV infected patients are aimed to have access to treatment for their diseases including monitoring of therapeutic efficacy during treatment. Reduction in any cost may affect the success of the project. To help in reducing the budget for laboratory monitoring, the authors developed a less expensive assay to calculate HIV viral load for use in resource-limited countries.

Material and Method

The standards: The supernate of HIV culture was diluted by human plasma and was used as standard virus in the study. The HIV RNA in it was quantitated by ROCHE AMPLICOR HIV-1 Monitor Assay which was used as a gold standard assay in the present study. The serial dilutions of 250, 1000, 5000, 15000, 50000 and 2000000 copies/mL were used to create a standard curve for each real time PCR assay (Figure 1).

Preparation of clinical samples and standards: One hundred and five clinical blood samples were collected in the EDTA from HIV-infected adults and children. Plasma was separated from the clinical samples by centrifugation within 4 hours of collection and aliquots were stored at - 80˚C until use. RNA from both clinical samples and standard were extracted by Nuclisens Isolationtion Kit Automated (bioMérieux, The Netherlands) according to the manufacturer guidance.

Measurement of the HIV-RNA by TaqMan real-time PCR and Amplicor: The new assay measured viral RNA in the sample by using Real time PCR with TaqMan probe. It was called 'In-house One-tube-one-step HIV Viral Load Assay' (IOVA). The HIV-1 RNA was reverse transcribed and number of HIV-1 RNA was measured in the same tube by using a combined reagent from

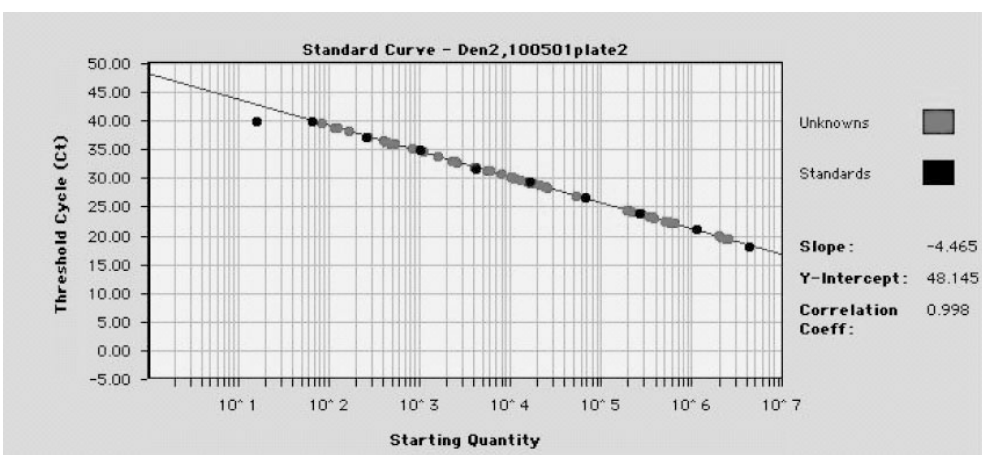

Fig. 1 The standard curve from each experiment created from the reference virus in various dilutions (black dots) and the reported correlation coefficient (r) from the software in the real time PCR machine at the right lower corner of the figure. The acceptable cut point in each run must have correlation coefficient (r) higher than 0.95

QIAGEN OneStep RT-PCR Kit (QIAGEN, Germany) and TaqMan PCR Core Reagent Kit (Applied Biosystems, CA) with universal forward and reverse oligonucleotide primers to amplify DNA and universal internal oligonucleotide TaqMan probe to detect the specific amplified products. An internal oligonucleotide probe is labeled with 2 different fluorescent dyes. A fluorescent reporter dye, FAM, is covalently coupled to the 5'-end of an oligonucleotide probe and a fluorescent quencher molecule, TAMRA is coupled to the 3'-end of the probe. The close proximity of reporter and quencher results in suppression of reporter fluorescence. The TaqMan subtype-specific probe hybridizes to a specific target sequence within the PCR products. When the polymerase encounters the 5'- end of TaqMan probe, the 5'-3' nuclease activity of Taq Polymerase will cleave the TaqMan probe and release the reporter dye. The separation of the reporter from the quencher resulted in an increased fluorescence of the reporter that can be detected by photocell in the ABI Prism^{R} 7700 Sequence Detection System (Applied Biosystems, CA). This process occurs in every cycle. The amount of fluorescence in a sample is proportional to the amount of specific PCR product generate. The number of cycles before the fluorescent emitted passes a fixed time called threshold cycle (Ct) and the log number of targets initially present is proportional to the Ct. The Ct of each sample comparing to the Ct in standard curve allows us to calculate the amount of viral copies in the sample.

The primers and probe were designed from the conserved sequences on pre-gag region and were aimed for all subtypes and circulating recombinant forms (CRFs) of HIV-1. The forward primer was VL1 (5'-TTTGACTAGCGGAGG CTAGA-3), the reverse primer was VL2 (5'-TCCCCCTGGCCTKAACCGAAT-3') and the probe was VL-pr (5'-TACTGACGCTCTCGCACCCAT CTCTC-3'). The primers were chosen to amplify a 150 bp fragment. The TaqMan probe was synthesized by Biosource (Biosource International, CA). The 25 uL PCR mixture comprised high-performance liquid chromatography-purified primers VL1 and VL2 (200 nM concentration of each); 500 nM TaqMan probe; dATP, dCTP, dTTP and dGTP (200 nM concentration of each); 7.5 x mixed buffer (half of 5x buffer of QIAGEN OneStep RT-PCR Kit and half of 10x buffer of TaqMan PCR Core Reagent Kit); 5 nM MgCl2 and 1 μL enzyme of QIAGEN OneStep RT-PCR Kit. Using one-tube-one step real time PCR, step of RNA reverse transcription was separated from the amplification by heating temperature in the real time PCR machine. The cycles were consisted of 1 cycle of 50 ˚C 30 min; 1 cycle of 95 ˚C 10 min, 3 cycles of 95 ˚C 1 min, 55 ˚C 1 min and 72 ˚C 1 min; 45 cycles of 95 ˚C 10 sec and 55 ˚C 30 sec. Measurement of the HIV-1 RNA by ROCHE AMPLICOR was applied to all samples at the manufacturer-certified site and was used as a gold standard method for comparison with the new developed assay.

The percent coefficient of variation (%CV) was used to validate intra and inter assay. Correlation coefficient (r) and regression analysis of viral load by IOVA and ROCHE AMPLICOR were applied to describe the relationship. A p-value of < 0.05 was considered statistically significant.

Results

Reproducibility of the new assay: Ten independent assay runs using HIV-1 RNA ranging from 25 to $2x10^6$ were performed. The input standard RNA templates and a plot of Ct values showed that the assay could detect viral copy number in the sample ranging from 125 to $2x10^6$ copies/ mL with 100% sensitivity when using standard RNA as template.

Intra and inter assay validation:

The standard virus was used in various concentrations. Intra-assay validation of IOVA was evaluated by testing five sets of samples of five different dilutions in the same experiment. The percent of coefficient variation (%CV) calculated from their Ct value were 0.68%, 0.99%, 2.60%, 0.98% and 4.43% for the concentrations of 250, 500, 1000, 15,000 and 100,000 copies/mL respectively.

Inter-assay validation was calculated by testing four sets of six different dilutions which were the same number using for the standard curve were tested by new assay in four repeated experiments. The %CV calculated from their Ct values were 6.37%, 7.89%, 5.77%, 1.9%, 4.57% and 4.56% for the concentrations of 250, 1000, 5000, 15000, 50000 and 2000000 copies/mL respectively.

Comparison of HIV RNA quantitation between by new assay and by ROCHE AMPLICOR.

All of 105 clinical samples were quantitated for the viral RNA by ROCHE AMPLICOR and were used as the gold standard. Their viral load ranged from undetectable by <50 copies/mL to 6.86 $x10^5$ copies/mL with a mean and a median of $1.08x10^5$ and $2.56x10^4$ copies/mL respectively. RNA from all of them were extracted and processed for quantitation by new assay as indicated in the above method. The standard curve from each experiment was reported with correlation coefficient (r) by the software in the real time PCR machine. The acceptable cut point in each run must have correlation coefficient (r) higher than 0.95 (Figure 1). Since the clinical samples were kept for several months before being used in the present study, four samples detected as higher than 125 copies/mL by ROCHE AMPLICOR (200, 156, 269, and

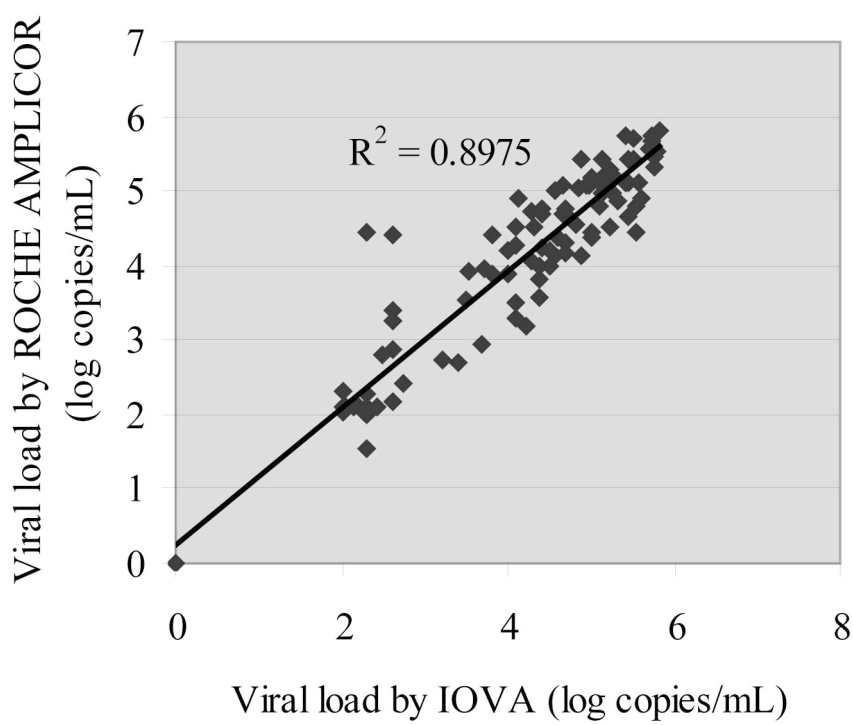

Fig. 2 Regression analysis showed that IOVA had linear association with the AMPLICOR (reference assay). The parallel quantitative analysis of clinical samples showed a high correlation (r = 0.95) between the two methods ($p<0.005$)

135 copies/mL) were undetectable by IOVA. However, two samples of 100 copies/mL by ROCHE AMPLICOR were detected as 207 and 104 copies/mL by IOVA. Regression analysis showed that the new assay had linear association with the reference assay at the range between 125 and $2x10^6$ copies/mL. The parallel quantitative analysis of clinical samples showed a high correlation (r = 0.95) between the two methods ($p<0.005$) (Figure 2). The mean and standard deviation calculated from the differences between IOVA results and ROCHE AMPLICOR results were 0.07 and 0.49 log-copies/mL respectively.

Table 1. Comparison of the results of HIV-1 quantification from the clinical samples by using the cut point at 50 copies/mL for ROCHE AMPLICOR and cut point at 125 copies/mL for IOVA. The sensitivity, specificity, positive and negative predictive values of IOVA were 91.9%, 100%, 100% and 79.5% respectively

HIV RNA quantitation By IOVA	HIV RNA quantitation by AMPLICOR	
	>50 copies/mL (or positive)	<50 copies/mL (or negative)
>125 copies/mL (or positive)	91	0
<125 copies/mL (or negative)	8	31

Sensitivity and specificity

To increase the statistical power of detection for diagnostic test, 20 HIV-negative plasma samples were added into the panel of 105 samples. All of these added negative samples were undetectable by IOVA. Using the cut point at 50 copies/mL by ROCHE AMPLICOR and cut point at 125 copies/mL by IOVA, the results from both assays were compared (Table 1). The sensitivity, specificity, positive and negative predictive values of IOVA were 91.9%, 100%, 100% and 79.5% respectively.

Discussion

There are several in-house quantitative HIV-1 assays using different technique based such as Gen-Probe[6] or LightCycler[7,8] with SYBR Green. Each assay has some advantages over the other assays. Real time PCR based with TaqMan probe is one of the most popular techniques to be used in viral quantitation. It was chosen for the present study because it showed promising results in quantitation of several viruses as mentioned in the previous reports such as the assays for severe acute respiratory syndrome coronavirus (SARS-CoV)[9], hepatitis B virus[10], hepatitis C virus[11], cytomegalovirus[12], Epstein-Barr virus[13], human papilloma virus[14], HIV-2[15] and Simian Immunodeficiency virus (SIV)[16]. The primers and probe were designed from the conserved genome regions of more than 200 viral sequences in order that they could hybridize to all current HIV-1 subtypes and circulating recombination forms (CRF). This made IOVA a fascinating assay. The sensitivity of detection from samples containing standard HIV-1 RNA 125, 50 and 25 copies/mL were 100%, 70% and 10% respectively (data not shown) therefore the cut point of the lower limit of IOVA was determined from the lowest value with highest sensitivity. IOVA was developed during most commercial viral load assays could measure HIV-1 RNA as low as 400 copies/mL. They are currently improved for this threshold sensitivity to less than 50 copies/mL and were called ultrasensitive viral load assays[17,18]. The improvement of the presented assay has been underway to achieve the lower threshold sensitivity. To improve this cut point, the authors are testing with the pelleting virus particles in plasma of centrifugation prior to RNA extraction.

The reproducibility of IOVA was good as demonstrated by the low number of %CV from both inter and intra assay validation. The results of clinical plasma samples obtained by IOVA and reference ROCHE AMPLICOR HIV-1 MONITOR test with a panel of 105 samples were in good

agreement (mean difference, 0.07 +/- 0.49 log units). The results from both assays showed a linear range between 125 and $2x10^6$ copies of viral RNA with a good correlation (r = 0.95). Other in-house real time PCR assays could have different correlations according to what technique was used for reference. The major factor that the difference between tests were not comparable was no standard HIV RNA or standard HIV quantitative assay available[3,4]. Even the different commercial assays could provide significantly different viral copy numbers[19-22]. The authors thought that a higher correlation could be achieved if the fresh samples were applied on both assays at the same time.

The in-house one tube real time PCR assay allows quantitation of HIV-1 RNA in 200-400 samples per operator per week. The cost is 1/4 to 1/8 of those commercial assays. It has been applied on a follow up cohort in Phramongkutklao hospital.

Since the incidence of HIV infected patients is going up worldwide, we are facing a great number of samples for HIV monitoring during therapeutic process. The test automation to improve practicability and reduce costs will be the next step[4]. Since HIV has a significant genetic diversity against time, surveillance of HIV genetic characterization should be performed. Sequence mismatches can provide a significant effect on reaction efficiency so the probe and primers need to be evaluated periodically[23].

Conclusion

A one step real time PCR assay using TaqMan probe for quantification of HIV-1 viral load was developed. It had excellent dynamic range of 6 logs, sensitivity of 125 copies, good intra- and inter-assay reproducibility. The new system was more affordable than the commercial method.

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การวัดปริมาณสายพันธุกรรมของเอชไอวีโดยการทำ RT PCR ในเครื่อง real time PCR และใช้ TaqMan probe

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วัตถุประสงค์: *พัฒนาวิธีการตรวจวัดปริมาณสายพันธุกรรมของเอชไอวี (viral load) ในราคาถูกลง*
วัสดุและวิธีการ: *พัฒนาวิธีการวัดปริมาณสารพันธุกรรมของเอชไอวีขึ้นโดยใช้วัสดุส่วนใหญ่ที่มีในประเทศด้วยวิธีการทำ reverse transcriptase (RT) PCR ด้วยเครื่อง real time PCR ในขั้นตอนและหลอดทดลองเดียว การออกแบบ TaqMan probe และ primers ได้ออกแบบมาจากลำดับเบสที่คงที่จากทุกสายพันธุ์ของเอชไอวี นำไวรัสที่เป็นมาตรฐานมาทำเป็นหลายความเข้มข้นนำมาตรวจหาจำนวนไวรัสและใช้สร้าง standard curve ได้ทดสอบวิธีใหม่นี้กับน้ำเหลืองของผู้ป่วยติดเชื้อเอดส์จำนวน 105 รายและน้ำเหลืองของคนปกติจำนวน 25 ราย โดยนำไปเปรียบเทียบกับผลที่ได้จากวิธี ROCHE AMPLICOR*
ผลการศึกษา: *วิธีใหม่ที่พัฒนาขึ้นสามารถตรวจสายพันธุกรรมของเอชไอวีได้ตั้งแต่ 125 ถึง $2x10^6$ copies/มล. มีความไวเป็นร้อยละ 92 ความจำเพาะร้อยละ 100 positive predictive value ร้อยละ 100 และ negative predictive value ร้อยละ 79.5 ค่าที่ได้จากการตรวจโดยวิธีใหม่มีความสัมพันธ์เป็นเส้นตรงกับค่าที่ได้จากการตรวจโดยวิธี AMPLICOR มีค่าสัมประสิทธิ์ของสหสัมพันธ์ (correlation coefficient) เป็น 0.95*
สรุป: *ได้พัฒนาวิธีการตรวจวัดปริมาณสายพันธุกรรมของเอชไอวีขึ้น ค่าที่ตรวจได้โดยวิธีใหม่นี้มีความน่าเชื่อถือและมีราคาค่าตรวจถูกลง*