

Evaluation of NucliSens EasyQ™ HIV-1 assay for quantification of HIV-1 subtypes prevalent in South-east Asia

H.Y. Lam^a, J.H.K. Chen^a, K.H. Wong^b, K. Chan^b, P. Li^c, M.P. Lee^c,
D.N. Tsang^d, K.Y. Yuen^a, W.C. Yam^{a,*}

^a Department of Microbiology, Queen Mary Hospital, The University of Hong Kong, Hong Kong SAR, China

^b Integrated Treatment Centre, Special Preventive Programme, Centre of Health Protection, Department of Health, Hong Kong SAR, China

^c Department of Medicine, The Queen Elizabeth Hospital, Hong Kong SAR, China

^d Department of Pathology, The Queen Elizabeth Hospital, Hong Kong SAR, China

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Abstract

Background: Monitoring anti-retroviral therapy requires that viral load assays for human immunodeficiency virus type 1 (HIV-1) be applicable to diverse HIV-1 subtypes.

Objectives: To evaluate NucliSens EasyQ™ HIV-1 assay for quantitation of common HIV-1 subtypes prevalent in South-east Asia.

Study design: One hundred and nineteen plasma samples collected in Hong Kong and Cambodia were used to compare the performance of NucliSens EasyQ™ HIV-1 and COBAS Amplicor™ HIV-1 Monitor version 1.5 assays. Viral RNA extracted from the NucliSens MiniMAG™ was also used for HIV-1 subtyping.

Results: Performance of NucliSens EasyQ™ correlated well with COBAS Amplicor™ ($r=0.777$, $p<0.001$) and the small mean difference ($0.0462 \log_{10}$ IU/mL) obtained in the Bland and Altman model indicated good agreement between two assays. The NucliSens EasyQ™ assay demonstrated a 95% sensitivity at 500 IU/mL and 100% specificity. Reproducibility of this assay was within \log_{10} 2–4 IU/mL and had a coefficient of variation between 2.3% and 10.4%. Among the 109 specimens included in the analysis, HIV-1 subtyping identified 64 CRF01_AE, 38 subtype B, 3 subtype C, 3 CRF07_BC and 1 subtype G viruses.

Conclusions: Performance of NucliSens EasyQ™ was comparable to COBAS Amplicor™ for HIV-1 viral load monitoring. RNA extracts from NucliSens MiniMAG™ could be used for HIV-1 viral load monitoring, subtyping and drug resistance mutations detection. Our findings highlight the versatility of both NucliSens EasyQ™ and COBAS Amplicor™ in monitoring prevalent subtypes and rare circulating recombinant forms (CRFs) in the South-east Asia region.

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Keywords: Viral load monitoring; HIV-1; NucliSens EasyQ™; COBAS Amplicor™; Subtype diversity

1. Introduction

Plasma HIV-1 RNA level monitoring is highly recommended as an integral part in the implementation of anti-retroviral (ARV) therapy and predictor of disease prognosis (Mellors et al., 1997; O'Brien et al., 1997; Powderly et al., 1999). The introduction of highly active antiretroviral therapy (HAART) in clinical practice often results in rapid decline of

plasma HIV-1 RNA. Highly sensitive viral load monitoring assays with a broad linear range are essential for the assessment of to ARV therapy (Raboud et al., 1998). Currently, various commercial assays including COBAS Amplicor™ HIV-1 Monitor version 1.5 (Roche Diagnostics, Branchburg, NJ), Versant HIV-1 RNA 3.0 assay, (Bayer Diagnostics, Tarrytown, NY) and NucliSens HIV-1 QT test (BioMérieux Inc., Boxtel, The Netherlands) are available for viral RNA quantitation in plasma samples, utilizing reverse-transcription polymerase chain reaction (RT-PCR), branched DNA signal amplification (bDNA) and nucleic acid sequence-based

* Corresponding author. Tel.: +852 28554892; fax: +852 28551241.

E-mail address: wcyam@hkucc.hku.hk (W.C. Yam).

amplification (NASBA), respectively (Dyer et al., 1999; Ginocchio et al., 1999; Murphy et al., 2000).

HIV-1 is characterized by a high genetic diversity and is classified into three distinct groups (M, N and O). Group M virus strain is further divided into subtypes and circulating recombinant forms (CRFs) (Los Alamos HIV database [<http://hiv-web.lanl.gov/>]). In recent years, subtypes B, C and CRF01_AE have played the major roles in the HIV-1 pandemic in the South-East Asia region (Oelrichs and Crowe, 2003). Emergence of CRF07_BC and CRF08_BC in China raised concern about the evolution of second generation inter-CRF recombinants (Yang et al., 2003), but data regarding the performance of commercially available HIV-1 monitoring assays for the detection of HIV-1 subtypes and CRFs prevalent in South-East Asia is very limited.

In the present study, a new HIV-1 RNA quantitative assay, NucliSens EasyQ™ HIV-1 assay version 1.1 (BioMerieux Inc., Boxtel, The Netherlands) and the manual NucliSens MiniMAG™ (BioMerieux Inc., Boxtel, The Netherlands) extraction system were compared with COBAS Amplicor™ HIV-1 Monitor version 1.5 assay (Roche Diagnostics, Branchburg, NJ) on clinical plasma samples. NucliSens EasyQ™ assay is the first commercial HIV-1 viral load monitoring assay applying NASBA technology together with the real-time molecular beacons detection method (Van Beuningen et al., 2001).

2. Materials and methods

2.1. Patient samples and sample collection

A total of 119 specimens were randomly collected in EDTA tubes from 102 HIV-1 infected patients in Hong Kong (72) and Cambodia (30). Both treatment-naïve and treatment-experienced patients were included. Plasma samples were separated by centrifugation at $1100 \times g$ for 10 min within 24 h after collection and stored at -80°C .

2.2. Quantitation of HIV-1 RNA

Quantitation of HIV-1 RNA was performed following the manufacturers' instructions.

2.2.1. COBAS Amplicor™ (RT-PCR)

This assay applies RT-PCR technology targeting the *gag* p24 region of HIV-1 (Sun et al., 1998). Using 200 μL of plasma, the linear range is 400–750,000 copies/mL (Sun et al., 1998). For plasma with viral loads above the upper detection limit, samples were diluted 100-fold with normal human plasma and re-tested.

2.2.2. NucliSens EasyQ™ (NASBA)

This assay has a dynamic range from 50 to 3,000,000 IU/mL (Yao et al., 2005). One milliliter of plasma sample, together with the NucliSens EasyQ™

internal calibrator, was processed using the semi-automated NucliSens MiniMAG™ extractor. HIV-1 RNA and calibrator were subjected to co-amplification with the same primer but hybridized with different fluorescent-labeled probes. The calibrator and HIV-1 RNA differ by a short nucleotide sequence which is identified by sequence-specific molecular beacon probes (Weusten et al., 2002). Amplicons were monitored with a real-time NucliSens EasyQ™ analyzer. Similar to COBAS Amplicor™, NucliSens EasyQ™ assay uses the *gag* gene as the target site for detection.

2.3. Subtype diversity

For subtype determination using HIV *pol* gene analysis, the same RNA extract from NucliSens MiniMAG™ extractor was amplified by RT-PCR followed by direct sequencing of the PCR amplicons (Yam et al., 2006). The Pregap4 and Gap4 modules of Staden Package (Medical Research Council-Laboratory of Molecular Biology, England) were used for sequence assembly and the HIV-1 subtype was determined using Stanford HIVdb database (<http://hivdb6.stanford.edu>).

2.4. Reproducibility, sensitivity and specificity

The reproducibility and sensitivity of NucliSens EasyQ™ HIV-1 assay was assessed using the World Health Organization (WHO) HIV-1 standard (WHO 1st International Standard 1999, 97/656), which was a lyophilized HIV-1 subtype B virus at 100,000 IU/mL after reconstitution. The standard was diluted in normal human plasma to concentrations of 100, 500, 1000 and 10,000 IU/mL. For each concentration, five aliquots were extracted and assayed in triplicate. The mean, standard deviation and percentage of coefficient of variation (CV%) were estimated to determine the intra- and inter-run assay variations. Specificity of NucliSens EasyQ™ HIV-1 assay was evaluated with 25 HIV-1 serologically negative plasma samples from 10 healthy blood donors, 10 Hepatitis B virus (HBV)-infected and 5 Hepatitis C virus (HCV)-infected patients. All samples were confirmed to be HIV-1 RNA negative by COBAS Amplicor™ HIV-1 Monitor version 1.5.

2.5. Statistical analysis

All statistical analyses were performed using \log_{10} -transformed values. According to the NucliSens EasyQ™ instruction booklet, the COBAS Amplicor™ value was converted from copies/mL to IU/mL by multiplying a factor of 0.51. The linear range of COBAS Amplicor™ was estimated between 204 and 382,500 IU/mL. Correlation was used to assess the degree of linear association between two assays. Agreement between the two assays was analyzed by the Bland and Altman model (Bland and Altman, 1999). All statistical analyses were performed with the SPSS version 11.0 software (SPSS Inc., Chicago, IL).

3. Results

3.1. General description of data set

Performance of NucliSens EasyQ™ assay and COBAS Amplicor™ version 1.5 test was compared using 119 specimens. Ten specimens (8.4%) were excluded for further analysis: three specimens yielded undetectable viral RNA in both assays; one specimen had a viral load of 340 IU/mL with NucliSens EasyQ™ and below the lower detection limit with COBAS Amplicor™; six specimens were reported as result invalid in NucliSens EasyQ™, but yielded results with COBAS Amplicor™, ranging from 2652 to 102,000 IU/mL. There was insufficient sample volume to retest these 10 specimens. For the remaining 109 specimens, the mean viral load was 4.56 log₁₀ IU/mL (3.08–6.41 log₁₀ IU/mL) for NucliSens EasyQ™ compared to 4.51 log₁₀ IU/mL (2.61–6.35 log₁₀ IU/mL) for COBAS Amplicor™. Among these 109 specimens, 9 exhibited a viral load beyond the detection limit of COBAS Amplicor™ (>382,500 IU/mL) and were re-tested with 100-fold diluted samples. Repeated assays demonstrated between 408,000 and 1,428,000 IU/mL.

3.2. Performance comparison of NucliSens EasyQ™ and COBAS Amplicor™

The HIV-1 viral load results of 109 specimens were used to evaluate both assays performance. A good linear correlation was found between NucliSens EasyQ™ and COBAS Amplicor™ (correlation coefficient (*r*): 0.777, *p* < 0.001). In Fig. 1, the fitted regression line is described by the equation: $y = 0.7323x + 1.2544$ and the coefficient of determination (*R*²) indicates that 60.3% of the data can be represented by this equation.

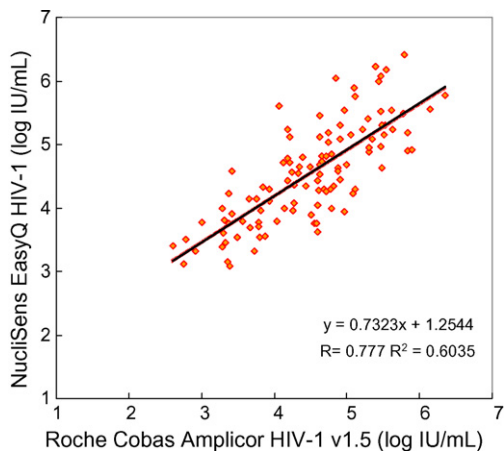


Fig. 1. The linear relationship between NucliSens EasyQ™ and COBAS Amplicor™. The solid line describes the equation: $y = 0.7323x + 1.2544$; 60.35% specimens in the graph can be represented by this equation.

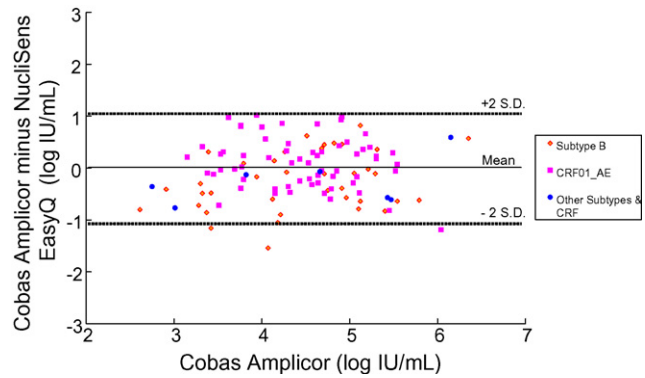


Fig. 2. The linear relationship between NucliSens EasyQ™ and COBAS Amplicor™. The Bland–Altman plot shows the difference in measurement between the two assays for same specimen (COBAS Amplicor™ minus NucliSens EasyQ™, log₁₀ IU/mL) vs. the result of COBAS Amplicor™ for each specimen (used as a comparative reference in this study). The mean difference (0.0462 log₁₀ IU/mL, solid line) and limits of agreement (mean ± 2 S.D., 1.028 to −1.12 log₁₀ IU/mL, dotted line) are displayed in the plot. Other subtypes and CRF included subtypes C, G and CRF07_BC.

The agreement between two assays was studied using the Bland–Altman model (Bland and Altman, 1999). Fig. 2 shows that mean difference of NucliSens EasyQ™ against COBAS Amplicor™ was 0.0462 log₁₀ IU/mL and the standard deviation of mean difference was 0.537 log₁₀ IU/mL. The limits of agreement of plot analysis were 1.028 and −1.12 log₁₀ IU/mL (mean ± 2 standard deviations [S.D.]). Specimens with mean differences greater than 0.5 and 1 log₁₀ IU/mL comprised 23% (36/109) and 4.6% (5/109), respectively. The mean difference for 3 samples had a higher NucliSens EasyQ™ value than COBAS Amplicor™ value. Subtype analysis revealed these three specimens were subtype B (two) and CRF01_AE (one).

3.3. Impact of subtype diversity on the NucliSens EasyQ™ assay

The distribution of HIV-1 subtypes was analyzed by submitting the *pol* gene sequence to the web-based Stanford HIVdb database. The major subtype population was CRF01_AE (64), followed by subtype B (38), comprising 93.5% of total sample size. Among 37 Cambodian specimens, 36 were identified as CRF01_AE. Other HIV-1 subtypes were subtype C (3), CRF07_BC (3) and subtype G (1). The correlation study and Bland–Altman analysis was used to verify subtype specificity with the NucliSens EasyQ™ assay. This indicated that the correlation coefficient was similar for both subtype B and CRF01_AE when assayed on NucliSens EasyQ™ and COBAS Amplicor™ assays. However, Bland–Altman plot analysis showed a better agreement between the two assays for CRF01_AE. The mean difference of subtype B was −0.258 (S.D. = 0.59) and the limit of agreement of plot analysis was 0.922 to −1.438. For CRF01_AE, the mean difference was 0.096 (S.D. = 0.485) and the limit of agreement was 1.066 to −0.874.

Table 1

The reproducibility and sensitivity studies of the NucliSens EasyQ™ assay using a panel of the WHO HIV-1 RNA standard

WHO HIV-1 standard panel (IU/mL)	Expected mean value (log ₁₀ IU/mL)	Results of five different runs ^a					Observed mean value (log ₁₀ IU/mL)	S.D. (log ₁₀ IU/mL)	Coefficient of variance (%)
100 ^b	2	<LDL	<LDL	2.06	2.4	1.68	NA	NA	NA
500	2.69	2.18	2.33	2.11	2.2	2.09	2.18	0.094	4.3
1,000	3	2.29	2.32	2.63	2.79	2.88	2.58	0.27	10.4
10,000	4	3.87	3.66	3.77	3.81	3.87	3.8	0.087	2.3

LDL: lower detection limit; NA: not available.

^a Result of each run was the mean value of triplicates.^b There were two values of the triplicates that were undetectable for each run labeled “<LDL”; all replicates were positive in the other three runs.

3.4. Reproducibility, sensitivity and specificity of the NucliSens EasyQ™ assay

The reproducibility and sensitivity of NucliSens EasyQ™ assay were evaluated (Table 1). The mean observed values of five separate assays run in panel 2–4 (500, 1000 and 10,000 IU/mL) were 2.18, 2.58 and 3.8 log₁₀ IU/mL, respectively, with CV% (defined as the standard deviation over the observed mean value) ranging from 2.3% to 10.4%. For 100 IU/mL input, only three out of five separate runs yielded results in all triplicates and the other two runs exhibited results of “below the lower detection limit”. The observed 50% and 95% detection rate of the WHO HIV-1 standard panel was 100 and 500 IU/mL, respectively. The specificity of NucliSens EasyQ™ assay was assessed by 25 HIV-negative plasma specimens and were negative in the NucliSens EasyQ™ assay.

4. Discussion

We found an overall good agreement between NucliSens EasyQ™ and COBAS Amplicor™ assay using randomly selected clinical specimens. The high correlation ($r=0.777$, $p<0.001$) and small mean difference between the two assays (0.0462 log₁₀ IU/mL) are in agreement with recent studies: (1) NucliSens EasyQ™ versus COBAS Amplicor™: $r=0.874$; mean difference = 0.0668 (Stevens et al., 2005), (2) NucliSens EasyQ™ versus NucliSens QT™: $r=0.878$; mean difference = 0.28 (Yao et al., 2005), (3) NucliSens EasyQ™ versus Versant bDNA™: $r=0.866$ (de Mendoza et al., 2005). Six of 119 specimens (5.04%) showed invalid results in NucliSens EasyQ™ due to poor amplification during the NABAS reaction which had been reported previously (Stevens et al., 2005). As NucliSens EasyQ™ and COBAS Amplicor™ work on different principles, they are affected by inhibitors to different extent. This may account for the invalid result in NucliSens EasyQ™, but quantifiable result in COBAS Amplicor™. One specimen showed discordant result between the two assays (positive in NucliSens EasyQ™ but negative in CobasAmplicor™), which may be the result of the lower limit of detection of COBAS Amplicor™. In addition, nine specimens had viral loads greater than the detection limit of

COBAS Amplicor™, as demonstrated when their 100-fold diluted specimens were re-tested. These findings indicate the broader dynamic range of NucliSens EasyQ™ over COBAS Amplicor™ (NucliSens EasyQ™ 50–3,000,000 IU/mL; COBAS Amplicor™ 204–382,500 IU/mL), thus reducing running cost of NucliSens EasyQ™ in routine application. This feature of NucliSens EasyQ™ is also important for management of ARV therapy (Yeni et al., 2004).

Three specimens (two subtype B and one CRF01_AE) showed a mean difference outside the limit of agreement in the Bland–Altman plot analysis, indicated this is not subtype specific. Both assays are suitable for monitoring prevalent subtypes and rare circulating recombinant forms (CRFs) in the South-east Asia region.

Our study is also concordant with previous findings that the dominant HIV-1 subtypes in Asia are B, C and CRF_01AE (Ruxrungham et al., 2004). Similar correlation coefficient and mean difference was observed between the two assays for CRF01_AE and subtype B. A recent study in South Africa revealed that the performance of NucliSens EasyQ™ assay is comparable to COBAS Amplicor™ for subtype C isolates (Stevens et al., 2005). In the current study, the detection of CRF07_BC, which is commonly circulating among the intravenous drug users in China, emphasizes the versatility of NucliSens EasyQ™ on different CRFs of HIV-1 (Su et al., 2000), and indicates that both assays are suitable for viral load determination of diverse HIV-1 subtypes.

RNA extracts from NucliSens MiniMAG™ extractor can also be used for subtype determination. RNA extracts of a few samples were also applied successfully to monitor drug resistance mutations in *pol* gene sequence as described previously (data not shown) (Yam et al., 2006). The sensitivity of NucliSens EasyQ™ assay in this study, reported as the detection limit and the 95% positive rate, is comparable to the reported value of COBAS Amplicor™ (Sun et al., 1998). As HIV-1 patients are frequently co-infected with HBV or HCV, the high specificity of NucliSens EasyQ™ is essential for monitoring the HIV-1 viral load in the presence of other blood-borne viruses. We were able to process 48 specimens using the NucliSens EasyQ™ system within an 8-h working day. The semi-automatic NucliSens MiniMAG™ extractor significantly reduced the hands on time for nucleic acid isolation and the NucliSens EasyQ™ analyzer can accommodate 48 specimens per run. By manual extraction of nucleic acid,

a single run of COBAS Amplicor™ can only complete 18 specimens in a working day. NucliSens EasyQ™ provides a desirable alternative to existing commercial assays for HIV-1 viral load monitoring.

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