Available online at www.sciencedirect.com
ScienceDirect
Journal of Virological Methods 146 (2007) 178–187
Journal of Virological Methods
www.elsevier.com/locate/jviromet

Evaluation of two commercially available alternatives for HIV-1 viral load testing in resource-limited settings

Kim Steegen[a,b,c,*], Stanley Luchters[a,b], Nancy De Cabooter[c], Jacqueline Reynaerts[c], Kishor Mandaliya[d], Jean Plum[c], Walter Jaoko[e], Chris Verhofstede[c], Marleen Temmerman[a]

[a] *International Centre for Reproductive Health, University Hospital, Ghent, Belgium*
[b] *International Centre for Reproductive Health, Mombasa, Kenya*
[c] *Aids Reference laboratory, Ghent University, Ghent, Belgium*
[d] *Coast Provincial General Hospital, Mombasa, Kenya*
[e] *Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya*

Received 20 February 2007; received in revised form 22 June 2007; accepted 27 June 2007
Available online 7 August 2007

Abstract

There is an urgent need for low-cost assays for HIV-1 quantitation to ensure adequate follow-up of HIV-infected patients on antiretroviral therapy (ART) in resource-limited countries. Two low-cost viral load assays are evaluated, a reverse transcriptase activity assay (ExavirLoad v2, Cavidi) and a real-time reverse transcriptase PCR assay (Generic HIV viral load, Biocentric). Both tests were compared with the ultrasensitive HIV Amplicor Monitor assay. Samples were collected in Mombasa, Kenya, from 20 HIV-1 seronegative and 150 HIV-1 seropositive individuals of whom 50 received antiretroviral treatment (ART). The ExavirLoad and the Generic HIV viral load assay were performed in a local laboratory in Mombasa, the Amplicor Monitor assay (version 1.5, Roche Diagnostics) was performed in Ghent, Belgium.

ExavirLoad and Generic HIV viral load reached a sensitivity of 98.3% and 100% and a specificity of 80.0% and 90.0%, respectively. Linear regression analyses revealed good correlations between the Amplicor Monitor and the Generic HIV viral load ($r = 0.935$, $p < 0.001$) with high accuracy (100.1%), good precision (5.5%) and a low percent similarity coefficient of variation (5.4%). Bland–Altman analysis found 95% of the samples within clinically acceptable limits of agreement (−1.19 to 0.87 log copies/ml). Although, the ExavirLoad also showed a good linear correlation with the Amplicor Monitor ($r = 0.901$, $p < 0.001$), a problem with false positive results was more significant. The cost per test remains relatively high (US$ 30 for ExavirLoad and US$ 20 for the Generic HIV viral load). Hence, false positive results and the need for an expensive PCR instrument for the Generic HIV viral load assays still limit the implementation of these tests in less equipped, less experienced laboratories.

Keywords: HIV-1; Low-cost viral load assay; Resource-limited countries

1. Introduction

Programs to scale-up ART in resource-limited countries have received a lot of attention in the last 3 years (WHO, 2006a). Currently, almost 1.5 million people in these countries are receiving ART and the numbers continue to grow. 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 (Petti et al., 2006). 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 resistant mutations and the selection of viruses with broad cross resistance to antiretrovirals. An increasing number of patients carrying drug resistant virus will inevitably lead to a spread of these resistant strains in the population. Moreover, recent studies have shown that determination of the viral load, in combination

* Corresponding author at: Aids Reference Laboratory, University Hospital, De Pintelaan, 185, Blok A, B-9000 Gent, Belgium. Tel.: +32 9 240 51 61; fax: +32 9 240 36 59.
E-mail addresses: kim.steegen@UGent.be (K. Steegen), stanley.luchters@icrhk.org (S. Luchters), nancy.decabooter@UGent.be (N. De Cabooter), jacqueline.reynaerts@UGent.be (J. Reynaerts), kishor@nyalimsa.com (K. Mandaliya), jean.plum@UGent.be (J. Plum), wjaoko@kaviuon.org (W. Jaoko), chris.verhofstede@UGent.be (C. Verhofstede), marleen.temmerman@UGent.be (M. Temmerman).

0166-0934/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2007.06.019

with an adherence intervention, can assist patients in maintaining their first-line regimen, preventing unnecessary switches in treatment (Boulle et al., 2006; Reynolds et al., 2006). In addition, viral load assays, when cheaper and more user-friendly, could be used to assess ART program quality, and are a useful tool for early diagnosis of perinatally infected children (Delamare et al., 1997; Lambert et al., 2003; Rouet et al., 2001; Simonds et al., 1998).

Broadly used commercial viral load assays such as the Roche Amplicor HIV-1 Monitor, Bayer Versant HIV-1 RNA, BioMérieux Nuclisens HIV-1 QT and the Abbott real-time HIV-1 PCR assays remain too expensive (US$ 50–100 per test) (Fiscus et al., 2006; Stephenson, 2002). Recently, two more affordable viral load assays became available: the ExavirLoad assay which determines the activity of the reverse transcriptase (RT) enzyme as a marker of retroviral replication, and the Generic HIV viral load test which is a real-time PCR assay, measuring the HIV-RNA burden in plasma. The performance of these assays was compared with the Roche Amplicor HIV-1 Monitor 1.5 assay. Both alternative viral load assays were evaluated in Coast Province General Hospital in Mombasa, Kenya. The Roche Amplicor Monitor assay was performed in Ghent, Belgium.

2. Materials and methods

2.1. Patients and samples

All study participants were recruited from Coast Province General Hospital in Mombasa, the second largest public hospital in Kenya. A total of 150 adult HIV-1 infected patients attending the HIV comprehensive care centre (CCC) were selected at random. The CCC was set-up to launch the ART program in Mombasa. Of the selected patients, 50 were on ART and 100 were treatment naïve. Additionally, 20 HIV-seronegative patients were selected at random after voluntary testing and counselling at the CCC or the antenatal clinic. Written informed consent for participation in the study was obtained from all 170 study participants. The study was approved by the ethics review committee of the University of Nairobi.

The mean age of the HIV-1 seronegative subjects was 27.5 years (S.D. ± 5.9), 16 (80.0%) of them were women. The mean age of the HIV-1 infected patients was 36.9 (S.D. ± 9.2) years, 99 (66.0%) were women and 51.3% of all infected patients were in clinical stage 3 or 4 (WHO, 2006b). Median CD4 cell counts, available for 143 of the HIV-infected patients, was 243 cells/mm^3 (IQR 138–405 cells/mm^3). Patients on ART were on treatment for a mean of 13 months (ranging from 2 weeks to 33 months).

Ten millilitres of EDTA blood was collected for CD4 cell count (FACScount Becton Dickinson Immunocytometry, Oxford, UK). The remainder of the EDTA blood was centrifuged to collect the plasma which was stored in three different aliquots at −80 °C until processing. Two aliquots were used in Mombasa to perform the ExavirLoad and the Generic HIV viral load test. One aliquot was shipped to the AIDS Reference Laboratory at the Ghent University Hospital, Belgium, where the Amplicor Monitor assay (Roche Diagnostics, Basel, Switzerland) was performed.

2.2. ExavirLoad version 2 Cavidi assay

HIV-1 RT activity in plasma samples was assessed in Mombasa, using the ExavirLoad version 2 kit (Cavidi Tech AB, Uppsala, Sweden, Lot Nos. 05052 and 06018) following manufacturers' instructions. Results were evaluated using the ExavirLoad Analyser version 1.1 software. The assay measures the activity of the HIV RT enzyme in converting RNA to cDNA. A virus binding gel is added to 1 ml of plasma to purify the virus particles. After removing inhibitors such as RT inhibitor drugs and antibodies by washing steps, the viruses are lysed. The lysates are subsequently transferred to a 96-well plate for the RT activity assay. During an overnight incubation, the RT enzyme incorporates BrdUTP into a DNA strand complementary to a polyA template bound to the wells. An anti-BrdUTP antibody conjugated to alkaline phosphatase is added and the amount of incorporated BrdUTP is detected using a substrate. The colour intensity of each well is read using a standard plate reader at 405 nm. Results are extrapolated against a standard curve, expressing the HIV RT activity in fg/ml. The RT activity is automatically converted to HIV RNA copies/ml equivalents using the ExavirLoad Analyser software. The lower detection limit (LDL) of this assay is 400 copies/ml.

2.3. Generic HIV-1 viral load Biocentric assay

The Generic HIV viral load assay (Biocentric, Bandol, France, Lot Nos. 1298307 and 1401633) was also performed in Mombasa. The principle of real-time PCR is based on a fluorogenic 5′ nuclease assay: a probe with a reporter dye at the 5′-end and a quencher dye at the 3′-end is cleaved during the amplification reaction. As the reporter dye gets separated by the quencher dye, an increased fluorescence of the reporter is detected. The fluorescence is directly proportional to the initial amount of RNA present in the sample. The software produces a threshold cycle (C_t-value) from each raw fluorescence data and extrapolates that against a standard curve.

HIV-1 RNA was extracted from 200 μl of plasma using the QIAamp Viral RNA mini kit (QIAgen, GmbH Germany, Lot No. 1014795). The volumes of AVL buffer and absolute ethanol were increased as per manufacturers' instructions. Final elution was done in 60 μl of Molecular Biology Grade water (Sigma–Aldrich, Bornem, Belgium).

The Generic HIV viral load kit was used for further RT-PCR amplification. The RT-PCR targets a conserved consensus region in the long terminal repeat (LTR) region of HIV-1. Samples were first tested according to the manufacturer's instructions (referred to as 'standard method'), in a total reaction volume of 50 μl containing 20 μl of RNA, 25 μl master mix and 1 μl each of the two primers, the probe, the reference dye and the enzyme. To reduce costs, a modified version of the assay, in which the volume of all reagents was reduced by half, was also evaluated (referred to as 'modified method').

A standard curve was constructed with serial 10-fold dilutions (from 5,000,000 to 500 copies/ml) of the standard sample included in the kit. The cycling conditions were 30 min at 48 °C and 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification and data collection were carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, South Africa). The Generic HIV viral load assay has a LDL of 300 copies/ml.

2.4. Roche Amplicor HIV-1 Monitor 1.5 assay

The Ultrasensitive Cobas Amplicor HIV-1 Monitor Test version 1.5 (Roche Diagnostics, Basel, Switzerland, Lot No. H02784) with a lower detection limit of 50 copies/ml was used as a reference method and performed according to the manufacturer's instructions (Sun et al., 1998). All Cobas Amplicor Monitor analysis were run in Ghent, Belgium.

2.5. Subtyping

Protease (PR) and reverse transcriptase (RT) gene sequences were obtained using a home-made sequencing assay as described earlier (Steegen et al., 2006). Direct sequencing of both sense and antisense strands was done with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). Sequencing reaction products were analysed on an ABI310 or an ABI3130XL Genetic Analyser (Applied Biosystems). All validations and subsequent manipulations of the sequencing results as well as the interpretations of the genotyping data and the subtyping, were performed using the Smartgene™ HIV software packages (Integrated Database Network System, Smartgene, Zug, Switzerland).

2.6. Statistical analysis

All statistical analyses were performed using SPSS 15.0 (SPSS, Illinois, USA). In order to avoid bias caused by different upper and lower detection limits viral load results above 100,000 copies/ml were equalized to 100,000 copies/ml (5log copies/ml). The lower viral load values obtained with Amplicor Monitor were adapted according to the comparator assay. When the Generic HIV viral load data set was analysed, the Amplicor Monitor viral load results <300 copies/ml (LDL of Generic HIV viral load) were equalized to 300 copies/ml. When the ExavirLoad data set was analysed, all Amplicor Monitor results <400 copies/ml were equalized to 400 copies/ml (LDL of ExavirLoad). All results below the LDL of an assay, were set at half the LDL value, i.e. 150 copies/ml (log = 2.2) for Generic HIV viral load and 200 copies/ml (log = 2.3) for ExavirLoad. The normality of data distribution was examined by the Smirnov–Kolmogorov test. Direct correlations between the different data sets were measured with a two-tailed Spearman rank correlation. A Bland–Altman difference plot was generated for bias and agreement measurements, including limits of agreement (Bland and Altman, 1999). A percent similarity model was applied to determine accuracy, precision and an overall agreement between two assays (Scott et al., 2003).

Table 1
Number of patients with detectable and undetectable viral load results in the different assays

	Amplicor Monitor		ExavirLoad		Generic HIV viral load (50 μl)		Generic HIV viral load (25 μl)	
	Undetectable VL (<50 c/ml)	Detectable VL	Undetectable VL (<400)	Detectable VL	Undetectable VL (<300 c/ml)	Detectable VL	Undetectable VL (<300 c/ml)	Detectable VL
HIV negative (n = 20)	20 (100%)	0 (0%)	16 (80%)	4 (20%)	19 (95%)	1 (5%)	18 (90%)	2 (10%)
HIV positive, ART naïve (n = 100)	1 (1%)	99 (99%)	1 (1%)	99 (99%)	4 (4%)	96 (96%)	5 (5%)	95 (95%)
HIV positive, ART treated (n = 50)	31 (62%)	19 (38%)	15 (30%)	35 (70%)	35 (70%)	15 (30%)	30 (60%)	20 (40%)
Total HIV positive (n = 150)	32 (21%)	118 (79%)	16 (11%)	134 (89%)	39 (26%)	111 (74%)	35 (23%)	115 (77%)

Patients are grouped according to HIV and treatment status. VL = Viral load, c/ml = copies/ml, ART = antiretroviral therapy.

To assess a possible influence of different subtypes on the outcome of the viral load assays, an independent two tailed *t*-test was used.

3. Results

3.1. Specificity and sensitivity of the assay

Only the Amplicor Monitor assay gave an undetectable viral load result for all 20 samples from HIV-negative individuals. Positive results were obtained in one patient by the standard Generic HIV viral load (1432 copies/ml), in two patients by the modified Generic HIV viral load (673 and 724 copies/ml) and in four patients in the ExavirLoad (407, 432, 900 and 3174 copies/ml), resulting in a specificity of 95.0% (95% CI: 73.1–99.7%), 90.0% (95% CI: 66.9–98.2%) and 80.0% (95% CI: 55.7–93.4%), respectively (Table 1).

Of the 31 HIV seropositive patients on ART with an undetectable viral load in the Amplicor Monitor assay (Table 1), 30 and 27 had a viral load below the detection limit by the standard and modified Generic HIV viral load, respectively. Only 12 patients had an undetectable viral load by ExavirLoad. Of note, three out of four patients for whom a positive result was obtained with the Generic HIV viral load also scored positive in ExavirLoad. For most of the samples the viral load remained low, ranging from 413 to 3833 copies/ml in Generic HIV viral load and from 404 to 3410 copies/ml in ExavirLoad. One treatment naïve patient had an undetectable viral load in all three assays.

Of the 150 specimens from HIV-infected individuals, 118 (78.6%) had a detectable viral load in the Amplicor Monitor assay (Table 1). Of those, 111 and 113 were detectable with the standard and the modified Generic HIV viral load assay, respectively. In the ExavirLoad, 113 samples were detectable. The seven samples with a detectable viral load in the Amplicor Monitor assay that remained undetectable in the Generic HIV viral load assay, had Amplicor Monitor viral load results below the detection limit of the Generic HIV viral load assay (55, 73, 74, 81, 128, 159 and 294 copies/ml) and therefore can not be considered as false negative. The five samples with a detectable viral load in the Amplicor Monitor assay but with an undetectable result in ExaVirLoad had an Amplicor Monitor viral load of 55, 73, 74, 9790 and 15,300 copies/ml. The latter two were clearly above the detection limit of the assay and must be considered as false negative in ExaVirLoad. This results in a sensitivity of 100% (95% CI: 95.9–100%) and 98.3% (95% CI: 93.2–99.7%), for Generic HIV viral load and ExavirLoad, respectively.

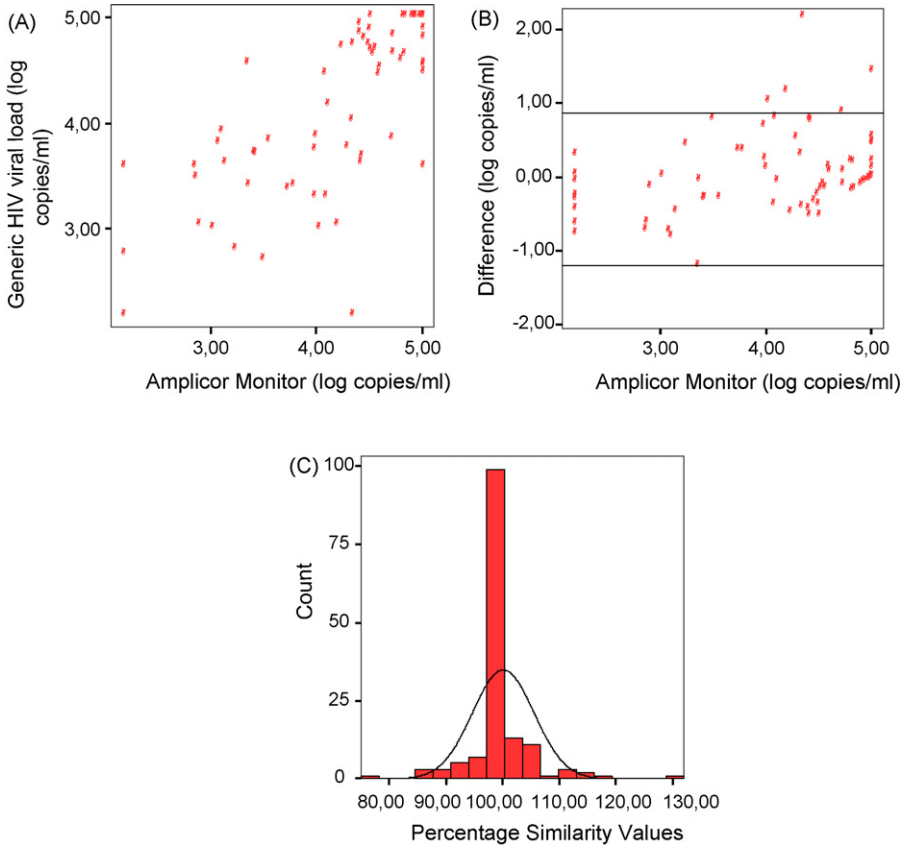

Fig. 1. Comparison between the results of the Generic HIV viral load assay (standard method) and the Amplicor Monitor assay. All samples with a viral load <300 copies/ml were equalized to 150 copies/ml (2.2log). (A) Direct assay correlation between Amplicor Monitor and Generic HIV viral load. Spearman correlation $r = 0.935$ ($p < 0.001$). (B) Bland–Altman difference plot with the difference between Amplicor Monitor and Generic HIV viral load (*Y*-axis) against the Amplicor Monitor results (*X*-axis). The bias on the difference is −0.16 (S.D.: 0.54) with limits of agreement between −1.19 and 0.87. (C) Percent similarity plot with 100.1% accuracy, 5.5% precision and a coefficient of variance of 5.4%.

Table 2
Summary of the results of the statistical analysis

	Direct assay correlation		Bland–Altman plot			Percent similarity plot		
	Spearman coefficient	*p*-Value	Bias (mean)	S.D.	Limits of agreement	Accuracy (mean)	Precision (S.D.)	Overall agreement (CV)
Generic HIV viral load (50 μl) $n = 150$	0.935	<0.001	−0.16	0.53	−1.19 to 0.87	100.1%	5.5%	5.4%
Generic HIV viral load (50 μl) $n = 63$ (trimmed)	0.835	<0.001	0.03	0.54	−1.02 to 1.08	100.3%	7.1%	7.1%
Generic HIV viral load (25 μl) $n = 150$	0.939	<0.001	0.01	0.29	−0.56 to 0.58	100.6%	4.8%	4.8%
Generic HIV viral load (25 μl) $n = 63$ (trimmed)	0.902	<0.001	0.02	0.40	−0.77 to 0.80	100.8%	6.2%	6.1%
ExavirLoad $n = 150$	0.901	<0.001	−0.03	0.51	−1.03 to 0.96	100.8%	7.8%	7.7%
ExavirLoad $n = 63$ (trimmed)	0.787	<0.001	−0.15	0.58	−1.29 to 0.98	98.9%	8.1%	8.2%

S.D.: Standard deviation, CV: coefficient of variance.

3.2. Correlation between Generic HIV viral load and Amplicor Monitor assays

Standard Generic HIV viral load results and Roche Amplicor results were available for all 150 patients. Forty-nine had a viral load >100,000 copies/ml and 31 were undetectable in both (with Amplicor Monitor LDL set at 300 copies/ml). In 40 samples a higher viral load value was found by Generic HIV viral load compared to Amplicor Monitor, whereas in 30 of the samples the Generic HIV viral load result was lower. Differences in log copies/ml were <0.5 and thus within the normal range of the test variability for most of the samples ($n = 49$). For 21 samples, the difference ranged from 0.50 to 2.16 log copies/ml. As shown in Fig. 1A, a good correlation was found between the results of both assays (Spearman correlation coefficient of $r = 0.935$; $p < 0.001$). In the trimmed data set, with exclusion of results above and below the detection limit, the correlation decreased to $r = 0.835$, but remained statistically significant. The agreement between the two assays was assessed by a Bland–Altman plot and the percent similarity model. Results are shown in Fig. 1B and C. When the modified version of Generic HIV viral load assay was compared to the Amplicor Monitor assay a comparable correlation coefficient was found ($r = 0.939$). The results of additional statistical analysis are summarized in Table 2.

3.3. Correlation between ExavirLoad and Amplicor Monitor assays

ExavirLoad and Amplicor Monitor results were available for all 150 patients. Forty-two had a viral load above 100,000 copies/ml and 16 were undetectable in both assays (with LDL Amplicor Monitor set at 400 copies/ml). In 56 samples, the difference in log copies/ml between both results was <0.5. In 11 patients the difference exceeded 1.0 log. For five of these, the viral RNA was either undetectable or above the HDL in one of the two assays. Overall, the results of both assays correlated well, with a Spearman correlation coefficient of $r = 0.901$ ($p < 0.001$) for the untrimmed data (Fig. 2A) and $r = 0.787$ ($p < 0.001$) for the trimmed data set, with exclusion of results above and below the detection limit. The results of the Bland–Altman and percent similarity plot are shown in Fig. 2B and C and in Table 2.

3.4. Correlation between Generic HIV viral load assay, standard and modified version

All samples from the 150 seropositive patients were run both with the standard Generic HIV viral load assay and a modified version with a reaction volume reduced to 25 μl. Thirty-nine and 33 samples were found with an undetectable viral load in the standard and modified assay, respectively. For 26 samples, the log difference between the viral load results of both versions was greater than 0.5, but the difference exceeded 1.0log in only 5. A good correlation was found between the results of both assay versions, with a Spearman correlation coefficient of 0.937 ($p < 0.001$) (Fig. 3A). Fig. 3B and C show the results of additional statistical analysis.

3.5. HIV-subtype distribution and influence of the HIV subtype on the results of the viral load assays

Sequencing data of the protease (PR) and (RT) gene were available for 56 out of the 60 patients for whom the sequence analysis was attempted, including all 50 patients on ART and 10 selected at random from the 100 treatment naïve patients. Proviral DNA was used for the sequencing reactions, allowing the subtyping of samples irrespective of the RNA viral load. Results revealed a subtype distribution as follows: subtype A, 55.4% ($n = 31$), D, 12.5% ($n = 7$), CRF16_AD, 12.5% ($n = 7$), C, 10.7% ($n = 6$), G, 1.8% ($n = 1$) and recombinations of mainly D, A, C and CRF_AE 7.1% ($n = 4$).

Table 3 shows the results of the three viral load assays for the 56 patients for whom a subtyping was performed. For some samples an undetectable viral load was observed in the Amplicor Monitor and the Generic HIV viral load while a positive result was obtained in ExaVirLoad. This discordance was observed more frequently in samples with a subtype D (2 out of 2) or C infection (5 out of 6), than in samples with subtype A virus (5 out of 12). An independent, two-tailed *t*-test, comparing the results of subtype A samples ($n = 31$) and non-subtype A samples ($n = 25$) showed no statistically significant subtype influence

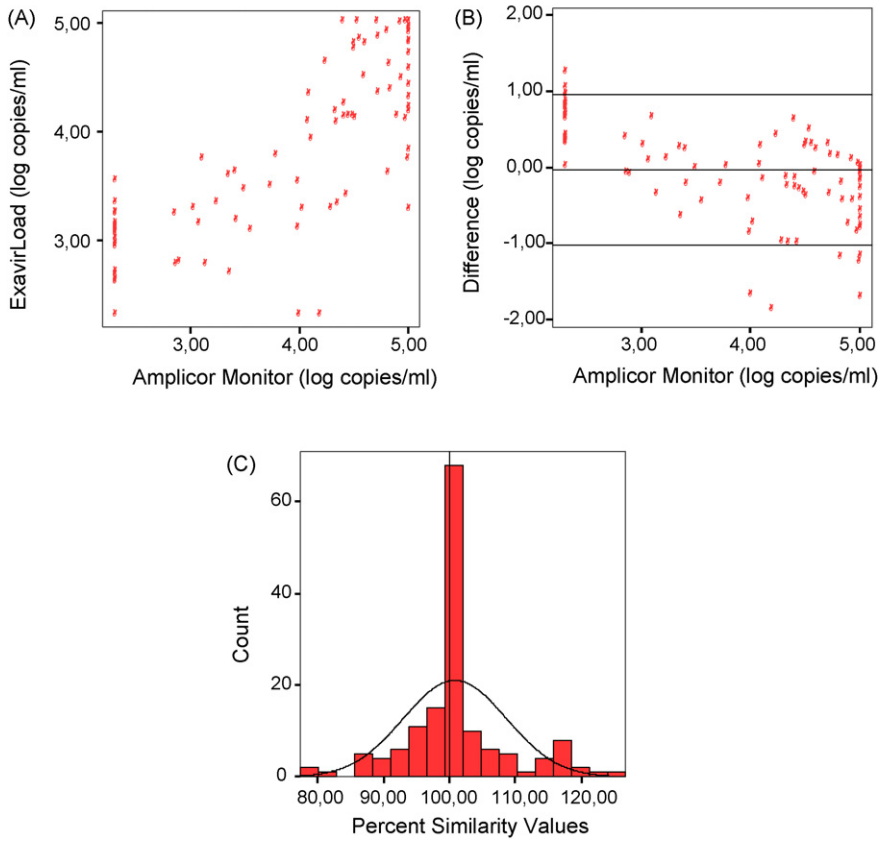

Fig. 2. Comparison between the results of the ExavirLoad assay and the Amplicor Monitor assay. All samples with a viral load <400 copies/ml were equalized to 200 copies/ml (2.3log). (A) Direct assay correlation between Amplicor Monitor and ExavirLoad. Spearman correlation $r = 0.901$ ($p < 0.001$). (B) Bland–Altman difference plot with the difference between Amplicor Monitor and ExavirLoad (Y-axis) against the Amplicor Monitor results (X-axis). The bias on the difference is −0.03 (S.D. = 0.51) with limits of agreement between −1.03 and 0.96. (C) Percent similarity plot with 100.8% accuracy, 7.8% precision and a coefficient of variance of 7.7%.

($p = 0.645$ for the Generic HIV viral load and $p = 0.699$ for the ExavirLoad).

4. Discussion

The challenge of how to appropriately monitor patients on antiretroviral treatment in resource-limited settings was only raised a few years ago with the increasing availability of ART in these regions (Majchrowicz, 2003). In Western countries, follow-up of HIV infected individuals includes at least regular CD4 cell counts and viral load quantitations, supplemented with genotypic resistance testing in case of treatment failure (CDC, 2006). Implementation of this procedure in resource-limited settings will far exceed the available budgets. In some countries, the combined cost of a CD4 cell count and a viral load test is higher than the cost of 2 months ART therapy (Stephenson, 2002). A number of alternative methods for viral load testing, including p24 antigen detection, reverse transcriptase activity testing and real-time PCR assays for viral RNA quantitation, have been evaluated by others (Fiscus et al., 2006) and good correlations were found between these assays and standard commercial viral load tests. However, most of these studies were performed in high-income countries and therefore did not take into account the possible logistical problems that might be encountered in resource-limited settings. In the study presented here, the assays were performed on-site, in the Coast Province General Hospital, a public provincial referral hospital in Mombasa. Despite the fact that all reagents had to come from overseas, delivery went fairly well, which is partly due to the fact that reagents for the assays can be bought together as a complete kit. Only the QIAgen extraction kit for the Generic HIV viral load assay had to be purchased separately.

The results of the assessed assays correlated well with the Amplicor Monitor, with Spearman correlation coefficients of $r = 0.935$ for the Generic HIV viral load and $r = 0.901$ for the ExaVirLoad. The Generic HIV viral load showed a smaller bias and narrower limits of agreement, compared to the ExavirLoad assay. Besides, the precision and overall agreement with the Amplicor Monitor was higher for the Generic HIV viral load compared to the ExavirLoad assay. Large confidence intervals for specificity of the evaluated assays compared to the Amplicor Monitor were mainly due to the small sample size of HIV-negative individuals. This must be seen as a limitation of the study.

A relatively high number of false positive results were observed, both in the small group of HIV negative individuals and in the group of patients on a successful ART regimen according to the Amplicor Monitor viral load results. Only 38.7% of

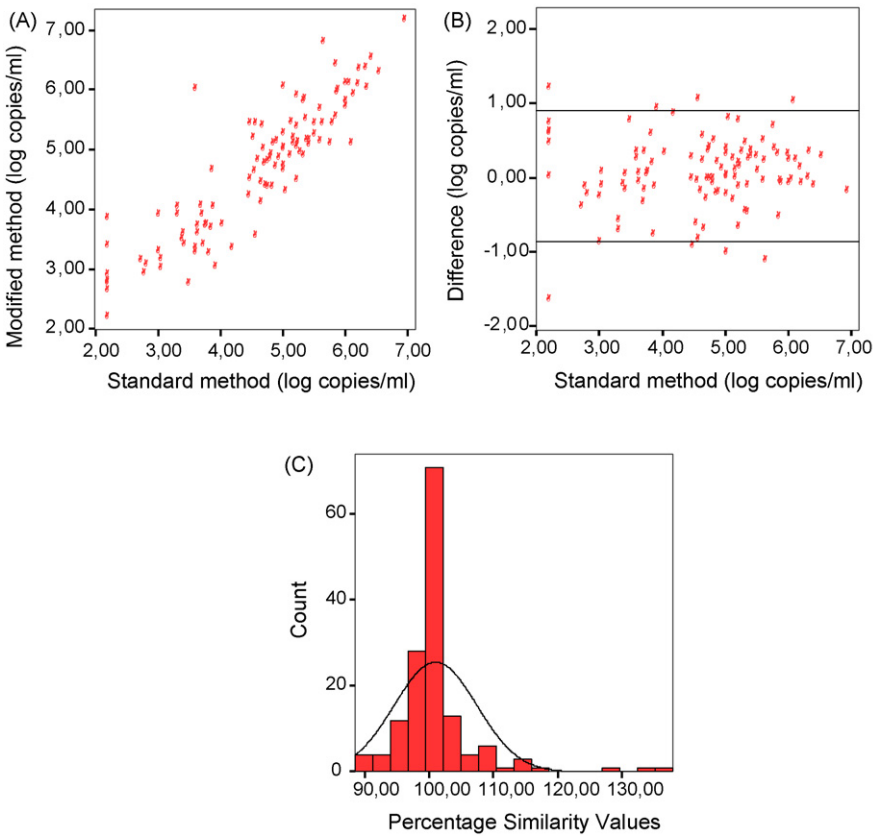

Fig. 3. Comparison between the results of the Generic HIV viral load assay, using the standard and modified method. All samples with a viral load < 300 copies/ml were equalized to 150 copies/ml (2.2log). (A) Direct assay correlation between methods 1 and 2. Spearman correlation $r = 0.937$ ($p < 0.001$). (B) Bland–Altman difference plot with the difference between the standard and modified method (*Y*-axis) against the results of the standard method (*X*-axis). The bias on the difference is 0.01 (S.D.: 0.40) with limits of agreement between −0.84 and 0.85. (C) Percent similarity plot with 100.9% accuracy, 6.5% precision and a coefficient of variance of 6.4%.

the samples from treated patients in which the viral load was <50 copies/ml in Amplicor Monitor, had an undetectable viral load in the ExavirLoad. Ninety-seven percent (96.8%) remained undetectable in the Generic HIV viral load. Further analysis of the specificity of both tests and especially of the ExavirLoad assay, on a larger number of HIV negative and successfully treated individuals, is needed. False positive results were low viral loads in the majority of the cases.

Since the primary aim of viral load testing is the evaluation of treatment efficiency, the possible occurrence of false positive results in successfully treated patients is an important drawback. Increasing the cut off of the assay might be a possibility to avoid patients being falsely classified as treatment failures, but this would prevent the detection of real, early virological failure. False positive results occurred less frequently in the Generic HIV viral load assay. The Generic HIV viral load assay is an amplification-based assay and does not contain an internal decontamination step comparable to the UNG-decontamination in the Amplicor Monitor. Although amplification and detection is performed in a single, sealed plate, which reduces the risks for contamination, false positive results due to PCR contamination cannot be excluded, especially in laboratories where strict separation between sample processing area, pré-PCR area and amplification area is not obvious.

When developing assays for resource-limited countries, special attention should be paid to the high genotypic diversity of HIV in these regions. Subtyping was 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. The subtype distribution found was similar to what was previously described for the same region (Neilson et al., 1999; Yang et al., 2004). No subtype-related differences were expected for the ExavirLoad assay, as the measurement of the RT activity is supposed to be independent of the HIV-subtype (Braun et al., 2003; Jennings et al., 2005; Malmsten et al., 2003, 2005; Seyoum et al., 2006; Sivapalasingam et al., 2005; Stevens et al., 2005). Nevertheless, infections with subtype D and C virus were associated with five and two false positive results, respectively. Due to a small sample size for subtype D, C, CRF16_AD, F and the recombinant forms, statistical analyses of single subtype specific differences was not possible. When grouping all non-A subtypes, no statistically difference between the performance of the ExavirLoad assay for subtype A and non-A was observed ($p = 0.699$). Additional studies are needed to evaluate the performance of the ExavirLoad assay for each of the subtypes individually.

For the Generic HIV viral load assay, no effect of HIV subtype on the performance of the test could be observed. This is in

Table 3
Results of viral load assays according to subtype

Study no.	Subtype	Amplicor Monitor		Generic HIV viral load		ExavirLoad	
		VL (c/ml)	VL (log c/ml)	VL (c/ml)	V (log c/ml)	VL (c/ml)	VL (log c/ml)
020	A	>100,000	>5.00	>100,000	>5.00	>100,000	>5.00
133	A	>100,000	>5.00	>100,000	>5.00	>100,000	>5.00
116	A	>100,000	>5.00	>100,000	>5.00	79,150	4.90
018	A	35,400	4.55	55,272	4.74	68,850	4.84
139	A	26,300	4.42	4,811	3.68	2,526	3.40
028	A	21,600	4.33	55,472	4.74	11,655	4.07
011	A	16,900	4.23	51,926	4.72	42,350	4.63
141	A	10,400	4.02	1,000	3.00	1,866	3.27
016	A	5,330	3.73	2,367	3.37	3,056	3.49
106	A	3,520	3.55	6,802	3.83	1,186	3.07
144	A	2,520	3.40	5,152	3.71	4,104	3.61
132	A	2,260	3.35	2,524	3.40	485	2.69
127	A	1,690	3.23	630	2.80	2,119	3.33
102	A	1,250	3.10	8,196	3.91	5,490	3.74
113	A	1,030	3.01	1,000	3.00	1,914	3.28
145	A	294	2.47	<300	<2.48	2,140	3.33
039	A	159	2.20	4,811	3.68	1,179	3,07
142	A	128	2.11	<300	<2.48	463	2.67
021	A	73	1.86	<300	<2.48	<400	<2.60
101	A	<50	<1.70	<300	<2.48	<400	<2.60
111	A	<50	<1.70	<300	<2.48	<400	<2.60
119	A	<50	<1.70	<300	<2.48	<400	<2.60
121	A	<50	<1.70	<300	<2.48	<400	<2.60
130	A	<50	<1.70	<300	<2.48	<400	<2.60
136	A	<50	<1.70	<300	<2.48	<400	<2.60
143	A	<50	<1.70	<300	<2.48	<400	<2.60
122	A	<50	<1.70	<300	<2.48	3,410	3.53
134	A	<50	<1.70	<300	<2.48	1,390	3.14
148	A	<50	<1.70	<300	<2.48	1,301	3.11
118	A	<50	<1.70	<300	<2.48	834	2.92
138	A	<50	<1.70	<300	<2.48	408	2.61
030	C	66,300	4.82	43,205	4.64	40,125	4.60
105	C	21,000	4.32	10,576	4.02	14,690	4.17
120	C	81	1.91	<300	<2.48	1,272	3.10
131	C	<50	<1.70	<300	<2.48	1,723	3.24
117	C	<50	<1.70	<300	<2.48	494	2.69
103	C	<50	<1.70	3,833	3.58	<400	<2.60
108	D	74	1.87	<300	<2.48	<400	<2.60
115	D	<50	<1.70	<300	<2.48	<400	<2.60
129	D	<50	<1.70	<300	<2.48	1,265	3.10
150	D	<50	<1.70	<300	<2.48	1,113	3.05
110	D	<50	<1.70	<300	<2.48	456	2.66
112	D	<50	<1.70	<300	<2.48	421	2.62
109	D	<50	<1.70	<300	<2.48	404	2.61
126	G	<50	<1.70	<300	<2.48	989	3.00
002	CRF16_AD	>100,000	>5.00	>100,000	>5.00	>100,000	>5.00
147	CRF16_AD	98,200	4.99	>100,000	>5.00	5,480	3.74
114	CRF16_AD	15,300	4.18	1,080	3.03	<400	<2.60
107	CRF16_AD	55	1.74	<300	<2.48	<400	<2.60
104	CRF16_AD	<50	<1.70	<300	<2.48	<400	<2.60
125	CRF16_AD	<50	<1.70	<300	<2.48	1,136	3.06
140	CRF16_AD	<50	<1.70	<300	<2.48	490	2.69
010	A +D	9,790	3.99	7,415	3.87	<400	<2.60
123	A + D	3,060	3.49	518	2.71	2,810	3.45
137	AE + D	<50	<1.70	<300	<2.48	<400	<2.60
128	A + C	<50	<1.70	<300	<2.48	892	2.95

VL = Viral load, c/ml = copies/ml.

agreement with other studies evaluating an in-house real-time PCR assay that targeted the same Long Terminal Repeat (LTR) region. 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 (Drosten et al., 2006; Rouet et al., 2007, 2005).

The strength of the study presented here is that the assays were evaluated in 'field conditions' and in an adequate number of both ART naïve and experienced patients. Only few other studies have addressed the use of the ExavirLoad or the Generic HIV viral load assay or a comparable in-house LTR based real-time PCR assay for the follow-up of adult patients on ART (Braun et al., 2003; Greengrass et al., 2005; Stevens et al., 2005). They concluded that both assays could be suitable to monitor treatment in resource-limited countries. Sample sizes were, however, very small and all these studies were conducted in well-equipped laboratories which might explain the better performance of these assays compared to the results presented here.

The observation of some false positive results in HIV-negative patients might impair the use of the Generic HIV viral load assay for diagnosis of perinatal HIV infection although an in-house LTR based real-time PCR assay has also shown to be highly sensitive and specific for early pediatric diagnosis (Rouet et al., 2005).

When evaluating viral load tests in resource-limited settings, one should not only take into account the performance of the test, but also recurrent costs, availability of trained laboratory staff, logistics for reagent delivery, accessibility of instrument servicing and availability of external quality control programs. The ExavirLoad assay has the advantage of a relatively cheap start-up cost (US$ 3000) and maintenance free equipment. However, the turn around time of the assay is long (2.5 days for 30 samples) and the test is very labor intensive. Even though the cost per test (US$ 30) is lower than the current commercially available assays, it remains relatively high. To our knowledge, there is no external quality control program available which includes the ExavirLoad assay. A possible advantage of the test is the option of using the left-over of the extracts in a kit for phenotypic resistance analysis (HIV Phenotype RT kit) (Shao et al., 2003; Tuaillon et al., 2004).

The turn around time of the Generic HIV viral load assay is low, allowing 48 samples to be run in one day. The cost per test is about US$ 20 but can safely be reduced to US$ 10 by reducing the recommended volume reagent as appeared from the results described, although a more thorough evaluation of this adaptation is needed. Purchasing and servicing of the PCR equipment, however, remains a high cost to overcome. An advantage might be that the same equipment can be used to perform other real-time PCR assays like assays for the detection of other sexually transmitted infections.

In conclusion, both the Generic HIV viral load assay and the ExaVirLoad assay could be suitable tools for viral load determination in resource-limited settings, yet both tests have their limitations and should not be implemented without a thorough on-site evaluation. Further research is needed to address the influence of different subtypes on the performance on the evaluated viral load assays, especially for ExavirLoad. More research is needed to optimize the sensitivity and specificity of low-cost viral load assays and to develop assays that are easy to handle and require only a minimum of laboratory infrastructure and training. Meanwhile manufacturers of commercial viral load assays should commit themselves to further simplify their assays, reduce the prices and provide the necessary support in infrastructure for their use in resource-limited settings.

Acknowledgements

We would like to thank Dr. Khadija Shikely for giving us the opportunity to conduct this study at the hospital, the study participants, the counsellors and the phlebotomists. We are grateful to Mercy Mutie, Mary Ndinda John, Els Demecheleer, Kenny Dauwe and François Rouet for their technical assistance in the laboratory.

Kim Steegen is supported by the Flemish Interuniversity Council (VLIR).

References

Bland, J.M., Altman, D.G., 1999. Measuring agreement in method comparison studies. Stat. Methods Med. Res. 8, 135–160.

Boulle, A., Van Cutsem, G., Coetzee, D., Hilderbrand, K., Goemaere, E., Maartens, G., 2006. Regimen durability and tolerability to 36-month duration on ART in Khayelitsha, South Africa (Abstract 66). Conference on Retrovirusses and Opportunistic Infections.

Braun, J., Plantier, J.C., Hellot, M.F., Tuaillon, E., Gueudin, M., Damond, F., Malmsten, A., Corrigan, G.E., Simon, F., 2003. A new quantitative HIV load assay based on plasma virion reverse transcriptase activity for the different types, groups and subtypes. AIDS 17, 331–336.

CDC, 2006. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, vol. 2007. Centers for Disease Control and Prevention.

Delamare, C., Burgard, M., Mayaux, M.J., Blanche, S., Doussin, A., Ivanoff, S., Chaix, M.L., Khan, C., Rouzioux, C., 1997. HIV-1 RNA detection in plasma for the diagnosis of infection in neonates. The French Pediatric HIV Infection Study Group. J. Acquir. Immune Defic. Syndr. Hum. Retrovirol. 15, 121–125.

Drosten, C., Panning, M., Drexler, J.F., Hansel, F., Pedroso, C., Yeats, J., de Souza Luna, L.K., Samuel, M., Liedigk, B., Lippert, U., Sturmer, M., Doerr, H.W., Brites, C., Preiser, W., 2006. Ultrasensitive monitoring of HIV-1 viral load by a low-cost real-time reverse transcription-PCR assay with internal control for the 5′ long terminal repeat domain. Clin. Chem. 52, 1258–1266.

Fiscus, S.A., Cheng, B., Crowe, S.M., Demeter, L., Jennings, C., Miller, V., Respess, R., Stevens, W., 2006. HIV-1 viral load assays for resource-limited settings. PLoS Med. 3, e417.

Greengrass, V.L., Turnbull, S.P., Hocking, J., Dunne, A.L., Tachedjian, G., Corrigan, G.E., Crowe, S.M., 2005. Evaluation of a low cost reverse transcriptase assay for plasma HIV-1 viral load monitoring. Curr. HIV Res. 3, 183–190.

Jennings, C., Fiscus, S.A., Crowe, S.M., Danilovic, A.D., Morack, R.J., Scianna, S., Cachafeiro, A., Brambilla, D.J., Schupbach, J., Stevens, W., Respess, R., Varnier, O.E., Corrigan, G.E., Gronowitz, J.S., Ussery, M.A., Bremer, J.W., 2005. Comparison of two human immunodeficiency virus (HIV) RNA surrogate assays to the standard HIV RNA assay. J. Clin. Microbiol. 43, 5950–5956.

Lambert, J.S., Harris, D.R., Stiehm, E.R., Moye Jr., J., Fowler, M.G., Meyer III, W.A., Bethel, J., Mofenson, L.M., 2003. Performance characteristics of HIV-1 culture and HIV-1 DNA and RNA amplification assays for early diagnosis of perinatal HIV-1 infection. J. Acquir. Immune Defic. Syndr. 34, 512–519.

Majchrowicz, M., 2003. Beyond antiretroviral access: low-cost laboratory tests needed for the developing world. AIDS 17 (Suppl. 4), S13–S15.

Malmsten, A., Shao, X.W., Aperia, K., Corrigan, G.E., Sandstrom, E., Kallander, C.F., Leitner, T., Gronowitz, J.S., 2003. HIV-1 viral load determination based on reverse transcriptase activity recovered from human plasma. J. Med. Virol. 71, 347–359.

Malmsten, A., Shao, X.W., Sjodahl, S., Fredriksson, E.L., Pettersson, I., Leitner, T., Kallander, C.F., Sandstrom, E., Gronowitz, J.S., 2005. Improved HIV-1 viral load determination based on reverse transcriptase activity recovered from human plasma. J. Med. Virol. 76, 291–296.

Neilson, J.R., John, G.C., Carr, J.K., Lewis, P., Kreiss, J.K., Jackson, S., Nduati, R.W., Mbori-Ngacha, D., Panteleeff, D.D., Bodrug, S., Giachetti, C., Bott, M.A., Richardson, B.A., Bwayo, J., Ndinya-Achola, J., Overbaugh, J., 1999. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. J. Virol. 73, 4393–4403.

Petti, C.A., Polage, C.R., Quinn, T.C., Ronald, A.R., Sande, M.A., 2006. Laboratory medicine in Africa: a barrier to effective health care. Clin. Infect. Dis. 42, 377–382.

Reynolds, S., Kagaayi, J., Nakigozi, G., Makumbi, F., Opendi, P., Nakamya, P., Gray, R., Wawer, M., Quinn, T., Lutalo, T., Serwadda, D., 2006. Early immunologic and virologic responses to ART in rural Rakai. In: Uganda Program and Abstracts of the XVIth International AIDS Conference.

Rouet, F., Chaix, M.L., Nerrienet, E., Ngo-Giang-Huong, N., Plantier, J.C., Burgard, M., Peeters, M., Damond, F., Ekouevi, D.K., Msellati, P., Ferradini, L., Rukobo, S., Marechal, V., Schvachsa, N., Wakrim, L., Rafalimanana, C., Rakotoambinina, B., Viard, J.P., Seigneurin, J.M., Rouzioux, C., 2007. Impact of HIV-1 genetic diversity on plasma HIV-1 RNA quantification: usefulness of the Agence Nationale de Recherches sur le SIDA Second-Generation Long Terminal Repeat-Based Real-Time Reverse Transcriptase Polymerase Chain Reaction Test. J. Acquir. Immune Defic. Syndr (Epub ahead of print).

Rouet, F., Ekouevi, D.K., Chaix, M.L., Burgard, M., Inwoley, A., Tony, T.D., Danel, C., Anglaret, X., Leroy, V., Msellati, P., Dabis, F., Rouzioux, C., 2005. Transfer and evaluation of an automated, low-cost real-time reverse transcription-PCR test for diagnosis and monitoring of human immunodeficiency virus type 1 infection in a West African resource-limited setting. J. Clin. Microbiol. 43, 2709–2717.

Rouet, F., Montcho, C., Rouzioux, C., Leroy, V., Msellati, P., Kottan, J.B., You, B., Viho, I., Dabis, F., 2001. Early diagnosis of paediatric HIV-1 infection among African breast-fed children using a quantitative plasma HIV RNA assay. AIDS 15, 1849–1856.

Scott, L.E., Galpin, J.S., Glencross, D.K., 2003. Multiple method comparison: statistical model using percentage similarity. Cytometry 54, 46–53.

Seyoum, E., Wolday, D., Girma, M., Malmsten, A., Meselle, T., Gronowitz, J.S., Britton, S., 2006. Reverse transcriptase activity for quantitation of HIV-1 subtype C in plasma: relation to RNA copy number and CD4 T-cell count. J. Med. Virol. 78, 161–168.

Shao, X.W., Malmsten, A., Lennerstrand, J., Sonnerborg, A., Unge, T., Gronowitz, J.S., Kallander, C.F., 2003. Use of HIV-1 reverse transcriptase recovered from human plasma for phenotypic drug susceptibility testing. AIDS 17, 1463–1471.

Simonds, R.J., Brown, T.M., Thea, D.M., Orloff, S.L., Steketee, R.W., Lee, F.K., Palumbo, P.E., Kalish, M.L., 1998. Sensitivity and specificity of a qualitative RNA detection assay to diagnose HIV infection in young infants. Perinatal AIDS Collaborative Transmission Study. AIDS 12, 1545–1549.

Sivapalasingam, S., Essajee, S., Nyambi, P.N., Itri, V., Hanna, B., Holzman, R., Valentine, F., 2005. Human immunodeficiency virus (HIV) reverse transcriptase activity correlates with HIV RNA load: implications for resource-limited settings. J. Clin. Microbiol. 43, 3793–3796.

Steegen, K., Demecheleer, E., De Cabooter, N., Nges, D., Temmerman, M., Ndumbe, P., Mandaliya, K., Plum, J., Verhofstede, C., 2006. A sensitive in-house RT-PCR genotyping system for combined detection of plasma HIV-1 and assessment of drug resistance. J. Virol. Methods 133, 137–145.

Stephenson, J., 2002. Cheaper HIV drugs for poor nations bring a new challenge: monitoring treatment. JAMA 288, 151–153.

Stevens, G., Rekhviashvili, N., Scott, L.E., Gonin, R., Stevens, W., 2005. Evaluation of two commercially available, inexpensive alternative assays used for assessing viral load in a cohort of human immunodeficiency virus type 1 subtype C-infected patients from South Africa. J. Clin. Microbiol. 43, 857–861.

Sun, R., Ku, J., Jayakar, H., Kuo, J.C., Brambilla, D., Herman, S., Rosenstraus, M., Spadoro, J., 1998. Ultrasensitive reverse transcription-PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma. J. Clin. Microbiol. 36, 2964–2969.

Tuaillon, E., Gueudin, M., Lemee, V., Gueit, I., Roques, P., Corrigan, G.E., Plantier, J.C., Simon, F., Braun, J., 2004. Phenotypic susceptibility to nonnucleoside inhibitors of virion-associated reverse transcriptase from different HIV types and groups. J. Acquir. Immune Defic. Syndr. 37, 1543–1549.

WHO, 2006a. Progress on global access to HIV antiretroviral therapy: a report on "3 by 5" and beyond, vol. 2007. World Health Organization, Geneva.

WHO, 2006b. WHO case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV related disease in adults and children, vol. 2007. World Health Organization, Geneva.

Yang, C., Li, M., Shi, Y.P., Winter, J., van Eijk, A.M., Ayisi, J., Hu, D.J., Steketee, R., Nahlen, B.L., Lal, R.B., 2004. Genetic diversity and high proportion of intersubtype recombinants among HIV type 1-infected pregnant women in Kisumu, western Kenya. AIDS Res. Hum. Retroviruses 20, 565–574.