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Current challenges to viral load testing in the context of emerging genetic diversity of HIV-1

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Introduction: One of the major characteristics of HIV-1 is its extreme genetic diversity. A key factor in assessing the sensitivity of a molecular-based assay measuring HIV-1 RNA viral load (VL) in plasma is its ability to detect/quantify all (or most of) relevant HIV-1 genetic subtype/recombinant forms accurately.
Areas covered: This review provides an overview of the current commercially available quantitative real-time assays (the Abbott RealTime HIV-1, Roche TaqMan HIV-1 versions 1.0 and 2.0, BioMérieux Nuclisens EasyQ HIV-1, Siemens VERSANT HIV-1 RNA 1.0 kinetic PCR, and Biocentric Generic HIV Viral Load assays). For each assay, studies from 2005 to 2010 assessing the impact of HIV-1 genetic diversity on the reliability of HIV-1 RNA quantification are described.
Expert opinion: In light of HIV-1 genetic diversity, a general recommendation to favor one test over the other cannot categorically be made. Larger field evaluations of HIV-1 RNA assays should be conducted in areas where HIV-1 genetic diversity is the highest. The large-scale implementation of HIV-1 VL testing is urgently required in the developing world to change HIV infection from a likely death sentence into a manageable chronic infection, as done in Northern countries.

Keywords: genetic diversity, HIV, HIV-1 RNA viral load, subtypes/clades

Expert Opin. Med. Diagn. [Early Online]

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) epidemic remains a major public health challenge. In 2009, UNAIDS estimated that a total of 33.4 million (31.1 – 35.8 million) of people are living with HIV worldwide and that 2.0 million (1.7 – 2.5 million) deaths are related to AIDS. Moreover, ~ 90% of individuals infected with HIV-1 live in developing countries [1].

One of the main characteristics of HIV is its tremendous genetic diversity. The continuously increasing diversity of HIV-1 strains circulating worldwide has consequences on all aspects of the management of this infection [2,3]. This genetic diversity has important practical implications on reliability of serological diagnostic assays [4,5], efficiency of antiretroviral (ARV) therapy [6], selection of ARV drug resistances [6,7], transmissibility and disease progression [8-10], and vaccine design [11,12].

In this review, the focus is on how the HIV-1 genetic diversity represents a challenge for the reliable detection and quantification of HIV-1 RNA in plasma [13]. Molecular HIV-1 RNA assays are urgently required for the early diagnosis of pediatric HIV-1 infection and to monitor efficiency of highly active antiretroviral therapy (HAART) in low- and middle-income countries. Tests are mainly sequence-based and are thus subjected to sequence variability constraints that may

10.1517/17530059.2011.566860 © 2011 Informa UK, Ltd. ISSN 1753-0059

Article highlights.

- A key factor in assessing the sensitivity of a molecular-based assay measuring HIV-1 RNA viral load in plasma is its ability to detect/quantify all (or at least most of) relevant HIV-1 genetic groups, subtypes/recombinants accurately, including non-B HIV-1 variants.
- By the end of 2010, four main manufacturers had commercialized six quantitative real-time HIV-1 RNA assays using closed platforms: The Abbott RealTi*me* HIV-1 assay (Abbott Molecular, Inc.), the Cobas TaqMan HIV-1 version 1.0 (v1.0) and Cobas TaqMan HIV-1 version 2.0 (v2.0) assays (Roche Molecular Systems), the Nuclisens EasyQ HIV-1 v1.2 and v2.0 assays (BioMérieux Clinical Diagnostics), and the VERSANT HIV-1 RNA 1.0 kinetic PCR assay (Siemens).
- One extra HIV-1 RNA RT-qPCR assay has been developed and evaluated by the Agence Nationale de Recherches sur le SIDA. This open system has been commercialized by Biocentric (Generic HIV Viral load Assay).
- From 2005 to 2010, each of the above-mentioned assays was assessed for its reliability regarding HIV-1 genetic diversity: 18 published studies for the Abbott kit, 15 for the Roche systems, 7 for the BioMérieux assays, 2 for the new Siemens test, and 3 for the Generic HIV Viral Load assay.
- In light of results of these evaluations, a general recommendation to favor one test over the other cannot be categorically drawn. Large field evaluations are lacking in areas where HIV-1 genetic diversity is the highest, such as in Central Africa.
- In developing countries, the use of ARV drugs without performing VL testing is totally empirical, and the risk of emergence and transmission of HIV-1 strains resistant to one or two or three classes of ARV drugs is real. If we want to change HIV infection from a likely death sentence into a manageable chronic infection, as in northern countries, the large-scale implementation of HIV-1 VL testing is urgently needed.

This box summarizes key points contained in the article.

significantly affect their performance and reliability by spoiling the hybridization of the primers and/or probe.

1.1 HIV classification and origins

One of the major characteristics of human immunodeficiency viruses is their extensive genetic variability attributed to the lack of proofreading and recombinogenic properties of the reverse transcriptase (RT) enzyme, rapid turnover of virions *in vivo*, and host selective immunologic pressures [14-16]. The HIV genome encodes nine open reading frames: *gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vpr*, *vif* and *vpu*. Phylogenetic analysis of major variations in these genes allow for HIV to be classified at several levels, beginning with distinctions between HIV-1 and HIV-2. The initial genetic diversity of HIV is tightly associated to its origins. HIV-1 and HIV-2 are the result of cross-species transmissions from different African primate species, that are, SIVcpz and SIVgor, from chimpanzees (*Pan troglodytes troglodytes*) and gorillas (*Gorilla gorilla*), respectively, for HIV-1 in West Central Africa, and SIVsmm from sooty mangabeys (*Cercocebus atys*) in West Africa [17-19]. SIVs from chimpanzees and gorillas from West Central Africa have crossed the species barrier on at least four occasions, leading to HIV-1 groups M, N, O and P in human beings [20-23]. Similarly, the eight groups of HIV-2, A – H, are the result from at least eight independent transmissions of SIVs infecting sooty mangabeys from West Africa. These HIV variants have different virological and epidemiological histories. Some have remained restricted to a few cases of human infections, whereas others have spread among humans.

HIV-1 group M strains are the source of most HIV infections worldwide, having infected > 60 million people since the recognition of the AIDS epidemic (with 70% of them in sub-Saharan Africa). HIV-1 group O remains restricted to West Central Africa and represents greater than or equal to ~ 1% of HIV-1-infected people in Cameroon. HIV-1 group N is confined to Cameroon, with < 20 infected individuals. HIV-1 group P has recently been identified in two subjects from Cameroon only [23,24].

Within group M, HIV-1 can be subdivided further into nine different subtypes (A – D, F – H, J, K), sub-subtypes (A1 – A5, F1, F2), and at least 48 circulating recombinant forms (CRFs) (**Figure 1**) [25]. In addition, multiple unique recombinant forms (URFs) have been described and a relatively substantial number of strains cannot be classified into current subtypes, especially in Central Africa [26,27]. Finally, recombinant strains may recombine further, leading to increasing complexity.

1.2 Geographic distribution of HIV-1 infection

The classification of HIV strains has helped in tracking the course of the HIV pandemic. The geographic distribution of the different HIV-1 M subtypes and CRFs is heterogeneous and is a highly dynamic process (**Figure 2**). It varies by region of the world, by country, and even within a country. Subtype B accounts only for ~ 10% of total infections worldwide [28], but is prevalent in industrialized countries, including Western Europe and North America. Therefore, most clinical and biological studies are based on patients infected with this clade, and diagnostic tools and ARVs have been mainly optimized on subtype B strains. The remaining subtypes and recombinant forms (grouped as 'non-B' variants) are less studied, even though they cause ~ 90% of the estimated 33 million HIV infections worldwide. Among them, subtypes C, A and CRF01_AE and CRF02_AG are the most prevalent and responsible for nearly 70% of all HIV infections.

Within Africa, CRF02_AG accounts for the bulk of infections in West Africa, subtypes A and D are common in East Africa, and subtype C predominates in Southern Africa as well as North East areas such as Ethiopia. The highest genetic diversity is observed in West Central Africa where all HIV-1 groups co-circulate. Within Asia, CRF01_AE is

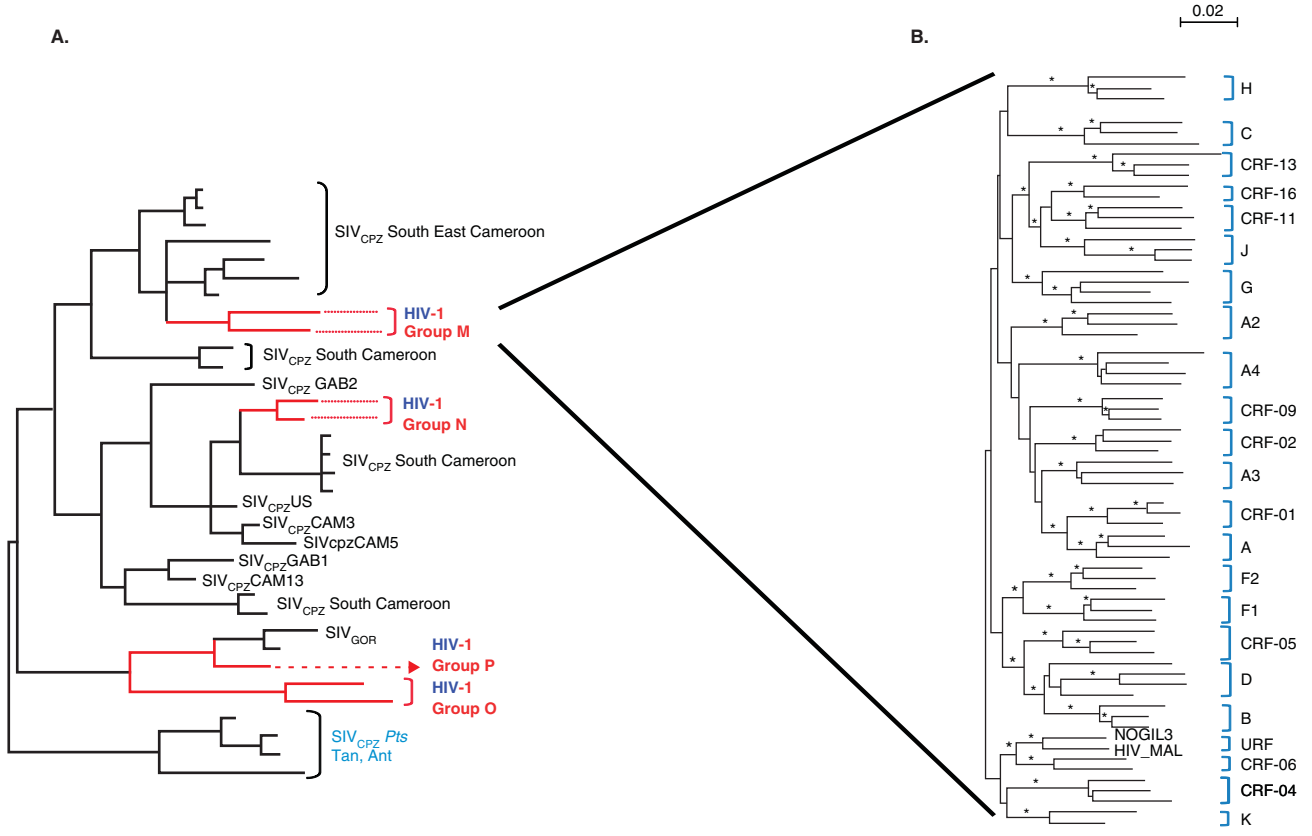

Figure 1. Phylogenetic tree of near full-length genome sequences representing the genetic diversity of the human immunodeficiency virus type-1/SIV_{CPZ}/SIV_{GOR} lineage.

Based on data from [25].

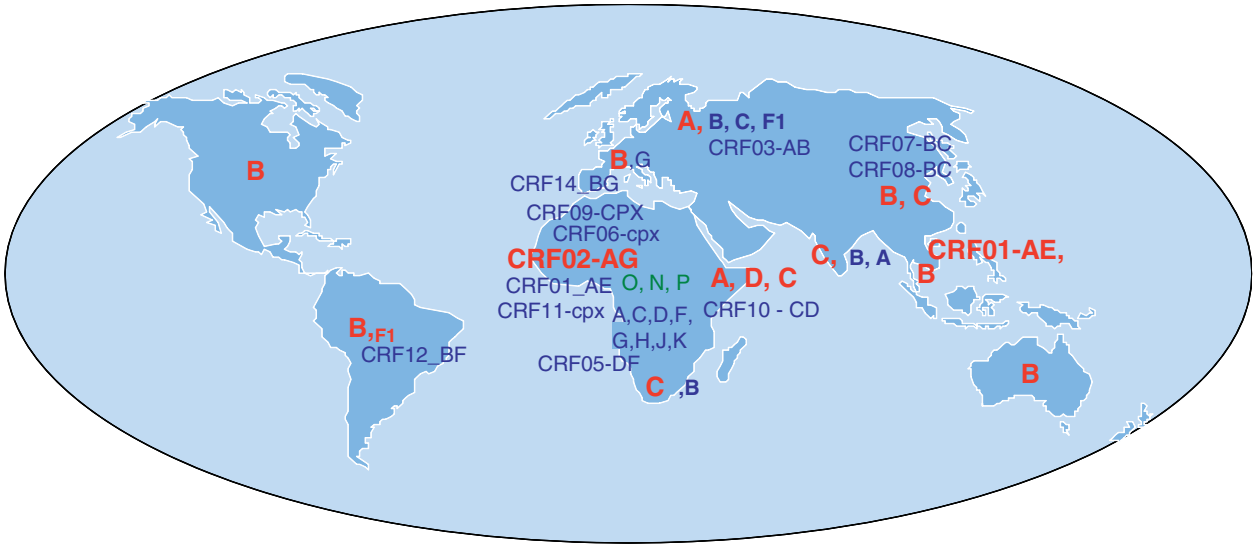

Figure 2. Schematic representation of the geographic distribution of HIV-1 group M (subtypes and major circulating recombinant forms), N, O and P worldwide.

Based on [28].

predominant in South-East Asia but originated from Central Africa. Subtype C is predominant in India and Nepal. Two BC clades, CRF07 and CRF08, co-circulate in China.

With increasing mobility and migration, HIV-1 variants inevitably intermix in different parts of the world and the distribution of different forms of HIV-1 in the world is thus a dynamic process. HIV-1 molecular epidemiology studies in industrialized countries showed that the number of new infections with non-B strains is increasing over time, especially in some European countries (such as France and Switzerland), where ~ 50% of newly diagnosed patients are infected with non-B variants [29-35].

2. HIV-1 viral load quantification

2.1 Definition

Viral load (VL) is defined as the concentration of a viral entity (either particle or provirus) in one specific organism compartment. In HIV-1 viral particles, VL can be assessed by measuring HIV-1 RNA or other components such as the RT enzyme activity or the p24 structural protein. HIV-1 RNA VL in plasma (the so-called VL) is directly correlated with the number of viral particles circulating in this compartment, thus reflecting the active peripheral HIV-1 replication in HIV-1-infected individuals; because of the wide range of HIV-1 viral particles that may circulate in plasma, results are expressed in HIV-1 RNA copies/ml of plasma with a $\log_{10}$ transformation. Genome equivalent per milliliter had been proposed, but was not accepted by the HIV Scientific community [36].

2.2 Fifteen-year overview of quantitative plasma HIV-1 RNA VL techniques

Assays that quantify plasma HIV-1 RNA copy number became available in the mid-1990s. For 10 years (1995 – 2005), quantitative HIV-1 RNA assays were initially based on end point target amplification techniques, such as reverse transcriptase polymerase chain reaction (RT-PCR) and nucleic acid sequence-based amplification (NASBA), with detection/amplification carried out after the amplification step by hybridization of amplified products with specific labeled probes and subsequent quantification by enzyme immunoassay or chemiluminescence. In addition, signal amplification-based techniques such as branched DNA (bDNA) were developed. Most of the tests were commercialized by four manufacturers (Abbott, Bayer, BioMérieux and Roche) [37-47]. Their sensitivity thresholds were ~ 300 – 500 copies/ml. They showed relatively low dynamic ranges. They were not fully automated and sensitive to contamination. These early tests were designed and optimized for the detection/quantification of HIV-1 subtype B, but could fail to detect non-B subtypes properly [48-57].

Since 2005, the same suppliers have modified the detection/quantification principle of their HIV-1 RNA assays, gradually switching from end point to quantitative real-time PCR (RT-qPCR) detection technologies that continuously monitor the fluorescence emitted by the amplification products [58]. The advantages of the fluorescence-based quantitative real-time PCR and NASBA technologies are numerous: they offer significant improvements to the quantification of the target because the fluorescence data are acquired during the exponential phase of the amplification. With these more attractive assays, the lower detection limit has been significantly improved (~ 20 – 50 copies/ml) by using larger volumes of samples. These technologies have considerably improved the post-amplification process by limiting the contamination issues in reducing the handling of amplified samples. Thus, their conceptual and practical simplicity, together with their speed, sensitivity and specificity within a homogenous assay, have made them the cornerstone for HIV-1 RNA quantification in a wide range of samples from numerous sources, including plasma specimens. They may be fully automated, including the extraction step [59]. This review focuses on these new-generation assays.

By the end of 2010 there were six real-time-based commercially available licensed HIV-1 RNA assays: The Abbott RealTi*me* HIV-1 assay (Abbott molecular, Inc.), targeting the highly conserved integrase region of the *pol* gene; the Cobas TaqMan HIV-1 version 1.0 (v1.0) and Cobas TaqMan HIV-1 version 2.0 (v2.0) assays (Roche Molecular Systems), targeting a conserved region in the *gag* gene (v1.0), and an extra conserved region in the *LTR* gene for v2.0 (dual-target strategy); the Nuclisens EasyQ HIV-1 v1.2 and v2.0 assays (BioMérieux Clinical Diagnostics), targeting a well-conserved *gag* region of HIV-1; and the VERSANT HIV-1 RNA 1.0 kinetic PCR assay (now Siemens, formerly known as Bayer and as bDNA assay from Chiron), targeting a highly conserved part of the HIV-1 *pol* gene.

These new RT-qPCR assays have been designed to improve their ability to detect and quantify accurately the wide range of HIV-1 variants belonging to group M (including non-B subtypes), mainly through a more adequate design of primers and probes that are well conserved across subtypes and CRFs. However, the genomes available in sequence banks for primer and probe selection are still biased towards subtype B, and do not reflect the entire picture of the HIV-1 genetic diversity worldwide. Their sensitivities may also be affected by point mutations within primer/probe target sequences. Thus, all HIV-1 (non-B but also B) clades may still have a negative impact.

Today, given that there is no single technique capable of quantifying the whole spectrum of HIV-1 strains circulating worldwide, at least 15 distinct in-house HIV-1 RNA real-time PCR techniques have also been developed by HIV researchers besides the above-mentioned commercial assays [60]. Some of these tests may be considered as academic reference tools, allowing an independent evaluation of licensed assays. They also allow for independent thought from established manufacturers' kits in which mismatches cannot be identified because the primers and probe sequences

are not known to customers. In this review, a RT-qPCR test developed by the Agence Nationale de Recherches sur le SIDA (ANRS) targeting a well-conserved LTR region is described [61,62]. This test has been commercialized (Generic HIV Viral Load assay, Biocentric).

3. Impact of HIV-1 diversity on HIV-1 RNA quantification

3.1 General methodology

Assessment of the impact of HIV-1 genetic diversity on the reliability of HIV-1 RNA quantification can be performed by using the following.

1) Panels (plasma or culture supernatant) with well-known HIV-1 genotypes [63]. These panels must be large and, if possible, genetically diverse. They can be prepared at no cost by reference virology laboratories or purchased from distinct companies. The World Health Organization (WHO) can also provide a genotyped reference panel (National Institute for Biological Standards and Control [NISBC] No. 01/466) at no cost.
2) Clinical plasma specimens harboring well-characterized HIV-1 subtypes/CRFs.
3) Clinical plasma samples from known and diverse geographical origins, reflecting locally circulating HIV strains.

Performance evaluation can be conducted in one or distinct laboratories (multi-center study). In terms of ability to detect HIV-1 subtypes/CRFs/URFs (equivalent to sensitivity), one assay can be compared versus another, or one assay can be evaluated versus two or more further tests. Pearson correlation coefficients are used to compare HIV-1 RNA results obtained by the two (or more) tests. Concordance between assays may be assessed by the Bland–Altman approach [64] and percentage similarity [65]. Usually, a 0.5 $\log_{10}$ copies/ml difference is a particularly stringent cutoff point, as it is normally used to detect clinically relevant variation between two values obtained with the same assay. A major discrepancy is usually defined by a difference of at least 1.0 $\log_{10}$ copies/ml of plasma.

3.2 Review of the literature (in a chronological order according to PubMed)

3.2.1 Evaluation of the Abbott RealTime HIV-1 assay

Foulongne *et al.*, in France [66], compared HIV-1 RNA concentrations obtained from 88 samples (58 B and 30 non-B) with the Abbott RealTi*me* HIV-1 assay, the Abbott LCx HIV RNA quantitative assay and the Roche Cobas TaqMan HIV-1 test v1.0. HIV-1 RNA could be quantified in 6 of 11 CRF02_AG-infected samples by the LCx assay whereas HIV RNA was quantified in 7 of 11 and 9 of 11 samples by the TaqMan HIV-1 assay and the Abbott RealTi*me* HIV-1 assay, respectively. HIV-1 RNA levels measured by the Abbott RealTi*me* HIV-1 assay were higher than those obtained with the two other assays in CRF02_AG samples but were lower in B-subtype specimens (**Table 1**).

Garcia-Diaz *et al.* [67] compared the Abbott RealTi*me* versus LCx HIV-1 assays on 89 clinical specimens from the UK. No significant difference in HIV-1 RNA levels was obtained.

Swanson *et al.* [68] evaluated the performance of the Abbott RealTi*me* HIV-1 assay on 89 specimens from Brazilian blood donors, mostly (~ 80%) harboring B subtypes. Values were compared with those obtained with the Abbott LCx. Overall, 87 samples were quantified by both assays with results in a similar range, whereas two subtype B specimens were marginally detected with the real-time technique only. The same team [69] measured VLs from 100 HIV-infected patients using the Abbott RealTi*me* assay. This panel included 26 subtypes A, 20 B, 27 C, 10 D, 1 CRF01_AE, 3 CRF02_AG and 13 recombinant viruses. Compared with results obtained with three other techniques (Versant HIV-1 RNA 3.0, Monitor v1.5 and LCx), the Abbott test provided reliable quantification of these genetically diverse strains.

Gueudin and collaborators, in France [70], compared the Abbott RealTi*me* assay versus the Roche TaqMan test v1.0 on 29 culture supernatants and 88 clinical samples (mostly CRF02_AG). The Abbott system quantified all 29 HIV-1 group M supernatants, whereas three of these samples (two G and one H) were underestimated or undetectable with the TaqMan technique. The Abbott RealTi*me* system quantified seven HIV-1 group O strains; none was detected by the Roche technique. Neither technique crossreacted with HIV-2. Similar HIV-1 RNA concentrations were obtained for subtype B and D strains, but four samples (including three CRF02_AG) were underquantified by > 1 $\log_{10}$ with the Roche assay.

Swanson *et al.* [71] evaluated the Abbott RealTi*me* assay on a serially diluted panel of 37 group M and 2 group O virus isolates. Values of four quantified panel members of group M differed by > 1 $\log_{10}$ when comparing RealTi*me* HIV-1 and Monitor v1.5. The group O viruses were reliably quantified by the Abbott test.

Braun and colleagues [72] used four distinct assays (Abbott RealTi*me*, TaqMan v1.0, Monitor v1.5 and Versant 3.0) to quantify HIV-1 RNA from a 12-member group M panel, 2 group O viruses and 97 clinical specimens from HIV-1-infected blood donors. The results of all four assays were highly correlated, but VL results derived from the Abbott RealTi*me* technique were generally lower (log 0.2 – 0.4) than with the Monitor assay v1.5. HIV group O samples were detected with the Abbott RealTi*me* test only.

Schutten and collaborators, in the Netherlands [73], conducted a multi-center evaluation of the Abbott RealTi*me* HIV-1 assay using clinical samples and the 2005 Quality Control for Molecular Diagnostics (QCMD) HIV panel. Comparison with other assays (TaqMan v1.0, Amplicor v1.5, Versant HIV-1 RNA 3.0 and LCx) revealed only minor

Table 1. Impact of HIV-1 genetic diversity on quantitative HIV-1 RNA results obtained with the Abbott RealTi*me* HIV-1 assay (results of 18 studies conducted between 2006 and 2010).

Authors	Year	Samples			Comparison with	Main results/conclusions	Manufacturer-independent study*
		N	HIV subtypes	Geographic origin			
Foulongne *et al.* [66]	2006	88	58 B, 30 non-B	France	LCx, TaqMan v1.0	Higher sensitivity and higher HIV-1 RNA levels obtained with the Abbott RealTi*me* for CRF02_AG strains but low concentrations for subtypes B	?
Garcia-Diaz *et al.* [67]	2006	89	Clinical samples (51 genotypes determined)	UK	LCx	No significant difference in HIV-1 RNA levels obtained with the two tests	Yes
Swanson *et al.* [68]	2006	89	69 B, 20 non-B	Brazil	LCx	No major discrepancy	No
Swanson *et al.* [69]	2006	100	20 B, 80 non-B	UK	Versant 3.0, Amplicor v1.5, LCx	No major discrepancy; good tolerance of the Abbott RealTi*me* test to nucleotides mismatches	No
Gueudin *et al.* [70]	2007	132	44 culture supernatants (29 group M, 8 group O, 7 HIV-2) 88 clinical samples (7 B – D, 16 A-CRF01, 49 CRF02, 16 others CRFs/subtypes)	France	TaqMan v1.0	Difference > 1 log10 for 12% of samples; 7/8 group O samples quantified by the Abbott test; no crossreactivity with HIV-2 with the Abbott test	?
Swanson *et al.* [71]	2007	39	37 group M, 2 group O	?	Versant 3.0, Amplicor v1.5	Four major discrepancies; reliable quantification of group O viruses by the Abbott RealTi*me* technique	No
Braun *et al.* [72]	2007	111	2 panels (12 M, 2 O) and 97 clinical samples	?	TaqMan v1.0, Amplicor v1.5, Versant 3.0	No major discrepancy; reliable quantification of group O viruses by the Abbott RealTi*me* technique	No
Schutten *et al.* [73]	2007	55 – 168	HIV-1 genotypes not predefined	?	TaqMan v1.0, Amplicor v1.5, Versant 3.0, LCx	Only minor differences between the four assays	?
Tang *et al.* [74]	2007	105	WHO genotype reference panel; 4-member group O panel; 90 well-characterized clinical specimens	Cameroon, Thailand, Brazil, UK	Amplicor v1.5, Versant 3.0	Excellent agreement between the three tests	No
Schutten *et al.* [75]	2007	77	WHO genotype reference panel; 66 clinical samples	Netherlands	Amplicor v1.5	Excellent agreement between the two tests	?

*The question mark indicates that authors acknowledged Abbott Molecular, Inc. in their publication for providing the assay reagents, and/or supplies, and/or instruments, and/or funding to support the study.
VL: Viral load.

Table 1. Impact of HIV-1 genetic diversity on quantitative HIV-1 RNA results obtained with the Abbott RealTi*me* HIV-1 assay (results of 18 studies conducted between 2006 and 2010) (continued).

Authors	Year	Samples			Comparison with	Main results/conclusions	Manufacturer-independent study*
		N	HIV subtypes	Geographic origin			
Xu *et al.* [76]	2008	269	171 genotypes available (67 B′, 89 BC, 15AE)	China	EasyQ	Good performance with clades B′ and BC; AE samples better detected/quantified with the Abbott test	Yes
Sloma *et al.* [77]	2009	99	Panels M, N and O; 87 clinical samples (genotypes not determined)	US	TaqMan v1.0	Mean difference between Abbott and Roche values = -0.34 log10 copies/ml	?
Crump *et al.* [78]	2009	100	Clinical specimens (genotypes not determined)	Tanzania	Amplicor v1.5	Higher values with the Abbott assay than the Roche test for low HIV-1 RNA concentrations; opposite results for high HIV-1 RNA concentrations	Yes
Wirden *et al.* [79]	2009	249	Only non-B subtypes (113 CRF02, 139 other non-B CRFs/subtypes)	France	TaqMan v1.0	Rare major discrepancies; difference 1 log in 3% of specimens, with higher values always with the Abbott test	Yes
Scott *et al.* [80]	2009	170	20-member HIV-1 subtype panel 150 patient samples (genotypes not determined)	South Africa	TaqMan v1.0, EasyQ v1.1, Monitor v1.5	Excellent performance of the Abbott test which tended to generate higher values than the other assays	?
Pyne *et al.* [81]	2009	219	Two subtype panels (n = 39) (one from Abbott and the WHO 01/466 panel); 180 clinical specimens (genotypes not determined)	US	Roche Amplicor v1.5	2 CRF02, 2 subtypes C, and 1 subtype B better quantified with the Abbott test; group O samples quantified with the Abbott test	?
Choi *et al.* [82]	2009	104	80 genotypes available (90% B subtypes)	South Korea	EasyQ HIV-1 v1.1	Significantly higher quantification with the Abbott kit	Yes
Babady *et al.* [83]	2010	1190	Samples previously found undetectable with the Roche TaqMan assay v2.0	US	Roche TaqMan v2.0	Lack of significantly discordant VL results	?

*The question mark indicates that authors acknowledged Abbott Molecular, Inc. in their publication for providing the assay reagents, and/or supplies, and/or instruments, and/or funding to support the study.
VL: Viral load.

differences. The Abbott RealTi*me* technique showed better performance on CRF02_AG strains.

Tang *et al.*, in the US [74], used the Abbott RealTi*me* HIV-1 assay against the WHO genotype reference panel (01/466), 4 group O isolates and 90 well-characterized clinical specimens representing group M subtypes A – D, F, G, CRF01_AE, CRF02_AG, and group O (10 each). The Abbott technique quantified all members of the WHO panel (including group O), but the group N specimen was underquantified (~ -0.9 $\log_{10}$), as compared with the value obtained with the Monitor v1.5 test. For the group M clinical specimens, relative to RealTi*me* HIV-1 test, 93 and 96% of values were within 1.0 $\log_{10}$ for Monitor v1.5 and Versant HIV-1 RNA techniques, respectively. The group O samples were accurately quantified by the Abbott technique only.

Schutten *et al.* [75] evaluated the Abbott RealTi*me* PCR test for its ability to quantify the WHO reference genotype panel (01/466) and 66 clinical specimens. The results for the Abbott test were relatively close to the calculated mean of the results for the WHO panel. Only one clinical specimen (one subtype B) showed a > 1 $\log_{10}$ difference between the Abbott RealTi*me* technique and the Cobas Amplicor HIV-1 Monitor test v1.5.

Xu *et al.*, in China [76], evaluated the performance of the Abbott RealTi*me* HIV-1 assay for measuring VLs for HIV-1 clades B′, BC and AE. The results were compared with those obtained with the EasyQ test. The mean difference between the Abbott technique and the EasyQ test for clade AE samples was higher (n = 13; +0.559 $\log_{10}$ copies/ml) than for clade B′ specimens (n = 58; -0.007 $\log_{10}$ copies/ml) and clade BC samples (n = 88; +0.314 $\log_{10}$ copies/ml).

Sloma *et al.*, in the US [77], used the Abbott RealTi*me* HIV-1 technique to quantify HIV-1 RNA from representative strains of HIV-1 group M (subtypes A – H) (n = 8), N (n = 1) and O (n = 3). All strains were quantifiable by the Abbott RealTi*me* test, whereas group M and N strains were quantifiable by the Roche Cobas TaqMan HIV-1 assay v1.0. When testing 87 clinical samples, a mean difference of -0.34 $\log_{10}$ between the Abbott and Roche assays was obtained.

Crump and colleagues, in Tanzania [78], compared the performance of the Abbott RealTi*me* HIV-1 assay against the Roche HIV-1 Monitor test v1.5 using samples collected among 100 HIV-1-infected blood donors from Tanzania. The Abbott assay produced higher values than the Roche test in the low HIV-1 RNA concentration samples, but lower values on the higher concentration HIV-1 RNA specimens.

Wirden and co-workers, in France [79], determined viral loads in 249 clinical samples from patients infected with non-B subtypes (mostly CRF02_AG strains) by both the Abbott RealTi*me* and Cobas TaqMan v1.0 assays. The differences exceeded 0.5 $\log_{10}$ for ~ 20% of samples and 1 $\log_{10}$ for 3%, with higher values obtained with the Abbott technique in the latter cases.

Scott *et al.*, in South Africa [80], evaluated the Abbott RealTi*me* HIV-1 assay to quantify HIV-1 subtypes C in South Africa. Its performance was compared with the performance of existing assays (including the COBAS TaqMan, v1.0, and the EasyQ HIV-1 assay, v1.1). Twenty samples from an HIV-1 subtype panel derived from blood donors in Cameroon, South Africa, Thailand and Uganda were also used. It consisted of subtypes A, D, F, G, CRF01_AE and CRF02_AG. The results obtained by Abbott and Roche assays were comparable. All assays were able to quantify all panel members but RealTi*me* HIV-1 tended to generate higher values than the other assays. All three systems adequately detected subtypes B, D and F. Only five samples (two G and three CRF02_AG) produced differences of > 1.0 $\log_{10}$ copies/ml between assays.

Pyne *et al.*, in the US [81], evaluated the Abbott RealTi*me* HIV-1 test with the Roche Amplicor HIV-1 Monitor test, v1.5. Two subtype panels were tested, including group M, N and O samples, and some common CRFs. For most samples, the assays showed good agreement. However, two CRF02_AG samples, two subtype C samples and one subtype B sample measured ~ 1.1, 0.8 and 0.6 $\log_{10}$ copies/ml higher in the RealTi*me* assay than in the Amplicor assay, respectively. The RealTi*me* assay was able to quantify all three group O samples (none by the Amplicor assay). For the group N sample available, the Amplicor assay gave results ~ 1 $\log_{10}$ higher than the Abbott test.

Choi and collaborators, in South Korea [82], evaluated the Abbott RealTi*me* HIV-1 assay in comparison with the EasyQ HIV-1 test v1.1 among 104 clinical samples (mostly B subtypes). The Abbott RealTi*me* kit showed significantly higher quantification than the EasyQ kit in both subtype B and subtype non-B samples.

Babady and colleagues, in the US [83], compared HIV-1 RNA levels obtained with the Abbott RealTi*me* HIV-1 assay on 1190 unique clinical plasma specimens and previously yielding 'target not detected' results by the Cobas TaqMan HIV-1 test v2.0. The authors obtained a very low degree of VL discordance, with a maximum VL difference of 150 copies/ml.

3.2.2 *Evaluation of the Roche Cobas TaqMan HIV-1 assays*

3.2.2.1 *Roche Cobas TaqMan HIV-1 assay version 1.0*

Katsoulidou *et al.*, in Greece [84], compared values obtained with the Cobas TaqMan HIV-1 assay v1.0 with results obtained with three other techniques (Monitor v1.5, LCx and Versant HIV-1 RNA 3.0), using clinical specimens with various VL levels (n = 240, 177, and 156 for the comparison TaqMan v1.0 versus LCx, TaqMan v1.0 versus bDNA v3.0, and TaqMan v1.0 versus Amplicor v1.5). A VL difference of at least 1 $\log_{10}$ was observed in 1.7% (n = 3) of the samples tested by TaqMan v1.0 and LCx (subtypes A, B and F), in one (0.5%) sample of the specimens assessed by TaqMan v1.0 and bDNA 3.0, and in one sample (0.8%) (belonging to subtype F) of the specimens tested by TaqMan v1.0 and Amplicor v1.5 (**Table 2**).

Table 2. Impact of HIV-1 genetic diversity on quantitative HIV-1 RNA results obtained with the Roche Cobas TaqMan HIV-1 assays (results of 15 studies conducted between 2006 and 2010).

Authors	Year	Samples			Comparison with	Main results/conclusions	Manufacturer-independent study[§]
		N	HIV subtypes	Geographic origins			
Katsoulidou* *et al.* [84]	2006	156 – 240	Unknown HIV-1 subtypes	Greece	Amplicor v1.5, Versant 3.0, LCx	Rare major discrepancies	Yes
Gomes* *et al.* [85]	2006	104	Subtypes B (n = 48) and non-B subtypes (n = 56)	Portugal	Versant 3.0	Three samples (1 B, 1 G, 1 CRF02) with major discrepancies	Yes
Perrin* *et al.* [86]	2006	80	One panel and 72 clinical specimens	?	Amplicor v1.5	No significant difference in HIV-1 RNA VLs obtained with the two assays	No
Schumacher* *et al.* [87]	2007	79	Cell culture solutions (subtypes A – H) and 71 clinical samples	?	Amplicor v1.5, Versant 3.0	No major discrepancies	No
Damond* *et al.* [88]	2007	140	B and non-B subtypes	France	Amplicor v1.5	24% of samples underquantified with the Cobas TaqMan HIV-1 test v1.0	Yes
Oliver* *et al.* [89]	2007	191	Clinical samples (24 known genotypes, 11 B and 13 non-B)	UK	Amplicor v1.5	Higher reliability of the TaqMan HIV-1 assay v1.0 for quantifying some non-B subtypes/CRFs	?
Holguin* *et al.* [90]	2008	55	Non-B subtypes only	Spain	EasyQ, Versant 3.0	4.8% of major discrepancies between the TaqMan v2.0 and v1.0	Yes
Scott[‡] *et al.* [92]	2009	137	Clinical specimens (genotypes not determined)	South Africa	TaqMan v1.0, RealTime HIV-1	Significant improvement with the v2.0 test compared with the v1.0 technique	Yes
Taylor[‡] *et al.* [93]	2009	160	Genotypes not fully determined	Austria	TaqMan v1.0, RealTime HIV-1	Superior HIV-1 group M subtype coverage including also group O detection with the Cobas TaqMan HIV-1 v2.0	Yes
Sizmann[‡] *et al.* [94]	2010	77	Clinical samples (n = 66, genotypes not determined) Group O isolates (10 culture supernatants, 1 patient specimen)	?	TaqMan v1.0, Amplicor v1.5, RealTime HIV-1	CRF02_AG member underquantified with the Cobas TaqMan HIV-1 v1.0; group N and O samples quantified only with the TaqMan v2.0 test	No
Pas[‡] *et al.* [95]	2010	11	WHO panel (01/466), 6 HIV-2 samples		TaqMan v1.0	Excellent quantification of the group M, group N and group O samples of the WHO panel; no crossreactivity with HIV-2	No

*Evaluation of the Roche Cobas TaqMan HIV-1 assay version 1.0.

[‡]Evaluation of the Roche Cobas TaqMan HIV-1 assay version 2.0.

[§]The question mark indicates that authors acknowledged Roche Molecular System in their publication for providing the assay reagents, and/or supplies, and/or instruments, and/or funding to support the study.

CRFs: Circulating recombinant forms; VL: Viral load.

Table 2. Impact of HIV-1 genetic diversity on quantitative HIV-1 RNA results obtained with the Roche Cobas TaqMan HIV-1 assays (results of 15 studies conducted between 2006 and 2010) (continued).

Authors	Year	Samples			Comparison with	Main results/conclusions	Manufacturer-independent study[§]
		N	HIV subtypes	Geographic origins			
De Bel[‡] *et al.* [96]	2010	375	Clinical specimens (B and non-B subtypes)	Belgium	TaqMan v1.0, Amplicor v1.5	Major underquantification in 20% of the tested samples with the TaqMan v1.0	?
Damond[‡] *et al.* [97]	2010	288	Clinical specimens (B and non- B subtypes)	France	TaqMan v1.0	Enhanced performance of the upgraded test v2.0	No
Janse van Rensburg[‡] *et al.* [98]	2010	117	Clinical specimens (B and non- B subtypes)	US and Africa	RealTi*me* HIV-1	Consistent higher VL with the Cobas TaqMan test v2.0	Yes
Bourlet[†] *et al.* [99]	2010	74	Clinical samples (mostly subtypes B and CRF02_AG)	France	RealTi*me* HIV-1, TaqMan v1.0, EasyQ v1.2	Means of difference null between TaqMan v2.0 and Abbott RealTi*me* for CRF02_AG but positive for subtypes B	No

*Evaluation of the Roche Cobas TaqMan HIV-1 assay version 1.0.
‡Evaluation of the Roche Cobas TaqMan HIV-1 assay version 2.0.
§The question mark indicates that authors acknowledged Roche Molecular System in their publication for providing the assay reagents, and/or supplies, and/or instruments, and/or funding to support the study.
CRFs: Circulating recombinant forms; VL: Viral load.

Gomes *et al.*, in Portugal [85], evaluated the performance of the Cobas TaqMan HIV-1 assay v1.0 on 104 samples previously assessed for HIV-1 RNA by the Versant HIV-1 RNA 3.0 assay. The 104 samples included subtype B (n = 48) and non-B subtypes (n = 56). Overall, there were three samples (one B, one G and one CRF02_AG) showing a difference in viral load of > 1 $\log_{10}$ copies/ml.

Perrin *et al.*, in Switzerland [86], compared the TaqMan 1.0 and Amplicor v1.5 assays on a BBI 8-member panel and 72 clinical specimens. All major M subtypes of the panel were accurately detected by both assays. No significant difference in HIV-1 RNA levels obtained for clinical samples by the two tests was found.

Schumacher *et al.*, in Germany [87], compared the TaqMan 1.0 with the Amplicor v1.5 and Versant 3.0 assays on cell culture supernatants and 71 clinical specimens. No major discrepancy was observed in this study.

Damond *et al.*, in France [88], compared plasma HIV-1 RNA VL values obtained on 140 specimens with the Cobas TaqMan HIV-1 assay v1.0 and the Amplicor HIV-1 v1.5 technique. Mean values were 2.9 (range < 1.6 – 4.54) and 4.2 (range 3.0 – 5.63) $\log_{10}$ copies/ml, respectively, showing the lack of agreement between the two assays. Differences between the Amplicor and the TaqMan assays ≥ 0.5 $\log_{10}$ copies/ml were observed for 34 specimens (24%). These 34 patients were infected by subtypes B (n = 9) and non-B subtypes, including subtypes A (n = 4), D (n = 1), CRF02_AG (n = 15), CRF13_cpx (n = 1), F (n = 1) and G (n = 3).

Oliver *et al.*, in the UK [89], evaluated the TaqMan v1.0 assay in comparison with the Amplicor v1.5 on 191 clinical samples. The mean difference between the two assays was 0.05 $\log_{10}$ copies/ml. Thirteen samples gave results with variances > 0.5 $\log_{10}$.

Holguin and colleagues, in Spain [90], compared the performance of the Roche Cobas TaqMan v1.0 with two other techniques (Nuclisens HIV-1 EasyQ and Versant HIV-1 RNA 3.0). They tested 55 plasma specimens from untreated patients carrying non-B subtypes and CRFs. HIV-1 RNA was undetectable in 32.7, 20 and 14.6% using Nuclisens, Versant and TaqMan, respectively. Only 32 (58.2%) samples were quantified by the three methods. Subtypes G and CRF02_AG showed more discrepancies in VL than others.

Korn and collaborators [91], by systematic sequence analysis of HIV-1 variants with RNA levels underestimated by the Cobas TaqMan HIV-1 assay, demonstrated that mutations at a single position of the downstream primer can lead to the underestimation of HIV-1 RNA concentrations by > 2 log and to false-negative results in minipool screening of blood donors. The underquantified viruses belonged to subtype B, sub-subtype A1, sub-subtype F1 and CRF02_AG.

3.2.2.2 *Roche Cobas TaqMan HIV-1 assay version 2.0*

To achieve accurate quantification of mismatch specimens, HIV-1 TaqMan test v2.0 targets and amplifies a

second HIV-1 region (5′ LTR) in addition to *gag* (dual target strategy).

Scott *et al.*, in South Africa [92], evaluated the Cobas TaqMan v2.0 HIV-1 assay on 137 plasma specimens from South Africa. Viral load results were compared with those obtained with TaqMan v1.0 and Abbott RealTi*me*. Overall, 4.8% of samples showed clinically major VL differences (> 1 $\log_{10}$) between the two distinct versions of the Roche test. The Roche test v2.0 compared well with the results obtained with the Abbott RealTi*me* technique.

Taylor and colleagues in Austria [93] compared HIV-1 RNA levels obtained by the Cobas TaqMan assays (v1.0 and v2.0) and the Abbott RealTi*me* kit. The new Roche TaqMan assay allowed accurate determination of VL levels in individuals infected with HIV-1 isolates who were initially found falsely negative with Roche v1.0.

Sizmann *et al.*, in Switzerland [94], compared the performance of the Cobas TaqMan HIV-1 test v2.0 with the TaqMan HIV-1 predecessor v1.0 in 66 patient specimens. Severe underquantification (by > 1 $\log_{10}$) was observed for HIV-1 TaqMan v1.0 in 20 (30.3%) samples, whereas 15 (22.7%) samples showed moderate underquantification (by 0.5 – 1.0 log).

Pas *et al.*, in the Netherlands [95], compared the performance of the Cobas TaqMan HIV-1 test v2.0, with the v1.0 test, against the WHO 1st International Reference Genotype Panel (01/466). All assays showed excellent performance for group M samples of the reference panel, except the version 1.0 test, which slightly underquantified the CRF02_AG strain. The group N and O samples were quantified only with the version 2.0 test.

De Bel and collaborators in Belgium [96] clearly demonstrated underquantification with the Cobas TaqMan assay v1.0 on 375 plasma specimens. Compared with the Amplicor v1.5, 36 (9.5%) samples (including B and non-B subtypes) were underestimated (by at least a 0.71 $\log_{10}$ difference) with the Cobas TaqMan v1.0, whereas no sample was underestimated with the v2.0 assay.

Damond *et al.*, in France [97], evaluated the performance of the upgraded v2.0 of the Roche Cobas TaqMan HIV-1 test using prospective (n = 263) and archived (n = 25) clinical samples initially underquantified by the Cobas TaqMan HIV-1 test v1.0. Among the 25 archived samples, the HIV-1 subtype distribution was the following: A (n = 3), B (n = 9), F (n = 1), G (n = 1), CRF02 (n = 10) and CFR06 (n = 1). Among the 263 prospective samples, HIV-1 subtype information was available for 159 patients, and the distribution was A (n = 10), B (n = 66), C (n = 4), D (n = 4), F (n = 3), G (n = 5), H (n = 1), J (n = 1), CRF01 (n = 6), CRF02 (n = 50), CRF04 (n = 1), CRF05 (n = 1), CRF06 (n = 3), CRF12 (n = 1), CRF18 (n = 1) and CRF36 (n = 1). For these highly diverse samples, the performance of the new test was improved significantly and most of the underquantification observed with the first version test was eliminated.

Janse van Rensburg *et al.*, in the UK [98], reported that the Roche TaqMan v2 assay consistently provided higher VL levels than the Abbott RealTi*me* kit on 117 samples (69 from the US and 48 from Africa provided by both companies). A difference of > 0.5 $\log_{10}$ was observed for 30 and 10.4% in the two groups of clinical samples, respectively.

Recently, Bourlet *et al.*, in France [99], conducted a study in 74 HIV-1-infected subjects. Samples were tested for HIV-1 RNA by at least four assays (Abbott RealTi*me*, EasyQ v1.2, and TaqMan v1.0 and 2.0). The authors observed a higher disparity for CRF02_AG than subtypes B.

3.2.3 *Evaluation of the BioMérieux Nuclisens HIV-1 EasyQ assay*

De Mendoza *et al.*, in Spain [100], described the performance of the Nuclisens HIV-1 EasyQ test v1.1 assessed in 1008 clinical specimens collected from individuals infected with clade B (n = 774) and non-B (n = 234) HIV-1 variants. Three reference panels containing different HIV-1 subtypes/groups from Boston Biomedica Incorporation (BBI), Walter Reed Army Institute of Research (WRAIR) and NIBSC were also tested. The results were compared with those obtained by the Monitor HIV-1 v1.5 and the Versant HIV-1 RNA 3.0 assays. The Nuclisens EasyQ test was able to detect/quantify 17% of samples with non-B subtypes that were missed by the Versant technique. HIV-1 group O specimens were detected only with the EasyQ test (**Table 3**).

Stevens and her team in South Africa [101] evaluated the performance of the Nuclisens EasyQ assay among 284 HIV-1-infected individuals from South Africa. HIV-1 RNA levels quantified by the Nuclisens EasyQ assay correlated significantly with those of the Amplicor Monitor v1.5 assay. Nine (3.2%) patient outliers (points beyond the limit of agreement) were obtained.

Yao and colleagues, in China [102], compared the performance of the EasyQ test with the Nuclisens HIV-1 QT assay. Comparisons were done on 98 clinical samples from China and on 2 BBI well-characterized HIV-1 RNA panels. Among the 98 clinical specimens, the two assays detected HIV-1 RNA in 81 samples, and neither detected HIV-1 RNA in 12 specimens. Three clinical specimens had detectable HIV-1 RNA using the EasyQ only, whereas two had detectable HIV-1 RNA using the end point Nuclisens technique. For the two panels, both assays were able to detect and quantify HIV-1 RNA derived from eight distinct clades. However, both assays appeared to underestimate the HIV-1 RNA copy number of subtypes A and C (> 1 $\log_{10}$ than the expected values).

McClernon *et al.*, in the US [103], evaluated the performance characteristics of the Nuclisens HIV-1 EasyQ test on specimens harboring subtypes B from subjects participating in North American clinical trials (n = 91), and on samples with non-B subtypes from subjects participating in clinical trials in North and South America (n = 44). Comparison was done with the Roche Cobas Amplicor v1.5 assay.

Table 3. Impact of HIV-1 genetic diversity on quantitative HIV-1 RNA results obtained with the BioMérieux Nuclisens HIV-1 EasyQ v1.1 assay (results of 7 studies conducted between 2005 and 2008).

Authors	Year	Samples			Comparison with	Main results	Manufacturer-independent study
		N	HIV subtypes	Geographic origins			
de Mendoza *et al.* [100]	2005	1008	Subtypes B (n = 774) and non-B subtypes/ CRFs (n = 234) Three distinct panels	Netherlands, France, Spain	Amplicor v1.5, Versant 3.0	Good performance of the Nuclisens EasyQ test against B and non-B subtypes	No
Stevens *et al.* [101]	2005	284	Genotypes not determined	South Africa	Amplicor v1.5	3.2% of major discrepancies	Yes
Yao *et al.*]102]	2005	98	98 clinical specimens (genotypes not determined) Two distinct panels	China	Nuclisens HIV-1 QT	5% of major discrepancies Subtypes A and C from the panels underestimated by the two tests	No
McClernon *et al.* [103]	2006	125	Subtypes B (n = 91) and non-B subtypes/ CRFs (n = 44)	North and South America	Amplicor v1.5, Nuclisens HIV-1 QT	No major discrepancy identified	Yes
Gottesman *et al.* [104]	2006	99	79 subtypes C 20 subtypes B	Ethiopia	Amplicor v1.5	Difference > 0.5 log obtained in 34% of the subtype C samples with higher values obtained with the Amplicor HIV-1 v1.5	Yes
Lam *et al.* [105]	2007	119	Clinical samples (including 64 CRF01_AE and 38 subtypes B)	Hong Kong, Cambodia	Amplicor v1.5	Three samples (2 B and 1 CRF02_AE) with higher levels with the EasyQ test	Yes
Wang *et al.* [106]	2008	153	Clades B′ (n = 48), B (n = 5), BC (n = 87) and AE (n = 13)	China	Amplicor v1.5, Nuclisens HIV-1 QT	Important discrepancies mostly with clades BC and AE	Yes

CRFs: Circulating recombinant forms.

In both cases (B and non-B subtypes), the authors reported excellent concordance between the two assays, with no major discrepancies.

Gottesman *et al.*, in Israel [104], compared the performance of the Nuclisens HIV-1 EasyQ assay with Amplicor assay v1.5 in 79 samples of subtype C-infected patients originating from Ethiopia. Twenty HIV-1 subtype B-infected patients served as controls. The disparity between the results of the two VL assays was highly significant in subtype C samples, with higher values mostly obtained with the Amplicor technique. No difference between the two assays was found in subtype B specimens.

Lam and colleagues, in China [105], evaluated the performance of the Nuclisens EasyQ HIV-1 assay for quantification of common HIV-1 subtypes prevalent in South-East Asia. Compared with Amplicor v1.5, three (of a total of 119) samples (two B and one CRF02_AE) showed higher values (difference > 1 $\log_{10}$) with the EasyQ test.

Wang *et al.* 106 studied the impact of HIV-1 genetic diversity in China on the measurement of VL by using three distinct techniques (Nuclisens EasyQ HIV-1, Nuclisens HIV-1 QT and Amplicor HIV-1 Monitor v1.5 assays). For this purpose, they assessed HIV-1 RNA levels with the 3 tests on 153 samples harboring clades B′ (n = 48), B (n = 5), BC (n = 87) and AE (n = 13) collected from different regions of China. Significant differences were found for the following VLs: clade BC measured by any two assays ($p < 0.001$); clade AE between Amplicor 1.5 and EasyQ ($p = 0.005$); and clade B′ between Amplicor 1.5 and EasyQ ($p = 0.04$).

3.2.4 *Evaluation of the Siemens Versant HIV-1 RNA version 1.0 kinetic PCR assay*

Ruelle and collaborators, in Belgium [107], analyzed 322 plasma samples from various HIV-1 subtypes with the Versant kinetic PCR (kPCR) assay previously tested with the Versant HIV-1 RNA v3.0 (bDNA) test. Further, 34 more samples randomly selected from a previous study on the Abbott RealTi*me* and the Nuclisens EasyQ assays were analyzed with kPCR. Among the 322 initial samples, 152 were found concordantly positive with the 2 assays. However, some specimens showed discordant results: 6 were not detected by kPCR in spite of a detectable VL with bDNA and 57 were detected by the kPCR test only. Discrepancies of > 0.5 $\log_{10}$ copies/ml were obtained with 36, 35 and 0% of the samples, respectively, assessed with the Nuclisens EasyQ, Versant bDNA 3.0 and Abbott RealTi*me* assays.

Troppan and collaborators, in Austria [108], evaluated the clinical performance of the Versant kPCR assay 1.0 on 196 samples. When compared with the Roche TaqMan assay v2.0, the median difference between the Siemens and the Roche assay was -0.6 $\log_{10}$ copies/ml. Seven samples (four CRF02_AG, one B, one C and one D) showed a discrepancy of > 1 $\log_{10}$ copies/ml, with higher values obtained with the Roche assay. The QCMD 2009 HIV-1 proficiency panel (including two subtypes B, four subtypes C and two CRF02_AG) was also assessed with this test. Six samples were found to have results with expected HIV-1 RNA concentrations, whereas two specimens (one subtype C and one CRF02_AG) were underquantified.

3.2.5 *Evaluation of the biocentric generic HIV viral load assay*

Rouet *et al.* [62], in France, evaluated the Generic HIV Viral Load assay on 898 specimens positive for HIV-1 RNA by the Amplicor v1.5 and/or Versant v3.0 assays. For this evaluation, 518 samples (from two panels and from patients) contained HIV-1 of known subtypes, whereas 380 samples were collected from 10 different countries (Argentina, Cambodia, Cameroon, Central African Republic, France, Ivory Coast, Madagascar, Morocco, Thailand and Zimbabwe). Overall, the sensitivity of the Biocentric test was 99.3% compared with the Amplicor v1.5. The differences between the values obtained using the Biocentric and Roche assays were + 0.10 and + 0.23 $\log_{10}$/ml for B and non-B subtypes, respectively. The group M and N members from the WHO panel showed similar concentrations by the Generic HIV Viral Load assay and the Amplicor v1.5 test.

Steegen *et al.*, in Kenya [109], compared the Generic HIV Viral Load assay with the Amplicor v1.5 and the reverse transcriptase activity assay (Exavir Load v2.0, Cavidi Tech) on 170 clinical samples. Despite the high genetic diversity of HIV-1 in East Africa, no impact of HIV-1 subtype on the performance of the Generic HIV viral load assay could be detected.

Rouet *et al.* [110] compared HIV-1 RNA levels obtained with the Generic HIV viral load assay with 2 assays (EasyQ and Amplicor v1.5) on 160 clinical specimens (from Burkina Faso, Kenya and South Africa) harboring non-B subtypes. Nine (5.6%) strains detectable with the Generic HIV viral load kit were not detected by either the Amplicor (n = 7) or the EasyQ (n = 2) test. One (0.6%) strain was missed with the Generic HIV viral load kit.

4. Conclusion

Reliable quantification of subgroup M, including non-B HIV-1 subtypes, is a critical feature of state-of-the-art HIV-1 quantification assays, owing to the continuously increasing genetic diversity and emergence of new atypical HIV-1 variants. The underquantification of HIV-1 RNA concentrations or even failure to detect some HIV-1 strains may have practical implications for the clinical management of HIV-1-infected patients. It can lead to an inappropriate strategy for HAART, and false-negative results for diagnosis of pediatric HIV-1 infection.

Facing the amazing HIV-1 genetic drift, it is likely that multiplexing strategy [111] (as used in the TaqMan v2.0 assay) will be integrated, sooner or later, in all closed and open HIV-1 RNA assays. The availability of new innovative technologies in the future will help to overcome the obstacles

posed by HIV-1 genetic diversity. Meanwhile, for optimal monitoring of HIV-1-infected people, each laboratory should initially compare different HIV-1 RNA tests and carefully choose the assay that performs best in terms of cost-effectiveness. Given population migration, traveling and viral recombination, assay evaluation must be a continual process. To obtain more relevant information on this critical topic, larger field evaluations of HIV-1 RNA assays, together with molecular epidemiology studies, should be specifically conducted in areas where HIV-1 genetic diversity is greatest, such as in Central Africa, when tests are available.

5. Expert opinion

5.1 What are the key findings and weaknesses of the work done in this field?

At present, it is not always easy to have clarity on the challenges faced for VL testing in the context of the emerging genetic diversity of HIV-1. Publications of manufacturer-independent studies are relatively rare. Field studies conducted locally in resource-limited countries, where ~ 90% of individuals infected with HIV-1 live, are very scarce. Results from the evaluations described above may appear contradictory, owing to differences in the following.

1) Study design (period, study sites, use of appropriate statistical methods, etc.). In fact, no international guidelines are available for describing appropriate method validation of HIV-1 RNA VL assays.
2) Sample size. Many of the studies described in this review were conducted with small sample size (N < 100). In the authors' opinion, a large number (at least > 100) of specimens is definitively required.
3) Specimen selection (panels and/or clinical samples, differences in proportion of B and non-B subtypes tested, etc.). By selecting predominantly subtype B specimens, the selection bias may conceal the real performance of the evaluated HIV VL tests on the whole spectrum of HIV-1 strains. Tested specimens have to include highly genetically diverse HIV-1 strains and/or must originate from distinct countries and continents.
4) Choice of the reference test. Many studies have previously used the Roche or Abbott or BioMérieux tests as gold standards. However, these 'reference' tests, even through their actual versions, may underquantify or even not detect some HIV-1 strains. For example, in South Africa where the BioMérieux Nuclisens EasyQ test is used to monitor HIV-1 RNA levels in individuals mostly (~ 97%) infected with homogeneous subtype C strains, subjects having ELISA HIV-positive samples with low CD4 cell count but no amplification with the EasyQ assay have been identified [112]. To the authors' knowledge, there is no publication showing an improvement with the EasyQ v2.0 test for HIV-1 RNA quantification of subtypes C, in comparison with the previous version 1.2. The difference between the two versions of this test remains unclear.
5) Tests dependent on targeted regions for HIV-1 genotyping and HIV-1 RNA quantification. HIV-1 genotyping is most often performed in a single region of the genome that is not necessarily the one targeted by primers and probe used in the quantitative HIV-1 RNA assay. Owing to recombination, some strains identified as subtypes B by genotyping (e.g., by using the *pol* gene) may belong to non-B subtypes according to the gene used for HIV-1 RNA quantification (e.g., the *gag* or *LTR* gene). This may explain why underquantification or lack of detection may not be associated with a particular subtype or CRF.

For all these reasons, it is not possible at present to recommend one test (over another) because so far no HIV-1 RNA molecular test is perfect. Further, HIV-1 genetic diversity does not change at the same speed worldwide. Some AIDS epidemics are relatively monomorphic (such as in South Africa), whereas others are caused by many heterogeneous B and non-B strains, even within one country. Consequently, virologic follow-up must be based on results obtained from the same assay, as much as possible. Physicians should not hesitate to request VL determination to be performed with two different techniques in order to highlight underestimation, notably in cases of discrepancy between VL and CD4 count results (e.g., undetectable VL with a CD4 count < 350 mm^{-3} in untreated HIV-1-infected individuals) and/or AIDS-defining clinical signs.

5.2 What about the clinical use of HIV-1 RNA VL testing in developed versus developing countries? A contrasting and unacceptable situation

Despite the extensive HIV-1 genetic diversity, the ultimate goal in this field is to enable any person on ARV treatment or any child born to a HIV-positive mother to get reliable viral load results, irrespective of where they live or with what HIV-1 subtype they are infected.

These goals are achieved in industrialized countries. Numerous studies have demonstrated that HIV-1 RNA levels are independent predictors of disease progression [113-119]. HAART significantly decreases HIV-related mortality and even reverses progression to disease [120-123]. The measurement of plasma HIV-1 RNA VL is performed routinely to decide treatment initiation or switch. The ultimate goal of HAART is the complete suppression of viral replication below the detection limit of the assays (< 20 – 50 copies/ml of plasma) in order to prevent the emergence of drug-resistant mutant strains. To explore viral reservoirs in ARV-treated patients with undetectable plasma HIV-1 RNA results, highly sensitive real-time PCR-based single-copy assays capable of

quantifying unspliced and multiply spliced HIV-1 RNA and proviral DNA in PBMC have been developed as research tools [124-126]. Measurement of HIV-1 RNA levels is also used for the early diagnosis of pediatric infection [127-130], and the diagnosis and prognosis of primary infection in adults [131].

The situation is clearly different in low- and middle-income countries. Since 2002, improved access to HAART through price reductions of patented medications, availability of generic drugs and significant increases in donor funding have resulted in an incredibly large number of HIV-1-infected individuals receiving ARV treatment in low- and middle-income countries. Today, ~ 5 million people are receiving HAART in these settings and it is essential to preserve the benefits obtained by increased access to care.

Unfortunately, most of the treated patients in developing countries are not monitored by HIV-1 RNA VL. Several studies in African settings have demonstrated that the association of clinical criteria and CD4 cell count data with treatment failure is limited [132-134]. By contrast, VL testing enables prolongation of the first-line regimen treatment using a targeted adherence strategy based on extra counseling and VL results. Treatment switches in patients who do not experience virological failure will increase treatment costs and may limit considerably therapeutic options in the future. Avoiding the unnecessary and premature use of second-line strategies is of paramount importance in sub-Saharan Africa because only one second-line ARV regimen (zidovudine, didanosine, lopinavir/ritonavir) is available in most countries. Alternatively, continuation of an inefficient treatment regimen will compromise, sooner or later, patients' clinical and immunological status. That is why it is essential to perform regular VL measurements to detect therapeutic failure early. It may limit the amount of acquired genotypic resistance and ensure better outcomes on second-line regimen as patients would have fewer nucleoside reverse transcriptase inhibitor mutations (such as thymidine analogue mutations). Without VL testing, the use of ARV drugs appears to be totally empirical, and the risk of emergence and transmission of HIV-1 strains resistant to one or two or three classes of ARV drugs is real. Finally, HIV-1 RNA (or DNA) VL testing is the most reliable virological tool that can perform an early diagnosis of pediatric HIV-1 infection. This early diagnosis is crucial to reducing the horrendous HIV-1-related pediatric mortality.

5.3 Why is HIV VL testing not performed in developing countries?

In fact, besides HIV-1 genetic diversity, numerous other obstacles (technical, logistical and economical) prevent HIV-1 RNA monitoring being implemented [135-137]. The first priority is thus to remove barriers due to cost, availability (including transport of specimens and of kits), complexity and accessibility. It is unfortunate that only four manufacturers (Abbott, BioMérieux, Roche and Siemens) share the highly profitable HIV VL market, by commercializing expensive assays (~ 50 – 100 US$/test). These assays require expensive closed equipment. These companies offer exclusively fully integrated packages including not only the reagents but also the equipment and related software. Registration to external quality control programs is an extra but necessary expense. In this context, these assays have rarely been evaluated locally in resource-limited countries. It is also unfortunate that no commercial assay is available to monitor plasma HIV-2 RNA levels in HIV-2-infected subjects in West Africa. At present, a RT-PCR licensing fee is hindering the entry of new players in the PCR market. It is highly anticipated that the expiration of the PCR patent (2011) will open new doors, bringing more competition, and could contribute to a decrease in unit price of VL testing. In particular, innovative low-cost approaches using 'open' systems constitute attractive alternatives that deserve to be evaluated further and eventually adopted by more African and Asian laboratories, including a dried blood spots sampling strategy [138].

In summary, HIV-1 VL testing is not seen as a high public health priority in developing countries. However, its implementation on a large scale is urgently needed if we want to reduce the epidemic in Africa and Asia. By achieving this, HIV infection will really change from a likely death sentence into a manageable chronic infection, as in northern countries.

Acknowledgments

The authors acknowledge C Rouzioux for carefully reviewing this manuscript.

Declaration of interest

F Rouet is supported by the French Ministry of Foreign and European affairs. The authors declare that they have no conflicts of interest and have received no payment for the preparation of this manuscript.

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