Quantitative HIV-1 proviral DNA detection: a multicentre analysis

Anita De Rossi[1], Marisa Zanchetta[1], Francesca Vitone[2], Guido Antonelli[3], Patrizia Bagnarelli[4], Luigi Buonaguro[5], Maria Rosaria Capobianchi[6], Massimo Clementi[7], Isabella Abbate[6], Filippo Canducci[7], Alessia Monachetti[4], Elisabetta Riva[8], Gabriella Rozera[6], Carolina Scagnolari[3], Maria Tagliamonte[5], Maria Carla Re[2] for the SIVIM (Italian Society of Medical Virology) group

[1]*Department of Oncology and Surgical Sciences, Oncology Section, Unit of Viral Oncology, AIDS Reference Centre, University of Padova, IOV-IRCCS, Padova, Italy;*
[2]*Retrovirus Laboratory, Section of Microbiology, Department of Haematology and Oncologic Sciences, University of Bologna, Bologna, Italy;*
[3]*Laboratory of Virology, Department of Molecular Medicine, Sapienza University of Rome, Italy;*
[4]*Department of Biomedical Sciences, Section of Microbiology, Virology Unit, Marche Politechnic University Medical School, Ancona, Italy;*
[5]*Laboratory of Molecular Biology and Viral Oncogenesis & AIDS Reference Centre, Istituto Nazionale Tumori "Fondazione Giovanni Pascale", Naples, Italy;*
[6]*National Institute for Infectious Diseases I.R.C.C.S. "Lazzaro Spallanzani", Roma, Italy;*
[7]*Laboratory of Microbiology and Virology, "Vita-Salute" San Raffaele University, Milan, Italy;*
[8]*Virology Section University Campus Bio-Medico, Rome, Italy*

SUMMARY

Despite the widespread use of molecular biology techniques, standardized methods for the measurement of HIV-1 proviral DNA are currently lacking and several discordant results are still present in different studies. To assess the clinical meaning of the proviral DNA load, a study group comprising seven different laboratories was set up to standardize a HIV-1 proviral DNA quantification method able to assess the DNA proviral load of the most relevant circulating HIV-1 subtypes. Reference samples (24 cellular samples infected with HIV-1 clade B, and 40 samples of peripheral blood mononuclear cells containing different concentrations of plasmids expressing different HIV-1 clades) were distributed and tested blindly. All laboratories employed hTERT gene as housekeeping gene and primers within the *gag* gene to quantify different HIV-1 clades. Inter-laboratory results did not differ statistically but showed only minor variations concerning HIV-1 DNA amounts and different HIV clades, with a good agreement among the laboratories participating in the study. Since test standardization represents a key step for future application in clinical practice, further studies of the patients' samples are in progress to establish the real meaning and utility of the proviral DNA load for clinical management of HIV-1 infected patients.

KEY WORDS: HIV, DNA detection, Standardization

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INTRODUCTION

The introduction of highly active antiretroviral therapy (HAART) has radically changed the natural history of HIV-1 infection. The implementation of new anti-retroviral therapeutic protocols between 1996 and 1999, resulting in a significant reduction of plasma viremia accompanied by arrested immune deterioration, generated cautious optimism also supported by mathematical models claiming the possibility of a complete eradication of HIV-1 infection (Perelson *et al*., 1997) However, subsequent studies demonstrated that not only is viral replication not completely interrupted by therapy (Clementi, 2000; Re *et al,*. 2005; Kulkosky *et al,*. 2006; Zanchetta *et al.,* 2006), but that the virus continues to evolve resulting in the emergence of resistant strains and an increasing spectrum of viral quasispecies in cell subpopula-

Corresponding author
Maria Carla Re
Retrovirus Laboratory
Section of Microbiology
Department of Haematology and Oncologic Sciences
University of Bologna, Bologna, Italy

tions hosting the infection (Noë *et al.,* 2005); Buonaguro *et al.,* 2007 Buonaguro *et al.*, 2007 b). The presence of latently infected cells, a virtually inexhaustible reservoir, has stimulated many studies on viral reservoirs and their significance.

Several data indicate a correlation between levels of proviral DNA and the outcome of treatment, with high levels of HIV-1 DNA associated with a faster progression to AIDS, an increased risk of death and a higher risk of HIV-1 RNA rebound (Galli *et al.,* 1998; Re *et al.,* 2005; Re *et al.,* 2006; Gibellini *et al.,* 2008; Palmisano *et al.,* 2008; Re *et al.,* 2009). Furthermore HIV-1 DNA seems to have a prognostic value as a marker of disease progression (Hatzakis *et al.*, 2004; Katzenstein *et al.,* 2002; Kostrikis *et al.,* 2002; Rouzioux *et al.,* 2005) in addition to other parameters, such as HIV-1 RNA viral load and CD4 count, highlighting a greater likelihood of achieving and maintaining long-term HIV-1 viral suppression in patients starting therapeutic protocols with low levels of HIV-1 DNA (Hatzakis *et al.*, 2004; Paraskevis *et al.*, 2009; Beloukas *et al.,* 2009a)

Despite the widespread use of molecular biology techniques, standardized methods for the measurement of the two most significant forms of HIV-1 DNA, integrated and non integrated, are currently lacking and discordant results persist due to unmatched selection of patients, extraction protocols and quantitative methods employed in the studies. The techniques available are either in-house procedures or involve troublesome modifications of commercial tests (Lillo *et al.,* 2004; Ometto *et al.,* 2002; Beloukas *et al.,* 2009a; Beloukas *et al.*, 2009b). To assess the clinical meaning of the proviral DNA load, proviral DNA must be quantified by standardized methods, whose results can be compared and applied in different clinical settings. To achieve this aim, a study group comprising seven different laboratories was set up to compare and standardize HIV-1 proviral DNA quantification methods able to assess the DNA proviral load of the most relevant circulating HIV-1 subtypes.

MATERIALS AND METHODS

A quantitative HIV-1 DNA test was performed on reference samples according to extraction and amplification methods currently in use in six virological centres with previous Polymerase Chain Reaction (PCR) experience (Lab 1, Lab 2, Lab 3, Lab 4, Lab 5 and Lab 6). Another Centre (Lab 7) prepared and sent coded plasmids (NIBSC, Programme Eva Centre for Aids Reagents, UK) expressing the HIV-1 *gag* gene of the most relevant HIV-1 clades (A, B, C, D, F, G, H, J, CRF01_AE and CRF02_AG).

Briefly, each laboratory analyzed coded reference samples [experimentally infected cells, C8166 and peripheral blood mononuclear cells (PBMCs) and coded plasmids]. Prior to testing, each laboratory provided details concerning testing procedures for comparative evaluation.

Cells and reference samples

The C8166 cell line was maintained in RPMI 1640 medium (Gibco, Grand Island NY) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Invitrogen Srl), 2 mM glutamine (Gibco Paisley, UK).

PBMCs were taken from the fresh blood of 4 healthy adult volunteers, after giving their informed consent following the Helsinki declaration. They were isolated by Ficoll-Paque (MP Biomedicals), seeded in flasks at the final concentration of $1x10^6$ cells in RPMI 1640 medium supplemented with 10% foetal calf serum and activated by addition of 5 µg/ml phytohemagglutinin (Sigma Aldrich) 60 h before use.

8E5LAV, a T-lymphoblastoid cell line containing a single proviral copy of HIV-1 LAV per cell (Folks *et al.,* 1986), was maintained in RPMI 1640 medium (Gibco, Grand Island NY) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Invitrogen Srl) and 2 mM glutamine (Gibco Paisley, UK).

HIV-1 negative CEM cells (Foley *et al.,* 1965), maintained in RPMI 1640 medium supplemented with 10% FCS, were used to construct a standard curve obtained by adding tenfold serial dilutions of 8E5LAV (from 10^2 to 10^6 8E5LAV to $2x10^6$ CEM cells). Samples were sent as frozen cell pellets to the laboratories participating in the study.

Virus. HIV-1_{IIIB} was obtained from HIV-1 infected C8166 cells co-cultivated with non-infected C8166 cells for 6 days at 37°C. Virus-containing supernatant was stored at -80°C. To establish the titer of virus stock, eightfold serial dilutions of the stock were assayed in C8166 cells in a 96-well plate. The 50% tissue culture infectious dose

($TCID_{50}$) was calculated by the Reed and Muench statistical method (Hierholzer *et al.,* 1996), by counting syncytia in six replicate wells under low-power magnification after 72 h incubation at 37°C.

Virus infected cell preparation. C8166 and PBMCs ($1x10^6$) were infected as previously described (Gibellini *et al.,* 2007) with 1ml of HIV-1_{IIIB} virus, previously titrated. After 3 and 5 days for C8166 and 6 and 11 days for PBMCs, cells were harvested and the cell pellets ($5x10^6$ /vial) were prepared, coded and stored at -80°C until distribution and testing.

Non-B HIV-containing PBMCs preparations. Coded plasmids containing the HIV-1 *gag* gene of the most relevant HIV-1 clades (A, B, C, D, F, G, H, J, CRF01_AE and CRF02_AG:) were obtained by Lab 7 from the NIBSC AIDS repository (Heteroduplex Mobility Analysis HIV-1 gag Subtyping Kit, ARP964). Three dilutions (10^2, 10^3 and 10^5 plasmid copies) were prepared and added to $1x10^6$ human PBMCs from healthy donors and sent to all the other laboratories participating in the study.

DNA extraction. Four laboratories (Labs 1,2,3, and 5) isolated the total DNA from each sample (C8166 and PBMCs infected and uninfected, and PBMCs containing different HIV-1 clades) by manual extraction using a QIAamp DNA blood kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's recommendations. In Labs 4 and 6, 1×10^6 cells of each coded sample were lysed in 125 µl of TE buffer (10 mM Tris-HCL [pH 8] and 0.1 mM EDTA) containing 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, and 600 mg/ml proteinase K as previously described (De Rossi *et al.* 1996).

Primer and probe sets. HIV-1 primers and probe sets (Table 1) were designed using Primer Express Software and were located in the highly conserved *gag* gene (Ometto *et al.* 2002; Vitone *et al.* 2005) (HIVPV22 genome, Gen Bank accession: K02083) and *pol* region as already described (Désiré *et al.* 2001).

In particular, to detect HIV-1 DNA proviral load in experimentally infected cells (C8166 and PBMCs), Laboratories 1, 2 and 3 utilized *pol* primers, while Laboratories 4, 5 and 6 utilized *gag* primers. Analysis of PBMC /plasmids containing *gag* gene of different HIV-1 clades were performed by all the laboratories using *gag* primers, even if with different target sequences (Table 1).

Human telomerase reverse transcriptase (hTERT) located in the 5p15.33 (Gen Bank accession: AF128893) (Sozzi *et al.* 2003) was employed as housekeeping gene and amplified in parallel with HIV-1 genes to quantify the total number of cellular genomes.

Standard/Reference curve. Serial tenfold dilutions of 8E5LAV in CEM cells (from 10^2 to 10^6 8E5LAV to $2x10^6$ CEM cells) or commercial HIV-1 gag plasmid (Clonit, Alfa-Wasserman S.p.A Diagnostic, Milan, Italy) ranging from 10 to $1x10^5$ copies, were used as quantitative calibrators.

HIV-1 DNA real time PCR Assay. HIV-1 DNA levels were determined by Taqman real-time quantitative PCR assay (Labs 1,2,3,4,6). The methodological approach used for quantification,

TABLE 1 - *Oligonucleotide sequences used for HIV-DNA detection in the laboratories partecipating in the study.*

Laboratory number	*Gene*	*Primers target*	*Probe*	*Target lenght*
1, 2, 3	pol*	P1-5'-TGGCATGGGTACCAGCACA-3', P2-5'-CTGGCTACTATTTCTTTTGCTA-3'	5'- FAM-TTTATCTACTTGTTCATTTCCTCCAATTCCTT-3' TAMRA	199 bp
1, 2, 3,4, 6	gag	FW5'–TTAAGTGTTTCAATTGTGGCAAAGA-3' RW5'-AAAAAATTAGCCTGTCTCTCAGTACAATCT–3'	5'–FAM CCCTAGGAAAAAGGGCTGTTGGAAATG–3'- TAMRA	166 bp
5	gag	SK431-5'- TGCTATGTCAGTTCCCCTTGGTTCTCT -3' SK462 -5'-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3'		142 bp
1, 2, 3, 4, 5, 6	hTERT	FW, 5'–GGCACACGTGGCTTTTCG-3' RV, 5'–GGTGAACCTCGTAAGTTTATGCAA3'	5'–VIC-TCAGGACGTCGAGTGGACACGGTG–3'-TAMRA	98 bp

*Gene target used on the first experiment.

as reported in Table 2, was performed by using 2X TaqMan Universal PCR Master Mix (Applied Biosystem), forward and reverse primers, fluorogenic probe and 5-10µl of extracted DNA or cell lysate. The thermal cycling conditions were 2 min at 50°C, 10 min at 95°C, and 45 cycles each of 95°C for 15 s and 60°C for 1 min. The reaction was performed in a spectrofluorometric thermal cycler (ABI PRISM 7700 or 7000 Sequence Detector, PE Applied Biosystems and Biorad IQcycler, Bio-Rad Laboratories).

The SYBR Green real-time PCR (Gibellini *et al.*, 2008; Re *et al.*, 2009; Vitone *et al.*, 2005) assay was only employed by laboratory 5. The assay was performed on 20 µl PCR mixture volume of 2×Quantitect SYBR Green PCR Master Mix (Qiagen) containing HotStarTaq DNA polymerase, 200 nM of each primer (SK431, SK462, Table 2) and 10µl of DNA. All samples were analysed in duplicate. The amplification protocol for HIV-1 on the LightCycler instrument (Roche, Mannheim, Germany) was as a follows: 95°C for 15 min and 45 cycles each of: 94°C for 10 s, 60°C for 30 s, 72°C for 30 s, 78°C for 3 s.

All samples were run in triplicate and mean values were sent to the reference centre.

Statistical methods and data analysis

The data were first converted to their logarithmic (log_{10}) value. The mean, standard deviation (SD) and median were calculated for all positive samples. The variability was evaluated by descriptive statistics for the different sets of samples and expressed as a coefficient of variation (CV) on the basis of log_{10} value of HIV DNA proviral. The ANOVA test was performed to compare results obtained by the laboratories and results obtained using commercial and laboratory-developed standard curve.

RESULTS

Proviral HIV-1 DNA detection in experimentally infected cells

In the first series of experiments, 24 different samples obtained by mock infected and HIV-1 infected cells, were distributed to the laboratories for the evaluation of HIV-1 DNA load with their own established PCR-based techniques and procedures (standard curve, sample preparation, sequences target of amplification). The C8166 cells showed at 3 days post infection a mean value of HIV-1 DNA of 2.87 log_{10} (±0.44 SD) and at 5 days post infection a mean value of 4.60 log_{10} (±0.24 SD) (Table 3). HIV-1 experimentally infected PBMCs analyzed at 6 and 11 days post-infection showed a mean value of 5.04 log_{10} (±0.79 SD) and 6.24 log_{10} (±0.52 SD) respectively (Table 3). The inter-laboratory coefficients of variation (CV) were 15.19 % and 15.66% for $C8166_{IIIB}$ and $PBMC_{IIIB}$, respectively when the analysis was performed 3 and 6 days after infection (Table 3). A better concordance was observed for samples containing higher HIV-1 DNA levels, i.e obtained at later times post infection; indeed, the CV were 5.25 % and 8.25% for

TABLE 2 - *Comparison of procedural characteristics among different laboratories.*

	Platform	*DNA imput (µl)*	*Cell lysate imput (µl)*	*Reaction volume (µl)*	*Gene target*	*Primer concentration (FW/REV nM)*	*Probe Concentration*	*Type fluor*	*LOD*** copies/ 10⁶ cell*
Lab1	ABI 7700	10	-	50	pol* gag	300/300	200/300	FAM/TAMRA	<40
Lab2	ABI 7000	5	-	25	pol* gag	200/200	200	FAM/TAMRA	<10
Lab3	ABI 7700	10	-	50	pol* gag	300/300	200/300	FAM/TAMRA	<3
Lab4	ABI 7700	10	10	50	gag	300/900	200	FAM/TAMRA	<10
Lab5	LightCycler 5,3,2	10	-	20	gag**	200/200	-	SYBR green	<10
Lab6	Biorad IQcycler	-	5	25	gag	300/900	200	FAM/TAMRA	<10

*gene target used on the first experiment; **gag sequence SK431, SK462; ***LOD= Limit of Detection.

$C8166_{IIIB}$ and $PBMC_{IIIB}$, at 5 and 11 days post infection, respectively. No significant differences were observed between the mean values of Labs l, 2, 3 which quantitated HIV-1 DNA by using *pol* as target gene, and Labs 4, 5, 6 which quantitated HIV-1 DNA by using *gag* as target gene (Table 3).

Accuracy and sensitivity of the HIV-1 DNA assay

As a second step, the accuracy and sensitivity of the HIV-1 DNA assay were evaluated on a panel of non-B HIV-1 containing PBMCs experimentally generated by adding two different concentrations of plasmid DNAs (10^3 and 10^5 copies/10^6 PBMCs) containing *gag* genes of different HIV-1 genetic subtypes. To align with the genome target contained in these spiked preparations, all laboratories used *gag* primers (Table 1).

Analysis of samples containing 10^3 copies plasmid DNA/10^6 PBMCs showed a variation of values within a ±0,50 log_{10} for most (84%) samples, with the remaining 16% of values within a ±1,00 log_{10} range from median value (Figure 1A).When the analysis of non-B HIV-DNA content was focused on the second set of samples (10^5 copies/10^6 PBMCs), 60% of values resulted within the range of ±0.50 log_{10} (Figure 1B), while 27% were within a range of ±1.00 log_{10} (Figure 1B). HIV-1 DNA load of HIV-1 subtypes C, D, G and J were overestimated in 3 laboratories.

HIV-1 DNA detection by two calibration curves

To evaluate the sensitivity of detection of low levels of HIV-1 DNA, we compared results obtained using two different concentrations (10^2 and 10^3 plasmid copies/10^6 PBMC) of different HIV-1 clades with two calibration curves generated from a commercial HIV-1 gag plasmid and the 8E5LAV cell line.

As shown in Figures 2A and 2B, all laboratories detected all the HIV-1 clades and slight but not statistically significant differences were observed between quantitative results obtained using the two different calibration curves.

In particular, most samples of different HIV-1 clades (78% and 73% using the 8E5LAV cell line and the commercial HIV-1 *gag* plasmid reference curve, respectively) showed values within ±0,50 log_{10} at plasmid concentration of 10^2 copies/10^6 PBMC.

Similar results were obtained at higher (10^3 plasmid copies/10^6 PBMCs) plasmid concentration: 78% and 67% of samples showed values within ±0,50 log_{10} by 8E5LAV cell line and commercial HIV-1 *gag* plasmid reference curve, respectively. The results obtained at 10^3 plasmid copies/10^6 PBMCs (Figure 2B) were similar to those obtained in the previous experiments using different sample preparations and 8E5LAV cell line as reference curve (Figure 1A).

TABLE 3 - *HIV DNA (log10copies/106cells) in C8166 HIV infected cells and PBMCs at different time post infection.*

Lab	*C8166 HIV-1 infected HIV DNA (log_{10} copies/10^6 cells)*		*PBMCs HIV-1 infected HIV DNA (log_{10} copies/10^6 cells)*	
	3 days p.i.	*5 days p.i.*	*6 days p.i.*	*11 days p.i.*
Lab1	3.18	4.54	6.11	6.78
Lab2	2.00	4.36	3.65	5.30
Lab3	2.94	4.91	5.08	6.23
Lab4	3.04	4.57	5.08	6.11
Lab5	2.94	4.36	5.20	6.43
Lab6	3.11	4.88	5.08	6.56
mean± SD(CV%	2.87±0.44 (15.19)	4.60±0.24 (5.25)	5.04±0.79(15.66)	6.24±0.52(8.25)
median (range)	2.99(2.00-3.18)	4.56(4.36-4.91)	5.08 (3.65-6.11)	6.33(5.30-6.78)

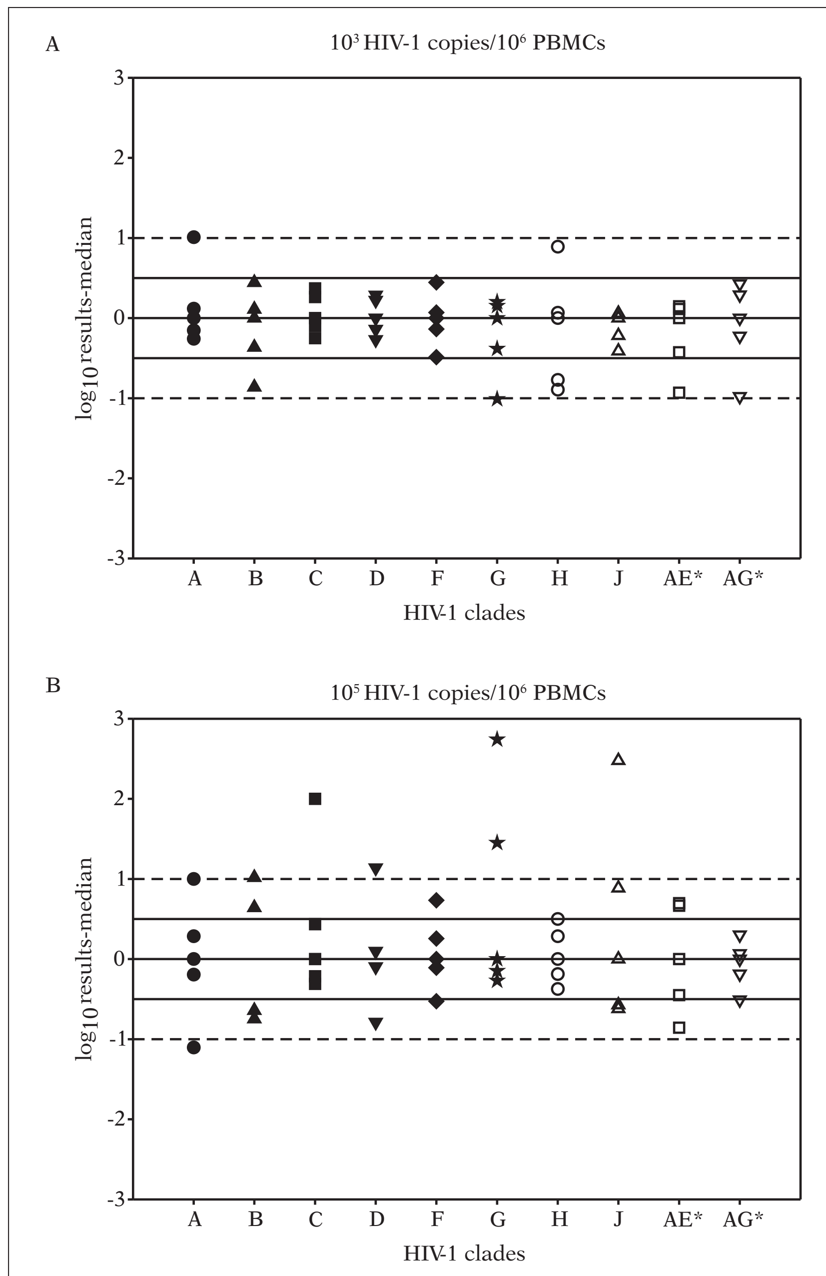

FIGURE 1 - *Variation of HIV-DNA results for different HIV-1 clades at concentration 10^3 copies/10^6 PBMCs (A) and 10^6 copies/10^6 PBMCs CB.*

DISCUSSION

In addition to CD4 cell count and plasma viremia, quantification of HIV-1 DNA in peripheral blood cells may be extremely important for disease monitoring, especially in HAART-treated patients (Re MC *et al.*. 2009; Ramratnam *et al.*. 2000; Sharkey *et al.* 2000; Butler *et al.*, 2002; Garbuglia *et al.*, 2004; Rozera *et al.*, 2009, d'Ettorre *et al.*, 2010) when plasma viral RNA can be undetectable by current techniques (Cara *et al.*, 2002; Sarmati *et al.*, 2005; Ngo-Giang-Huong *et al.*, 2001; Kostrikis *et al.*, 2002) . Several assays based on real time PCR are currently used to quantify HIV-1 DNA levels showing good accuracy and reproducibility. Nevertheless, several contrasting reports have raised uncertainty as to the meaning of viral DNA load probably related to the different methods used to detect HIV-1 DNA load, and different patient selection criteria (group size,

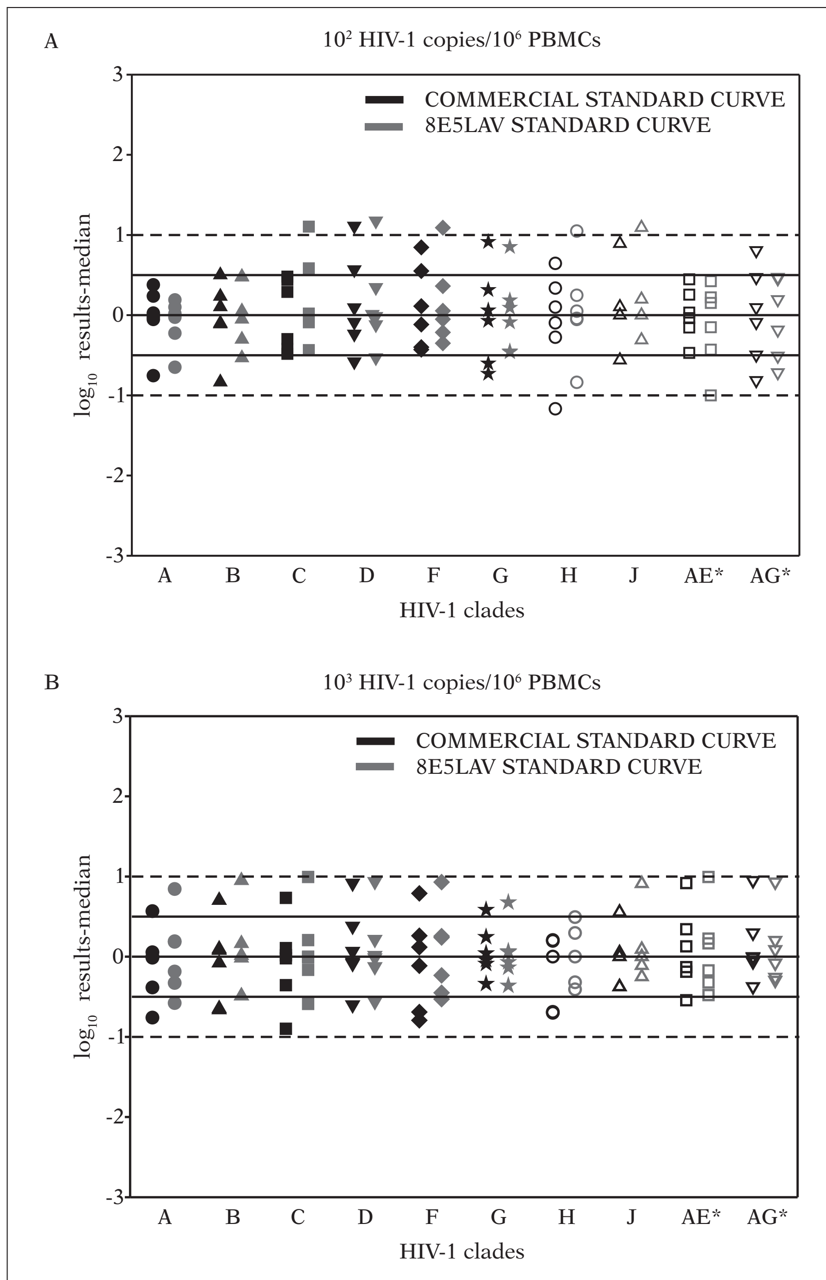

FIGURE 2 - *Variation of HIV-DNA results for different HIV-1 clades at concentration 10^2 copies/10^6 PBMCs (A) and 10^3 copies/10^6 PBMCs (B) using a commercial plasmid (black) and 8 E5LAV (grey) reference curve.*

CD4+ cell count thresholds, matching of the selected patients).

Despite the widespread use of molecular biology techniques, a universal format is not employed to measure the level of cells associated with HIV-1 DNA, and available techniques are either in-house procedures or troublesome modifications of commercial tests (Ometto *et al.*, 2002; Lillo *et al.,* 2004; Beloukas *et al.,* 2009b; Kabamba-Mukadi *et al.,* 2005).

To assess the meaning of the proviral DNA load, proviral DNA must be quantified by a standardized method and results compared and applied in different clinical settings. To achieve this aim, we organized a network of national laboratories with established experience in the virological field to standardize the methodology currently used and to compare results. Results showed that regardless of the procedures they choose to employ all laboratories involved in the network were able

to detect HIV-1 DNA in experimentally infected cells. In a first set of experiments, aimed to quantitate the HIV-1 DNA in C8166 cells and PBMCs experimentally infected with HIV-1_{IIIB}, all laboratories obtained comparable results, with coefficients of lower variation when the proviral HIV-DNA levels were higher. In the second phase, HIV-1 DNA was quantified in a panel of cellular samples constructed to simulate the infection by different genetic subtypes. Contrary to what was observed with infected cells, these results revealed a higher variability at higher plasmid concentrations. Indeed, 84% and 60% of samples were within a ±0.50 log_{10} at low (10^3 copies/10^6 PBMCs) and high (10^5 copies/10^6 PBMCs) viral load, respectively. Further studies are in progress to fully understand the unduly high discordant results obtained for C, D, G and J subtypes.

To evaluate the inter-laboratory agreement to detect low levels (10^2 and 10^3 HIV-1 copies/10^6 PBMCs) of HIV-1 DNA load, we compared results obtained with two calibration curves generated from a commercial HIV-1 *gag* plasmid and 8E5LAV cells. Results showed that all the laboratories were able to successfully identify all the HIV-1 clades without significant differences. This is an important finding, even if HIV-1 subtypes are present with low frequency in the Italian population. Epidemiological evidence suggests that only 10% of the viruses transmitted through heterosexual contact could potentially belong to non-B subtypes and CRFs, but HIV-1 isolates genetically related to novel subtypes have recently been increasingly identified and described (Tagliamonte *et al.,* 2006; Buonaguro *et al.*, 2008; Bruselles *et al.,* 2009).

Summing up, our study suggested that:

1) all laboratories detect and quantitate HIV-1 DNA load in a comparable manner;
2) no statistically different results were obtained using two different calibration curves;
3) all the HIV-1 subtypes were always detected even though higher levels of HIV-1 DNA load were often detected by some laboratories.

Since test standardization is a key step for future application in clinical practice, our study has the final aim to achieve reproducible results, irrespective of laboratory performing the test. A rational choice of test and a better knowledge of the diagnostic procedures used in individual laboratories will improve the use of HIV-1 DNA load also providing crucial information to clinicians.

This study demonstrated good agreement among the six laboratories for HIV-1 DNA quantification that could be used as a predictive marker of disease outcome and to monitor the efficacy of antiretroviral treatment. Further studies are in progress to rule out ongoing differences and minimize inter-laboratory variability.

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