Inter- and Intra-laboratory Variability in HTLV-1 Provirus Load Quantification Using Real-Time Polymerase Chain Reaction Assays: A Multi-Center Study

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Summary

Human T-cell leukemia virus type-1 (HTLV-1) provirus load (VL) is an important determinant of viral pathogenesis and malignant evolution. Although VLs have been quantified by in-house real-time quantifiable polymerase chain reaction (qPCR) technology around world, little is known about the harmonization of VL in mutual assays. We evaluated intra- and interlaboratory variability of HTLV-1 VL at six laboratories using DNA samples seropositive for HTLV-1 in a two-step manner. The 1st study measured 60 samples by original in-house assays, showing that the intralaboratory coefficient of variation (CV) was almost constant at around 50% across laboratories, but interlaboratory CV (%) was very wide by sample. The interlaboratory correlation coefficients ranged from 0.760 to 0.875, indicating that VLs were measured with good precision in each laboratory, but interlaboratory regression slopes differed from 0.399 to 2.206, indicating that VLs were measured with big variation in some laboratories. To examine the effect of standard reference materials (RM) on VL variability, we performed a 2nd study using 20 samples only by substituting RM for plasmid HTLV-1 provirus. The median interlaboratory CV of raw pX copy number before normalization was reduced significantly from 66.9% to 35.3%; however, no improvement in interlaboratory CV for VL was statistically observed (59.9% versus 48.2%). In conclusion, each in-house assay system worked well with good precision, but harmonization of VL was insufficient by only standardizing RM. The relevant choice of not only RM, but also internal control genes for data normalization, is expected to be realistic to standardize the VL.

Abreviations;HTLV-1; Humam T-cell Leukemia Virus type-1, VL;Virial load, CV;coefficient variation, RM; Reference Material, Adult-T-cell Leukemia;ATL, the Joint Study on Predisposing Factors of ATL Development;JSPFAD, quantifiable polymerase chain reaction;qPCR, peripheral blood mononuclear cells;PBMCs, internal control;IC

Introduction

Quantification of the Human T-cell leukemia virus type-1 (HTLV-1) provirus copy number in the genome has contributed to understanding of the pathophysiology of the infected cells [1, 2, 3]. Cells infected with HTLV-1 are generally thought to carry one provirus genome in their chromosomal DNA, indicating that one copy is equivalent to one cell [4]. Accordingly, since HTLV-1 provirus load (VL) directly reflects the number of HTLV-1-infected cells, it is useful and relevant to monitor dynamic changes in infected cells in individual carriers [5]. To clarify a causative relation between the pre-leukemic state of adult T-cell leukemia (ATL) and kinetics of infected cell burden, we established a nationwide cohort study for asymptomatic HTLV-1 carriers in Japan, designated as the Joint Study on Predisposing Factors of ATL Development (JSPFAD) in 2002 [6]. The main purpose of the cohort study was to measure the HTLV-1 VL of asymptomatic HTLV-1 carriers using a quantifiable polymerase chain reaction (qPCR) over a long period of time. For the HTLV-1 VL measurement by real-time qPCR, a variety of methods have been reported, but the gold standard method for the measurement has not been identified and validated to date because there is no standard reference material (RM) that is essential for absolute real-time qPCR for proviral DNA. The variability in the VL measurement may be influenced by both biological characteristics and technical variables. From a technical point of view, some studies used a plasmid provirus as RM for a standard calibration curve to measure the raw pX copy number before normalization, but others used HTLV-1-inected cell lines, such as a TARL-2 cell line carrying one provirus derived from rat-infected cells. A variety of internal controls (IC), β-actin, β-globin, RNase-p, and CD81 have been also used for the qPCR assay system. These different methods for the measurement of HTLV-1 VL may introduce bias into the association between VL and disease outcome.

To address this issue, it is necessary to evaluate intra- and interlaboratory variability of real-time qPCR assays to quantify HTLV-1 VL. The present study demonstrated intra- and

interlaboratory variability of HTLV-1 VL in six research laboratories, and an approach to standardization.

Materials and Methods

Samples:

We used a total of 80 DNA extraction samples from peripheral blood sero-positive for HTLV-1, which were stored in the core laboratory of JSPFAD, the University of Tokyo. The genomic DNA was isolated using a QIAGEN Blood Kit (Qiagen, Hilden, Germany) from peripheral blood mononuclear cells (PBMCs). All samples were taken under the approval of the respective ethical committees.

Study laboratories

Six research laboratories (one core laboratory, T, and five laboratories, A, B, C, D, and E), all of which were members of JSPFAD, participated in this study. In each laboratory, HTLV-1 VL was routinely measured by its own in-house (home-brew) real time qPCR system. Equipment, target sites within the pX region for amplification, probe sites, standard RM, and internal control genes differed by laboratory (Table 1). The original protocol in each laboratory is referred to in references.[3,7,8,9,10,11] All assay systems were basically constructed based on the same concept considering the characteristics of the HTLV-1 genome. As summarized in Table 1, two types of RM were used for the standard calibration curve to measure the raw pX copy number before normalization; a plasmid provirus in the four laboratories and a TARL-2 cell line carrying one provirus derived from rat-infected cells in two laboratories. For internal controls (IC), two laboratories used β-actin, one used CD81, one used β-globin, and two used RNase P. In order to make a comparison between laboratories, we used a common procedure to express HTLV-1 VL values as pX copy numbers per 100 PBMCs, based on the assumption that infected cells harbored one pX

copy of the integrated HTLV-1 provirus per cell and 2 IC gene copies per cell [5].

Study design

After receiving the sample DNAs, each laboratory diluted them for its own in-house (home-brew) real time qPCR system. We performed two studies to evaluate variations in HTLV-1 VL copy number per 100 PBMCs, pX copy number per PCR reactions containing 50ng genomic DNA adjusted from respective assay systems, and the IC gene copy number per PCR reaction with 50ng genomic DNA. In the 1st study, 60 samples were measured by routine in-house (home-brew) qPCR systems in each of the six laboratories. In the 2nd study, 20 samples were measured by the in-house assay systems only by substituting individual RMs for the plasmid HTLV-1 provirus (9.0 kb provirus inserted in pUC19) that was used in the core laboratory T. The 2nd study was performed at same laboratories as the 1st study excepting for laboratory E. Each laboratory performed duplicate assays for each sample in both studies. All measured data were sent to laboratory A, and then the intra- and interlaboratory variation were evaluated in each study.

Statistical analysis

Intralaboratory variability was evaluated by calculating the mean, standard deviation (SD), median, ranges, and coefficient of variation (CV=100 x SD/mean, %) for the measured HTLV-1 VL in each laboratory. In the 1st study, intralaboratory variability was evaluated by dividing by ascending order into three different VL levels (low, intermediate, and high) based on measured VLs at the core laboratory T. Each population of different VL level included 20 samples. Intralaboratory variability in each VL level was expressed as box plots for measured VLs and the CV%, and calculated intralaboratory CVs.

Interlaboratory variability, precision, and accuracy of the quantitative measurements of VL were evaluated by calculating interlaboratory CV (%) for each sample, and by fitting

linear regressions. Linear regression analyses were performed between measured VLs of the core laboratory T as standard and those of each of the five laboratories, from which the linear slope, Spearman's correlation coefficient (rs), and the coefficient of determination (R2) were evaluated. In this study, a Spearman's rank (rs) correlation coefficient > 0.8 was defined as indicative of good precision, and a regression slope of 1.0 was indicative of 100% accuracy; thus, VLs measured in the laboratories were equal to those in the core laboratory T. In the 2nd study, we examined whether interlaboratory CV for VLs, raw pX copies and IC gene copies was improved by only standardizing RM.

The Mann-Whitney test was used to evaluate whether there was any significant difference between measurements. A statistically significant level was set at 0.05.

Results

1. Intralaboratory variability of VLs in the 1st study

In the 1st study, the six laboratory assay systems were detectable for all 60 samples with a wide rage of VLs. For the 60 samples, the median VL values for 6 laboratory assays, T, A, B, C, D, and E, were 5.4, 5.7, 12.6, 5.3, 12.3, and 3.4 copies per 100 PBMCs, respectively. There was a large variation in the median VLs among laboratories. The maximal difference in the median VL between laboratories was 3.7 fold (D vs. E).

Figure 1A shows the intralaboratory variability of actually measured VLs at each VL level. We confirmed that all 5 laboratories accurately measured samples with low VLs with laboratory T being low, as well as high VLs at laboratory T being high.. However, the box plots show that distribution of measured VLs differed across laboratories in each VL level. In the low and intermediate VL levels, there was almost quantitative accordance in the median VL between laboratories T, A, C and E (no statistical differences), but the median VL values were significantly higher in laboratories B and D than the core laboratory T ($P<0.05$). In the high VL levels, although a wider distribution of measured values was

observed in all laboratories, there was almost quantitative accordance in the median VL between laboratories T, A, and C, but the median VLs were significantly higher in laboratory B and D than the core laboratory T, whereas they were lower in laboratory E than in T ($P<0.05$).

Figure 1B shows intralaboratory CVs (%) at three VL levels. In the low VL level, the range of intralaboratory CV was somewhat different across laboratories. At the intermediate VL level, the range of intralaboratory CVs was similar in 4 laboratories (T, B, C, and E), but was greater in laboratory D ($p<0.05$). In the high VL level, the range of intralaboratory CVs was similar across laboratories, except for laboratory E, where the intralaboratory CV was very large ($p<0.05$). The median intralaboratory CV at the low, intermediate, and high VL levels were 44.1% (range, 32.3-58.9%), 36.0% (range, 25.4-44.5%), and 55.0% (range, 51.6-71.8%), respectively, indicating there was no statistically significant difference, although the intralaboratory CV showed a tendency to increase as the VL level increased from low to high. These results demonstrated that intralaboratory variability was almost constant at around 50% across VL levels in the 5 laboratories, except for laboratory E, despite a wide distribution of actually measured VLs values.

2. Interlaboratory variability of VLs in the 1st study

The overall median interlaboratory CV for 60 samples was 59.9% (ranges 34.2–93.4%), which indicates that the interlaboratory variability of quantitative measurements across all laboratories was very wide. Figure 1C shows the interlaboratory CV (%) of measured VL for individual samples in ascending order. There was a wide variability in interlaboratory CV by sample. The median interlaboratory CV at the low, intermediate, and high VL levels were 67.4% (range, 35.7-82.3%), 57.4% (range, 41.2-87.4%), and 54.9% (range, 34.2-77.6%), respectively, indicating that interlaboratory CV decreased as the measured-target value (i.e. VL) increased from low to high. Although interlaboratory CV (%) was very wide by sample,

there was a good correlation in the scatter plots of each VL between values measured in the core laboratory T and those measured in the five laboratories. The fitted linear regression curves are shown in Figure 2. The interlaboratory correlation coefficients ranged from 0.760 to 0.875, indicating that VLs were measured with good precision in each laboratory. However, interlaboratory regression slopes differed among laboratories. The slopes of laboratories A and C were close to 1.0 (0.992 and 0.984, respectively), indicating that the measured VLs in the two laboratories were similar to values that were measured in the core laboratory T. The slopes of laboratory B and D were greater than 1.0 (rs=1.393, and rs=2.206, respectively), whereas the slope of laboratory E was less than 1.0 (rs=0.399), indicating that the VLs measured in these 3 laboratories were out of alignment from the values measured in the core reference laboratory T. However, it is well recognized that the difference in slope influences all samples equally as a systemic error [15]. To standardize data, we calculated an “adjusting coefficient”, which is defined as an inverse value of the slope (1/slope) of each laboratory. The “adjusting coefficient” for each laboratory ranged from 0.453 to 2.51 (Table 2).

3. Inter-laboratory variability after sharing RM in the 2nd study

The findings from the 1st study revealed that a systemic error and a specific bias were probably involved in the poor harmonization. The large variation among assay systems in the 1st study was thought to result from some factor affecting universal and equal measurements of samples such as RM and normalization, which are essential for qPCR. Therefore, we conducted the 2nd study by partially standardizing each in-house assay system according to a core laboratory T.

In the 2nd study, the median VLs of 5 laboratories (T, A, B, C, and D) was 4.9, 6.6, 2.7, 4.5, and 3.4 copies per 100PBMCs. The maximal difference in the median improved from 3.7 fold in the 1st study to 2.4 fold. As to the median interlaboratory CVs for VLs, raw pX

copy number before normalization, and IC gene copy number, as shown in Figure 3-A, -B and -C, the median CVs for only raw pX copy number was reduced significantly from 66.9% to 35.3% ($p<0.05$), whereas those of both VLs and IC gene copy number remained statistically unchanged. The data are summarized in Table 3. We assume that the marked reduction in the median interlaboratory CV of the raw pX copy number despite no statistical change in interlaboratory CV of VL may have been influenced by the variation in IC gene copy measurements. Therefore, we next examined the effect of IC gene copy assays on the accuracy and precision of interlaboratory CV of VLs.

3. Quality by IC gene copy assays

To confirm that a large variation in IC gene copy measurements is involved in the lack of improvement of standardization of VLs in each assay system, we further evaluated accuracy and precision by IC gene assays. The “IC accuracy” was defined as the measured copy number relative to the expected copy number with an input genomic DNA dose of 50ng. This amount corresponds to about 16,600 copies based on one copy per 3ng of genomic DNA. The “IC precision” was evaluated by using median CV (%) of 60 measurements by qPCR with the respective IC gene. The results are summarized in Table 4, showing that both “IC accuracy” and “IC precision” were superior in RNase P, β-globin and CD81 compared to β-actin.

Discussion

Many studies have reported that VL is linked to pathogenesis of a virus[12,13]. HTLV-1 VL is thought to be equivalent to the HTLV-1-infected cell number. However, in contrast to RT-qPCR for a large amount of transcripts, it is difficult to accurately discriminate a small concentration of the HTLV-1 provirus at a level of $10^{-2 \text{ to } -3}$.[14] The reliability of any in-house assay systems have usually been verified, but little information is known about the intra- and interlaboratory variations in HTLV-1 VL measured by different in-house qPCR systems set up independently at each laboratory. It is desirable that all assay systems can measure VLs accurately anytime and anywhere[15,16], but to our knowledge there is no information about this.

During a longitudinal follow-up of HTLV-1 carriers in our nationwide cohort study (JSPFAD), we were confronted with unreasonable fluctuations and a large range of VLs from laboratory to laboratory. Accordingly, to clarify the current status of harmonization of VLs measured by home-brew tests, this study was conducted in 6 research laboratories, all of which were members of JSPFAD.

The 1st study revealed three interesting findings. First, we found a large difference in measured VLs between laboratories with a maximal difference of 3.7 fold, although intralaboratory CVs were almost constant as around 50% across laboratories, except for one laboratory. Second, in the scatter plot of VLs between each 5 laboratory and the core laboratory T, the inter-laboratory correlation coefficients were good or excellent. These results indicate that each in-house assay system works well individually, although there was somewhat a difference in inter-laboratory measurements. Fortunately, this type of systemic error can be adjusted with an additional factor to standardize data. In our study, we used an inverse value of the slope as an “adjusting coefficient” to standardize data as shown in Table 2. Third, an extremely wide variation in HTLV-1 VL values was seen, especially in the group of high VL samples, such as 100 copies or more per 100 PBMCs or much lower

rather than expected values, which is rarely observed. This is probably be explained by the biological characteristics of HTLV-1 virus, such as defects or mutations in regions of primers and probes or multiple integration of the proviral genome[17].

In our 1st study, we inferred that main cause of the universal difference in intralaboratory VLs may be due to the difference in standard RMs because precision in each of the five laboratories was good. To confirm our inference, a 2nd study was performed using the same in-house assay systems modified by standardizing RMs with plasmid provirus DNA. Consequently, interlaboratory CV for raw pX copy number before normalization was reduced significantly, whereas interlaboratory CV for VL and that of that IC gene copy number remained unchanged. This shows that a variation in IC gene measurements is also important as an approach to standardization. In our study, we found that both “IC accuracy” and “IC precision” were superior in RNase P, β-globin and CD81 compared to β-actin (Table 4). Nevertheless, the most relevant IC gene for provirus quantification remains unclear.

Now, the question is what grade of CV is acceptable for measurement of VL? The data variations by qPCR are affected by many factors, such as biological variations, process variations, systemic variations, and other biased variations. Biologically, even the theory of one pX copy per cell remains to be elucidated. In general, the grade of CV has been reported to be from 20% to 100% in transcripts and less than 20% in genomic genes[5]. As a result, is the CV level of around 50% obtained by the present study relevant or suitable in order to evaluate pathophysiological events in HTLV-1 infections? So far, most healthy carriers have low VLs of less than 3%[18]. In our study, as expected, interlaboratory CV of VLs decreased as the measured value increased from low to high (Figure 1C), which presumably reflects the effects of stochastic phenomena operative at a low input template copy number. On the other hand, it is considered that a VL of 5-10% is the critical level to be at risk for adult T-cell leukemia[19,20]. This demands high accuracy and precision for real

time qPCR for proviral DNA, equivalent to the infected cell number. We now expect that about 10 to 20 % of CV is acceptable for HTLV-1 VL measurement.

In conclusion, through this study we have shown that there is still wide variability between real-time qPCR assay systems for quantifying HTLV-1 VL in both inter- and intra-laboratory variability of VL. To improve the accuracy and precision for the quantification of HTLV-1 VL, standardization of HTLV-1 VL is expected to be realized by using appropriate RM (plasmid DNA) and relevant IC genes.

Acknowledge: This work was supported by the Japanese government.

Author's contribution: YK, TW and SK organized this study. DS, YY, MS, AO, KU, RK, and SI analyzed VL data. MI, DS, and SK analyzed variability. SK wrote the manuscript. All authors read and approved the manuscripts.

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Figure Legends

Figure 1: Variation in HTLV-1 viral loads (VLs) quantification at the three VL levels in the 1st study. The three VL levels were divided based on VLs that were measured at a core laboratory T. Ranges of VL values at each level were as follows: low level (0-2.1, n=20), intermediate level (3.0-7.7, n=20), and high level (10.2-43.2, n=20). (A) Intralaboratory variability of measured VLs at each VL level. The box plots show the median (horizontal line), interquartile range (box), and range (whiskers) in each laboratory (T, A, B, C, D, and E). The y-axis shows measured VL copy numbers per 100PBMCs. (B) Intralaboratory coefficient of variations (CVs) (%) at three VL levels: The CV values were calculated based on measured VLs in each laboratory. (C) Interlaboratory CV (%) for individual samples: Each CV value was calculated based on measured VLs in 6 laboratories. PBMCs= peripheral blood mononuclear cells, CV= coefficient of variation

Figure 2: Interlaboratory comparison of HTLV-1 VL in linear regression analysis. Scatter plot of VL of 60 samples between the core laboratory T and the other five laboratories. T, A, B, C, D, and E indicate each laboratory. VL indicates HTLV-1 viral load. PBMCs indicate peripheral blood mononuclear cells.

Figure 3: Comparison of interlaboratory CV in individual samples for VL, raw pX copy number before normalization and IC gene copy in the 1st study and the 2nd study. The median CV for only raw pX copy number significantly decreased compared to that of the 1st study (66.9 versus 35.3%, $p<0.05$). *;scale over, 144.7%

Figure 1

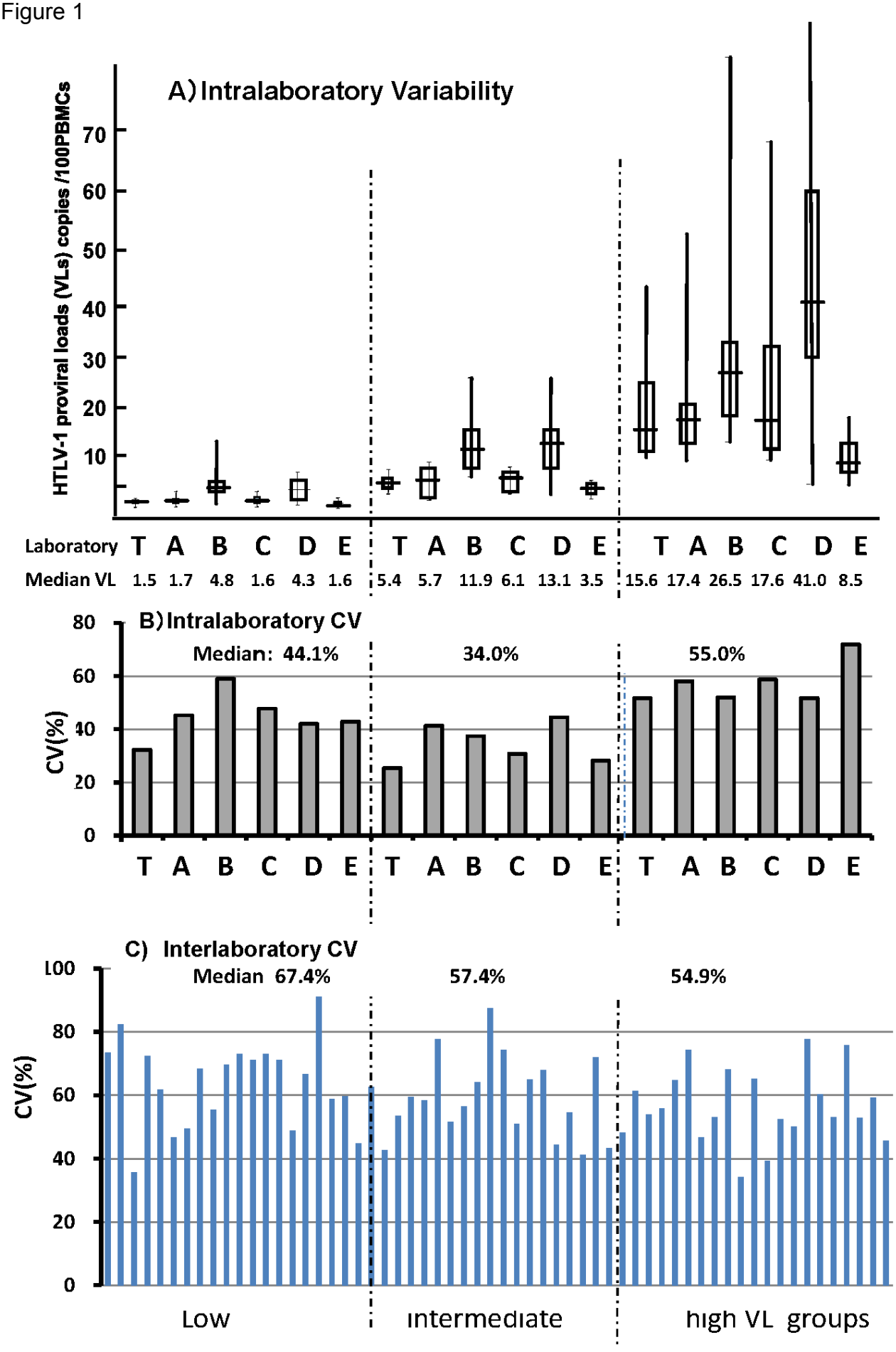
A) Intralaboratory Variability
HTLV-1 proviral loads (VLs) copies /100PBMCs
70
60
50
40
30
20
10
Laboratory T A B C D E T A B C D E T A B C D E
Median VL 1.5 1.7 4.8 1.6 4.3 1.6 5.4 5.7 11.9 6.1 13.1 3.5 15.6 17.4 26.5 17.6 41.0 8.5
B) Intralaboratory CV
Median: 44.1%
34.0%
55.0%
CV(%)
80
60
40
20
0
T A B C D E T A B C D E T A B C D E
C) Interlaboratory CV
Median 67.4%
57.4%
54.9%
CV(%)
100
80
60
40
20
0
Low
intermediate
high VL groups

Figure 2

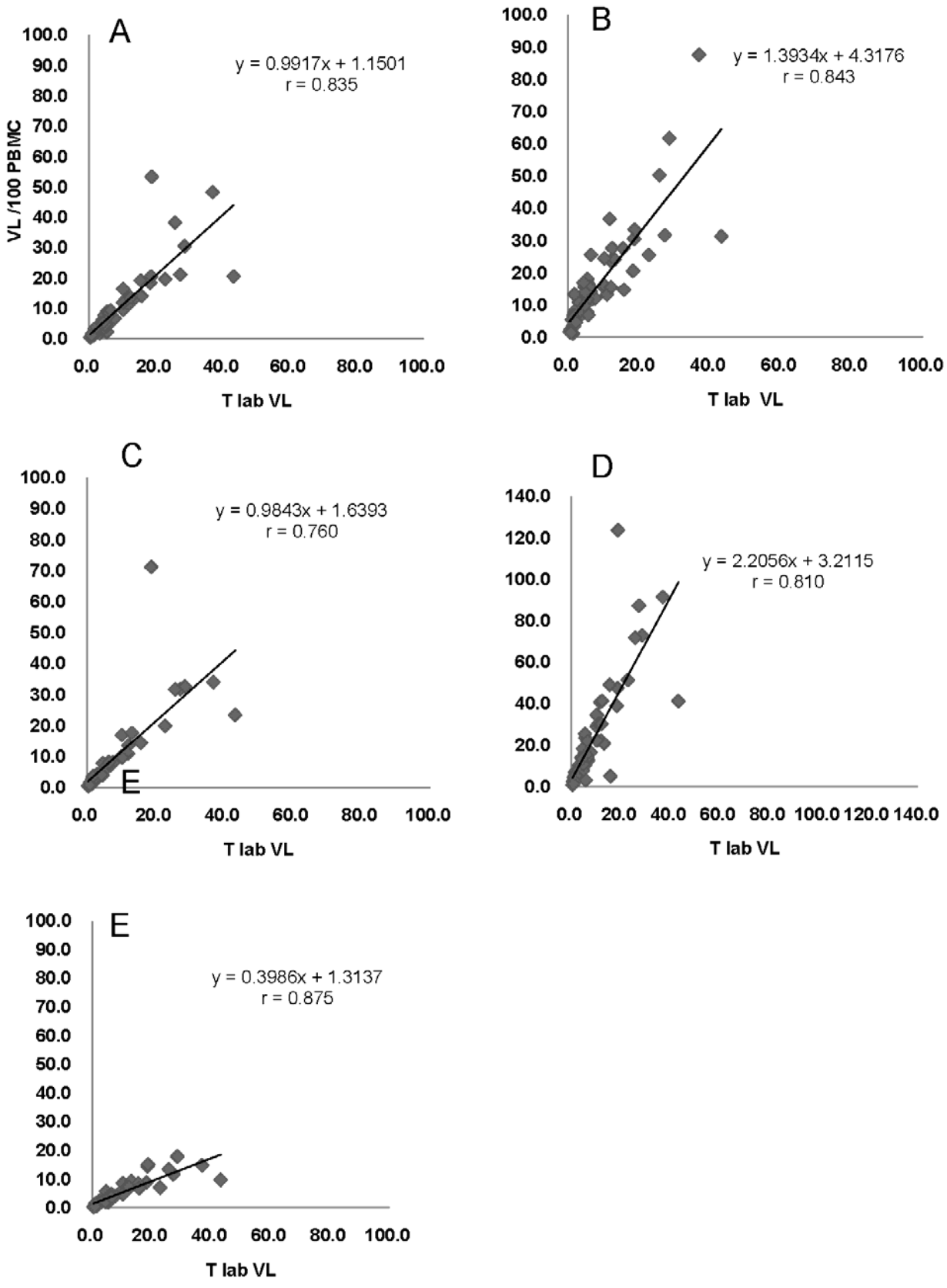
A
y = 0.9917x + 1.1501
r = 0.835
VL /100 PBMC
T lab VL
B
y = 1.3934x + 4.3176
r = 0.843
T lab VL
C
y = 0.9843x + 1.6393
r = 0.760
T lab VL
D
y = 2.2056x + 3.2115
r = 0.810
T lab VL
E
y = 0.3986x + 1.3137
r = 0.875
T lab VL

Figure 3

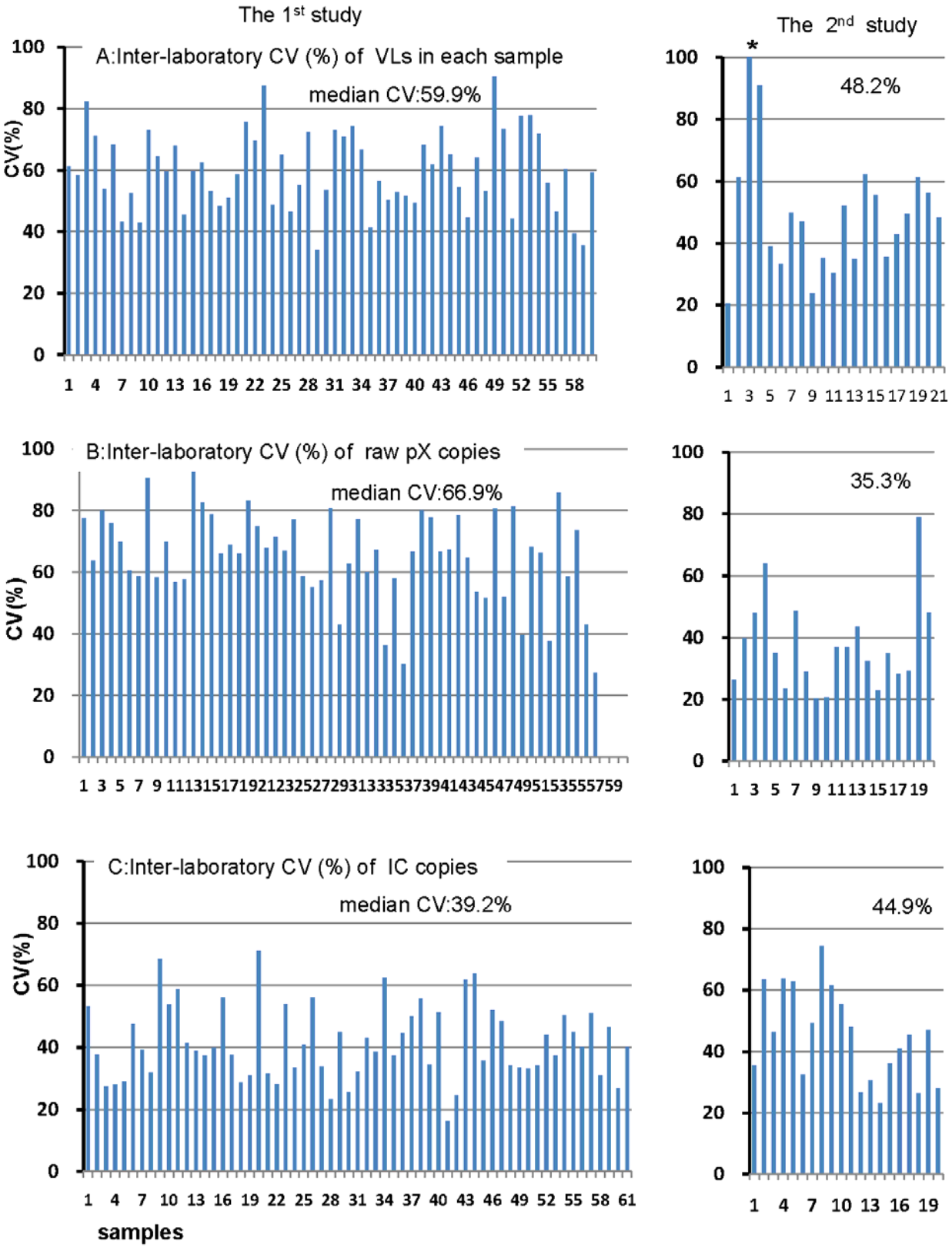
The 1st study
A:Inter-laboratory CV (%) of VLs in each sample
median CV:59.9%
CV(%)
The 2nd study
48.2%
B:Inter-laboratory CV (%) of raw pX copies
median CV:66.9%
35.3%
C:Inter-laboratory CV (%) of IC copies
median CV:39.2%
44.9%
samples

Table 1. Six in-house real time qPCR systems

Institute	platform	RM for pX	primer site	probe	template dose	IC gene	RM for IC
T	ABI Prism7000	9.0 kb provirus inserted in pUC19	pX(tax)	Hydrolysis	50ng	RNase-P	purified human genome
A	LC480	9.0 kb provirus inserted pBR322	pX(tax)	Hydrolysis	30ng	β-globin	TaqMan Control DNA
B	ABI Prism7700	TARL-2 rat cell line infected	pX(tax)	Hydrolysis	50ng	β-actin	normal human DNA
C	LightCycler	PCR product inserted in pGEM-T	pX(tax)	Hydrolysis	100ng	RNase-P	normal human DNA
D	ABI Prism7500	TARL-2 rat cell line infected	pX(tax)	Hydrolysis	100ng	β-actin	normal human DNA
E	ABI Prism7900	cloning pX-fragment inserted in plasmid	pX(tax)	Hydrolysis	1000ng	CD81	CD81 PCR product inserted plasmid

Table 2. An example おf transferability of VLs using an additional factor in each laboratory calculated in the 1st study.

	T lab	A lab	B lab	C lab	D lab	E lab
correlation	Base line	0.835	0.843	0.760	0.810	0.875
slope	1.000	0.992	1.393	0.984	2.206	0.399
additional factor =1/slope	1.000	1.008	0.718	1.016	0.453	2.506

Table.3: Summarized results from the 1st and 2nd surveys

		1st Study	2nd Study
Inter-laboratory variations (median)			
VL	(CV%)	59.9(34.2-93.3)	48.2(20.-144.3)
raw pX copy/50ng DNA dose	(CV%)	66.9(30.1-91.6) *	35.7(20.5-79.0) **
IC gene copy/50ng DNA dose	(CV%)	39.2(16.2-71.2)	44.9(23.0-740)
Correlation between the core Lab and the other			
VL			
r	(mean)	0.824	0.815
slope	(mean)	1.195	1.050
pX raw copy/50ng DNA dose			
r	(mean)	0.761	0.811
slope	(mean)	1.513	1.055
PCR			
PCR Efficiency for pX		1.976	1.981
PCR Efficiency for IC		1.967	1.980

VL; Normalized data based on the formula (raw pX copy numbers/IC gene copy number/2)
* Vs **; p<0.05, (-); range

Labo	T(Core)	C	B	D	A	E
IC gene	RNP	RNP	β-actin	β-actin	β-globin	CD81
Accuracy measured/ expected	1.345	0.869	2.522	1.816	1.051	1.041
Precision intra-sample CV	11.8%	13.2%	35.05%	32.60%	11.4%	13.8%

Table 4. Comparison of accuracy and precision of internal control genes used in each laboratory.
Rnase-P(RNP), b-globin and CD81 were expected to be at a tolerable level, but not b-actin.
Definition of accuracy and precision: Accuracy; practically measured data relative to calculated IC gene copy number (ng genomic dose/2). Precision; mean of CV.