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Development of C-TILDA: A modified TILDA method for reservoir quantification in long term treated patients infected with subtype C HIV-1

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ABSTRACT

A better characterization of the HIV reservoir is pivotal for the development of effective eradication strategies. Accurate quantification of the latent reservoir remains challenging. Starting from a regular blood draw, the Tat/Rev induced limiting dilution assay (TILDA) combines serial dilution of $CD4^+$ T cells with a PCR-based detection of HIV-1 spliced mRNA produced upon cell stimulation. Here we adapted the original protocol for HIV-1 subtype B to detect tat/rev mRNAs transcribed from reactivated latently infected cells in long term suppressed patients infected with HIV-1 subtype C. Given the heterogeneity of global HIV epidemiology, it is pivotal to develop assays with optimal performances also in patients infected with non-B subtypes. We observed that, in these patients infected with subtype C virus, the HIV reservoir quantified by TILDA correlates with both the time of virological suppression and CD4/CD8 ratio.

1. Introduction

The existence of a latent reservoir, constituted by integrated viral DNA that is replication-competent despite being transcriptionally silent, represents the main obstacle for HIV eradication (Chun et al., 1997, 1995; Richman et al., 2009). The latent viral reservoir is established early during the course of infection (Chun et al., 1997, 1995), and allows persistence of HIV even in presence of potent combination antiretroviral therapy (cART) that can effectively block viral replication (Chun et al., 2010; Davey et al., 1999). During prolonged suppressive cART, cells harboring silent provirus are extremely rare (Eriksson et al., 2013). Nevertheless, in the absence of viral transcription and virus production, the immune system is not able to recognize them and cannot target them for elimination (Rasaiyaah et al., 2013). As a consequence, in patients who interrupt suppressive antiretroviral therapy, the relatively few latent viruses are reactivated leading to plasma viral rebound within a few weeks (Chun et al., 2010; Davey et al., 1999).

The growing awareness of the importance of reservoir size assessment for the tailored management of HIV-1 infected patients sets the need for assays that can accurately quantify the number of cells harboring replication-competent virus in vivo. Evidence-based studies enrolling elite and post-treatment controllers, i.e. individuals who control viral replication in absence of antiretroviral therapy, have shown that the size of the latent reservoir is related to the likelihood of and the time to viral rebound (Sáez-Cirión et al., 2013; Walker and Yu, 2013). Consistent with a role of cART in reducing reservoir size and improving patient survival (Archin et al., 2012; Palella et al., 1998), early start of treatment is associated with lower levels of proviral DNA and, in turn, with longer time to rebound and reduced risk of virological failure (Avettand-Fènoël et al., 2016; Williams et al., 2014; Yerly et al., 2004).

Over the last two decades, different methods for reservoir quantification have been developed to cope with the low number of cells harboring replication-competent virus, which challenges standard molecular techniques. The quantitative viral outgrowth assay (QVOA) determines the number of resting $CD4^+$ T cells releasing active virus upon stimulation, and is considered the gold standard to determine reservoir size in HIV infected patients during cART (Eriksson et al., 2013). Nevertheless, QVOA is time-consuming and requires a large

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amount of blood (Eriksson et al., 2013; Ho et al., 2013). Quantification of total HIV-1 DNA by means of real-time PCR or digital droplet PCR is frequently used as an alternative method for reservoir assessment in clinical studies (Alidjinou et al., 2015). Despite being a relatively simple technique that can be performed starting from a variety of biological samples (Avettand-Fènoël et al., 2016; Williams et al., 2014; Yerly et al., 2004), quantification of HIV-1 DNA overestimates the size of the reservoir as the vast majority of detectable HIV-1 genomes is not replication-competent (Ho et al., 2013). Quantification of cell-associated HIV-1 RNA (CA-HIV RNA) produced upon in vitro stimulation may represent an additional reservoir biomarker (Pasternak et al., 2013). However, gag-pol RNA in stimulated cells may be the result of transcription starting from an upstream promoter, revealing the presence of a defective provirus located into an activated host gene rather than an actual latent provirus that has been reactivated (Bullen et al., 2014). A recently developed assay combines a FISH method targeting gag-pol mRNAs with the detection of p24 protein, improving specificity and limiting the issue of false positive events (Baxter et al., 2018, 2017; Grau-Expósito et al., 2017; Martrus et al., 2016). However, the method is still in the early phases of development and further investigation is required to determine its accuracy. Detection of multiply-spliced RNA (msRNA) molecules has been proposed as an alternative method to improve specificity in identifying reactivation of a competent virus, as spliced viral RNA is frequently missing in defective viruses due to deletions that encompass *tat* and *rev* genes (Ho et al., 2013). Based on these observations, Procopio and colleagues established the Tat/rev Induced Limiting Dilution Assay (TILDA), a PCR-based method that provides an estimate of the frequency of latently HIV-1 infected $CD4^+$ T cells producing tat/rev msRNA (Procopio et al., 2015). This assay couples the sensitivity of a molecular method with a limiting diluting assay, thereby providing accurate quantification of the clinically relevant reservoir starting from a regular blood sample.

HIV-1 has an enormous genetic variability that played a crucial role in its worldwide spread (Hemelaar, 2013, 2012). HIV-1 diversity results in the presence of groups, subtypes and recombinant forms that significantly differ in terms of geographical distribution and pathogenesis (Hemelaar, 2013, 2012). Changes in nucleotide composition between different subtypes were estimated at around 15 %, although region and subtype accessed can significantly influence genotypic and phenotypic diversity (Li et al., 2015). Historically, subtype B has been predominant in Europe and North America, while subtype C is the most dominant subtype in Africa. However, in the past decade, as a result of the increase in migratory flows, Europe has seen a remarkable increase in the number of cases diagnosed with HIV-1 subtype C (Hemelaar et al., 2019; Lai et al., 2014).

Considering the impact of sequence diversity, changes in subtype distribution can impact on the performances of molecular methods for HIV-1 detection. Sequence differences between subtype B and C point towards the inability to detect clade C viruses using TILDA, which has been designed based on subtype B viruses (Procopio et al., 2015). Here we adapted the TILDA assay to obtain a technique to quantify HIV-1 reservoir size in long-term suppressed patients infected with subtype C.

2. Methods

2.1. Participants and samples

Samples were collected after receiving approval from the Erasmus MC Medical Ethics Committee and St. Orsola Hospital (489/2018/Sper/AOUBo) Ethics Committee. All enrolled patients were ≥18 years and provided written informed consent; patients infected with either subtype B or C HIV-1 were cART-treated for at least 2 years at the time of the study, with undetectable viral load (detection limit of 20 copies/ml of plasma) for at least one year. PBMCs were collected from blood samples obtained from HIV-1 infected patients that were referred to the Infectious diseases Unit of the St. Orsola Hospital or from leukapheresis obtained from patients that were referred to the Infectious Disease Section of the Erasmus University Medical Center. PBMCs were isolated by Ficoll Paque (GE Healthcare) density centrifugation according to the manufacturer's instructions. Plasma HIV-RNA load was detected by standard commercial viral RNA detection assay (COBAS® AMPLICOR, Roche Molecular Systems, Inc., Branchburg, NJ, USA). $CD4^+$ and $CD8^+$ T lymphocyte count was determined by flow cytometry (FACScan, Becton & Dickinson, USA) using commercially available monoclonal antibodies (Perfetto and McCrary, 1999); specifically, $CD4^+$ T cells were stained with PE anti-human CD3 antibody (Beckton Dickinson, cat. No. 7347) and FITC anti-human CD4 (Beckton Dickinson, cat. No. 347413), whereas $CD8^+$ T cells were stained with PE anti-human CD3 antibody (Beckton Dickinson, cat. No. 7347) and FITC anti-human CD8 (Cymbus Biotechnology, cat. No. CBL131 F). Subtype characterization was determined according to the HIV-1 *pol* gene sequence (Bon et al., 2010; Musumeci et al., 2016).

2.2. Quantification of total HIV-1 DNA

DNA was purified from PBMCs (Quick DNA Microprep Kit, Zymo Research) and quantified using Nanodrop (Labtech, Ringmer, UK). Total HIV-1 DNA levels were measured with the HIV-1 DNA qPCR kit (Diatheva, Fano, Italy) following manufacturer's instructions. The number of HIV-1 copies was normalized to the number of $CD4^+$T cells present in the sample calculated based on the percentage of $CD4^+$ T cells of the same blood sample (Massanella et al., 2015).

2.3. $CD4^+$ T cells isolation

$CD4^+$ T cells were isolated from PBMCs by negative magnetic selection (EasySep™ Human $CD4^+$ T Cell Isolation Kit, Stemcell Technologies). Upon isolation, $CD4^+$ T cells were cultured in RPMI 1640 (Euroclone) supplemented with 2 mM L-Glutamine (Euroclone), 10 % Fetal Bovine Serum (Gibco, Life Technologies) and 1 % Penicillin Streptomycin (Euroclone) for 3 h at 37 °C and 5 % CO_2. After 3 h, cells were stimulated with 100 ng/ml PMA (SigmaAldrich) and 1 μg/ml ionomycin (Sigma Aldrich) for 12 − 16 hours.

2.4. C-TILDA

Subtype C specific TILDA (C-TILDA) primers and probe were design based on subtype C alignments available in Los Alamos Database using WebLogo (https://weblogo.berkeley.edu/logo.cgi). Primers and probes were designed in the most conserved regions of the tat-rev exons, selecting the more represented nucleotides in the alignment (Table 1, Supplementary Figure S1).

$CD4^+$ T cells obtained from HIV-1 infected patients were stimulated overnight with 100 ng/ml PMA and 1 μg/ml ionomycin. After stimulation, cells were counted and serially diluted in culture media to 54×10^6 cells/ml, 27×10^6 cells/ml, 9×10^6 cells/ml and 3×10^6 cells/ml. From each serial dilution, 1 μl of the cell suspension was plated in a 96-well plate as described by Procopio and colleagues (Procopio et al., 2015). 18–24 replicates of each serial dilution were tested. Pre-amplification was performed using SuperScript III Platinum One-Step qRT-

Table 1
Sequence of subtype C specific primers and probe.

Primer/Probe	Sequence (5'-3')	Genomic location*
TAT1FC	GAA GAA GCG GAG ACA GCG A	5977-5996
TAT2FC	GCA GTG AGG ATC ATC AAA ATC CTA TAT CAA AGC A	6012-6045
REV C	GGA TCT GTC TCT GTC TTG CTC TCC	8436-8459
TILDA C	[6FAM] CTT CTT CTT CGA TTC TTC CGA GCC TGT CGG GT [TAM]	8402-8433

* Relative to the sequence of HXB2 reference strain of HIV-1.

PCR Kit (Life Technologies) as previously described (Procopio et al., 2015) and was run in a GeneAmp PCR system 9700 instrument (Applied Biosystem). Pre-amplification was carried out with primers TAT1FC and REVC (final concentration of 0.25 μM each). At the end of the pre-amplification run, PCR products were diluted in 40 μl of water. The subsequent real-time PCR was performed on the same day using PrecisionPLUS qPCR Master Mix (PrimerDesign). 10 μl of 2X buffer were added together with primers TAT2FC and REVC (final concentration of 0.25 μM each), the probe TILDA C (final concentration of 0.1 μM), 1 μl of pre-amplification product and H_2O to a final volume of 20 μl. The real-time reaction was carried out in a VERSANT kPCR Molecular System (Stratagene) using the following program: 5 min at 95 °C followed by 45 cycles of 95 °C 10 s and 60 °C for 60 s and acquisition of the fluorescent signal at the end of each cycle. The frequency of cells expressing inducible msRNA of tat/rev was calculated as described by Procopio and colleagues with the maximum likelihood method (Procopio et al., 2015).

Each run included a calibration curve to control for the accuracy of the PCR method. The curve was prepared using dilutions of the sequence of tat/rev msRNA of subtype C (gBlocks Gene Fragment, IDT) which was ligated in a pGEM-T Easy vector (Promega) using a T4 ligase (T4 DNA Ligase, Promega) and subsequently cloned into competent bacterial cells. The TILDA plasmid obtained (pTatRevC) was isolated with High Pure Plasmid Isolation Kit (Roche) and stored at -20 °C. The plasmid was diluted to obtain the following calibration scale: 5×10^4 copies/reaction, 5×10^3 copies/reaction, 5×10^2 copies/reaction, 5×10^1 copies/reaction, and 5×10^0 copies/reaction. Each plasmid dilution was tested in duplicate.

2.5. Evaluation of cross-reactivity between subtype B and C assays

$CD4^+$ T cells obtained from patients infected with HIV-1 subtype B or C were stimulated according to the protocol described in section 2.3. RNA was extracted using the Hybrid-R miRNA (GeneAll) following manufacturer's instruction and eluted in 50 μl. Pre-amplification was carried out using SuperScript III Platinum One-Step qRT-PCR Kit (Life Technologies) starting from 1 μl of RNA. Pre-amplification and the real-time PCR subsequent steps were performed as described in section 2.4. Each patient sample was run in quadruplicate and tested both with subtype B primers and probe and subtype C primers and probe.

2.6. Statistical analysis

Statistical analyses were carried out using Graphpad Prism 5 software. Pearson test (two-tailed) was used to analyze data and a $p < 0.05$ was considered as the level of significance.

3. Results

3.1. Optimization of TILDA for the detection of C-subtype tat/rev

The significant increase in prevalence of non-B subtypes in Europe and Noth America, suggests that the molecular epidemiology of HIV-1 is changing (Lai et al., 2014; Mazzuti et al., 2019). Here we modified the published protocol for TILDA (Procopio et al., 2015) to detect subtype C viruses and be able to quantify reservoir in long-term subtype C HIV-1 infected patients, who constitute a growing population among HIV-1 infected patients.

First, we designed a new set of primers and probe based on the consensus subtype C HIV-1 sequence available on the Los Alamos HIV database. In order to enhance the sensitivity of the primers, we aligned and compared 382 sequences, identifying the regions carrying the lower variation rate and, where variation was not avoidable, selecting the most conserved nucleotides (Fig. 1).

Second, we changed the original dilution panel (18×10^6 cells/ml, 9×10^6 cells/ml, 3×10^6 cells/ml and 1×10^6 cells/ml) by performing the following dilutions: 54×10^6 cells/ml, 27×10^6 cells/ml, 9×10^6 cells/ml and 3×10^6 cells/ml. As a result, using the same number of reactions, we tested a higher number of cells ($7{,}44 \times 10^5$ vs $2{,}232 \times 10^6$). We evaluated the effect of increasing the number of cells/well in two patients, one infected with subtype B and the other with subtype C: in both cases, we were able to quantify the reservoir only using the modified dilution panel (Supplementary Table S1).

The sensitivity of the PCR method was assessed using serial dilutions of a plasmid containing the target sequence. After extraction and real-time RT-PCR, the assay sensitivity limit, defined as the dilution where 100 % of replicates was detectable at Ct values ≤25, was set at 5 copies/reaction. The Ct values showed linear reference curves for pTatRevC plasmid (Fig. 2, $r^2 > 0.95$). The inter-assay coefficient of variability of the Ct values ranged from 1.04 % to 1.25 %. The median slope was –3.22 (range, -2.83 to -3.43) and the median efficiency was 106 % (range, 95.6%–125.8%).

We characterized the cross-reactivity between subtype B and subtype C primers and probe sets using RNA extracted from stimulated $CD4^+$ T cells obtained from six patients, three infected with HIV-1 subtype B and three with HIV-1 subtype C. In all samples, total RNA was retrotranscribed and amplified following the TILDA RT-PCR method using either subtype B or subtype C primers and probe. Only subtype B primers and probe were able to amplify tat/rev msRNA in samples obtained from subtype B infected patients. Similarly, RNA extracted from HIV-1 subtype C samples was successfully amplified with subtype C primers and probe, while no signal was detected using subtype B primers and probe (Supplementary Table S2).

Finally, when we performed the assays on cells derived from a subtype C infected patient, we were able to detect msRNA and quantify viral reservoir only using the C-TILDA (Table 2, row 3). Interestingly, we could not detect any signal with subtype B TILDA even when increasing the number of replicates to reach 4 million cells total (data not shown), further supporting the absence of cross-reactivity between assays.

3.2. C-TILDA effectively quantifies reservoir in C infected patients

To confirm the performances of C-TILDA, we performed the assay on samples derived from routine clinical practice, which are more likely to reflect the real-life heterogeneity of the HIV-1 population. We isolated $CD4^+$ T cells from 12 patients, three infected with a subtype B virus and nine infected with subtype C. At the time of collection, plasma viral (RNA) load was determined for all patients to confirm viral suppression. Table 2 shows the frequency of $CD4^+$ T cells expressing tat/rev msRNA detected by TILDA using the original assay developed by Procopio and colleagues (Procopio et al., 2015), for subtype B samples, and the newly developed C-TILDA assay, for subtype C samples. As expected, the original assay successfully quantified the cells producing msRNA in subtype B infected patients. On the other hand, the C-TILDA was able to detect the target in eight out of nine tested subtype C infected patients, with the number of infected cells per million $CD4^+$ T cells ranging from 1 to 24 (Table 2). Consistent with the high level of viral suppression in the patients selected to test the assay, in whom viremia had been below the limit of detection for at least one year, we detected a median frequency of tat/rev msRNA producing $CD4^+$ T cells of less than 10 per million of $CD4^+$ T cells. Our method was not able to detect any tat/rev msRNA producing cell only in one patient who had undetectable plasma viremia for more than three years (Table 3).

3.3. The number of CD4 + T cells producing tat/rev msRNA correlates with immunological and virological parameters

We performed an exploratory analysis to assess whether the results of C-TILDA assay correlated with patient immunological and virological parameters (Table 3). Patient 3 was excluded from the analysis due to the lack of clinical and immunological information.

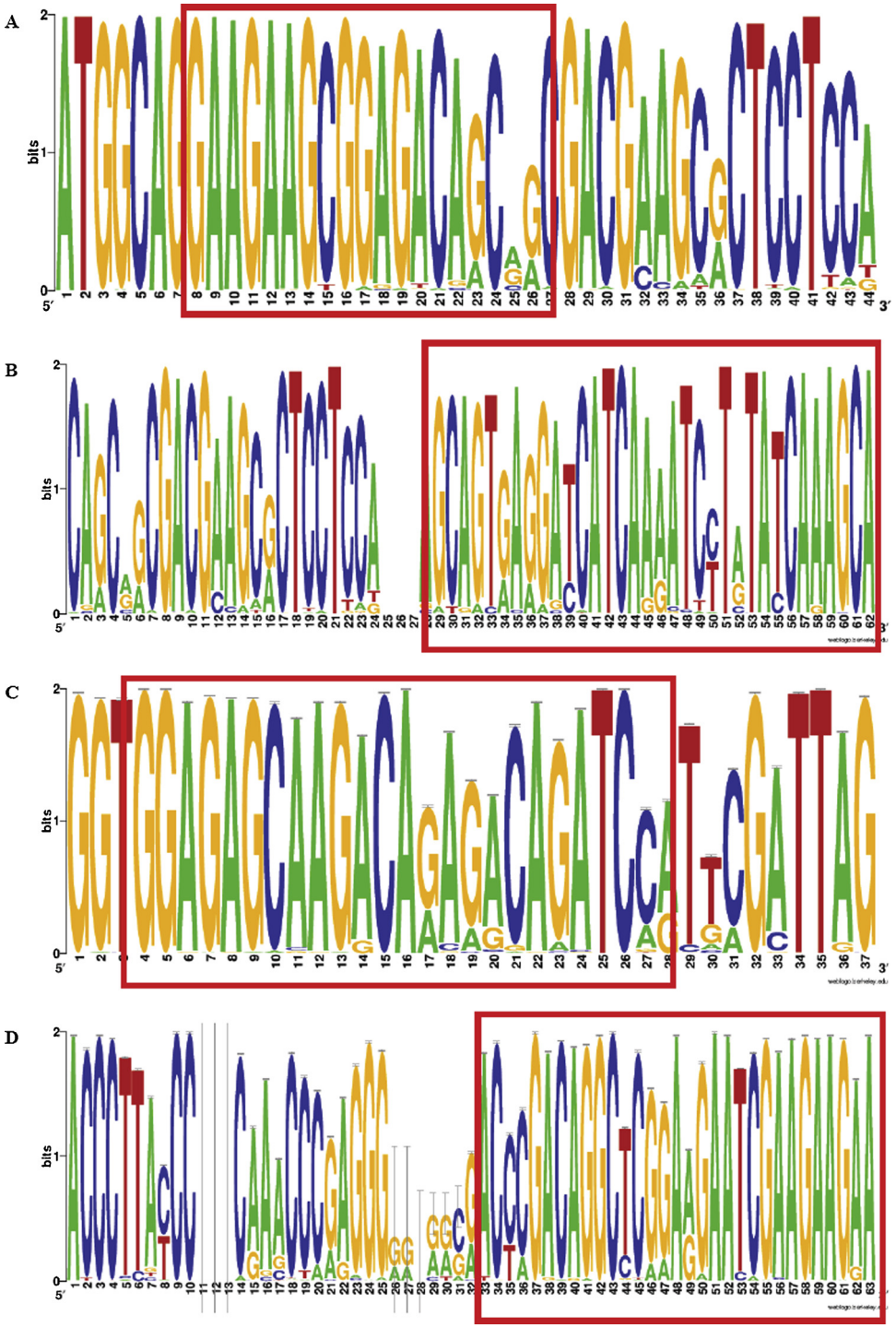

Fig. 1. Graphic representation of the target regions of subtype C-specific primers and probe set. The height of each nucleotide is proportional to the degree of conservation of the nucleotide among subtype C sequences available in Los Alamos HIV database. The red squares highlight the sequence of C-TILDA primers A) TAT1FC, B) TAT2FC, C) REV C and D) TILDA C probe (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

We compared the results of the C-TILDA with the duration of viral suppression and total HIV-1 DNA. Given that plasma viral RNA was below the limit of quantification in all patients included in the study, we considered the time since reaching viral suppression as a surrogate marker of viral control. Consistent with a role of suppressive cART in promoting reservoir decay (Chun et al., 2007; Siliciano et al., 2003), TILDA results significantly correlated with the time since virological suppression (Fig. 3A, $r^2 = 0.505$, $p = 0.048$).

Similarly, HIV-1 DNA is frequently used as a biomarker of the viral reservoir since it has a slower decay than plasma HIV-1 RNA, whose levels can be reduced below the limit of detection of clinical assays within a few months after cART initiation. Nevertheless, the biological meaning of HIV-1 DNA in long-term virosuppressed patients is still debated, given that the vast majority of infected cells harbor defective proviruses and thus are not targeted by antiviral therapy. In this study, we observed a significant positive correlation between the number of cells harboring replication-competent virus estimated by C-TILDA (patients 5–11, Fig. 3B, $r^2 = 0.573$ $p = 0.049$) and the amount of HIV-1

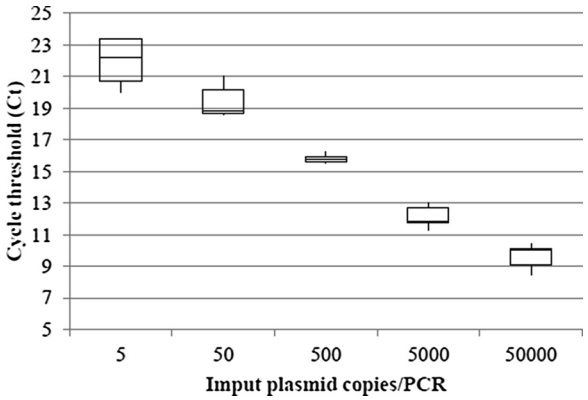

Fig. 2. Standard curve of pTatRevC using the set of primers and probe specific for subtype C HIV-1. Boxes show median and interquartile ranges of Ct values obtained from three different runs performed in duplicate.

Table 2
Evaluation of the reservoir size by TILDA in long-term cART treated patients (confidence interval 95 %). TND: target not detected. NA: not analyzed.

Patient	Subtype	B-TILDA		C- TILDA	
		Cells expressing tat/rev msRNA/10^6 $CD4^+$ T cells	Confidence Interval (95 %)	Cells expressing tat/rev msRNA/10^6 $CD4^+$ T cells	Confidence Interval (95 %)
1	B	12	8-17	NA	
2	B	3	1-6	NA	
3	C	TND	TND	24	17-33
4	B	136	93-197	NA	
5	C	NA		7	4-12
6	C	NA		1	0-4
7	C	NA		14	8-25
8	C	NA		16	7-35
9	C	NA		7	4-14
10	C	NA		6	2-14
11	C	NA		11	3-37
12	C	NA		0	0

DNA within the $CD4^+$ T cell population.

Importantly, we found that C-TILDA results significantly correlated with the CD4/CD8 ratio, a key parameter reflecting the functionality of the immune system (Fig. 3C, r^2 = 0.504 p = 0.049), supporting the notion that recovery of immune system function is linked to the reduction of reservoir size.

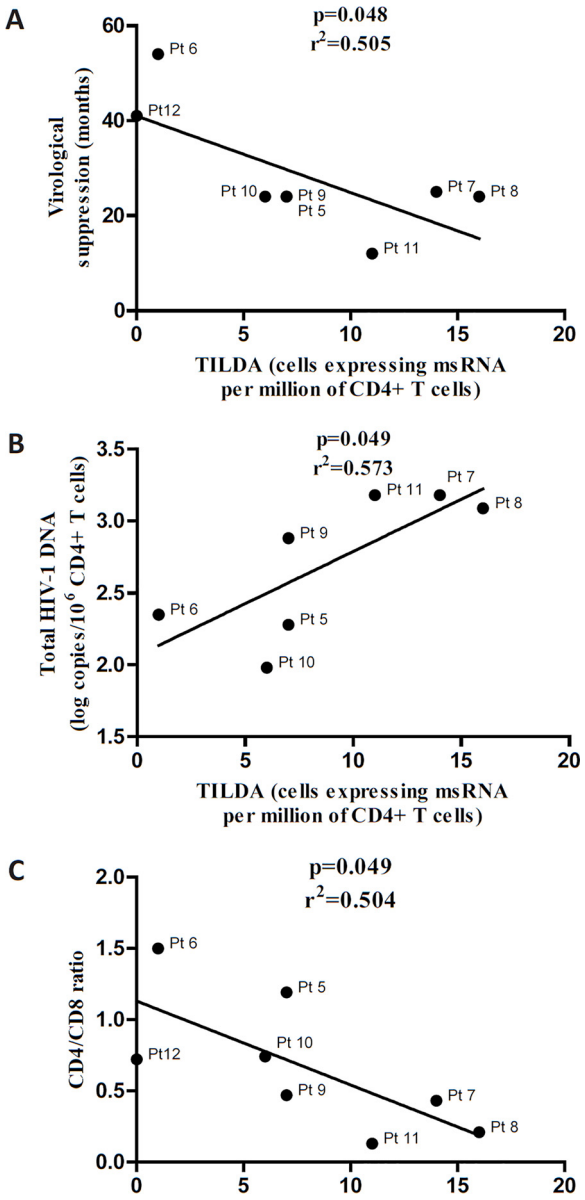

Fig. 3. Association between the reservoir size measured by TILDA and A) time from virological suppression (r2 = 0.505; p = 0.048); B) HIV-1 DNA proviral load (r2 = 0.573, p = 0.049), due to insufficient material, patient 12 was not tested for total HIV-1 DNA; and C) current CD4/CD8 ratio (r2 = 0.504, p = 0.049). Statistical analysis was performed using Pearson test (two-tailed), and setting 0.05 as the level for significance.

4. Discussion

The presence of latently infected cells precludes viral eradication in patients receiving cART (Archin et al., 2012; Siliciano et al., 2003). Hence, reaching a better understanding of viral dynamics during chronic infection is critical in order to properly understand the efficacy of available regimens against cellular reservoirs and establish the

Table 3
Clinical data of patients infected with subtype C. NA: not analyzed.

Patient	Time since diagnosis (years)	Time since virological suppression (months)	$CD4^+$T cells/μl	CD4/CD8 ratio	Total HIV-DNA (copies/10^6 $CD4^+$T cells)	tat/rev $msRNA^+$ $CD4^+$T cells/10^6 $CD4^+$T cells
5	3	24	2025	1.19	190	7
6	5	54	1484	1.50	226	1
7	2	25	739	0.43	1521	14
8	2	24	287	0.21	1236	16
9	2	24	1041	0.47	765	7
10	2	24	1044	0.74	95	6
11	5	12	386	0.13	1530	11
12	5	41	645	0.70	NA	0

optimal clinical and therapeutic strategies for patient management. In patients on prolonged suppressive cART, it is estimated that the frequency of latently infected cells within the resting memory CD4 + T population, which is considered the more relevant compartment for latency, is 1 in 10^6 cells (Eriksson et al., 2013; Ho et al., 2013).

Quantification of HIV-1 reservoir remains challenging: several techniques targeting different viral targets have been proposed to assess its size, with variable degrees of concordance (Ho et al., 2013; Sharaf and Li, 2017). TILDA is a PCR-based method that quantifies the reservoir size based on the ability of the reactivated $CD4^+$ T cells to produce tat/rev msRNA. In this study, we modified the original protocol allowing the successful quantification of the viral reservoir in long-term cART treated patients infected with subtype C virus.

As suggested by Procopio (Procopio et al., 2015), TILDA assay may not detect tat/rev msRNA in patients infected with HIV-1 non-B subtypes. Consistently, in our hands, the assay could not detect tat/rev msRNA in samples from patients infected with clade C virus.

The implementation of the assay for HIV-1 subtype C detection offers the chance of further evaluating the reservoir size in subtype C infected patients. The high prevalence of HIV-1 subtype C in the southern African countries along with India and Ethiopia, where it is involved in more than 94 % of HIV-1 infections, as well as the increasing migratory flows are fueling subtype C global expansion. Currently, HIV-1 subtype C represents 46.6 % of the infections worldwide and its frequency in western and central Europe and North America is around 4 % (Hemelaar et al., 2019). Therefore, the development of tools that can quantify subtype C virus is pivotal for the advent of new strategies aiming at curing HIV-1 infection.

Although QVOA currently represents the gold standard for reservoir quantification, TILDA has the advantage of requiring a consistently lower amount of blood and being less time-consuming compared to QVOA that requires from 7 to 21 days (Wang et al., 2018).

Currently, most of the clade C virus analyses are PCR-based and can involve ddPCR (Bosman et al., 2018) or NGS (Rodgers et al., 2017), although the use of RNAscope ISH technique has been recently reported (Zhang et al., 2018). Despite overestimating its true size (Sharaf and Li, 2017), HIV-1 DNA is one of the most common markers used to assess the viral reservoir. Several studies highlighted the importance of choosing the proper PCR-based assay to guarantee an optimal measurement of HIV-1 DNA, regardless of the subtype (Bosman et al., 2018; Rutsaert et al., 2018). Viral diversity can limit DNA quantification to a variable extent, depending on the target region of the different assays. Bosman and colleagues observed that LTR (Aitken et al., 2013) and JO (*pol*) assays (Benki et al., 2006; Pankau et al., 2018; Rousseau et al., 2004) can sensitively and reliably measure HIV-1 DNA, whereas Gag assay (Bosman et al., 2015) can detect only specific subtypes (Bosman et al., 2018; Thomas et al., 2019).

A new method combines single-cell sorting and ddPCR enabling high-throughput characterization of a specific cell population infected with the virus. This new technique has been employed both for HIV-1 DNA analysis and unspliced and multiple spliced HIV-1 RNA quantification (Araínga et al., 2017; Dunay et al., 2017; Gibellini et al., 2018; Morón-López et al., 2017; Yucha et al., 2017). Nevertheless, the possibility that changes in the viral sequence may influence the sensitivity of the assay has not been explored yet.

In this complex scenario, we focused on TILDA assay as it combines the accuracy in detecting cells harboring replication-competent virus with limited technical complexity and the possibility to scale up for high throughput testing.

Notwithstanding the advantages offered by TILDA, the assay still offers room for improvement. In a recent publication, Pezzi and colleagues (Pezzi et al., 2018) proposed to increase TILDA sensitivity using a bead-based method for RNA extraction to remove background cell-derived material. Moreover, the production of msRNA is associated with cell vitality and, therefore, correct handling and rapid processing of the sample before and after stimulation are pivotal for the accuracy of the assay. Hence, as suggested by Chatel and colleagues, we avoided the storage of the pre-amplification product at – 20 °C to prevent the loss of sensitivity of TILDA (Châtel et al., 2018).

We designed a new set of primers and probe to recognize tat/rev msRNA of clade C viruses, enabling the detection of the target in patients infected with subtype C viruses. Furthermore, we focused on the analysis of viral reservoir in long-term virosuppressed patients that are considered promising candidates for structured treatment interruption. In these patients, the frequency of latently infected cells is very low (Buzon et al., 2014), and thus, using the published TILDA protocol, it would be necessary to increase the number of wells tested in each run. We instead increased the number of $CD4^+$ T cells per well, thereby incrementing the likelihood of finding latently infected cells without impacting the cost of the assay. Although, in our hands, the new dilution panel allowed to detect HIV-1 msRNA in two samples that tested negative using the original dilution set, we are aware that increasing the number of cells per well could increase the background and reduce the sensitivity (Châtel et al., 2018). It is worth noting, however, that three out of four of the cell dilutions used in our experimental setting (3×10^6 cells/ml, 9×10^6 cells/ml, and 27×10^6 cells/ml) fall within the range of concentrations that did not significantly impact amplification in the experiments performed by Chatel et al. Furthermore, the observations reported by Chatel and colleagues have been obtained in a different experimental setting, where RNA extracted of ACH-2 cells is added to either monocytes or $CD4^+$ T cells. In these conditions, the decrease in sensitivity may be due to not only to reaction inhibition but also to RNAse activity on free RNA.

The size of the latent reservoir was measurable in eight out of the nine subtype C infected patients included in the analysis, with only one patient remaining undetectable possibly due to the long period of viral suppression (41 months). Although primers and probes were designed in the regions with the lowest sequence variation, it is not possible to exclude that mismatches prevented amplification in this patient. Indeed, suboptimal detection due to sequence variability is common in HIV-1 molecular assays and has been reported also for diagnostic tools (Rouet et al., 2007). Furthermore, although the virus was identified as subtype C based on *pol* gene sequence, recombination may occur among different subtypes, generating chimeric viruses which represent a further challenge for diagnostic methods (Ramirez et al., 2008). Future studies, performed on larger cohorts of patients will allow us to estimate precisely the proportion of subtype C viruses which may escape detection with C-TILDA.

In long-term suppressed patients infected with subtype C, the frequency of $CD4^+$ T cells expressing tat/rev msRNA ranged from 1 to 24 per million $CD4^+$ T cells. Our data are in line with a recently published work where reservoir size was determined using HIV-Flow, a flow cytometry-based assay to quantify $p24^+$ cells (Pardons et al., 2019), and compared with the results of other assays assessing the size of the latent reservoir, including TILDA. Using TILDA, the authors reported a median of 20.1 tat/rev $msRNA^+$ $CD4^+$T cells/10^6 cells, while the frequency of p24 producing cells, as assessed by HIV-flow, was $4.3/10^6$ cells (Pardons et al., 2019). Moreover, they showed a significant correlation between HIV-Flow and TILDA assay. Hence, TILDA could be a solid alternative option when the starting material does not have a sufficient number of cells, considering that HIV-Flow requires at least 5×10^6 $CD4^+$ T cells (Pardons et al., 2019).

The fate of the viral reservoir under suppressive therapy is still debated. Although several authors suggested that effective antiretroviral therapy leads to a progressive decay of the reservoir (Chun et al., 2007; Siliciano et al., 2003), recent works have questioned this view, showing that despite a rapid decline during the first months after starting cART, viral reservoir remains stable (Crooks et al., 2015; Honeycutt et al., 2017; Nikolaus et al., 2018) and that intensification of antiretroviral regimens does not significantly influence residual viremia (Gandhi et al., 2010). Our exploratory analysis highlighted a correlation between TILDA results and time of viral suppression, supporting a

role of suppressive cART in promoting reservoir decline. These data, however, need to be confirmed in longitudinal studies in which patients will be followed over time since starting of therapy. In this scenario, the TILDA might provide a better estimate of the viral reservoir than proviral DNA. Here we showed a significant correlation between reservoir size assessed by TILDA and total HIV-1 DNA in $CD4^+$ T cells, similarly to what previously observed by Procopio (Procopio et al., 2015) in subtype B viruses. Interestingly, although both methods may overestimate the size of the reservoirs, TILDA it is likely to be more accurate because of the lower chances to observe tat/rev msRNA production from defective viruses. Consistently, Pardons et al. recently reported that the prediction of reservoir size obtained with TILDA is 50 times more accurate than the one based on HIV-DNA (Pardons et al., 2019).

Finally, among the patients included in the analysis, the size of the reservoir assessed by TILDA correlated with the CD4/CD8 ratio, a known marker of immune recovery (Han et al., 2018; Mussini et al., 2015). Although obtained on a limited number of samples, these results further support the accuracy and clinical meaning of the assay.

In conclusion, we report the successful implementation of C-TILDA to detect tat/rev transcripts of subtype C HIV-1, emphasizing the clinical relevance of adapting molecular assays to the changing epidemiology of the virus and the need to make available the most advanced molecular assays to the entire HIV-1 positive population. This is essential to drive forward cure research and comply with the UN aim to end the HIV epidemic by 2030. Future challenges may involve the implementation of this attractive technique extending its application to additional non-B subtypes.

Ethical statement

Samples were collected after receiving approval from the Erasmus MC Medical Ethics Committee and St. Orsola Hospital (489/2018/Sper/AOUBo) Ethics Committee. All patients were enrolled provided written informed consent.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.113778.

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