Journal of Virological Methods 188 (2013) 1–5

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Generation of dried tube specimen for HIV-1 viral load proficiency test panels: A cost-effective alternative for external quality assessment programs

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Article history:
Received 11 May 2012
Received in revised form 9 November 2012
Accepted 19 November 2012
Available online 7 December 2012

Keywords:
Dried tube specimen
HIV
Viral load
DTS
Proficiency testing

A B S T R A C T

Participation in external quality assessment programs is critical to ensure quality clinical laboratory testing. Commercially available proficiency test panels for HIV-1 virus load testing that are used commonly in external quality assessment programs remain a financial obstacle to resource-limited countries. Maintaining cold-chain transportation largely contributes to the cost of traditional liquid proficiency test panels. Therefore, we developed and evaluated a proficiency test panel using dried tube specimens that can be shipped and stored at ambient temperature. This dried tube specimens panel consisted of 20 μl aliquots of a HIV-1 stock that were added to 2 ml tubes and left uncapped for drying, as a preservation method. The stability of dried tube specimens at concentrations ranging from 10^2 to $10^{6.5}$ RNA copies/ml was tested at different temperatures over time, showing no viral load reduction at 37 °C and a decrease in viral load smaller than 0.5 Log_{10} at 45 °C for up to eight weeks when compared to initial results. Eight cycles of freezing–thawing had no effect on the stability of the dried tube specimens. Comparable viral load results were observed when dried tube specimen panels were tested on Roche CAPTAQ, Abbott m2000, and Biomerieux easyMAG viral load systems. Preliminary test results of dried proficiency test panels shipped to four African countries at ambient temperature demonstrated a low inter assay variation (SD range: 0.29–0.41 Log_{10} RNA copies/ml). These results indicated that HIV-1 proficiency test panels generated by this methodology might be an acceptable alternative for laboratories in resource-limited countries to participate in external quality assessment programs.

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1. Introduction

Because of the contribution of several donors including the U.S. President's Emergency Plan for AIDS Relief, the Global Fund for AIDS, Tuberculosis and Malaria, and others, several RLC[1] continue to scale-up HIV ART[2] to infected patients (Holmes et al., 2010; Klausner et al., 2011). Patients enrolled in the ART programs of RLC are monitored by CD4 count testing according to guidelines of the World Health Organization (Chalamilla et al., 2012; Hawkins et al., 2011; WHO guidelines, 2010). However, many RLC have also initiated HIV-1 VL[3] testing to monitor patients and evaluate their ART programs (Filler et al., 2011). Without quality assurance such as method validation, staff training, equipment maintenance, and participation in EQA[4] programs that include testing of PT[5] panels, VL test results are not reliable. Incorrect VL results could induce wrong clinical management decisions and unreliable program evaluations (Chalermchan et al., 2007; Goguel, 1991; Hannon et al., 1989; Hofherr et al., 1992; Jackson et al., 1993). Commercially available EQA programs that use PT panels for HIV-1 VL are limited in number and cost-prohibitive to most RLC programs. Difficulties in logistics for shipping frozen PT panels to remote locations and possible interruption of cold-chain transportation are also complicating factors in many RLC.

To address these challenges, we adapted a methodology describing the use of PT panels for HIV serology made of DTS[6] (Parekh et al., 2010) to develop PT panels for HIV-1 VL. The advantages of DTS over traditional liquid samples are lower costs of preparation and shipment, stability at ambient temperature, and reduced biosafety risks. A stepwise approach to determine the optimal reagents and

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[1] Resourse-limited countries.
[2] Antiretroviral therapy.
[3] Viral load.
[4] External quality assessment.
[5] Proficiency testing.
[6] Dried tube specimens.

0166-0934/$ – see front matter Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.jviromet.2012.11.036

conditions for preparing and processing DTS is described in this study.

2. Materials and methods

2.1. Virus stock

An aliquot of HIV-1, 97USNG30 (subtype C strain), was obtained from the NIH AIDS Reagent Program (Germantown, MD catalog # 4115). This virus was propagated following routine peripheral blood mononuclear cells (PBMCs) co-cultivation techniques for HIV-1 isolation (Dezzutti et al., 2004), generating the virus stock used in this study. Virus load titers of the cell culture were monitored every three days, reaching approximately 3×10^8 RNA copies/ml on day 12 post infection, as measured on the CAPTAQ instrument (see below). The same cell culture supernatant was collected and filtered through Nalgene 0.45 micron filter membranes (catalog # 09-741-08, Fisher Scientific, Pittsburgh, PA). Filtered cell culture supernatant aliquots of 1 ml of were stored at −80 °C.

2.2. VL assays

The COBAS AmpliPrep/COBAS Taqman (CAPTAQ) HIV-1 Test (Roche Diagnostic, Indianapolis, IN catalog # 03542998190) was used in all experiments, except where specified. The Abbott Real-Time HIV-1 assay (Abbott Molecular, Des Plaines, IL catalog # 6L18-90), used with the Abbott m2000 system, and the NucliSENS EasyQ HIV-1 v2.0 assay (Biomerieux, Durham, NC catalog # 285033), used with the Biomerieux easyMAG instrument were also employed to verify the variability of VL results. All VL assays were performed according to manufacturers' instructions.

2.3. Preparation of DTS

The HIV-1 stock was diluted in PBS[7] 0.1 M, pH 7.4 with or without 0.2% of a green liquid food dye, obtained at a local grocery store (The Kroger, Cincinnati, OH) to concentrations in the linear range of VL assays (10^2–$10^{6.5}$ RNA copies/ml). Food dye was added to PBS to facilitate the visual confirmation of the presence of DTS in the tubes. Based on a description of DTS prepared as PT panels for HIV-1 serology (Parekh et al., 2010), 20 μl of different concentrations of virus suspension were added to uncapped 2 ml screw-cap cryogenic vials and left inside a biosafety cabinet with the laminar air flow turned on to dry overnight. On the following day, samples were inspected visually for confirmation of desiccation and the DTS were capped and stored at 4 °C for later use. Prior to VL testing, DTS were rehydrated and processed as regular clinical samples as described in experiments below. A graphical representation of DTS preparation can be seen elsewhere (Parekh et al., 2010).

2.4. Optimization of conditions for DTS rehydration

DTS at different concentrations were used to determine optimal incubation parameters for DTS rehydration prior to VL testing. Temperature, time, and mixing conditions were evaluated. In addition, several resuspension buffers were also assessed. In order to resuspend DTS, 1.1 ml of the following solutions were added independently to the vials as follows: water, PBS, PBS with 0.05% Tween 20 (Sigma–Aldrich, St. Louis, MO), Roche SPEX and Roche wash buffer (used in the CAPTAQ HIV-1 Test), Nuclisens lysis buffer (used in the NucliSENS EasyQ HIV-1 v2.0 assay), Promega lysis buffer, Promega wash 1, and Promega wash 2 (used in the Abbott Real-Time HIV-1 assay). Following incubation of DTS with respective resuspension buffers, samples were centrifuged briefly to remove condensation from the caps. Reconstituted samples were transferred to test tubes and VL testing was performed according to manufacturer's recommendations. Promega lysis buffer was used as negative control.

2.5. Determination of DTS thermal stability

In order to evaluate stability, DTS containing different concentrations of the virus stock were incubated at 37 °C, 45 °C, and 60 °C and tested weekly for up to eight weeks. Another set of DTS was subjected to eight cycles of freeze–thawing (−20 °C to 37 °C), prior to VL testing. After each incubation period, or each freeze–thawing cycle, DTS were reconstituted by incubation with 1.1 ml of Nuclisens lysis buffer in a thermomixer shaking at 1000 rpm/25 °C/15 min, prior to VL testing.

2.6. Preparation of a PT panel for HIV-1 VL testing using DTS

The VL PT panel was composed of five samples containing different virus stock concentrations that were diluted in PBS-dye solution and a negative control. DTS-1 and DTS-2 had a concentration of $10^{3.5}$ RNA copies/ml. DTS-3 was the negative control and DTS-4 and DTS-5 had concentrations of $10^{2.5}$ and $10^{4.5}$ RNA copies/ml, respectively.

PT panels were shipped at ambient temperature using a commercial courier service, taking an average of seven days for delivery to laboratories located in Ethiopia, Cote d'Ivoire, Namibia, and Mozambique, which agreed graciously to participate in this study by testing blinded samples. Each participating laboratory received one panel accompanied with one tube containing 13 ml of Nuclisens lysis buffer and its Material and Safety Data Sheet (MSDS), instructions and process checklist, and a result submission form. Participating laboratories were requested to submit VL results in Log_{10} copies/ml by email within three weeks upon receiving materials. Random identification numbers were assigned to each laboratory to maintain confidentiality.

3. Results

3.1. Dilution of virus stock

We observed that DTS prepared from dilutions of the virus stock in PBS or human plasma yielded similar VL results (data not shown). Therefore, for cost-savings and biosafety purposes, PBS was used to prepare dilutions of virus stock, which were the source of all DTS used in this study.

3.2. Rehydration conditions of DTS

Variations in incubation conditions for rehydrating DTS with Nuclisens buffer were evaluated for improving VL recovery. Results showed that extending incubation periods beyond 15 min did not influence recovery yields. Additionally, incubation at ambient temperature (25 °C) worked just as well as at 60 °C (data not shown). The incubation condition selected for DTS rehydration as shaking in a thermomixer at 1000 rpm/ambient temperature (25 °C)/15 min for the remainder experiments of this study. However, vortexing DTS for 30 sec provided similar yields of VL compared to incubation in the thermomixer as described above and could be used as an alternative method (data not shown).

Overall results shown in Fig. 1 demonstrated that all rehydration solutions used for DTS reconstitution provided similar VL results (range 2.05–2.65 Log_{10} RNA copies/ml; standard deviation = 0.19).

[7] Phosphate buffered saline (Sigma–Aldrich, St. Louis, MO).

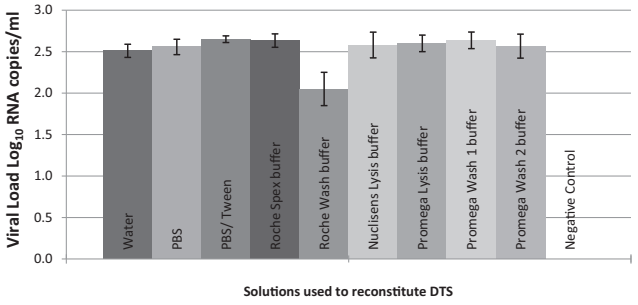

Fig. 1. Effects of different rehydrating solutions in the recovery yields of HIV-1 VL in DTS. DTS prepared from a single dilution of the virus stock was reconstituted in 1.1 ml of different solutions to determine which of these reagents would provide the highest yield of VL recovery. Each of these solutions were added to DTS vials and incubated in a thermomixer shaking at 1000 rpm/25 °C/15 min prior to VL testing according to manufacturer's instructions. All samples were tested in triplicates and the average VL was calculated and plotted. Error bars represent the SD of the average of VL results.

Of those solutions, Nuclisens lysis buffer was selected as the DTS rehydration reagent for the remainder of this study.

3.3. Stability of DTS

The potential interference of a visual aiding reagent (food dye used to in the preparation of DTS) in VL testing was evaluated. Two sets of DTS prepared with or without a green food dye harboring the same concentration of virus (2.0 Log_{10} RNA copies/ml) were incubated at 37 °C and tested weekly in triplicates for up to eight weeks. There was no decrease in VL during this period and the inclusion of the food dye had no effect on VL results. Results of DTS with food dye ranged from 1.88 to 2.23 Log_{10} RNA copies/ml, with a SD of 0.11, whereas VL results of DTS without dye ranged from 1.75 to 2.34 Log_{10} RNA copies/ml and a SD of 0.19 (data not shown).

The stability of DTS with 4 concentrations between 10^3 and 10^6 RNA copies/ml was further assessed by exposing these samples to 45 °C and 60 °C, over time. DTS incubated at 45 °C for eight weeks demonstrated an average VL reduction of 0.35 Log_{10} RNA copies/ml when compared to controls stored at 4 °C (Fig. 2a). This reduction did not seem to increase significantly over time, as the first three weeks were marked by an average reduction of 0.31 Log_{10} RNA copies/ml and the last three weeks showed an average VL decrease of 0.39 Log_{10} RNA copies/ml. The effect of temperature on DTS was more evident at 60 °C as expected. The average reduction of VL in this case averaged 0.51 Log_{10} RNA copies/ml in comparison to controls in the first three weeks. This reduction increased to an average of 0.71 Log_{10} RNA copies/ml in the last three weeks (Fig. 2b).

DTS stability was further evaluated by submitting samples carrying 10^3 RNA copies/ml to eight freeze–thaw cycles. The average VL of all samples including controls was 2.8 Log_{10} copies/ml, with SD of 0.08 (Table 1), with no evidence of VL loss due to these freeze–thaw cycles.

3.4. Variability of DTS VL results across different testing platforms

A preliminary evaluation to determine a strain of HIV-1 that would provide similar VL results across different platforms was performed (data not shown). From the nine different HIV-1 strains obtained at NIH AIDS Reagent Program (Germantown, MD), 97USNG30 (subtype C), was the virus that provided most similar VL results in Roche CAPTAQ, Abbott m2000, and Biomerieux easyMAG VL systems. VL results in the highest concentration ranged from 4.36 to 4.51 Log_{10} copies/ml (SD = 0.09). DTS in the middle concentration provided VL ranging from 3.17 to 3.46 Log_{10} copies/ml

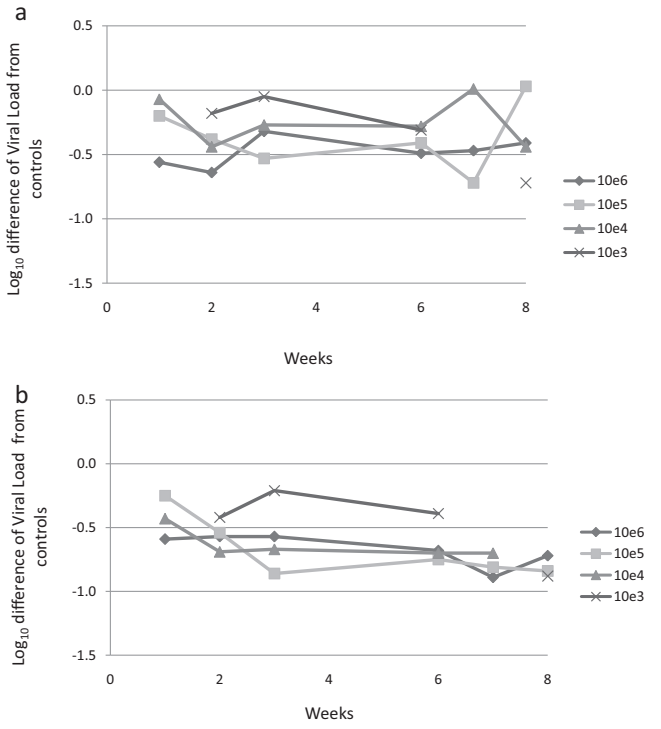

Fig. 2. (a) Stability of different virus concentrations of DTS at 45 °C2 2b Stability of different virus concentrations of DTS at 60 °C. Four different concentrations (10^3–10^6 Log_{10} RNA copies/ml) of the virus stock were used to prepare DTS containing a food dye, including negative controls. Samples were reconstituted with 1.1 ml of Nuclisens lysis buffer and incubated in a thermomixer shaking at 1000 rpm/25 °C/15 min prior to VL testing according to manufacturer's instructions. The difference in VL results between samples incubated at different temperatures and negative controls stored at 4 °C was plotted to estimate the stability of DTS over time, up to eight weeks. Weeks 4 and 5 were skipped. Absence of plotted data indicates not detectable VL.

(SD = 0.16), and samples at the lowest concentration ranged from 2.18 to 2.46 Log_{10} RNA copies/ml (SD = 0.14; Fig. 3).

3.5. Field evaluation of DTS PT panels

In order to evaluate the performance of DTS PT panel on field conditions, a pilot evaluation in which samples were shipped to four African laboratories for HIV-1 VL testing was executed. Data received from these laboratories demonstrated relative closeness

Table 1
Effects of freezing/thawing cycles on VL results of DTS samples.

−20 °C/37 °C cycles	Log_{10} copies/ml
8×	2.85
7×	2.85
6×	2.72
5×	2.82
4×	2.78
3×	2.95
2×	2.84
1×	2.69
[a]RT	2.75
[a]37 °C	2.75
average	2.80
SD	0.08
CV	0.03

RT represents ambient temperature.
DTS samples were prepared in PBS and reconstituted in Nuclisens lysis buffer. Samples were tested in triplicates and average VL was calculated.
[a] Samples stored for one week prior to VL determination.

Table 2
DTS proficiency test evaluated in 5 laboratories.

Samples	CDC	Laboratory 1	Laboratory 2	Laboratory 3	Laboratory 4	Average	SD
	CAPTAQ 48[a]	CAPTAQ 48	CAPTAQ 96[b]	High pure/Taqman 48[c]	Biomerieux EasyQ		
	Viral load results (Log_{10} copies/ml)						
DTS 1	3.21	3.43	2.82	3.31	3.6	3.27	0.29
DTS 2	3.31	3.3	2.76	3.19	3.85	3.28	0.39
DTS 3	BD[d]	BD	BD	BD	BD	NA[e]	NA
DTS 4	2.52	2.3	1.71	2.06	BD	2.15	0.35
DTS 5	4.21	4.67	4.41	4.53	5.3	4.62	0.41

[a] CAPTAQ 48: Cobas Ampliprep/TaqMan 48.
[b] CAPTAQ 96: Cobas Ampliprep/TaqMan 96.
[c] High Pure/Taqman 48: RNA extraction performed with Roche High Pure kit and RT-PCR performed on Cobas TaqMan 48.
[d] BD: below level of quantitation.
[e] NA: not applicable.

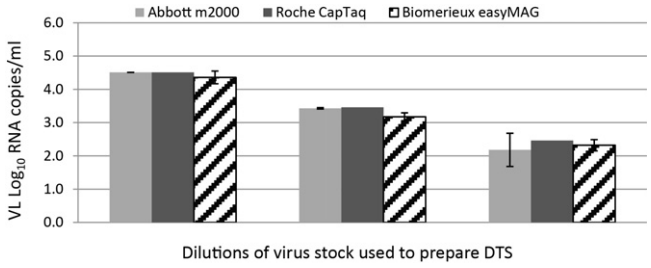

Fig. 3. Comparison of DTS VL results tested in Roche CAPTAQ, Abbott m2000, and Biomerieux easyMAG systems. VL testing was performed in DTS prepared from three different concentrations of virus stock with Abbott m2000, Roche CAPTAQ, and Biomerieux easyMAG platforms to assess variability of VL results in these VL assays. Samples were reconstituted with 1.1 ml of Nuclisens lysis buffer and incubated in a thermomixer shaking at 1000 rpm/25 °C/15 min prior to VL testing according to manufacturer's instructions. Error bars represent SD of samples tested in triplicates and duplicates with Biomerieux and Abbott instruments, respectively.

of VL results (Table 2). All results were within three SD from the average, with the exception of DTS-4 that was determined as VL below detection by Laboratory # 4 that tested this panel member with a Biomerieux system. When a 0.5 Log_{10} range above and below the average VL for each DTS concentration was calculated, few outliers were observed. Laboratory # 4 determined VL in DTS-2 and DTS-5, 0.6 and 0.68 Log_{10} above the average, respectively.

4. Discussion

The recent development of a DTS PT panel for HIV-1 serology targeted to RLC (Parekh et al., 2010) triggered the development of DTS described in this study for HIV-1 VL. Clinical samples from HIV-infected individuals do not contain VL in a concentration that would allow generating DTS PT panels as described in this study. This is due to the fact that volumes larger than 20 μl did not completely dry by exposure to air overnight (up to 16 h). Therefore, HIV-1-infected cell culture supernatants reaching concentrations in the range of 10^8 RNA copies/ml were the only available source of virus to generate DTS by this methodology. The DTS methodology to produce PT panels has several advantages over traditional liquid samples: (1) DTS is cheaper to produce, as it is made of 20 μl virus suspension in PBS. (2) DTS is cheaper to transport at ambient temperature than traditional PT samples that require dry ice for cold chain. (3) DTS provides additional biosafety protection, as spills are avoided by shipping and handling dry samples and for utilizing a lysis buffer that contains guanidine thiocyanate for reconstitution, which has virucidal activity (Blow et al., 2004). Among the lysis buffers tested here, Nuclisens buffer was chosen due to procurement reasons. However, the DTS methodology might not work well with other types of HIV-1 quantification assays, such as the ones that measure RT activity or p24 Ag concentration. Although the ultimate goal is to eventually transfer this methodology to RLC, the capacity to perform HIV-1-infected cell culturing might be a hindering factor in some of those laboratories.

The HIV-1 VL PT program offered by CAP[8] segregates results of participating laboratories according to types and/or manufacturers of VL assays. This approach is used to avoid generating biased passing and failing criteria due to the variability of VL test results observed in different VL assays (Lin et al., 1998). This situation is more likely to occur when a large number of results from one type of assay are compared to a small number of results from another type of assay. Previous studies have also shown differences in HIV-1 VL assay results when different platforms tested different strains of virus (Scott et al., 2009; Bourlet et al., 2011). Therefore, in order to minimize the variability of VL test results, we identified among few HIV-1 strains analyzed (data not shown), the 97USNG30 strain that provided similar VL results across HIV-1 VL assays used commonly such as Roche, Abbott, and Biomerieux testing platforms. These three platforms provided almost identical VL results in all DTS dilutions tested. However, the number of samples analyzed was too small to determine any statistical significance of these data. Analysis of results obtained from the international pilot evaluation of DTS, demonstrated that Biomerieux EasyQ assay provided higher VL readings on samples at higher concentrations and a result below detection on the sample with the lowest concentration, in comparison to the other laboratories. In addition to these inter-assay differences, laboratory # 2, which used a CAPTAQ 96 system provided lower VL results in all panel samples in comparison to the other laboratories that also used Roche-based systems. The significance of these differences cannot be calculated at this point due to the few number of samples analyzed. However, it is possible that effects of shipping, handling, and storage conditions of the panel, as well as procedural or instrumentation differences of testing might play role in this variability. In order to obtain significant data that would enable a reliable calculation of outliers and identification of testing problems as part of an EQA program, a larger number of participating laboratories is indispensable. The analysis of data from a larger number of participant laboratories will also determine the need to segregate participants by type of VL assay used, as it is managed currently by the CAP PT program.

5. Conclusions

Participation in EQA programs, such as the proficiency testing program of HIV-1 VL provided by CAP is one of the essential elements to ensure quality of services in clinical laboratories. In fact,

[8] College of American Pathologists, Northfield, IL.

clinical laboratories can only obtain ISO15189 or CAP accreditation by demonstrating proficiency in EQA programs. In summary, the data described in this study indicated that DTS is an easy, reliable, and cost-effective alternative to commercially available liquid PT panels for HIV-1 VL, as long as exposure of DTS to high temperatures (60 °C and above) for a period greater than three weeks is avoided. Once sufficient data are compiled and data analysis procedures are established from a sufficient number of participating laboratories, criteria for determining pass or fail results will be established avoiding biased and either excessively stringent or too flexible PT evaluations. These next steps will include offering a PT program for HIV-1 VL using DTS free of charge to laboratories in RLC based on the pilot evaluation described in this study.

Acknowledgments

We thank Drs. Bharat Parekh and Amilcar Tanuri for their invaluable contribution to the concept and organization of this manuscript.

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