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Addressing false negatives in viral diagnostic polymerase chain reactions: A new approach

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ABSTRACT

Viral infectious diseases represent an important global public health problem with high incidence of mortality and morbidity every year. Since viral infections account for greater than 60% of the burden from other infectious agents, efforts to achieve accurate screening of such viral pathogens by improving the sensitivity of the detection methods has been the major focus of many researchers. In this study, a method to concentrate virus particles such as Human Immunodeficiency virus1, Hepatitis B virus and Hepatitis C virus has been re-evaluated and the optimal protocol for concentrating the viruses from low-titre specimens has been determined. To concentrate virus particles, 1 mL of the clinical sample was centrifuged at 16,627 × g at 4°C for 2 h. The pelleted viral particles were then processed for DNA and RNA preparation and tested by quantitative polymerase chain reaction (PCR) for the respective viral load. Recovery efficiencies of >100% was achieved for HIV-1 particles with possible lack of PCR inhibitory compounds. The described methodology enhances the limit of detection of mini-pool (MP) testing by almost 20 to 50 folds and could be applied by blood banks engaged in viral testing of blood samples.

INTRODUCTION

It has been demonstrated that enzyme-linked immunosorbent assay (ELISA) is a specific, quick and economical diagnostic method that could replace the serum neutralization test (Ahsani et al., 2010a, b). However, polymerase chain reaction (PCR) is the most modern practical technology in diagnostics. It has been shown to be more rapid, and also in comparison to classical techniques (Ahsani et al., 2010a; Mohammadabadi et al., 2004, 2011). Genotyping, based on a more stable marker, DNA, is not dependent on gene expression and the discriminatory power of DNA-based methods is superior to that of phenotypic methods. The ability to distinguish between genomes is important to several disciplines of microbiological research, for example in studies on population genetics and microbial epidemiology (Ahsani et al., 2011; Zandi et al., 2014; Shahdadnejad et al., 2016). Of great importance when choosing a method for genotyping are the typing ability, reproducibility, discriminatory power and also the ease and cost of performing the analysis.

Nucleic acid testing (NAT) is a molecular technique, highly sensitive and specific, used to detect pathogens such as a virus or a bacterium in a specimen of blood or other tissue or body fluids. This technique is based on amplification of the RNA or the DNA of the pathogen since it exponentially amplifies the target DNA by many orders of magnitude by cycling the temperature of the reaction several times.

NAT in blood donor screening reduces the risk of transfusion-transmitted viral infections (TTIs) and presently around 33 countries in the world have implemented NAT for human immunodeficiency virus

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(HIV) and around 27 countries for hepatitis B virus (HBV) and hepatitis C virus (HCV) (Roth et al., 2012). Since NAT shortens the window period, a time between when a patient has been infected and when they show up as positive by antibody tests such as ELISA for HIV, HBV and HCV infections, it is well-suited for screening blood donations providing an additional layer of safety for the patient. NAT can detect HIV-1 infection approximately 12 days before antibody positivity (Morris et al., 2010). Individuals with recent HIV acquisition may be more infectious than individuals with established infection (Wawer et al., 2005) and with HIV, NAT assays are more useful for detecting early infection, although the issue of low viral RNA concentration persists (Minno et al., 2016). Hence, the numbers of public health programs that screen for acute HIV infection with pooled HIV-NAT is increasing (Stekler et al., 2007).

The HIV epidemic remains a serious public health problem globally. An estimated 4.2 million people living with HIV globally are youth of 15–24 years and the burden is highest among females (Mafigiri et al., 2017). Reports suggest that people diagnosed with HIV infection over the age of 50 have higher rates of morbidity and mortality. Hence, the accurate detection of HIV infection during acute and early stages may help to control the HIV epidemic.

Chronic HBV infection is common worldwide, with almost 350 million people afflicted with the disease and approximately 600,000 deaths annually (Weinbaum et al., 2008). Hepatitis B surface antigen (HBsAg) is a serological marker that indicates active infection, while detection of anti-HBs, a neutralizing antibody, indicate immunity to HBV infection.

In India, blood screening for HBV, HIV and HCV is done by serological tests for HBsAg and antibodies to HIV-1 and 2 and HCV. Blood safety is a challenging task in India and that presently uses around 10 million units of blood every year. Hence, screening of blood samples by NAT for large volumes of samples becomes cumbersome and expensive and hence many transfusion centres around the world have introduced a technique called mini-pool (MP).

Although, MP testing involves additional time for sample pooling and adds effort to the process with careful record keeping, it is certainly cost-effective since it reduces the reagent costs that are required for processing individual samples. One of the disadvantages of viral testing, using MP, is that while pooling plasma samples with viral loads of 10-20 IU/ml, the samples become diluted to fall within the limit of detection (LOD) of the test employed. In such a scenario, detection of virus by PCR becomes challenging with a possibility of false negative result that is dangerous to the recipient who is in need of blood components. Hence, to address this aspect, we employed a simple yet cost-effective method to concentrate the viral particles in the MP which reduces the chances of getting false negatives in the uniplex PCR (ID).

Chatterjee et al. (2014) have compared the sensitivity of NAT by uniplex PCR and MP testing where samples with high viral loads were detected by all dilutions, but 67% of samples of low viral load were missed by MP-NAT. It was concluded by this study that ID-NAT (Uniplex PCR) is an ideal methodology for TTI screening (Hans and Marwaha, 2014).

False negatives in antigen based detection of Hepatitis B are also reported by several research workers. Matsuda et al. (2011) reported a case where a Mab for HBsAg could not detected in the serum of this patient when the assay was done using a single antibody versus two different antibodies. Case reports of false-negative diagnostic results due to HBsAg mutants have been described in blood banks (Levicnik-Stezinar, 2004; Zaaijer et al., 2001) and hospital settings (Koyanagi et al., 2000).

There still exists a high prevalence of false-positive results in HCV samples, especially among immune compromised patients or populations without liver diseases, leading to unnecessary cost-effective health expenditures and confusing diagnostic challenges. In the light of the above reports, accurate diagnostics of all these viruses appears critical for proper drug treatment (Kesli, 2011).

In case of HBV infections, due to rampant use of hepatitis B vaccine, there have been concerns about false positive HBsAg reactivity. Bernstein et al. (1994) show that 65% of the infants that tested positive, exhibited HBsAg results negative after 18 days. Use of quantitative real time PCR (Q-PCR) in such cases would certainly avoid the appearance of false positive tests since Q-PCR detects only active viral infections.

To address the false negative tests for all these viruses, we realised that in majority of the cases, it is the viral load that determines the success rate of any diagnostics and hence, we envisaged that if one is able to develop a methodology that does not get limited by the low viral loads, there will be an assurance of achieving tests that are fool-proof and would be free from the confusing diagnostic challenges. The experimental data generated in this regard is presented in this article.

Reports on usage of centrifugal devices to concentrate viruses exist. However, all these methods involve the use of an ultracentrifuge, which are expensive and not accessible to all labs especially in diagnostic labs of developing countries. Also, the centrifugal devices that can withstand high speed are costly making the cost of the diagnostic assays not attractive. In this article, we attempted to concentrate viruses by simple centrifugation and demonstrate the effectiveness of the protocol by showing viral recovery of >100% and discuss the feasibility of application of such a protocol for samples with low viral load. The objective of this study was to

investigate the effect of dilution on the sensitivity of tests employed.

MATERIALS AND METHODS

Routine Diagnostic Test (RDT) kits for qualitative diagnosis of HIV, HBV and HCV were purchased from J. Mitra and Co. Pvt. Ltd, New Delhi, India. SuperScript™ III One-Step RT-PCR System with Platinum Taq High Fidelity kit and Thermo Maxima SYBR Green master mix were procured from Invitrogen, USA. All other reagents used for this study was of analytical grade, unless otherwise mentioned. pTZ57R/T vector was from Thermo Fisher Scientific, USA. Lyophilized powders of T4 and MS2 phages and *Escherichia coli* C 3000 were purchased from ATCC, USA while *E. coli* BL21 was from Aristogene Life Sciences, Bangalore, India.

Patients and clinical samples

Patients who visited Kuppam Medical College, Andhra Pradesh, Bowring Hospital, Bangalore, Rangadore Memorial Hospital, Bangalore, India, St. John's hospital, Bangalore and Vikram hospital, Bangalore for viral testing of HIV, HBV and HCV during the period of March 2016 to March 2017 served as the study material. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) coated tubes and plasma was collected by centrifuging the samples at 2000 × g for 10 min. The plasma samples were stored at -20°C, until further use.

Nearly 740 samples from a Stem Cell Bank company in Bangalore (labelled as SCB, henceforth) were received at Cancyte technologies Pvt. Ltd., Bangalore for testing for these three viruses during the period October 2016 to December 2016 and these have also been used as sample for the MP testing described in this study. Informed consent forms, duly signed by the patients were collected at the above mentioned centres and have been preserved for all references.

Isolation of nucleic acids from clinical samples

150 µl of plasma from positive samples of HIV-1, HBV and HCV were processed for isolation of nucleic acids using NucleoSpin® Dx Virus kit (Germany) according to the manufacturer's instructions. Viral nucleic acids were eluted from the filter column with 50 µl of elution buffer and stored at -20°C until further use.

Serological test for collected plasma samples by RDT

Plasma samples were analysed for HIV, HBV and HCV by RDT as per manufacturer's instructions. The test kits employed included the HIV-1 TRI-DOT kit, HEPACARD test kit and HCV TRI-DOT test kits. Antigens gp41 and C terminus of gp120 of HIV-1 was detected using HIV-1 TRI-DOT test, while qualitative detection of Hepatitis B surface antigen (HBsAg) in human serum or plasma was detected using the HEPACARD kit reagents. The 4th Generation HCV TRI-DOT is a rapid, visual, sensitive and qualitative *in vitro* diagnostic test for the detection of antibodies to hepatitis C virus in human plasma that has been designed for the putative core (structural), protease/helicase NS3 (non-structural), NS4 (non-structural) and replicase NS5 (non-structural) regions of the HCV particles.

Construction of reference standards

The PCR fragments of *gag* gene of HIV-1, *S* gene of HBV and the 5' untranslated region (UTR) region of the HCV were cloned into the pTZ57R/T vector (Invitrogen Corp., Carlsbad, CA.) according to the manufacturer's instructions. These constructed plasmids provided a reproducible source of reference DNA, for all the PCR's. By using these plasmids, we avoided spectrophotometric quantification errors, which are inevitable when plasmids of different sizes are used.

Uniplex PCR of HIV-1, HBV and HCV

The gene specific primers used for uniplex PCR of HIV-1, HBV and HCV are given in Table 1. Uniplex PCR's for HIV-1, HBV and HCV were carried out using primers specific for the target viruses. For HIV-1 detection, primers that target the HIV-1 *gag* gene of the Indian subtype HIV specific strains was based on the paper by Acharya et al. (2014) while for HCV, primers that targeted the 5' UTR region of genotypes 1 to 6 was designed as per the method described by Pripuzova et al. (2012). Since the 5' UTR of the HCV genome is the most conserved region (Han et al., 1991), this served as an ideal template for HCV PCR.

For HBV, 25 sequences of HBV genotypes published in NCBI database, were taken and based on the conserved sequences, the primers that targeted the *S* gene of HBV were designed. 304 samples that were collected in various hospitals in and around Bangalore, during the period March 2016, to June 2017, were subjected to uniplex PCR and the results were compared with the RDT results carried out for these samples.

Q-PCR for all the three viruses was done using SuperScript™ III One-Step RT-PCR System with Platinum® Taq High Fidelity kit (Invitrogen, USA). Briefly 10 µl of the PCR mix containing 1 x Superscript buffer, 0.2 µl superscript III reverse transcriptase, primers for HIV-1, HBV and HCV at a final concentration of 0.2 µM

Table 1. Oligonucleotides used in this study.

S/N	Target gene	Primer direction	Primer sequence (5' to 3')
1	HIV-1 gag	Forward	ACATCAAGCAGCCATGCAAAT
		Reverse	TACTAGTAGTTCCTGCTATGTC
2	HBV S	Forward	CCCCCACTGGCTGGGGCTTGGT
		Reverse	AGGACGTCCCGCGCAGGATC
3	HCV 5' UTR	Forward	CRGAAAGCGYCTAGCCATGGCGT
		Reverse	ACTCGCAAGCACCCTATCAGGCA
4	T4 phage rIIa	Forward	CGCTGGGAAAAGAGGAATTATTTA
		Reverse	TCAATATATCGTTTAGCTGAA
5	MS2 phage replicase	Forward	CTCTGAGAGCGGCTCTATTGGT
		Reverse	GTTCCCTACAACGAGCCTAAATTC

final concentrations and nuclease-free water. PCR cycling conditions included 30 min incubation at 50°C with an initial denaturation at 95°C for 5 min followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, 45 s at 72°C and a final extension of 10 min at 72°C. For construction of PCR positive controls, the amplicons of the *gag* gene of HIV-1 (150 bp), *S* gene fragment of HBV (200 bp) and 3' UTR region of HCV (240 bp) were cloned in the InsTA clone PCR cloning kit pTZ57R/T vector (Thermo Fisher Scientific, USA) following manufacturer's instructions. The recombinant plasmids were purified and the identity of the cloned inserts was confirmed by DNA Sanger sequencing.

Bacteriophage nucleic acid spiking studies in MP samples

T4 and MS2 bacteriophages have been successfully used as an internal control (IC) for DNA and RNA viruses by Ninove et al. (2011). Since it is practically impossible to avoid the presence of PCR inhibitors in clinical samples, use of such IC's is extremely useful to avoid false-negative results. Hence, for detection of the DNA virus such as HBV, T4 phage DNA was spiked in all the plasma samples and the T4 PCR was perfomed along with HBV PCR. Oligonucleotide primers for the rIIa of the T4 phage and for the replicase gene of MS2 phage were designed based on the published sequence of the T4 bacteriophage (GenBank accession number, NC_000866) and MS2 phage (GenBank accession number, NC_001417).

For the RNA viruses such as HIV-1 and HCV, MS2 phage RNA was used for the spiking studies. The T4 and MS2 phages were propagated in bacterial hosts *E. coli* BL21 and *E. coli* C3000 respectively using Luria Bertani broth as the propagation medium. The phages were tested by routine test dilutions (RTD) and the total plaque forming units (pfu) per litre of broth was calculated. T4 phage DNA was prepared following the protocol described (Paul et al., 2011) while MS2 phage RNA was prepared using the DxVirus kit.

Construction of reference standards for HIV-1 viral load quantification

HIV-1 viral load represents a basic marker for evaluation of the rate and severity of HIV disease and to monitor the effectiveness of treatment. A cost-effective SYBR green-based real-time RT-PCR (SYBR green real-time RT-PCR) revealed by Light Cycler technology was evaluated for quantitation of HIV-1 RNA viral load in plasma of HIV-1 sero-positive patients.

In order to generate a standard curve, pTRZ5R/T-HIV-1 plasmid was serially diluted and calibrated against reference standards of the US Food and Drug Administration (FDA) approved Artus HIV-1 RG RT-PCR kit (Qiagen, Germany). A five dilution series of the reference standard was prepared with copy numbers as follows: 10^1, 10^2, 10^3, 10^4 and 10^5 HIV-1 viral RNA copies/ml.

SYBR Green I based one-step real time quantitative PCR was performed using a Rotor Gene Q-PCR System (Qiagen, Germany). 25 µl of a reaction mixture comprised of 12.5 µl of 2 x Thermo Maxima SYBR Green master mixes (Thermo Fisher Scientific, USA), each primer (forward and reverse) at a final concentration of 5 pmoles per reaction.

The performance of the SYBR green real-time PCR was assessed on arbitrarily selected 20 HIV-1 positive patients' plasma samples from the study panel described here. PCR cycling conditions were 30 min at 50°C, with initial denaturation of 5 min at 95°C followed 40 cycles of 20 s at 95°C, 20 s at 55°C, 30 s at 72°C and final elongation of 10 min at 72°C. The LOD of the Q-PCR test protocol was found to be one copy/reaction or 66 copies/ml of plasma.

Concentration of virus particles by centrifugation

Concentration of HIV-1 particles was firstly examined using centrifugation of three HIV-1 positive samples mentioned in this study. To sediment the viral particles, the samples were subjected to centrifugation at 16,627 ×g, for 2 h at 4°C in a fixed angle rotor (Eppendorf, USA). After centrifugation, the supernatant was aspirated carefully and the obtained pellet was subjected to nucleic acid isolation using Dx-virus kit. After centrifugation, the pellet was re-suspended with 150 µl fresh sero-negative human plasma and processed for nucleic acid isolation.

To evaluate sedimentation of viral particles other than HIV-1, four HBV positive plasma samples and three HCV positive plasma samples were used to pellet the viruses as described above. After pelleting, the nucleic acids obtained were subjected to uniplex PCR with virus specific primers.

Determination of minimum amount of HIV-1 virions that can be pelleted by centrifugation

Different amounts of estimated HIV-1 virions from a known HIV-1 positive sample were log diluted with a sero-negative negative plasma to get HIV-1 virions of 1.5×10^3/ml to 1.5×10^1/ml. These were subjected to concentration by centrifugation as described above followed by RNA isolation and Q-PCR for HIV-1 *gag* gene. Suitable standards of HIV-1 plasmid with 50000, 5000, 500 and 50 viral copies/ml was run simultaneously. The experiment was repeated three times and the data of a representative example is presented. The PCR amplicons were examined on 2% agarose gel stained with ethidium bromide and the respective Ct values were noted.

Preparation of mini-pools for NAT with known amounts of HIV-1 virions

The cost of viral test monitoring is usually achieved by reducing the number of assays needed to screen a population receiving drug treatments by MP strategy. The MP matrix approach with 8 or 10 pool size was the most efficient as suggested recently (Kim et al., 2013). In the light of the above, 3 sets of samples were made, with each set containing 200 µl of a known quantity of HIV-1 particles that mimics a MP testing of 5 different samples per pool. 150 µl of the sample from each set was then directly processed for HIV-1 RNA isolation on one side while 850 µl of the same solution was centrifuged at 16,627 g for 2 h on the other side. The pellet obtained from the latter set was processed for nucleic acid isolation.

Testing of samples from SCB by MP testing

740 donor samples were screened by a 5-sample MP (that is, 148 pools) for HIV-1, HBV and HCV testing. 300 µl plasma of 5 samples were pooled and the entire 1.5 ml pooled plasma was centrifuged at 16,627 for 2 h and the pellet obtained was processed for nucleic acid isolation.

RESULTS

Serological testing results

Table 2 elaborates the RDT data for all the clinical samples obtained from different hospitals of Bangalore, India and compares the RDT and the PCR data for all the three viruses tested in this study. Our results on uniplex PCR for the entire samples tested positive by RDT were close to 90% in all the cases. There were nearly 5 to 12% of samples that were PCR negative but RDT positive. This could be due to the possibility of presence of viral antibodies post-infection or the persistence of viral antigens post infection in such patients. Since the Q-PCR detects only the active state of viral infection, we believe that these results would have no negative implications on the disease outcome in such patients. In a few samples, we could get PCR positive and RDT negative and these could be due to samples with low viral loads since PCR is more sensitive than RDT.

Construction of reference standards for HIV viral load quantification

Figure S1 shows the plasmid maps of all the reference standards used in this study (See Appendix). Dhanasekaran et al. (2010) have found that the copy numbers of standards vary primarily due to degradation of the PCR amplicons (when used as standards) over a period of time during storage at 4°C and -20°C, which in turn affects the PCR efficiency and the results significantly. Since the plasmid versions of the cloned target sequences were noticeably more stable than the PCR product, the plasmids used by us served as stable controls for the Q-PCR experiments carried out in the study.

Uniplex PCR of HIV-1, HBV and HCV

Results depicted in Table 2 show that for HIV-1, 93.7% of the samples matched with the RDT results while for HBV and HCV, 86% and 88.6% matched with the RDT results. The sensitivity of uniplex PCR for HIV, HBV and HCV were 10 copies, 25 copies and 20 copies per reaction, respectively.

Table 2. Comparison between RDT and PCR of samples for HIV-1, HBV and HCV.

S/N	Hospital	No. of samples	HIV-1		HBV		HCV	
			RDT	PCR	RDT	PCR	RDT	PCR
1	Rangadore Hospital, Bangalore	31	0	0	3	3	2	2
2	St. Johns Hospital, Bangalore	12	2	2	2	2	2	2
3	Vikram hospital, Bangalore	15	12	12	1	1	0	2
4	Bowring Hospital, Bangalore	30	5	5	25	23	0	0
5	Kuppam Hospital, Andhra Pradesh	215	77	71	98	82	40	33
	Total	303	96	90	129	111	44	39

Table 3. Viral load of arbitrarily selected HIV-1 samples before and after spinning.

S/N	Sample ID	Age/Sex	Viral Load /ml before spinning	Viral Load /ml after spinning	% Recovery
1	6438	39/Male	3.8×10^4	5.8×10^4	152
2	7308	35/Male	4.0×10^4	1.3×10^5	325
3	4631	32/Female	6.1×10^5	1.0×10^6	164

HIV-1 viral load estimation by Q-PCR

The linearity of the assay was determined using a recombinant plasmid harbouring the amplified *gag* gene fragment of HIV-1. The calibration curve obtained with the inclusion of the HIV-1 plasmids as standards gave a regression line with an r^2= 0.992 and the linear regression of the observed and reference concentrations yielded a correlation coefficient of 0.998. A standard curve was generated by plotting the threshold cycles of reference standards versus the log concentrations. The dissociation curve of all reference standards showed a melting temperature of 82.5°C (data not shown). The LOD of SYBR green assay as 66 copies/ml is close to the observations of LOD of 50 virus copies/ml for HIV-1 by Acharya et al. (2014).

Twenty arbitrarily selected clinical samples subjected to Q-PCR yielded HIV-1 viral load in the range of 1.3×10^2 to 6.8×10^6/ml (See Appendix for Table S1).

Concentration of virus particles by centrifugation

The results presented in Table 3 clearly show that one could recover more than 100% of the HIV-1 particles by the way of centrifugation as adopted by us. We were successful in concentrating not only for HIV-1 viral particles by the centrifugation method but also the HBV and HCV particles. Figure 1A shows the agarose gel pictures of the PCR of HIV-1 virions before and after spinning while Figures 1B and C show the pattern for HBV and HCV respectively. The signal intensities of the samples before and after concentration of the viruses were found to be similar.

We observed that a minimum volume of 150 µl of plasma samples to 2 ml plasma could be subjected to centrifugation with no change in % recovery of the pelleted HIV-1, HBV and HCV virus (data not shown) making the suggested protocol attractive in limited sample availability cases too.

Determination of minimum amount of HIV-1 virus that can be pelleted by centrifugation

We wished to examine on the minimum amount of HIV-1 particles that can be pelleted by centrifugation since that aspect would decide on the use of the method for low viral load samples. Results indicated in Figure 2A, clearly indicates that PCR signal was seen in the all the viral dilutions taken up for estimation of HIV-1 viral load using SYBR green chemistry. It is encouraging to see that one could easily pellet almost 15 particles HIV-1 under the experimental conditions described. The Ct values decreased with the decreased amount of viral particles as expected (Figure 2B). In this experiment also, the % recovery of the HIV-1 particles was more than 100% (Table 4).

Preparation of mini-pools for NAT test with known amounts of HIV-1 particles with and without spinning

Dilutions of a HIV-1 positive sample were performed with

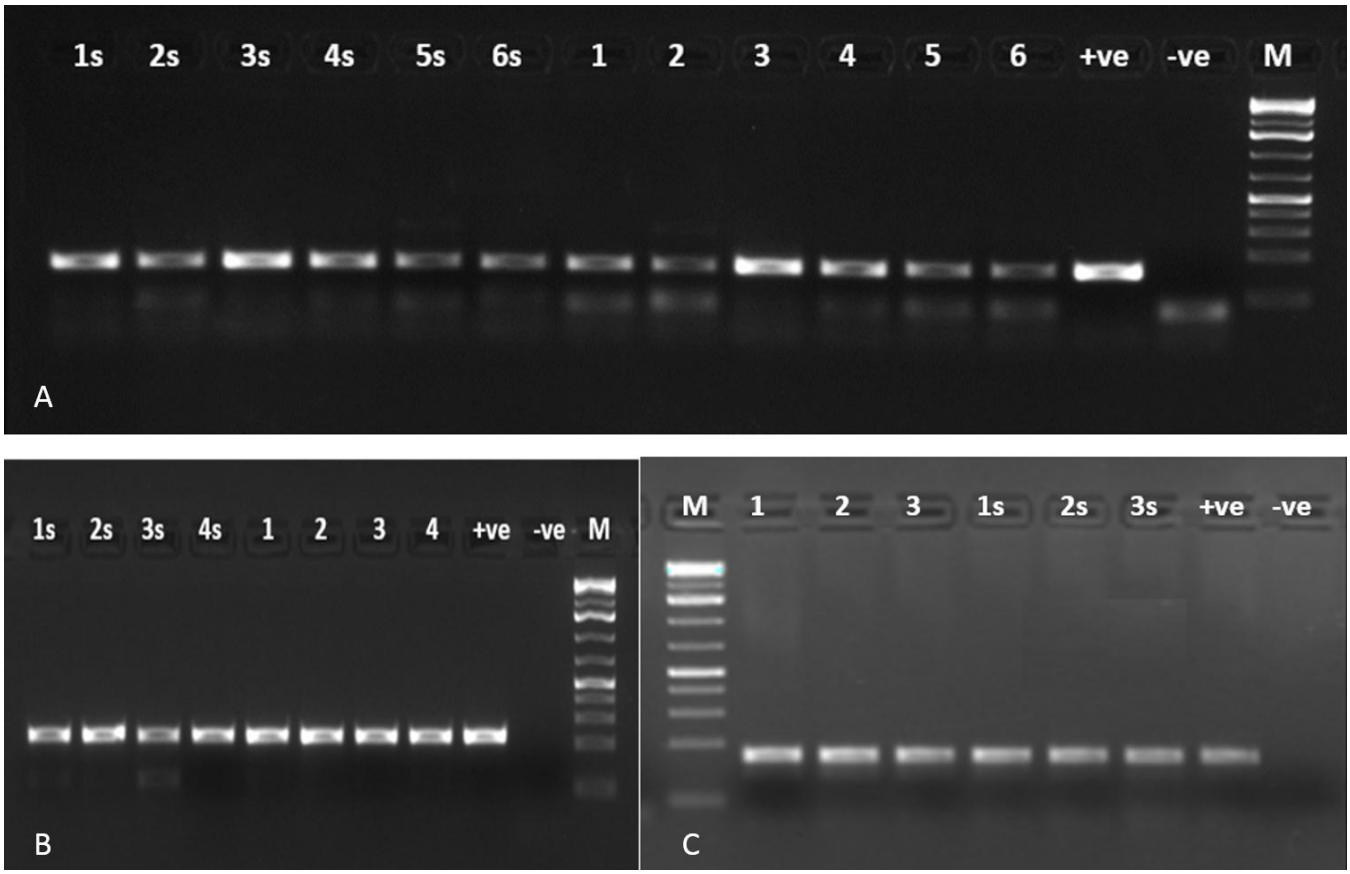
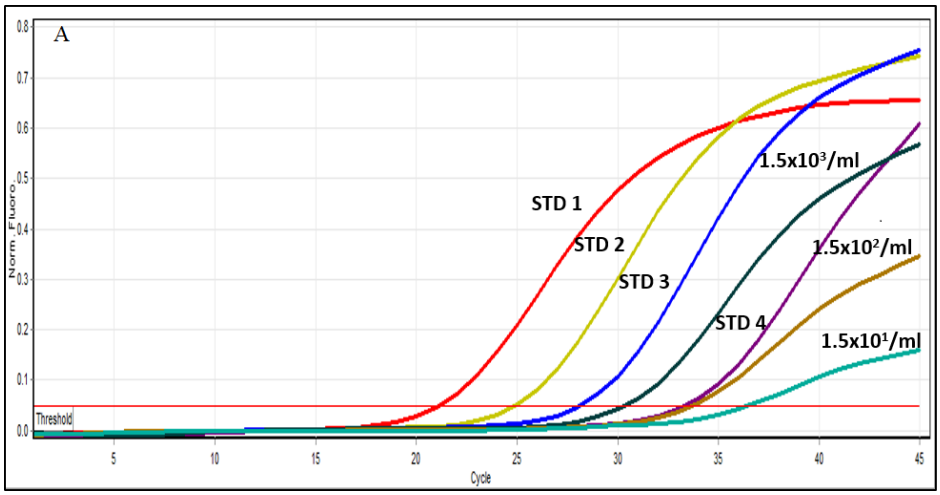


Figure 1. Effect of spinning of HIV-1, HBV and HCV positive plasma samples on PCR signals. Figure 1A shows the intensity of the PCR signal of HIV-1 *gag* gene (150 bp) for 6 samples randomly chosen from our HIV-1 plasma library. Letter 's' here refers to spinning; Lane 1s to 6s refers to HIV-1 plasma samples 1 to 6 subjected to spinning while lanes 7 to 12 shows samples 1 to 6 not subjected to concentration by spinning. Figure 1B shows the PCR of HBV *S* gene (200 bp) while Figure 1C denotes 3 samples of HCV PCR of 5'UTR region (240 bp) that were subjected to spinning (1s to 3s-lanes 4 to 6) and 3 samples untreated (lanes 1 to 3). +ve denotes PCR with positive plasmid DNA while -ve refers to PCR without template respectively.



B

S.N	Samples	Ct
1	STD 1	21.11
2	STD 2	24.93
3	STD 3	28.13
4	STD 4	33.3
5	$1.5x10^3$/ml	30.37
6	$1.5x10^2$/ml	33.65
7	$1.5x10^{1/}$ml	36.49

Figure 2. Determination of minimum amount of HIV-1 virus pelleted by centrifugation. Note that the PCR signal is observed with a sample with 15 virus particles/reaction. 2B shows that the Ct value is inversely proportional to the viral copies in the sample as expected.

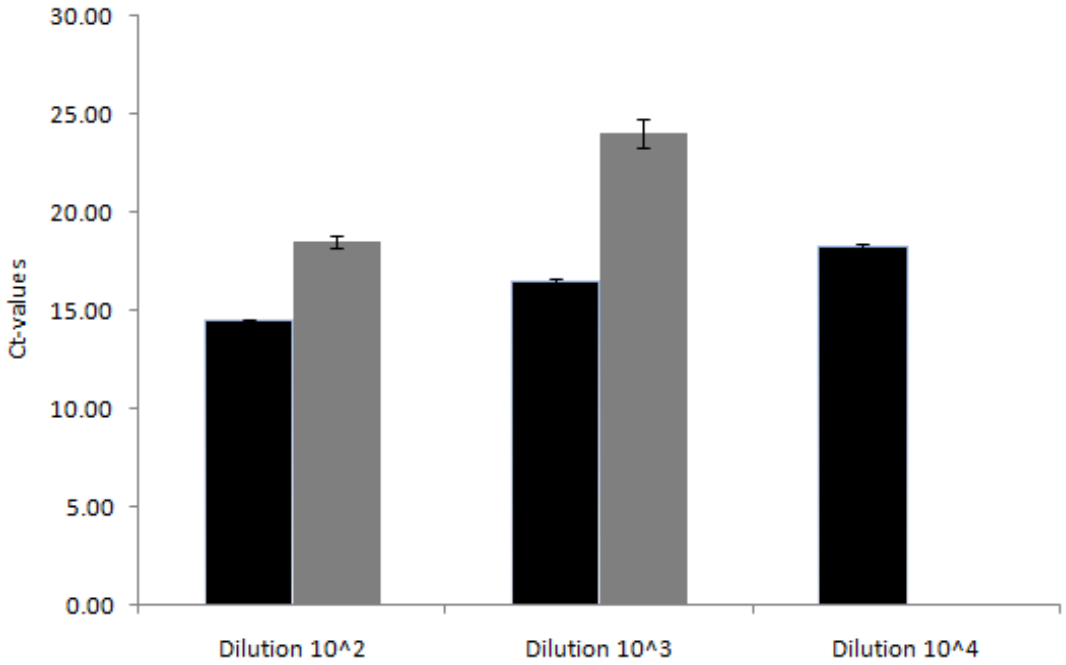


Figure 3. Histogram showing Ct values of Q-PCR for HIV-1 *gag* gene at different dilutions with and without spinning. The x axis denotes the dilutions used in the study while the Y axis denotes the Ct fluorescent values. The first, second and the third dilution had 2000, 200 and 20 virions/ml. It is clear from this graph that samples of < 20 copies/ml would be missed in a PCR test when processed by the original method while upon concentration prior to nucleic acid extraction would give the required PCR signal. The black histograms denote samples tested after spinning while the gray bars denote the same samples tested without pelleting. The values are mean ± SD of experiments carried out in triplicates.

Table 4. Determination of minimum amount of HIV-1 particles sedimented by centrifugation.

S/N	Expected viral copies/ml	Achieved viral copies/ml	% Recovery
1	1500	1480	98.66
2	150	286	>100
3	15	33	>100

known viral load to simulate the MP NAT scenario. The Ct value of the sample with 200 virions/ml without spinning (Figure 3, dilution 10^3 -grey bar) amounted to 3 copies/reaction, which was detected using Q-PCR while the same sample after spinning resulted in 20 copies/reaction (Figure 3, dilution 10^3- black bar) that is detectable with a much lower Ct value indicating an enhancement in the sensitivity of the assay method employed by centrifugation of the sample prior to nucleic acid extraction. The data presented in Figure 3 clearly indicates that the mini-pooled samples are missed by Q-PCR especially for samples with lower viral load (0.333 viral particles/reaction) when processed the regular way of using 150 µl plasma for nucleic acid extraction followed by uniplex PCR for HIV-1 using SYBR chemistry. Interestingly enough, the sample of the same dilution after concentration by pelleting could be easily detected by Q-PCR (with 1.8 virus copies/reaction). It is evident from Figure 3 that the Ct values increased with samples of lower viral loads in comparison to the samples with high viral loads. This data clearly offers an advantage of the proposed method of concentrating viruses in a MP sample for successful diagnosis of low viral load samples.

A flow chart of the 5 sample MP testing followed is depicted in Figure 4. While Figure 4A shows the pattern

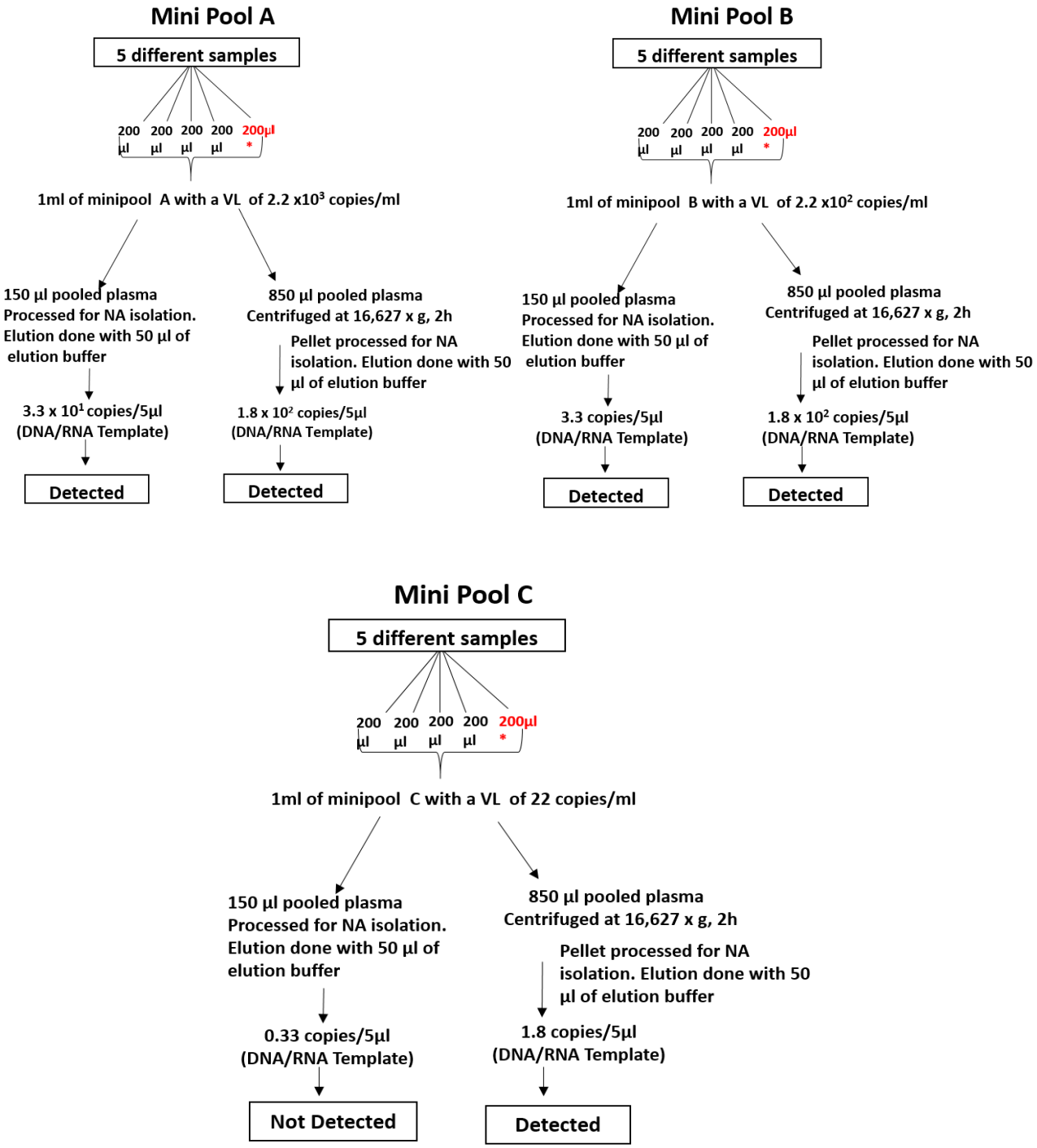


Figure 4. Flow chart showing impact of viral loads on the screening testing efficiency of minipool format. Mini Pool A and B clearly shows that when the viral load of samples is 2.2×10^3 to 2.2×10^2 copies/ml, in a 5- sample minipool format would show successful PCR signals. The samples with a viral load of ≤22 copies/ml would fail to show the required PCR, unless spun and tested (Mini Pool C).

of MP A, Figures 4B and C shows the flow of events for a MP B and MP C respectively. It is clear from these figures that when the viral loads is in the range of 2.2×10^3 copies/ml to 2.2×10^2 copies/ml and the MP comprises of 200 µl plasma pooling from every sample, the test is positive by PCR while for a MP sample with a

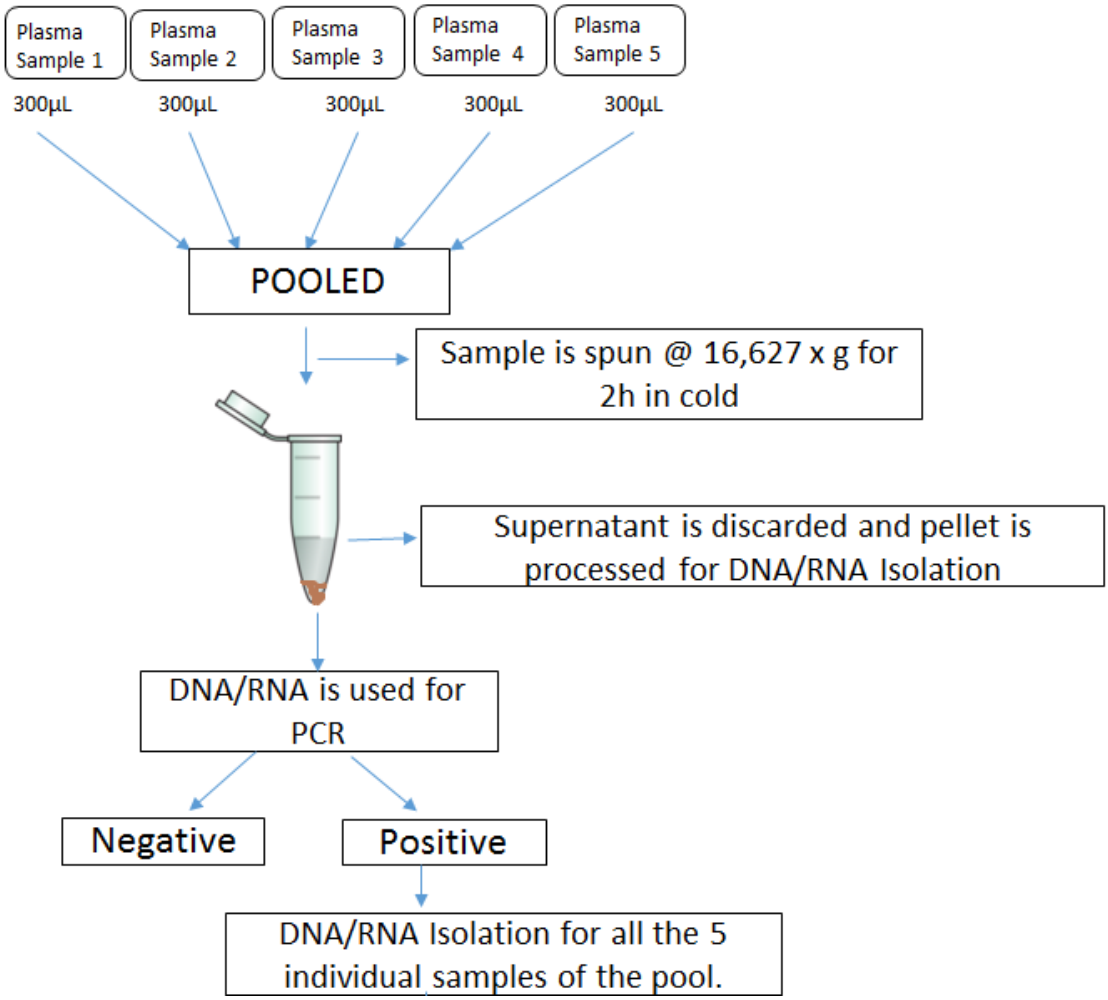


Figure 5. Schematic diagram of the strategy of mini-pooling method employed for this study. For the 148 MPs of 5 MP format of SCB samples, the pooling strategy followed is depicted as a flow chart.

sample of 22 viral copies/ml, in a 5 sample MP format, one could detect PCR signal for HIV-1 only when the sample is concentrated prior to nucleic acid extraction and not otherwise.

Testing of samples from SCB by MP testing and bacteriophage spiking studies

A secondary objective of this study was to determine the applicability of the proposed virus concentration method prior to NAT testing for screening samples in MPs. Thus, randomly selected 740 donor units were screened by both individual testing and in a 5-sample MP (that is, 148 pools).

Figure 5 shows the schematic diagram of the strategy of mini-pooling method employed for this study. Out of 740 samples (148 pools of 5 per MP) tested, pool # 87 (samples from CAN07/431 to CAN07/435) was positive for HIV-1 (Figure 6A) while pool #113 (CAN07/561 to CAN07/565) and #133 (CAN07/661 to 665) were positive for HCV (Figure 6C). Pool #145 (CAN07/721 to 725) was HBV positive (Figure 6E). Individual samples of the pool #87 (CAN07/435) was positive for HIV-1 (Figure 6B), CAN07/562 and CAN07/664 from pools #113 and #133 were positive for HCV (Figure 6D) while sample CAN07/723 was positive for HBV (Figure 6F).

Figures S2A and S2B show the plaques of T4 and MS2 phage enriched in *E. coli* BL21 and C 3000 cells respectively (See Appendix for figures). We performed phage nucleic acid spike studies for every MP that was negative for PCR for HIV-1, HBV and HCV to ensure that we did not come across any false negative results. All the samples that were negative for HBV PCR were positive for the spiked T4 phage DNA PCR and all the samples that were negative for PCR for HIV-1 an HCV showed

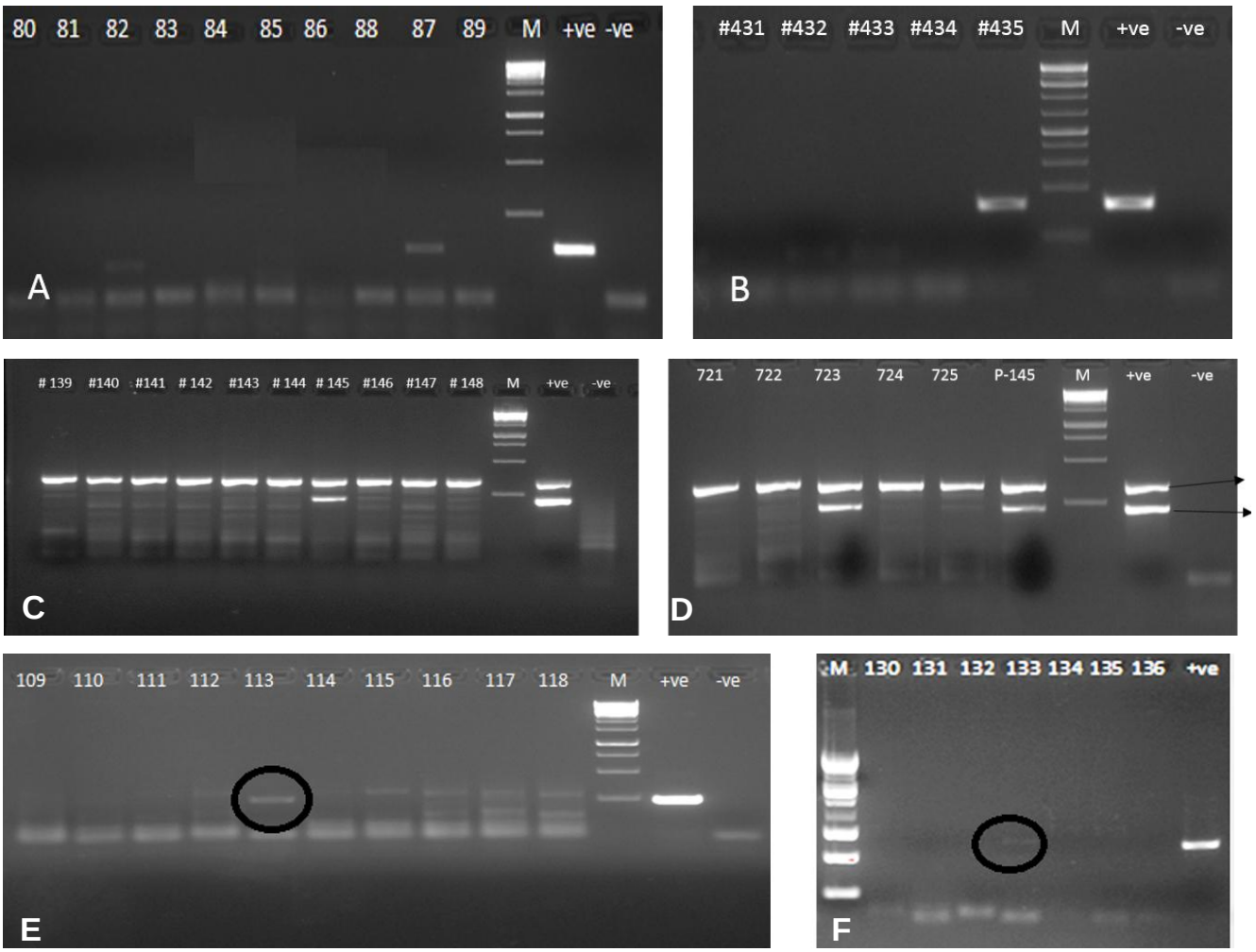


Figure 6. HIV-1, HBV and HCV PCR of the MP samples of SCB. Figure 6A shows the PCR of HIV-1 MPs from #80 to #89 while Figure 6B shows the PCR of individual samples of pool #87. Figure 6C indicates the PCR of HBV MPs from #139 to #148 while Figure 6D depicts the PCR of individual samples of pool #145. The top arrow denotes the T4 phage PCR (300 bp) while the lower arrow denotes the HBV PCR (200 bp). Figure 6E shows the PCR of HCV of MPs from #109 to #118 while Figure 6F shows the PCR of individual samples of pool #113. Since the PCR signal of HCV was faint, the PCR signals of interest have been circled for easy identification.

positive reactions with MS2 phage spiking studies. A representative gel picture for the PCR of the rIIa gene of the T4 phage (300 bp) is given in Figure 7A while the RT-PCR of the replicase gene of MS2 phage RNA (100 bp) is shown in Figure 7B indicating the possible absence of PCR inhibitors in the viral DNA templates and confirming the absence of any false negatives in the assay tests employed.

DISCUSSION

Whole-blood RDT is the standard of care tests employed in many resource-constrained countries due to their low cost, ease of use, and reliable performance (Mehra et al., 2014). False positive results with HIV RDTs have also been reported due to misinterpretation of the results, incorrect entries, cross-contamination between blood samples, persisting viral antigens post-infection and in some cases where patients are persistent carriers for viral antibodies (Klarkowski et al., 2014; Kukar et al., 2017). Shanks et al. (2015) reported that such false negative results in RDT is due to sero-conversion or misclassification by the lower sensitivity dilution test while Khuroo et al. (2014) reported that RDT's for detection of anti-HBsAg antibody is impaired due to reduced sensitivity with HBsAg *S* gene mutants. The HCV genotype differences is reported to affect the

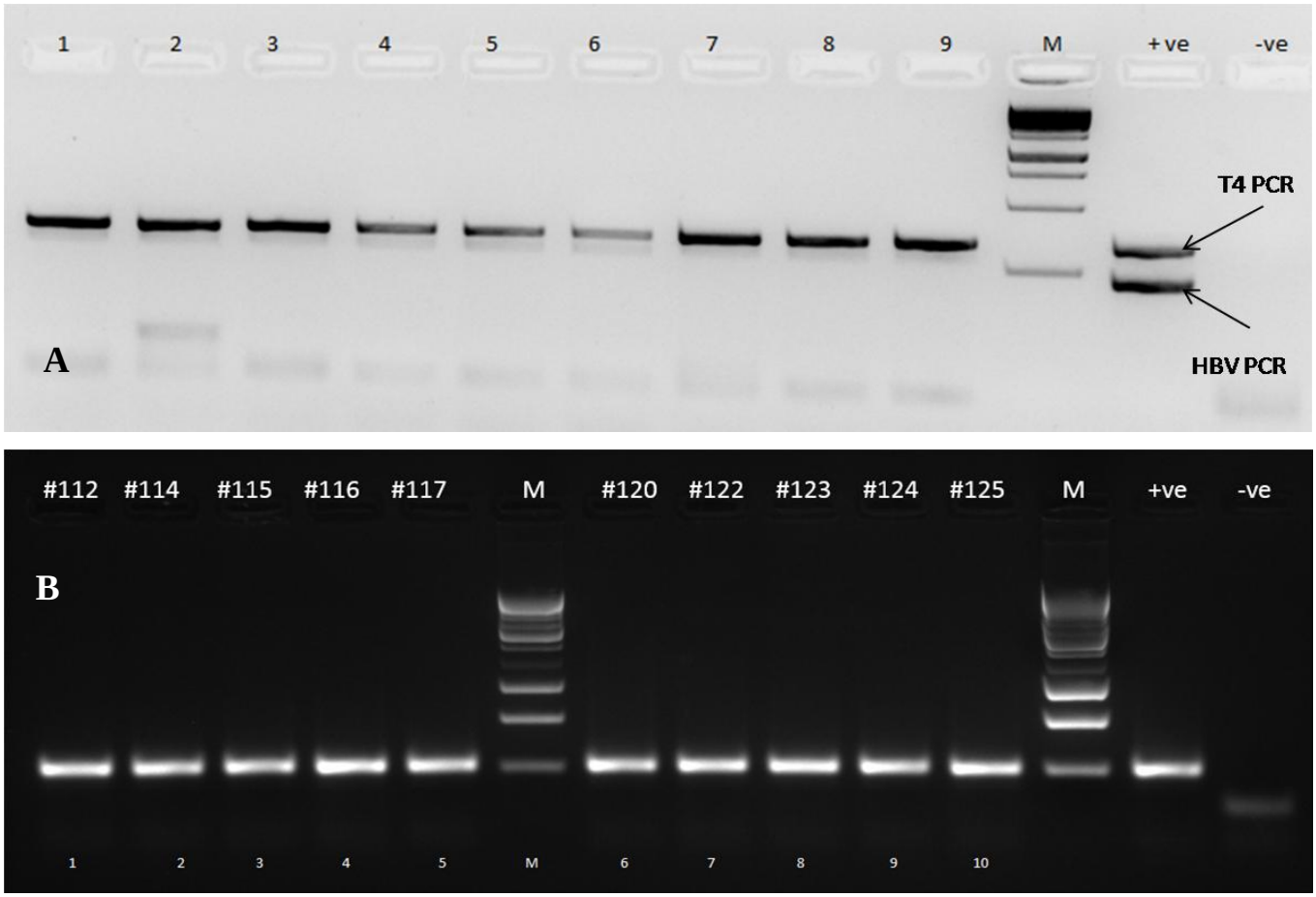


Figure 7. T4 and MS2 phage PCR's of randomly chosen negative MPs of HIV-1, HBV and HCV. We chose 10 MPs for testing T4 phage PCR while for MS2 phage RT-PCR, we chose 5 MPs each. It is clear from Figure 7A, that the samples that were negative for HBV showed clean PCR signal for the T4 phage DNA spiked studies. Lanes 1 to 10 indicate PCR with HBV negative samples used in this study. Figure 7B shows that the samples that were negative for RNA viruses such as HIV-1 and HCV did yield the expected PCR signal of the MS2 phage RNA spiked samples. Lanes 1 to 5 indicate the PCR signals achieved for HIV-1 negative samples (samples #112 to #117) while lanes 6 to 10 (samples #120 to #125) refer to the PCR signals for MS2 phage RNA with samples negative for HCV virus. +ve and -ve refer to positive and negative controls respectively. These data indicate the absence of PCR inhibitors in these RNA/DNA templates ruling out the possibility of false negative results in the tested samples.

diagnostic accuracy by RDT and HIV/HCV co-infection has been shown to show false positive HCV results for (Khuroo et al., 2015). For all these reasons, NAT appears a better alternative to RDT's (Smith et al., 2009; Shivkumar et al., 2012).

Anti-HBs is a neutralizing antibody, and its presence in plasma indicates immunity to HBV infection (Weber, 2005). The presence of both the anti-HBs and HBsAg has been documented in HBsAg positive patients, probably due to the incapability of antibodies to neutralize the circulating virions rendering them as carriers of HBV infection. In such a scenario, the NAT test is expected to be negative and this is the explanation one could give for our data on a few samples that showed negative results for the HBV PCR, although they were positive for HBV by RDT. It has been demonstrated that HBV DNA is a more reliable indicator of the presence of virus than HBe antigen (Mendy et al., 2006); hence our work presented in this article assumes significant importance.

Similarly, it is reported that a high proportion of blood donors labelled as anti-HCV antibody positive based on low antibody titres, may not be at increased risk of carrying HCV (Bar-Shany et al., 1996). This could be the reason for the observed differences in our PCR results in comparison to RDT's.

False positive results with HCV by RDT, is known to be due to cross-reactivity in anti-HCV EIA (Franzeck et al., 2013). It is possible to attribute such a reason for our observations on some samples showing positive results by RDT and negative results by PCR. Since the Q-PCR

is designed to work only on viruses that are active for causing infection, it appears that PCR results would be more reliable than the regularly employed immunoassays based RDT's or ELISA's.

MP testing is widely used and presently around 33 countries in the world have implemented NAT for HIV and around 27 countries for HBV (Stramer et al., 2013; Hans and Marwaha, 2014). In Japan and Germany, HBV NAT has been added to HIV/HCV MP-NAT blood donor screening and triplex PCR assays to detect all these three viruses are being developed (Busch, 2004). In a recent study, Yaseen et al. (2013), found 1.37% samples reactive on standard serology methods but non-reactive by NAT, which were later proved as false positive by confirmatory serological tests (Hans and Marwaha, 2014). Final dilution of the plasma samples by 1:8 has been shown to reduced detection of all targets to 50% or less (Elbjeirami et al., 2015), hence enhancing the sensitivity of the NAT assay appears extremely relevant and hence our work addressing this concern assumes critical importance.

Since NAT is being followed in blood centres of countries around the world such as the USA, Canada, Australia, New Zealand, South Africa, and some countries in Europe and Asia, there is emphasis to implement this method in India too. A number of private hospitals in Urban India have implemented NAT testing for testing viruses such as like HIV while the some of the government blood centres continue to carry out ELISA or RDT (Naidu et al., 2016). Also, since NAT reduces the window period of HBV to 10.34 days, HCV to 1.34 days and HIV to 2.93 days, it appears to be a better alternative for viral diagnosis.

In our study, one of the HIV-positive donor blood sample was arbitrarily selected and diluted five folds to achieve 22 copies/ml, which placed it much below the LOD of the Q-PCR employed (1 copy/reaction or 66 copies/ml), and therefore it was undetected by the MP assay. This was concerning as HIV-positive plasma with a viral load of less than 40 copies/ml has been demonstrated as the threshold for viremia that is indicative of ongoing virus replication associated with a risk of virologic rebound (Doyle et al., 2012). Furthermore, since reports on 67% of samples with low viral load missed by MP testing in comparison to the test by individual diagnostic test (IDT) (Chatterjee et al., 2012), the present methodology of centrifugation and analysis and detecting even virus samples of as low as 22 copies/ml in MP testing is encouraging and we are of the opinion that this strategy of viral concentration prior to extraction would be immensely useful for viral diagnostics.

Different approaches have been attempted by various researchers to concentrate different viruses from a variety of samples. A procedure to pellet HIV virus has been described by Kohno et al. (2002) that involves incubating equal volumes of virus samples and 20% PEG 20,000 in cold for 16 h followed by centrifugation at 17,860 × g for 20 min. Hjelmsø et al. (2017) adopted four steps to concentrate virions from the sewage samples that included protein precipitation with PEG, organic flocculation followed by filtration with positively charged filters. Concentration of pathogenic human viruses like adenoviruses and polyoma viruses has been achieved by alkaline treatment and ultracentrifugation (Ahmed et al., 2015). Various types of materials like magnetic beads and filters (Bidawid et al., 2000; Chen et al., 2010) and centrifugal devices (Yi, 2010) have been used to concentrate viruses. Satoh et al. (2008) concentrated of viruses by agglutination using poly-L-lysine in the presence of a bivalent metal, while Sanyal et al. (1991) and McHutchison et al. (1999) employed ultracentrifugation at 23,000 × g for concentrating HBV-DNA from serum. Other reports of virus concentration include concentration of HCV by centrifugation at 120,000 g for 1.5 h (Bull et al., 2016) and a sucrose gradient centrifugation (Fuscaldo et al., 1971). The virus purified using the low-speed centrifugation method contained as few contaminating proteins as the equivalent amounts of the lentivirus purchased commercially (Jiang et al., 2015). Damond et al. (2002) have mentioned the use of centrifugation of 1 ml plasma for enhancing the sensitivity of viral load of HIV-2.

Zhang et al. (2010) reported that the inhibition of RT-PCR in plasma samples occur due to PCR inhibitors in the plasma and the frequency of such an inhibition are between 0.34 to 2.4% in patients infected with HIV and hepatitis C virus, respectively. PCR inhibitors include heme, leucocyte DNA, EDTA, heparin, IgG etc. that are carried over when a higher volume of plasma (vRNA template) is processed. Our results on higher recovery of virus concentration after centrifugation reflect the possible removal of such soluble inhibitors by centrifugation. Hence, our proposed method of using 300 µl plasma for mini-pooling appears relevant and significant to achieve 100% accuracy in viral testing. In addition, the results demonstrated that centrifugation of plasma increased the sensitivity of detection by at least 20 to 50 folds irrespective of the size of the MP, which is interesting. This is important in studies requiring accurate and sensitive quantification of viruses when low copy numbers are anticipated. Kuhns et al. (2004) suggest that nearly 6% samples are negative by current MP HBV NAT methods and we strongly believe that such errors could be avoided by implementation of our proposed methodology in MP testing.

Centrifugation of plasma is reported to remove pathogenic substances such as auto antibodies, lipoproteins and circulating immune complexes from the plasma (Hafer et al., 2016). The lipids, that are PCR inhibitors, are also reported to be removed by high speed centrifugation. Our observations of higher recovery of

viruses especially with low viral counts could be attributed to these reasons.

The LOD of ID-NAT of a 10 × 10 matrix pools for HIV-1 is reported in the range of 500-1500 copies/ml and MPs of 5 was 250-500 copies/ml (Smith et al., 2009). By the methodology described by us, the LOD of the HIV-1 was ~10 copies/ml for any MP size be it 5 or 10 or 50 since the viral particles from all the samples are pelleted before processing for nucleic acid extraction. This data appears to be a major advancement in the field of viral diagnosis and would be of immense use in blood banks and other diagnostics centres which rely on MP testing.

The volume of plasma that is taken for mini-pooling is usually 20 µl (vanZyl et al., 2011) and some studies refer this as 500 µl (Chandrashekar, 2014) based on the intended pool size and the column used for extraction of nucleic acid from the virus containing samples. The sensitivity of the test is pool size dependent and is not obvious for several diagnostic centres according to Shyamala (2014), with the result that one come across false negative tests.

The Roche CobasAmpliPrep/CobasTaqMan real time HIV-1 test have lower limits of quantitation of 40 and 20 copies/ml, respectively while the Abbott Real Time HIV-1 assay (Real Time) (Abbott Molecular Inc., USA) and the Qiagen Artus HIV-1 QS-RGQ assay, have lower limits of quantitation of 40 and 45 copies/ml, respectively (Swenson et al., 2014). These kits, however, would fail to detect signals for samples with ≤ 20 copies/ml if the sample is in MPs of any pool size either 5 or 10 or 24. This is because even in a pool size of 5, the sample with ≤ 20 copies/ml is diluted 5 times resulting in a final viral load of ≤ 4 copies/ml. When 200 µl of such a pool is taken for nucleic acid extraction, the yield would be 0.06 copies/reaction which will be negative when tested by any of the above kit reagents. However, when we subject the same sample to centrifugation prior to nucleic acid extraction, one would have a minimum of 20 copies/200 µl which would be 2 copies/reaction that could be easily detected. The data described in this article, has indeed helped us to address our primary objective of determining utility, feasibility, and cost-effectiveness of MP-NAT for blood donor screening.

Low HIV-1 viral loads (LVL) (50 to 1,000 copies/ml) may predict virologic failure for HIV-infected patients viral therapy regime and may dictate a need for changes in the drug regimen (Amendola et al., 2014) and plasma HIV-1 viral load has been suggested as a better predictor of progression to AIDS and death over the number of CD4+ T cells (Mellors et al., 1996), hence, our work on detecting viral loads for samples < 20 viral copies/ml assumes critical importance.

We recognize that our study has limitations worth mentioning. The ideal condition to carry out a prevalence study is to sample the general population while here we have studied all the viral positive samples. Also, the number of positive samples that we have tested is not very high. In spite of the limitations discussed above, we believe that the present study may still contribute invaluable data for better viral diagnostics. Since NAT-based screening, till date, does not include a virus concentration step before nucleic acid extraction (Cardoso et al., 1998), our observations of successful pelleting viruses before nucleic acid extraction is novel and easily adaptable to individual NAT or even MP testing with assured success.

The false-negative results that occur infrequently during routine infectious-disease donor screenings can be avoided following this proposed protocol of an additional step of concentration of viruses prior to the isolation of their nucleic acid for detection. This method would prove useful to elucidate aspects that confer resistance to viral therapies, including mutations and genotype disparity studies. Although, the example of HIV is used throughout the article, the area of application is more general and can be applied to other viruses.

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Appendix

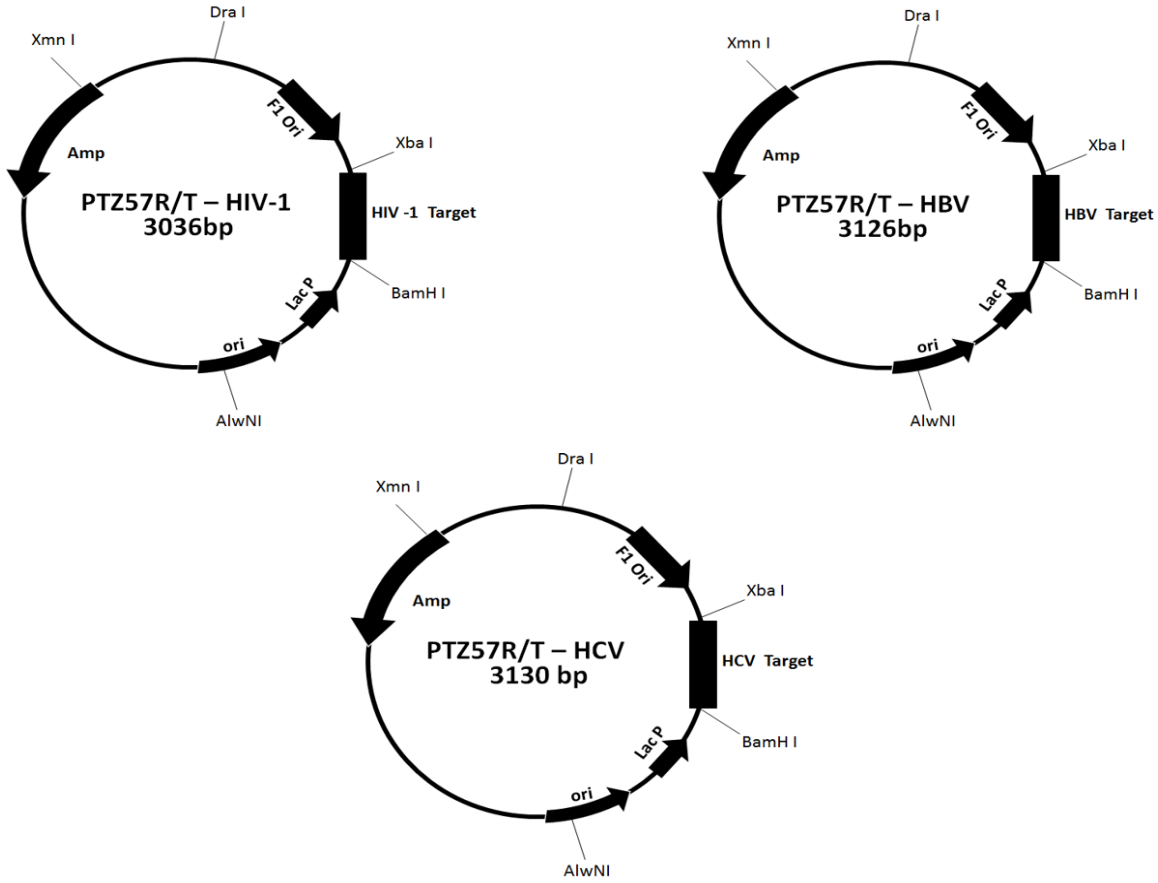

Figure S1. Plasmid maps of reference standards used in this study. Note that the fragment cloned in the TA cloning vector pTZ57R/T was 150 bp for HIV-1, 200 bp for HBV and 240 bp for HCV.

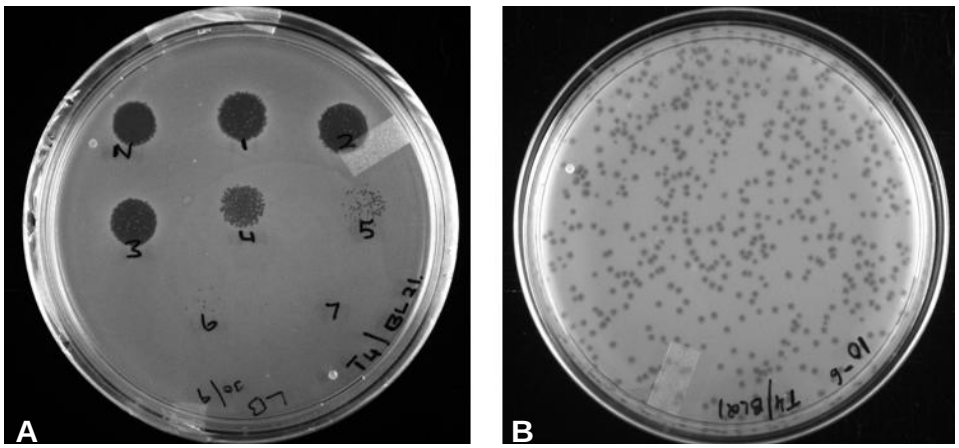

Figure S2. Morphology of T4 phage on lawn of *E. coli* BL21. Figure S2A shows the RTD of the enriched T4 phage to enumerate pfu/litre of the medium while Figure S2B shows the plaques of T4 phage on a lawn of BL21 cells.

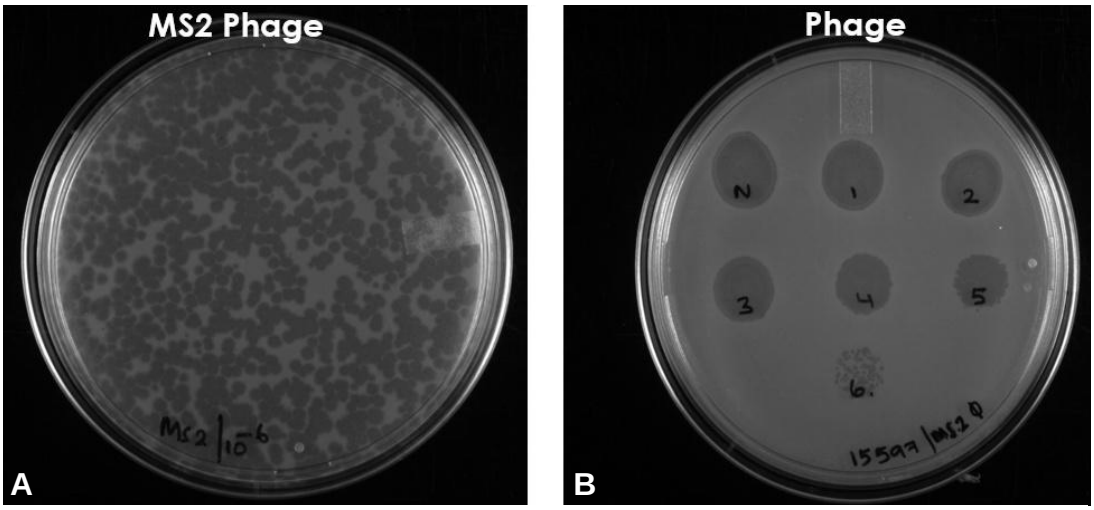

Figure S3. Morphology of MS2 phages on lawn of *E. coli* C 3000. Figure A shows the plaques of MS2 phage on a lawn of *E. coli* C3000 cells while Figure B shows the RTD of the enriched phage to enumerate pfu/litre of the medium.

Table S1. HIV-1 viral load by Q-PCR using SYBR green.

Sample No	Age	Sex	Viral load /ml
Sample 1	60	Female	1.9×10^{6}
Sample 2	42	Female	4.1×10^{5}
Sample 3	46	Male	6.8×10^{6}
Sample 4	45	Male	2.9×10^{5}
Sample 5	32	Female	6.1×10^{5}
Sample 6	49	Male	7.2×10^{2}
Sample 7	40	Male	6.6×10^{2}
Sample 8	50	Female	7.7×10^{4}
Sample 9	18	Male	5.8×10^{5}
Sample 10	45	Male	5.6×10^{4}
Sample 11	42	Male	2.1×10^{3}
Sample 12	35	Male	2.8×10^{4}
Sample 13	45	Male	3.0×10^{3}
Sample 14	70	Male	7.9×10^{2}
Sample 15	39	Male	3.8×10^{4}
Sample 16	48	Male	1.3×10^{2}
Sample 17	24	Female	5.9×10^{5}
Sample 18	40	Male	5.6×10^{5}
Sample 19	35	Male	4.0×10^{4}
Sample 20	50	Male	1.5×10^{4}