Technical Note

Random PCR and ultracentrifugation increases sensitivity and throughput of VIDISCA for screening of pathogens in clinical specimens

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Abstract

Introduction: Virus discovery based on cDNA-AFLP (VIDISCA) is a sequence-independent virus discovery method that was recently developed and successfully used to characterize unknown viruses in cell cultures. Its applicability, however, is limited by its low sensitivity.
Methodology: We evaluated whether the introduction of prior amplification of target sequences by random PCR (rPCR) increases the sensitivity of this method to improve its use on clinical specimens. In addition, ultracentrifugation was added to the protocol to allow for pooling of multiple samples, thereby increasing analytical throughput of the VIDSCA.
Results: We showed that rPCR enhanced the sensitivity of VIDISCA by 100-fold for two out of four viruses in different clinical samples, and that the ultracentrifugation step allowed for analyzing samples of large volumes (4 ml) and simultaneous processing of multiple (~40) clinical specimens.
Conclusions: We conclude that this modified VIDISCA protocol is a relatively easy method to use for screening of large numbers of clinical samples that are suspected to contain previously unrecognized pathogens, in settings where ultradeep sequencing platforms are not available.

Key words: random PCR; VIDISCA; ultracentrifugatiaon; virus discovery

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Introduction

The identification of several new viruses in the last decade shows that our list of viral pathogens is incomplete and stimulates the search for unknown pathogens [1-3]. Furthermore, Southeast Asia has been suggested to represent a hotspot for past and future emerging diseases [4], indicating that active surveillance for unknown pathogens is warranted in this region. The identification of new viruses, however, is challenging since viral load in clinical samples may be low, and the genetic sequences of unknown viruses are not available for specific primer design for the purpose of sensitive molecular detection methods.

Virus Discovery based on cDNA Amplified Fragment Length Polymorphism (AFLP) (VIDISCA) is a sequence-independent technique that has proven to be a successful tool for the detection of unknown viruses [5]. However, relatively low sensitivity (detection limit: 10^6 genome equivalents (GE) per ml for RNA virus [6]) limits its use to identify viruses already successfully cultured or abundantly present in clinical specimens [5,7,8]. Unfortunately, not all human viral pathogens can infect currently available laboratory cell lines, and at clinical presentation, viral titers in clinical specimens may not be sufficient for direct detection by VIDISCA.

Random-PCR (rPCR), utilizing a generic primer with a random hexamer 3'-end, has the capacity to enrich nucleic acids in clinical samples [9,10]. We evaluated the addition of rPCR to the VIDISCA method to increase its sensitivity and thereby allow for a broader application, *e.g.* on clinical samples. Additionally, we investigated whether the introduction of ultracentrifugation would allow for pooling of multiple clinical samples, thereby increasing the throughput of the VIDISCA method. Here, we show that the addition of rPCR and ultracentrifugation renders VIDISCA suitable for

Table 1. Oligonucleotide sequences used for rPCR and AFLP [5, 10]

Oligonucleotide sequences	Sequences (5' – 3')
FR26RV-N	GCCGGAGCTCTGCAGATATCNNNNNN
FR20RV	GCCGGAGCTCTGCAGATATC
HinP1-I standard	GAC GAT GAG TCC TGA CCG C
MseI standard	CTC GTA GAC TGC GTA CCT AA
HinP1-I-A	GAC GAT GAG TCC TGA CCG CA
HinP1-I-T	GAC GAT GAG TCC TGA CCG CT
HinP1-I-C	GAC GAT GAG TCC TGA CCG CC
HinP1-I-G	GAC GAT GAG TCC TGA CCG CG
MseI-A	CTC GTA GAC TGC GTA CCT AAA
MseI-T	CTC GTA GAC TGC GTA CCT AAT
MseI-C	CTC GTA GAC TGC GTA CCT AAC
MseI-G	CTC GTA GAC TGC GTA CCT AAG
HinP1-I anchor top strand	GAC GAT GAG TCC TGA C
HinP1-I anchor bottom strand	CGG TCA GGA CTC AT
MseI anchor top strand	CTC GTA GAC TGC GTA CC
MseI anchor bottom strand	TAG GTA CGC AGT C

high throughput screening of clinical samples for previously unrecognized pathogens.

Methodology

Patient samples and viral-load measurements

A plasma sample from a patient with laboratory-confirmed dengue virus type 1 (DENV-1) infection, a throat swab in viral transport medium (VTM) from a patient with laboratory-confirmed enterovirus encephalitis, and a nasal pharyngeal aspirate (NPA) in VTM from a patient co-infected with respiratory syncytial virus (RSV) and TTV-like mini virus infection were used. The concentrations of DENV-1 and EV in the clinical samples were quantified with previously described real-time RT-PCR methods [11,12]. For RSV, viral load measurement was done by using an in-house assay (manuscript in preparation).

As negative controls, we used a plasma specimen from a healthy volunteer for the DENV-1 positive plasma, and VTM for the respiratory samples collected in VTM.

Oligonucleotide sequences

Sequences of oligonucleotides used in this study (Table 1) were adapted from Froussard *et al.* [10] and van der Hoek *et al.* [5] and were synthesized by Sigma-Proligo (Singapore).

Sample pretreatment, RNA isolation and ultracentrifugation

Pretreatment of clinical samples was performed according to the VIDISCA protocol as described elsewhere [5]. In brief, eukaryotic cells were removed by centrifugation and the supernatant was subjected to DNase I treatment (New England Biolabs [NEB], Ipswich, MA, USA). Viral RNA was subsequently isolated with the easyMAG system (bioMérieux, Marcy l'Étoile, France), following the manufacturer's instructions.

Ultracentrifugation was done at $3.4 \cdot 10^5 \times g$ for 90 minutes at 4°C with use of an MLA-80 fixed angled rotor in an Optima MAX ultracentrifuge (Beckman Coulter, Inc., Fullerton, CA, USA).

Pre-enrichment of viral RNA by rPCR

Isolated RNA was pre-amplified by rPCR [9,10]. A two-step reverse transcription (RT) reaction was performed in a final reaction volume of 20 µl containing 10 µl of isolated RNA; 1 µM of primer FR26RV-N (Table 1); 0.5 mM of each dNTP (Roche Diagnostics GmbH, Mannheim, Germany); 5 mM DTT; 40 U of RNAout; and 200 U of Super Script III in 1X First Strand buffer (Invitrogen, Carlsbad, CA, USA). First 13 µl containing the isolated RNA, primer FR26RV-N, and dNTPs was incubated at 65°C for 5 minutes and immediately chilled on ice for at least 1 minute. The remaining 7 µl of DTT, RNAout, Super Script III and First Strand buffer were added to a final volume of 20 µl. The reaction was incubated at 25°C for 10 minutes and 37°C for 1 hour, followed by a denaturation- and enzyme-inactivation step at 94°C for 3 minutes. For double-stranded DNA synthesis of the resulting cDNA, 13 U of Sequenase T7 DNA polymerase version 2.0 (Amersham, Uppsala, Sweden) were added, followed

Table 2. The effect of rPCR on extracted viral RNA

Sample dilution	DENV-1 real time PCR result (Ct value)	
	Before rPCR	**After rPCR**
10^{-3}	27.5	10.9
10^{-4}	30.5	18.2

by incubation at 37°C for 1 hour and enzyme-inactivation at 75°C for 10 minutes.

Five µl of the reaction mixture was used for subsequent PCR with primer FR20RV (Table 1). PCR amplification was performed in a total reaction volume of 50 µl containing each dNTP (Roche Diagnostics) at 0.2 mM; 0.4µM of primer FR20RV; 2.5 mM of $MgCl_2$; 1X PCR buffer; and 2.5 U of hot start *Taq* DNA polymerase (QIAgen GmbH, Hilden, Germany). The PCR was carried out in a Tetrad PCR machine (BioRad, Hercules, CA, USA) with an enzyme-activation step at 95°C for 14 minutes and 30 seconds followed by 40 cycles of touch-down amplification consisting of 15 cycles of 95°C for 30 seconds, 65°C for 1 minute (decreasing by 1°C after each cycle), and 72 °C for 2 min; 25 cycles of 95°C for 30 seconds, 50°C for 1 minute, and 72°C for 2 minutes; and finally 72°C for 10 minutes.

After amplification, PCR products were purified using QIAquick PCR Purification Kits (QIAgen) and recovered in 30 µl of molecular grade water (Sigma Chemical Co., St Louis, MO, USA).

VIDISCA

The VIDISCA procedure was performed as described by van der Hoek *et al*. [5]. In short, isolated viral RNA was reversely transcribed using superscript III (Invitrogen, Carlsbad, CA, USA) and random hexamer primers (Roche Diagnostics GmbH, Mannheim, Germany), followed by a second-strand synthesis step with sequenase T7 DNA polymerase (Amersham, Uppsala, Sweden) and RNase H (NEB) [6]. Next either the obtained phenol/chloroform purified dsDNA or obtained rPCR product was digested by *Hin*P1I and *Mse*I (NEB), ligated to prepared *Hin*P1I and *Mse*1 adaptors by T4 DNA ligase (NEB), pre-amplified by PCR utilizing *Hin*P1I- and *Mse*I standard primers and, finally, amplified with primers *Hin*P1I-N and *Mse*I-N (Table 1), where N (A/T/C/G) represents one of four nucleotides at the 3' end of standard primers.

PCR products were separated on agarose gel. Fragments only present in lanes of patient samples were cloned into TOPO pCR2.1 (Invitrogen), and used to transform chemically competent TOP10 *Escherichia coli*, followed by amplification and sequencing (Applied Biosystems) with M13 primers and homology BLAST search (www.ncbi.nlm.nih.gov/blast).

Results

Viral RNA enrichment by rPCR

Because viral nucleic acids may be present in clinical samples at relatively low concentrations, we used rPCR, a sequence-independent amplification technique, to pre-amplify RNA from samples. In rPCR, a primer consists of a random hexamer at the 3' end and a second primer sequence at the 5' end. This second primer sequence is thus incorporated into the resulting double-stranded sequences during second-strand synthesis, and its complementary sequence serves as a primer binding site in the next amplification step (Figure 1).

To monitor the effect of rPCR on isolated viral RNA, a DENV-1 positive plasma sample was diluted in plasma derived from a healthy person. rPCR resulted in a substantial increase of target sequences as shown by reductions in cycle of threshold (Ct) values obtained from the DENV-1-specific real-time RT-PCR of samples after rPCR when compared to samples without prior random amplification (Table 2). Assuming a 10-fold difference in the number of target sequences for each 3 to 4 PCR cycles, the levels after rPCR are estimated to be 3 to 5 log higher than without preamplification.

Sensitivity enhancement of conventional VIDISCA by rPCR

AFLP using selected restriction enzymes allows for enrichment of viral sequences and facilitates easier differentiation of fragments [5]. While in conventional VIDISCA, dsDNA was used as input for AFLP, the end product of rPCR was used in the rPCR enhanced VIDISCA (Figure 1).

VIDISCA testing with and without prior rPCR of a dilution series of DENV-1 plasma (equivalent to viral loads of 7×10^7, 7×10^6, 7×10^5 and 7×10^4 GE per

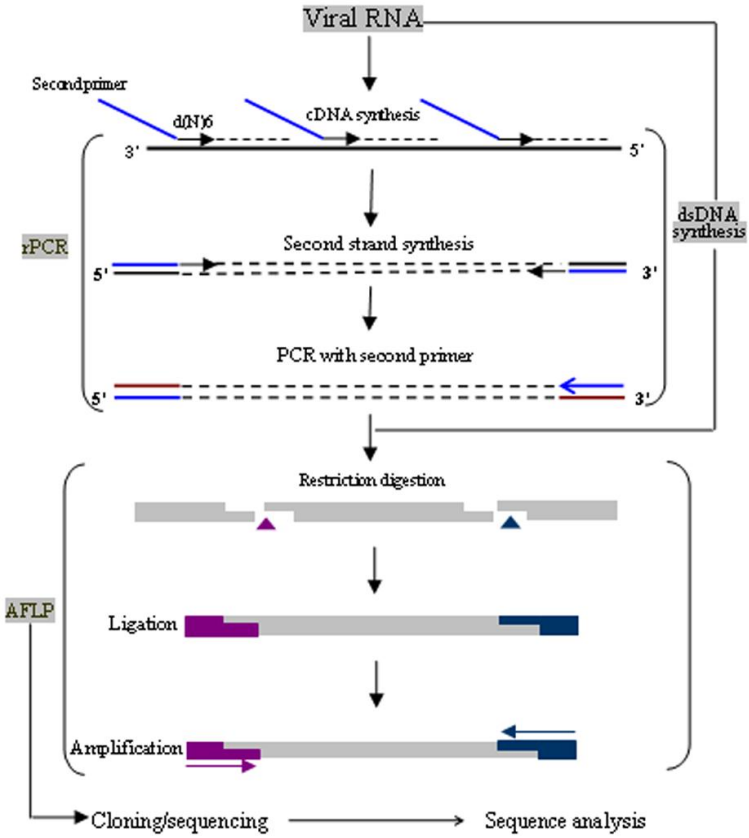

Figure 1.
An overview of VIDISCA/rPCR enhanced VIDISCA [5], and proposed mechanism of rPCR. Triangles indicate restriction enzymes (*Hin*P1 I and *Mse*I)

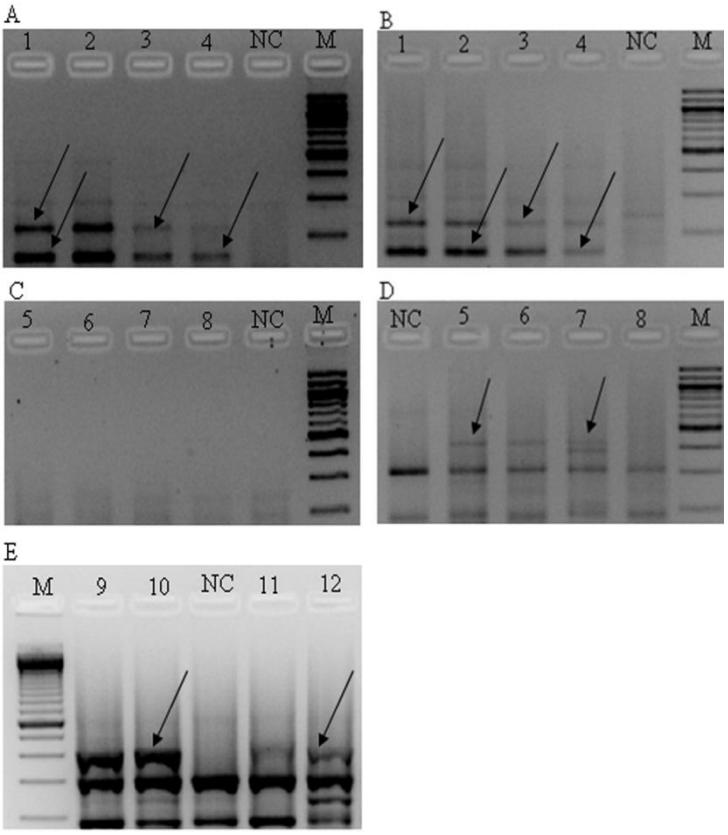

Figure 2.
Representative AFLP patterns of the DENV-1 positive plasma. DENV-1 amplified products are indicated by arrows. A and C are VIDISCA products of primer combinations: HinP1-I-T and MseI-C, and HinP1-I-G and MseI-C, respectively. B and D are rPCR enhanced VIDISCA products of primer combinations HinP1-I-T and MseI-C, and HinP1-I-G and MseI-C, respectively. E is rPCR enhanced VIDISCA products of primer combination HinP1-I-G and MseI-C obtained from 1:40-diluted plasma samples after ultracentrifugation. Lanes: 1 and 2: viral load: 7×10^7 GE/ml in duplicate; Lanes: 3 and 4: viral load: 7×10^6 GE/ml; Lanes: 5 and 6: viral load: 7×10^5 GE/ml; Lanes 7 and 8: viral load: 7×10^4 GE/ml; Lanes: 9 and 10: viral load 1×10^4 GE/ml; Lanes: 11 and 12: viral load 1×10^3 GE/ml

NC: negative control, M: molecular weight marker

Table 3. Sensitivity comparison between the VIDISCA and rPCR enhanced VIDISCA

Virus	Sample type	Sensitivity (GE/ml or sample dilution)	
		Normal VIDISCA	Enhanced VIDISCA
DENV1	plasma	7×10^6	7×10^4
TTV-like mini virus	NPA	10^{-2}	10^{-4}
RSV	NPA	2×10^6	2×10^6
Enterovirus	Throat swab	2×10^6	2×10^6

ml of plasma) showed a 100-fold reduction of the detection limit after prior rPCR (7×10^4 vs 7×10^6 GE/ml [Figure 2]).

When evaluating the dilution series of an NPA from a patient co-infected with a TTV-like mini virus and RSV, the TTV-like virus was likewise detected at 100-fold lower concentrations after prior rPCR (10^4 vs. 10^2 diluted sample [data not shown]), whereas the detection limits of VIDISCA with and without rPCR were similar for RSV (2×10^6 GE per ml (data not shown)). Finally, as for RSV, dilution series of an EV-positive throat swab from an encephalitis patient showed similar detection limits in both procedures (2×10^6 GE per ml (data not shown)). A summary on sensitivity comparisons between VIDISCA and rPCR-enhanced VIDISCA is presented in table 3.

Addition of ultracentrifugation to process multiple samples

We evaluated whether the introduction of an ultracentrifugation step would make rPCR enhanced VIDISCA more suitable for high throughput screening. Using 10 ml ultracentrifugation vials and 100 ul of clinical samples, 40 samples were analyzed at the same time. Testing of a DENV-1 positive plasma specimen showed that Ct values of a virus-specific real-time RT-PCR remained unchanged after ultracentrifugation of 1:40 diluted specimens, indicating no loss of target sequences and similar sensitivity of detection (Table 4). Likewise, VIDISCA patterns were unaffected compared to those from undiluted specimens (Figure 2E).

Practically, the entire protocol requires four days for completion, both for single or pooled samples.

Discussion

The diversity of human pathogens is far greater than previously appreciated [4]. Therefore, active surveillance for unknown emerging pathogens is crucial for preparedness and response to future infectious diseases. Because genomic information of unknown viral targets is not available for selection of specific primers; and because viral load might be low in clinical samples, an ideal virus discovery method should be sensitive and sequence independent.

Several methods for detection of unknown viruses exist, but virus discovery tools which depend on conserved sequences, such as generic PCRs targeted at virus families [13,14], pan-viral microarray [15] and multiplex PCR platforms utilizing multiple degenerate primers targeted at several different viral species (eg MassTag PCR) [16] are not useful when unknown pathogens are only distantly or not at all related to known viruses. Sequence-independent techniques such as random PCR followed by cloning and sequencing of PCR products might be sensitive but are laborious and have low efficiency [9]. Other sequence-independent methods that are based on high throughput, such as ultradeep sequencing technologies [17,18], provide promising means for detection and identification of unknown pathogens. However, current high costs and the requirement of appropriate bioinformatics support prevent their widespread use, particularly in resource-poor regions such as Southeast Asia where novel or previously unrecognized pathogens are likely to emerge [4]

VIDISCA is a sequence-independent method that does not require advanced technical expertise to operate, but applicability is narrowed by its low sensitivity [5,7,8].

In the present study, we have shown that combining VIDISCA with rPCR reduced the limit of detection in clinical specimens by 100-fold for two out of four viruses tested (DENV-1 and TTV-like virus). For the two other viruses (EV and RSV), the detection limit of VIDISCA was in accordance with that reported previously for other RNA viruses (appr 10^6 GE per ml) [6], but was not improved by the addition of prior rPCR.

In silico digestion of available RSV genomes (GenBank accession numbers: FJ614813.1,

Table 4. Validation of the recovery efficiency of ultracentrifuge

	DENV-1 positive samples			
Starting viral load (GE/ml)	4×10^5		4×10^4	
Sample pre-treatment	Non-diluted	1:40 dilution, ultracentrifugation	Non-diluted	1:40 dilution, ultracentrifugation
Viral load (GE/ml)	4×10^5	1×10^4	4×10^4	1×10^3
Sample volume for ultracentrifugation	NA	4ml	NA	4ml
RT PCR (Ct values)	27,5	28,0	30,5	31,0

NA: not applicable, GE/ml: Genome equivalent per ml

NC_001803.1, U39662.1, AY911262.1, AY353550.1, AF013254.1 and U39661.1) using the VIDISCA enzymes may provide an explanation for the limited sensitivity of detection: *In silico* digestion of RSV genomes only produced 0 – 4 fragments of different isolates in comparison with 17 fragments obtained from DENV-1 genome (GenBank accession number: NC_001477), (data not shown). As the AFLP part of VIDISCA relies on amplification of digestion fragments, the relatively low number of digestion fragments in the case of RSV likely reduces the chance of successful amplification. This suggests that to enable detection of the full range of viral pathogens, modifications on existing restriction enzymes of the VIDISCA protocol may be required. We do not, however, have explanations for the detection limit of EV.

In summary, combined with rPCR and ultracentrifugation, the VIDISCA method allows for sensitive and high throughput screening of novel or previously unrecognized pathogens. In this format, the protocol is most fit for retrospective use on batches of collected undiagnosed samples or for use in an outbreak setting. Because of the time it takes (~4days), routine real-time diagnostic use is not an option. The method can be performed in any laboratory that has the capacity for PCR and sequencing thereby increasing feasibility of pathogen discovery in relatively resource-limited regions, including those where new pathogens are most likely to emerge.

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