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Digital PCR provides absolute quantitation of viral load for an occult RNA virus

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Using a multiplexed LNA-based Taqman assay, RT-digital PCR (RT-dPCR) was performed in a prefabricated microfluidic device that monitored absolute viral load in native and immortalized cell lines, overall precision of detection, and the absolute detection limit of an occult RNA virus GB Virus Type C (GBV-C). RT-dPCR had on average a 10% lower overall coefficient of variation (CV, a measurement of precision) for viral load testing than RT-qPCR and had a higher overall detection limit, able to quantify as low as three 5′-UTR molecules of GBV-C genome. Two commercial high-yield *in vitro* transcription kits (T7 Ribomax Express by Promega and Ampliscribe T7 Flash by Epicentre) were compared to amplify GBV-C RNA genome with T7-mediated amplification. The Ampliscribe T7 Flash outperformed the T7 Ribomax Express in yield of full-length GBV-C RNA genome. THP-1 cells (a model of monocytic derived cells) were transfected with GBV-C, yielding infectious virions that replicated over a 120 h time course and could be infected directly. This study provides the first evidence of GBV-C replication in monocytic derived clonal cells. Thus far, it is the only study using a microfluidic device that measures directly viral load of mammalian RNA virus in a digital format without need for a standard curve.

1. Introduction

Viral load testing using nucleic acids has become more prevalent over the last decade due to the advent of new technologies such as reverse transcriptase quantitative PCR or RT-qPCR (Niesters, 2001). This technology has been very useful for the diagnosis of viral disease, monitoring antiviral therapy, and providing a clean blood supply (Bustin and Mueller, 2005). This sensitivity becomes crucial for the management of many viral pathogens such as the Hepatitis C Virus and the Human Immunodeficiency Virus Type-1 (HIV-1) (Kleter et al., 1993; Chun et al., 1997). RT-qPCR is highly sensitive for viral load testing, but major problems exist with assay variability, mainly due to mass-based standard curve construction (Bustin, 2000; Freeman et al., 1999). Mass-based standard curves do not allow the absolute detection of viral load. Downstream problems include lack of normalization between sample runs, and a high coefficient of variation/standard error of the mean (CV/SEM) between replicates (Rutledge and Côté, 2003; White et al., 2009).

Digital PCR (RT-dPCR), which stems from RT-qPCR, is a limiting dilution technique that physically separates individual nucleic acid molecules on a microfluidic chip. The real-time PCR products are administered into 770 individual reaction wells (Vogelstein and Kinzler, 1999; Bhat et al., 2009). This physical partitioning allows for positive PCR amplifications to be counted directly as the number of individual molecules per well at the reaction endpoint. The limiting dilution factor is chosen such that over 50% of the chip contains no template molecules per reaction well, giving a "0" (negative) result or a "1" (positive) result indicating one molecule per positive well. A microfluidic digital PCR chip (Fluidigm) with a capacity of 48 samples and a total scale of 36,960 nanoliter chambers were used to measure viral infection. The chip-based technology allows for standard real-time PCR chemistry assays (*i.e.* Taqman) without the use of an exogenous or endogenous standard and directly measures abundance of the amplicon of interest.

Occult viruses are infections in which the patient has viremia without a clinical disease (Piroth et al., 2008). However, infection with these viruses can be associated with other clinically diseased states. For example, when HIV-1 positive patients are co-infected with GB Virus Type-C (GBV-C), there is a decrease in the temporal progression to AIDS (Williams et al., 2004). Occult viruses are often not screened with common serological tests because they are unknown or novel and establishing their association with a particular disease may require extensive investigation. RT-dPCR provides an effective means for testing the viral load for occult viruses that

Abbreviations: GBV-C, GB Virus Type C; RT-qPCR, reverse transcriptase quantitative PCR; LNA, locked nucleic acid probes; RT-dPCR, reverse transcriptase digital PCR; CV, coefficient of variation; SEM, standard error of mean.

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could easily be overlooked. It can specifically detect the presence of any viral genome of interest early in infection with prior sequence information available and bear key information needed for comprehensive patient prognosis.

GBV-C is a positive-stranded RNA lymphotropic flavivirus. It shares a 30% amino acid homology to the Hepatitis C Virus (HCV), also a member of the *Flaviviridae*, but is related more closely to the GBV-A and GBV-B viruses (Simons et al., 2000). Although 3% of the population is infected with GBV-C, blood banks do not screen for its presence since no disease state has been associated with GBV-C infection (Simons et al., 2000; Xiang et al., 2000). Several studies have shown that patients infected with HIV-1 and co-infected with GBV-C show an improved survival from AIDS related infections (Nunnari et al., 2003; Xiang et al., 2001). GBV-C fits the definition of an occult virus and its potential importance in HIV-1 infection cannot be overlooked. Thus, GBV-C provides a perfect model of an occult viral system for measurement of absolute viral load with RT-dPCR.

2. Materials and Methods

2.1. In vitro transcription of GBV-C infectious clone

The GBV-C full-length 9.2 kb viral clone was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID NIH: pAF121950 from Dr. Jinhua Xiang and Dr. Jack Stapleton (Xiang et al., 2000). Sanger sequencing using M13 forward/reverse primers was used to confirm the Iowan GBV-C strain. (Iowan strain GBV-C clone accession no. AF009606.)

Five micrograms of pCR2.1-TOPO containing the GBV-C full-length cloned plasmid DNA (pAF121950) was linearized by *Spe* I restriction enzyme and then transcribed using T7 RNA polymerase (Promega, Madison, WI) for 2.5 h at 37 °C as a standard reaction. To eliminate plasmid DNA after *in vivo* transcription, Qiagen RNase-free DNase (10,000 U/mg) (Qiagen, Valencia, CA) digestion was completed twice for 30 min at 37 °C. The resulting full-length RNA genome of GBV-C (~9.2 kb) was gel purified and quantified using a Nanodrop spectrophometer (Thermo Scientific, Wilmington, DE). This standard method did not produce enough GBV-C genomic RNA for transfection studies. The yield of RNA transcription was compared from two commercial transcription kits, the T7 Ribomax Express (Promega, Madison, WI) and Ampliscribe T7 Flash (Epicentre, Madison, WI). The GBV-C viral clone was linearized by *Spe* I digestion and transcribed *in vitro* at both 37 and 42 °C according to manufacturer instructions. Both manufacturers stated that by increasing the temperature to 42 °C, the yield of the full-length product could increase by an additional 10%. Values were measured for A_{260}/A_{260} abundance using the Nanodrop.

2.2. Cell lines and transfections

Transfected Jurkat, THP-1 and PBMCs cells were used to determine viral load and detection limit. The cells were grown to a cell density of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM of L-glutamine and 1% Penicillin/Streptomycin overnight at 37 °C in 5% CO_2. No PHA, PMA or IL-2 induction was used. Two to seven million total cells were transfected with GBV-C RNA following the transmassager's transfection protocol from Qiagen. Purified PBMCs were provided by Astarte Biologics.

2.3. RNase protection assay for infectious virus

Seven days after transfection, cell supernatants were treated with RNaseA/DNase then passaged to uninfected cells. After the first successful passage of similar cell type, the supernatant was used to infect fresh cells and sampled for viral RNA at 48, 96, and 120 h. The passaged cells were again confirmed for total viral load via RT-dPCR seven days after the initial passage. This was repeated in three independent passages.

Table 1
Primer/probe sequences with Thermocycling Parameters.

GAPDH	
Forward	5′-AAATCCCATCACCATCTTCC-3′
Reverse	5′-GGACTCCACGACGTACTCAG-3′
Probe	5′-HEX-CATCGCCCCACTTGATTTTGGA-BHQ2-3′
GBV-C 5′-UTR	
Forward	5′-TAAACCGAGCCCATTACCC-3′
Reverse	5′-ACATTGAAGGGCGACGTG-3′
Probe	ROCHE UPL #61
Thermocycling parameters	
Initial denaturation	95°C – 3 min
Denaturation	95°C – 15 s
Annealing/extension	60°C – 60 s
Cycle	50

Primer/probe sequences along with thermocycling conditions 5′-UTR GBV-c UPL FAM-labeled LNA-based Taqman assay GAPDH standard Taqman HEX-labeled assay.

2.4. Total RNA isolation and cDNA synthesis

Using QIAamp® Viral RNA Mini Kit from Qiagen, total RNA from cell culture supernatants was isolated following the manufacturer's instructions (Qiagen, Valencia, CA). The total RNA was treated three times with DNase I (Stratagene, La Jolla, CA), gel-purified for high molecular weight transcript, and quality-checked for purity via an RNA Pico Chip 6000 from Agilent (Stratagene/Agilent, La Jolla, CA). All RNA used had an RIN (RNA integrity number) over 10. Any value greater or equal to seven ensures that RNA is of the highest quality. RNA was stored at −80 °C. cDNA synthesis of the total cellular RNA was carried out using random hexamers following the manufacturer's protocol for the Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA).

2.5. UPL Taqman assay design and reaction conditions

The 5′-UTR sequence from the Iowan GBV-C clone was uploaded to the Universal Probe Library (UPL) assay designer on the Roche diagnostics website, where it was processed to indicate the viral load assay. GAPDH was used to judge the quality of the cDNA and the fill rate required for the microfluidic chip. The reaction conditions were as follows; Universal Taqman Probe Master Mix (Roche) at 1× final concentration, 300 nM forward primer, 300 nM reverse primer and 150 nM of each probe (UPL #61 Roche for 5′-UTR of GBV-C (Table 1) were mixed in a multiplex reaction. ROX normalization dye was pre-mixed to correct for any optical bias. The primer, probe sequences and the thermal cycling parameters are presented in Table 1.

2.6. RT-qPCR on the Stratagene's Mx3005P

For testing purposes, a standard GBV-C cDNA plasmid control sample was created and serially diluted for calibration. To maintain the standard over time, the pooled cDNA sample was cloned into pCR2.1 (Invitrogen) and then transformed into DH5α cells. Plasmids containing standard pooled cDNA were harvested from mid-log phase DH5α cells and further isolated using Qiagen's QIAprep Spin Miniprep kit. The resulting plasmids were digested using *Spe* I, and gel purified. Plasmid DNA containing the infectious clone was serially diluted in tenfold increments in six replicates in order to obtain a coefficient of variation values.