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Validation and clinical use of a sensitive HIV-2 viral load assay that uses a whole virus internal control

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ABSTRACT

Background: Human immunodeficiency virus type 2 (HIV-2) is distantly related to the more widespread HIV-1. Although HIV-2 infection is rare in the U.S., cases are concentrated in the Northeast. No FDA-approved HIV-2 viral load assays exist. A clinically validated laboratory-developed assay is currently available in the U.S., however it is not currently approved for use on New York State patients.
Objective: To develop a sensitive viral load assay to quantify HIV-2 RNA in plasma and to validate it for clinical use.
Methods: The real-time RT-PCR assay simultaneously amplifies HIV-2 and a whole virus internal control, added during the lysis step. Two extraction volumes can be used. Results are reported in HIV-2 RNA International Units (IU).
Results: The assay has a limit of detection of 7 IU/mL and a lower limit of quantification of 29 IU/mL. The assay detects multiple strains of HIV-2 group A and B and generates reproducible results. Samples exchanged with a comparator laboratory produced similar viral load results, with 74% of positives differing by <0.5 $\log_{10}$ IU/mL. To date, we have tested 52 clinical specimens from 25 individuals. Twenty-eight (54%) specimens had measurable HIV-2 viral loads (range: 1.63–5.14 $\log_{10}$ IU/mL), 10 (19%) were positive but not quantifiable, and 14 were negative. HIV-2 RNA was detected in at least one specimen from 19 of 25 (76%) individuals tested.
Conclusions: We developed a sensitive and accurate HIV-2 viral load assay. Validation data indicate the assay is suitable for clinical use and its availability in New York State will improve clinical monitoring of HIV-2 infected patients.

1. Background

Human immunodeficiency virus, type 2 (HIV-2) was discovered in 1986 as a second lentivirus that causes acquired immunodeficiency syndrome (AIDS) [1]. HIV-2 is distantly related to HIV-1, the virus responsible for the global AIDS pandemic, because the viruses originated from separate transfers of simian immunodeficiency virus (SIV) into the human population (HIV-1 from chimpanzees and HIV-2 from sooty mangabeys) [2]. Although HIV-1 and HIV-2 have similar transmission routes and can cause immunodeficiency, patients infected with HIV-2 have lower viral loads than those infected with HIV-1 [3]. This leads to lower HIV-2 transmission rates and longer progression to clinical disease [4]. Although eight groups of HIV-2 have been identified, only Group A and Group B have had significant levels of human-to-human transmission [2]. HIV-2 infection occurs predominantly in West Africa and those areas with historical ties to West Africa [5]. In the U.S., HIV-2 is rare, with only 166 confirmed cases from 1988 to 2010 [6]. These cases are concentrated in New York City and surrounding areas due to the high numbers of immigrants from West Africa [6].

Viral load testing is an important tool used to monitor HIV disease progression and to detect treatment failures. Although several FDA-approved HIV-1 viral load assays exist, there are no FDA-approved assays to quantify HIV-2 RNA. Recently, a laboratory-developed HIV-2 viral load assay was clinically validated to run on the Abbott m2000 platform [7]. Although this is a sensitive and reliable assay, this automated robotic platform is not widely available in all settings and the assay is not currently approved for use in New York State (NYS). Under NYS regulations, clinical laboratories that perform testing on NYS residents must hold a NYS clinical laboratory permit and laboratory-developed tests must be reviewed and approved prior to use on NYS residents. Furthermore, public health reporting regulations require that laboratories electronically report all HIV nucleic acid test results, both

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qualitative and quantitative, to the NYS Department of Health. The lack of an HIV-2 viral load assay approved for clinical use in NYS impinged on both clinicians and surveillance programs.

2. Objective

Our objectives were to develop a sensitive HIV-2 viral load assay that includes a whole virus internal control to monitor assay performance and to validate the assay for clinical use on widely available real time PCR systems.

3. Study design

3.1. Controls and calibrators

A high titer HIV-2 virus stock was prepared by propagating H9 cells infected with HIV-$2_{MVP15132}$ (NIH AIDS Reagent Program) and harvesting viral supernatant. This virus stock was tested for cell-derived HIV-2 proviral DNA by testing extracted nucleic acid in the real time PCR assay without first performing reverse transcription. HIV-2 DNA makes up <0.2% of the total nucleic acid in the stock virus and therefore will not interfere with the quantification of HIV-2 viral RNA.

HIV-2 virus stock was diluted in fresh frozen negative human plasma (SeraCare Life Sciences, Milford, MA) to prepare the five HIV-2 calibrators and high and low positive controls. These calibrators and controls plus a negative control (negative human plasma) are extracted with each set of patient specimens.

The quantitative value of 10-fold serially diluted virus stock samples were determined by running them alongside the HIV-2 RNA International Standard (08/150, National Institute for Biological Standards and Control), which has an assigned value of 1000 international units (IU). Four separate extractions were performed, with three replicates of reverse transcription and PCR for each extraction. For each PCR replicate, virus dilutions were designated as 'standards' and the International Standards were designated as 'unknowns'. The IU values were adjusted, keeping the 10-fold difference between each virus dilution, until the difference between the actual and predicted IU values of the International Standards were at a minimum. The five HIV-2 virus calibrators were assigned values of 630,000, 63,000, 6300, 630 and 26 IU/200 μL; these calibrators included four of these 10-fold dilutions plus a low calibrator that was diluted and quantified separately.

Mouse hepatitis virus (MHV), an enveloped RNA virus in the Coronaviridae family, was used as a whole virus internal control; detection of MHV within a specified range signifies proper assay function and a lack of PCR inhibition. We obtained a high titer MHV virus stock (strain A59) and diluted it with molecular grade water to a concentration that would produce a Ct of ~30.

3.2. Validation samples

Samples used in the validation studies included negative human plasma spiked with HIV-2 stock virus, HIV-2 infected cells or infectious clones, and other viruses and pathogens. For the accuracy study, HIV-2 spiked plasma and remnant plasma samples from patients previously diagnosed with HIV-2 were tested with our HIV-2 viral load assay and a clinically validated HIV-2 viral load assay at University of Washington [7]. All samples were stored at <−20 °C prior to testing.

3.3. Real time PCR assay design

A duplex real time PCR was developed to simultaneously amplify HIV-2 and MHV. Primers and Taqman probes were designed using Primer Express 3.0 (Applied Biosystems, Foster City, CA) to amplify portions of the HIV-2 LTR repeat region and the MHV M gene (Table 1). The LTR repeat region occurs at the 5′ and 3′ ends of the HIV-2 genome; consequently, there are two targets per genome (Figure S1).

Table 1
HIV-2 and MHV oligonucleotide sequences.

Name	Sequence (5′ to 3′)	Nucleotide position[a]
HIV-2 amplicon size: 68 bp location: LTR repeat region (5′ and 3′)		
HIV-2 F27	TTGAGCCCTGGGAGGTTCT	545–563, 10006–10024
HIV-2 P48	*6-FAM*-CCAGCACTAGCAGGTAG-*MGBNFQ*	566–582, 10027–10043
HIV-2 R94	GGTGAGAGTCTAGCAGGGAACAC	612–590, 10073–10051
MHV amplicon size: 92 bp location: M gene		
MHV F435	GAC CCA TTA TTG AGG ATT ACC ATA CA	29378–29403
MHV P477	*HEX*-TTC GTG GCC ACC TCT ACA TGC AAG G-*BHQ-1*	29420–29444
MHV R526	AGA GAA ACC GGT GCC TAG CTT	29469–29449

[a] Based on SIVmm239 (#M33262) or MHV strain A59 (#AY700211).

3.4. Assay procedure

Total nucleic acid was extracted using the generic 2.0.1 protocol of the Nuclisens easyMAG system (bioMerieux, Durham, NC). Twenty microliters of MHV internal control was added to 2 mL lysis buffer along with 100–900 μL of each plasma sample and kept at room temperature for 1 h. One hundred microliters of magnetic silica diluted 1:1 in DEPC water was added to each sample. Extracted nucleic acid was eluted into 40 μL of elution buffer. For each sample, a single 20 μL reverse transcription reaction was performed using the iScript cDNA synthesis kit (Bio-Rad, Richmond, CA) according to manufacturer's recommendations with 10 μL of extracted nucleic acid added to each reaction. Two replicates of a 25 μL PCR reaction consisting of 5 μL cDNA, 12.5 μL Bio-Rad iQ Multiplex Powermix, 300 nmol/L each of two HIV-2 and two MHV primers, and 200 nmol/L each of HIV-2 and MHV probes were run for each sample. PCR reactions were run on an iQ5 real time PCR system (Bio-Rad) and incubated at 95 °C for 3 min followed by 45 cycles of 95 °C for 15 s and 59 °C for 1 min. Because the EasyMag extraction protocol extracts total nucleic acid, we also run a single 'no RT' 25 μL PCR reaction for each patient sample to detect contaminating HIV-2 proviral DNA. In this reaction, 2.5 μL of extracted nucleic acid and 2.5 μL of water are added instead of 5 μL cDNA. The assay was also validated on the Applied Biosystems 7500 Fast Real Time PCR system using the above procedures with one modification: 3.3 μL of ROX passive reference dye (Bio-Rad) was added to a full tube (1.25 mL) of iQ Multiplex Powermix before adding this reagent to the PCR mastermix.

3.5. Data analysis

Real time PCR data was analyzed using the Bio-Rad iQ5 Optical System Software Version 2.1 or the Applied Biosystems 7500 Software Version 2.0.5. Threshold values were set at 200 for both targets in the iQ5 software and 0.4 (HIV-2) and 0.2 (MHV) in the 7500 software. We used dynamic well factors in the iQ5 software and ROX as the passive reference dye in the 7500 software. In order for a run to be valid, the Ct values of the MHV internal control, low positive control and high positive control must be within an acceptable range and all negative control and NTC wells must be negative. The calibrator standard curve must have an efficiency of 90–110% and R^2 of > 0.99. In addition, the predicted viral loads of the Low and