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Review

Development of a contemporary globally diverse HIV viral panel by the EQAPOL program

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ABSTRACT

The significant diversity among HIV-1 variants poses serious challenges for vaccine development and for developing sensitive assays for screening, surveillance, diagnosis, and clinical management. Recognizing a need to develop a panel of HIV representing the current genetic and geographic diversity NIH/NIAID contracted the External Quality Assurance Program Oversight Laboratory (EQAPOL) to isolate, characterize and establish panels of HIV-1 strains representing global diverse subtypes and circulating recombinant forms (CRFs), and to make them available to the research community. HIV-positive plasma specimens and previously established isolates were collected through a variety of collaborations with a preference for samples from acutely/recently infected persons. Source specimens were cultured to high-titer/high-volume using well-characterized cryopreserved PBMCs from National y donors. Panel samples were stored as neat culture supernatant or diluted into defibrinated plasma. Characterization for the final expanded virus stocks included viral load, p24 antigen, infectivity (TCID), sterility, coreceptor usage, and near full-length genome sequencing. Viruses are made available to approved, interested laboratories using an online ordering application. The current EQAPOL Viral Diversity panel includes 100 viral specimens representing 6 subtypes (A, B, C, D, F, and G), 2 sub-subtypes (F1 and F2), 7 CRFs (01, 02, 04, 14, 22, 24, and 47), 19 URFs and 3 group O viruses from 22 countries. The EQAPOL Viral Diversity panel is an invaluable collection of well-characterized reagents that are available to the scientific community, including researchers, epidemiologists, and commercial manufacturers of diagnostics and pharmaceuticals to support HIV research, as well as diagnostic and vaccine development.

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1. Introduction

At the end of 2011, there were an estimated 34 million people living with human immunodeficiency virus type 1 (HIV-1) (UNAIDS, 2012). Extensive efforts to prevent transmission, diagnose infection, monitor disease progression and treat infected individuals are ongoing (WHO, 2011; O'Connell et al., 2012; Piot and Quinn, 2013). Standardized reagents and assays are critical to support these efforts as well as for evaluating immune responses to vaccine candidates.

The rapid evolution of HIV has given rise to multiple subtypes and recombinant strains of HIV-1 and HIV-2. Presently, HIV-1, the major source of the global pandemic, is divided into four groups: M (Major), O (Outlier), N (Non-M, Non-O) and P (Charneau et al., 1994; Simon et al., 1998; Corbet et al., 2000; Plantier et al., 2009). HIV-1 M group, which accounts for the vast majority of HIV-1 infections, has been further subdivided into nine subtypes (A–D, F–H, J, and K), 61 circulating recombinant forms (CRFs) and many unique recombinant forms (URFs) (Los Alamos HIV Sequence Database; URL: <http://www.hiv.lanl.gov>). The significant diversity observed within HIV poses serious challenges for vaccine development and for maintaining sensitive assays for screening, surveillance, diagnosis and clinical management.

Qualitative detection of HIV-1 RNA is employed in blood screening and for diagnosis of acute infections. Quantification of HIV-1 RNA viral load (VL) in plasma is essential for monitoring response to antiretroviral therapy, estimating the risk of disease progression and to establish diagnosis when a negative or indeterminate HIV-1 antibody test is present (Coombs et al., 1996; Mellors et al., 1996; O'Brien et al., 1996; Welles et al., 1996). The assays to detect and quantify HIV RNA have been developed and validated mainly using B subtype samples whereas non-B subtypes represent more than 89% of strains that are circulating with the proportion of CRFs and URF increasing (Hemelaar et al., 2011). Furthermore, assays are targeted to different genes in the viral genome using sequence-specific probes necessitating thorough evaluation of different viral load platforms across an array of subtypes. Differences between commercially available qualitative and quantitative viral load assays for non-B subtype strains have been noted (Antunes et al., 2003; Gottesman et al., 2006; Kim et al., 2007; Xu et al., 2010;

Amendola et al., 2011; Chudy et al., 2012). In addition, differences between subtypes in serological diagnostic and screening assays have been reported (Ly et al., 2007; Ly et al., 2012).

In addition to posing challenges for screening and HIV quantification, subtype-specific differences have been implicated in disparate rates of disease progression, development of drug resistance mutations and responses to antiretroviral therapies (Adazu et al., 2005). Recognizing the challenges that HIV diversity poses, HIV diversity panels have been previously assembled to support the development of HIV detection, treatment and vaccination (Huang et al., 2003; Brown et al., 2005). However, they were generated from source samples collected more than a decade ago and, therefore, do not adequately represent the current HIV-1 diversity. Furthermore, many of these samples have not been characterized with current molecular assays. Recognizing a need to develop a panel which encompasses the genetic and geographic diversity currently present worldwide, the National Institutes of Health/National Institute of Allergy and Infectious Diseases (NIH/NIAID) established a pilot program to develop a HIV subtype panel in collaboration with the Food and Drug Administration (FDA), Walter Reed Army Institute of Research (WRAIR), academia, and industry representatives (Manak et al., 2012). Through this pilot program, 30 viral isolates representing subtypes B, C, B/F, CRF04_cpx, and CRF02_AG were obtained from plasma samples. The viral strains in the pilot panel were well-characterized, and all of the data was compiled to be made available to researchers interested in those samples.

To expand on the important work initiated in the pilot panel program, the NIH/NIAID-sponsored External Quality Assurance Program Oversight Laboratory (EQAPOL) program was funded and includes as a major goal the continued development of contemporaneous HIV global diversity panels. The EQAPOL panel focused on identifying virus source specimens mainly from recently diagnosed individuals with acute/early stages of infection. Plasma samples were obtained through collaborations with blood banks worldwide taking advantage of their HIV nucleic acid technology (NAT) screening programs whereby HIV RNA positive/antibody-negative donors were detected and deferred. Additional samples were acquired from previously collected plasma from individuals who are part of recently HIV infected

cohorts. In some cases, it is difficult to export plasma/Peripheral Blood Mononuclear Cell (PBMC) samples from countries due to regulatory restrictions. Therefore, previously established viral isolates from these countries were also obtained to expand the overall diversity of the panel. Since the EQAPOL program's inception in 2011, 100 viral samples have been propagated to high-titer/high-volume and fully characterized.

2. Materials and methods

2.1. Viral source specimen collection and sources

2.1.1. NAT-yield plasma samples

2.1.1.1. Blood donor sample collection. Acquisition of samples from acutely infected blood donors for use in this study was coordinated by the Blood System Research Institute (BSRI). Samples were acquired through regular blood donations identified by individual or pooled screening as NAT-yield positive (RNA positive, antibody negative), and collected as whole blood donations processed into red blood cells and plasma components. All donations had been collected with ethical review and approval of the collection protocol and informed consent as previously established for the donor organization's collection activities.

2.1.1.2. Sample sources. Plasma samples were obtained from six countries on four continents; South Africa (South African National Blood Service (n = 148)), the U.S. (American Red Cross (n = 52)), France (Institut National de la Transfusion Sanguine (n = 5)), Brazil (Department of Infectious Disease/University of Sao Paulo, (n = 3)), Spain (Banc de Sang i Teixits (n = 13)), and Japan (Central Blood Institute, Blood Service Headquarters, Japanese Red Cross Society (n = 11)). All samples except those from Brazil and Spain were confirmed as NAT-yield (RNA positive, antibody negative) based on documented testing criteria at the collecting organizations. The HIV positive samples from Brazil (two Fiebig V and one Fiebig IV) and Spain were from recently infected donors as defined by very recent seroconversion, indeterminate Western blots or application of HIV recency assays with results indicating that the donations were given within 1–2 months of acquisition of infection (Busch et al., 2010).

2.1.1.3. Sample characterization. South African NAT-yield donors included in this study were identified from 2009 through 2011 by individual donation (ID) NAT screening using the Procleix Ultrio assay followed by HIV discriminatory transcription mediated amplification (TMA) (Gen Probe, San Diego, CA). All were negative by the Abbott HIV-1/2 ChLIA assay (Abbott Laboratories, Abbott Park, IL). Based on sequence analysis, the majority of these samples were clade C. U.S. NAT-yield donors were identified from 2001 through 2009 by mini pool (MP) NAT screening in pools of sixteen using Procleix followed by HIV discriminatory TMA (Novartis Diagnostics, Emeryville, USA). All were Abbott-HIV-1/2 EIA negative. The majority were clade B based on sequence analysis (Stramer et al., 2000; Stramer et al., 2004; Zou et al., 2010). French NAT-yield donors were identified from 2000 through 2011 by ID or MP NAT screening. All were negative by the Genscreen Ultra

Ag-AB (Genscreen Pty Ltd, Australia) and Innostest p24 antigen (Innogenetics NV, Gent, Belgium) assays. Descriptive information is not available for the Brazilian, Spanish, and Japanese NAT-yield and recently infected specimens.

2.1.2. Additional plasma and PBMCs samples

Plasma samples from 55 donors previously collected as part of the International AIDS Vaccine Initiative (IAVI) Protocol C were kindly provided by Pat Fast, Pauli Amornkul and Matt Price for inclusion in the EQAPOL program. The samples received by EQAPOL were collected at seven clinical research centers between 2009 and 2011: Kigali, Rwanda (Etienne Karita, Susan Allen and Eric Hunter); Lusaka and Copperbelt, Zambia (William Kilembe, Susan Allen, Mubiana Inambao, and Eric Hunter); Nairobi and Kilifi, Kenya (Eduard Sanders, Omu Anzala and Gaudensia Mutua); Entebbe and Masaka, Uganda (Anatoli Kamali and Eugene Ruzagira) (Price et al., 2011). At-risk volunteers were followed quarterly or monthly in large incidence cohorts, and it is estimated that samples were collected within 6 months of infection.

Additionally, Peripheral Blood Mononuclear Cells (PBMCs) and plasma from four donors in Russia were kindly provided by Hagen von Brisen at the Fraunhofer Institut für Biomedizinisch Technik (IBMT). Samples were collected in 2008 within an estimated 1–6 months of infection.

2.1.3. Viral culture supernatants and infectious molecular clones (IMCs)

Previously cultured viral supernatants from ten donors from China were kindly provided by Hong Shang, Wenqing Geng, Hualu Cui, and Hong Sun at the First Affiliated Hospital of China Medical University. Donor samples were collected between 2009 and 2011, and included a mix of supernatants obtained from recently infected (Fiebig IV and V) and chronically infected individuals as defined by the contributing laboratory. Thirty-three viral supernatants were obtained from the Centre for AIDS Reagents, NIBSC HPA UK, supported by the EC FP6/7 Europrise Network of Excellence, and NGIN consortia and the Bill and Melinda Gates GHRC-CAVD Project and were donated by Lucia Pérez Alvarez (Instituto de Salud Carlos III) (Fernandez-Garcia et al., 2009; Cuevas et al., 2010; Fernandez-Garcia et al., 2010; Delgado et al., 2012; Thomson et al., 2012; Diez-Fuertes et al., 2013). Twenty-three culture supernatants from donors in Cameroon, Spain, USA and Germany were kindly provided by Indira Hewlett (CBER, FDA) (Vallejo et al., 2003; Ragupathy et al., 2011; Zhao et al., 2012). Additionally, EQAPOL received 12 Infectious Molecular Clones (IMCs) made and contributed by the Beatrice Hahn and George Shaw Laboratories (University of Pennsylvania) from plasma collected from acute donors (four Fiebig I, two Fiebig II, one Fiebig III, and four Fiebig IV) in Uganda and Rwanda by IAVI and the Uganda Virus Research Institute-British Medical Research Council program on HIV/AIDS (UVRI/MRC) (Price et al., 2011; Tang et al., 2011; Price et al., 2012; Baalwa et al., 2013).

2.2. Feeder cell acquisition and permissivity testing

2.2.1. Feeder cell acquisition

PBMCs used as feeder cells for EQAPOL virus culture were collected from healthy donors by leukapheresis. All donors

were negative for HIV-1/2 Antibody, Hepatitis B Surface Antigen and/or Core, and cytomegalovirus (CMV). All donors were properly consented according to Duke University IRB, Federal and State regulations. Using the leukapheresis material, PBMCs were separated on Ficoll–Hypaque gradients and cryopreserved in a solution of 10% DMSO (Sigma-Aldrich, St. Louis, MO) in heat-inactivated fetal bovine serum (FBS) (Gemini Bio-products, West Sacramento, CA). PBMCs were slowly frozen using a computerized control rate freezer system and then quickly transferred into -180°C LN₂ freezers. All procedures were performed under Good Clinical Laboratory Practices (GCLP).

2.2.2. Donor PBMC permissivity

Donor PBMC permissivity was tested to select donors to pool for viral culture. Briefly, donor PBMCs were infected with a panel of six different viruses representing different subtypes, groups and coreceptors of HIV. Tissue Culture Infectivity Dose (TCID) in TZM-bl cells was measured on each of these donor/virus conditions 2, 4 or 6 days after infection (see below for TCID measurement). Five donors were selected to pool for all viral cultures based on the permissivity observed. Donors in the pool were replaced as needed.

2.3. Virus isolation and expansion

All work was carried out under BSL-3 guidelines in a BSL-3 environment at the Regional Biocontainment Laboratory at Duke University Medical Center using GCLP practices. Viruses were cultured in a two-step procedure termed the small and large-scale cultures. The protocol utilized from culture was adapted from previously published methods (Albert et al., 1987; Falk et al., 1987; Costa et al., 1996; Aguado-Martinez et al., 2009). Briefly, cryopreserved PBMCs pooled from five donors, termed feeder cells, were activated with α -CD28 at 100 ng/mL (BD Biosciences, San Jose, CA) and α -CD3 at 50 ng/mL (eBioscience, San Diego, CA) three days prior to the start of culture. On the culture start date, PBMCs were CD8-depleted using CD8 Dynabeads® (Invitrogen, Grand Island, NY) according to the manufacturer's instructions as removal of CD8+ T cells has been reported to increase HIV replication (Walker et al., 1986). When the source viral specimen was plasma, virus was first enriched by a 20% sucrose density cushion at 40,000 $\times g$ for 2 h. Virus isolated from plasma or virus culture supernatant (0.1–3 mL) was added to 2×10^7 CD3/CD28-stimulated, CD8-depleted PBMCs, centrifuged at 1200 $\times g$ for 2 h to enhance infection ("spinoculation" see (O'Doherty et al., 2000)), and cultured at 37°C and 5%CO₂ in a total volume of 20 mL in culture media (RPMI 1640 w/L glutamine, 20% FBS, 1% Pen Strep and rh IL-2 at 2 ng/mL).

Cells were cultured for two days, and then additional (2×10^7) CD8-depleted, activated feeder cells were added to the culture. Cell culture supernatant was removed, filtered, and frozen at -80°C on day 2 (prior to addition of supplementary cells), and then every two to three days through the life of the culture (usually less than 14 days). Spinoculation was performed at each harvest day. Representative aliquots from each harvest day were checked for viral load using the COBAS AmpliPrep/COBAS Taqman 48 HIV-1 test (Roche, Pleasanton, CA), and the culture was terminated after the viral load peaked. The culture supernatant isolated

from the peak culture day (approximately 30 mL) was thawed and aliquoted into LDMS-labeled vials at 0.5 mL/vial and stored at -80°C . This product was termed the "master lot" and used to start the subsequent large-scale culture.

For the large-scale culture, 0.5 mL of the master lot and 1×10^8 activated, CD8-depleted pooled PBMCs were spinoculated and cultured in a total volume of 100 mL. Similar to the small-scale culture, on day 2 the culture was further expanded by adding 1.75×10^8 activated, CD8-depleted pooled PBMCs and bringing the total culture volume to 250 mL. The process of expanding the culture was repeated every 2–3 days with the viral load closely monitored. When the culture reached high titer ($>1 \times 10^9$ cp/mL), neat culture supernatant was filtered then stored in 1 mL aliquots without any freeze/thaws prior to aliquoting. Virus was also stored by spiking defibrinated human AB plasma (Gemini Bio-Products, West Sacramento, CA) with culture supernatants to yield a variety of viral load concentrations.

2.4. Sample characterization

Source material (plasma, culture supernatants), intermediate culture supernatant products and final culture products (culture supernatant and HIV-spiked plasma) were characterized using a variety of assays. Testing included commercially available FDA-approved nucleic acid and antibody assays and a Research Use Only (RUO) p24 antigen assay. The Roche COBAS AmpliPrep/TaqMan HIV-1 Version 2.0 viral load assay was used to measure viral RNA in source material, intermediate culture supernatant products, and final culture products.

Anti-HIV reactivity of source plasma material was tested by the Bio-Rad Genetic Systems Anti-HIV-1/HIV-2 Plus O EIA and the Bio-Rad Genetic Systems HIV-1 Western blot methods (Hercules, CA). The PerkinElmer HIV-1 p24 Antigen Capture Assay (Waltham, MA) was used to measure supernatant antigen levels in final culture supernatant products. All assays were performed under GCLP and were performed according to manufacturer's instructions. Source viral plasma specimens were classified based on Fiebig stage as determined by the presence of viral RNA and antibody by EIA and specific bands on Western blot according to published methods (Fiebig et al., 2003). Testing of p24 antigen was not done to distinguish Fiebig stage I versus II.

Infectivity was determined using a TZM-bl-based TCID assay as previously described (Li et al., 2005). Briefly, TZM-bl cells (10,000 per well) were incubated with a 5-fold serial dilution of viral supernatant stock with four replicates per dilution. Cells were cultured for two days, and then infectivity was measured using Britelite Plus reagent (Perkin Elmer, Waltham, MA). Wells were considered positive for infection if the RLU was 2.5-fold higher than non-infected cells. Infectivity was calculated as the dilution at which half of the four wells per sample dilutions were positive. The assay was repeated as two independent replicate infectious titration studies, and the average TCID was reported.

2.4.1. Sterility testing

Selected viral source specimen and all final viral culture supernatant products were tested to ensure they were free

of adventitious agents, including bacterial contamination and mycoplasmas. Sterility testing included direct inoculation to detect bacterial contamination, a Mycoplasma test, and a qualitative Limulus amebocyte lysate (LAL) assay to detect endotoxin. Briefly, 0.5 mL of culture supernatant was added to 8 mL prepared BBL™ Fluid Thioglycollate Medium (BD, Franklin Lakes, NJ) or Trypticase™ Soy Broth with 6.5% sodium chloride (BD, Franklin Lakes, NJ) and incubated at $35 \pm 2^\circ\text{C}$ or $22.5 \pm 2.5^\circ\text{C}$, respectively. Samples were visually inspected for turbidity on days 3, 4, 7, and 14 after inoculation and were considered negative if no turbidity was observed during the 14 day culture period. Mycoplasma testing was done using a MycoAlert® Detection Kit (Lonza, Cologne, Germany) according to the manufacturer's instructions, and samples were reported as either positive or negative. Samples were tested for endotoxin using a qualitative gel clot LAL assay (Lonza, Cologne, Germany) according to the manufacturer's instructions. Prior to testing, samples were diluted 1/10 in culture medium to prevent inhibition of the clot reaction. If no clot was observed, then the sample was reported as containing less than 1.25 EU/mL endotoxin.

2.4.2. Genotyping based on near full-length HIV-1 genome sequences

Viral RNA was extracted from 100 μL of culture supernatant using the Qiagen EZ1 Virus mini kit on the BioRobot EZ1 (Qiagen, Valencia, CA) and used for cDNA synthesis using Superscript III Reverse Transcriptase (Life Technologies; Carlsbad, CA) and primers 1R3.B3R (5'-ACTACTGAAGCAC TCAAGGCAAGCTTTATTG-3' nt 9611–9642) and 07Rev9 (5'-C TTCCTGCCATAGGAGATGCCTAA-3' nt 5957–5980) for 3'- and 5'-half HIV-1 genome, respectively. Single genome amplification (SGA) was performed to obtain two overlapping half genomes for group M viruses as we previously described (Salazar-Gonzalez et al., 2009). For amplification of the 5'-half genome, the primers for the first round PCR were 1U5.B1F (5'-CCTTGAGTGCTTCAAGTAGTGTGTGCCGTGTGT-3' nt 538–571), 07Rev8 (5'-CCTARTGGGATGTGTACTTCTGAAC T-3' nt 5193–5219), and the primers for the second round PCR were UP1A (5'-AGTGGCGCCCGAACAGG-3' nt 634–650) and Rev11 (5'-ATCATCACCTGCCATCTGTTTCCAT-3' nt 5041–5066). For amplification of the 3'-half genome, the primers for the 1st round PCR were 07For7 (5'-CAAATTAYAAAAATT CAAAATTTTCGGGTTTATTACAG-3' nt 4875–4912) and 2.R3.B6R (5'-TGAAGCACTCAAGGCAAGCTTTATTGAGGC-3' nt 9607–9636), and the primers for the 2nd round PCR were VIF1 (5'-GGGTTTATTACAGGAGCAGCAGAG-3' nt 4900–4923) and Low2C (5'-TGAGGCTTAAGCAGTGGGTTC-3' nt 9591–9612). The same primers were used for amplification of both 5' and 3' halves of group O viral genomes, except the second round primers for the 5' half genome; O-LOW2 (5'-GAACCCGCTG CTTAACGCTCAATA-3' nt 507–530) and O-REV12 (5'-TGTCTG CTGTTTACAGGCTCAATCTGTGTT-3' nt 5325–5353). Eight PCR reactions were performed using the cDNA dilution that gave a PCR positive rate of 25% or less to obtain at least one 5' or 3' half SGA for each sample. The PCR amplification was performed using the Platinum Taq DNA polymerase High Fidelity (Life Technologies, Carlsbad, CA). The PCR thermocycling conditions were as follows: one cycle at 94°C for 2 min; 35 cycles of a denaturing step at 94°C for 15 s, an annealing step at 58°C for

30 s, an extension step at 68°C for 5.5 min; and one cycle of an additional extension at 68°C for 10 min.

SGA amplicons were sequenced directly by cycle sequencing and dye terminator methods using an ABI 3730xl genetic analyzer (Applied Biosystems, Foster City, CA). Individual sequences were assembled and edited using the Sequencer program 4.10 (Gene Codes, Ann Arbor, MI). Phylogenetic relationships of the newly characterized near full-length genome sequences were estimated from sequence comparisons with previously reported HIV-1 subtype, circulating recombinant form (CRFs) and group O virus reference sequences from the Los Alamos database. Sequences were added to an existing master alignment using the profile alignment option of CLUSTAL W and adjusted manually when necessary using SEAVIEW. Sites where there was a gap in any of the sequences, as well as areas of uncertain alignment, were excluded. Pairwise evolutionary distances were estimated by using the Kimura two-parameter model (Kimura, 1980). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987), and the reliability of topologies was estimated by performing bootstrap analysis with 1000 replicates. Recombination patterns were determined using the REGA Subtyping tool version 2 (<http://jose.med.kuleuven.ac.be/genotypetool/html/subtypinghiv.html>) and the jumping profile Hidden Markov Model (jpHMM) at GOBICS website (<http://jphmm.gobics.de/>) (Schultz et al., 2006; Schultz et al., 2009).

2.4.3. Coreceptor usage using cell culture

Coreceptor usage was determined on NP-2 cells which express CD4 along with CCR5 or CXCR4 as previously described (Shimizu et al., 2009; Jiang et al., 2011). Briefly, NP-2 cells (5×10^4) were seeded in 48-well plates the day before infection. Each virus (25 μL cell culture supernatant) was added to the well in triplicates. After absorption at 37°C for 3 h, cells were washed twice and maintained in complete medium at 37°C with 5% CO_2 . Virus replication was determined by measuring the p24 Antigen concentrations in culture supernatants collected at day 0 and day 7 with the Alliance HIV-1 p24 Antigen ELISA kit.

3. Results

3.1. Overview of EQAPOL Viral Diversity project process

For the EQAPOL Viral Diversity program, 50 HIV are cultured per year using a well-documented workflow for the culture and characterization process as depicted in Fig. 1. Briefly, source viral specimen samples were acquired from a variety of collaborators (see Materials and methods and Acknowledgments sections) and tested to select samples with characteristics desirable for culture. Preference was given for samples from acute infection (Fiebig Stage V or earlier) and samples that would expand the geographic or genetic diversity of the panel.

Separately, cryopreserved PBMCs were screened for HIV permissivity to a variety of HIV subtypes/coreceptors as different donors have different susceptibilities to HIV (Williams and Cloyd, 1991; Spira and Ho, 1995; Greco et al., 1998). PBMCs from five donors with the best permissivity were selected to be part of a Feeder Cell Pool to be used in all cultures. As needed, new donor

Fig. 1. Flow chart of EQAPOL Viral Diversity project. Viral source specimens were acquired and cultured in a two-step process to yield high-titer/high volume HIV culture supernatant. Virus is stored as neat culture supernatant and HIV spiked plasma at a variety of concentrations. Cultured viruses were extensively characterized and made available to approved interested sites using a secure web-based system.

PBMCs were added to the Feeder Cell Pool following HIV permissivity test.

Source viral specimen material was cultured with PBMCs in a two-step culture process to generate high-titer/high-volume culture stock, which was saved as neat culture supernatant and spiked into defibrinated plasma. Spiked plasma is made at approximately 1×10^7 , 5×10^7 and 1×10^4 cp/mL. Viral stocks were then extensively characterized and made available for order to approved laboratories via a web-based application (<https://eqapolapp.dhvi.duke.edu>). All products produced for distribution include a Certificate of Analysis (COA) signed by the EQAPOL Central Quality Assurance Unit, which monitors GLCP compliance in the EQAPOL program (see Todd et al., 2014).

All EQAPOL samples were named using a standardized naming convention based on a previously generated standard (Korber et al., 1994). The name provides information about the clade/group, country of origin, year of collection, and sample type (i.e. Culture supernatant, HIV-spiked plasma). As an example, in the name DEMB10US001.S1, DE indicates “Duke EQAPOL”, MB indicates Group M, Subtype B, 10 indicates the year the sample was collected, US is the two letter country code of origin, 001 indicates the sample number for that country and S1 indicates that the sample type is culture supernatant.

3.2. Viral source specimens and initial characterization

Since the start of the EQAPOL Viral Diversity program in September 2010, 370 viral source specimens have been

acquired as summarized in Table 1. Samples include 288 plasma specimens obtained through collaborations with BSRI and IAVI. To increase both genetic and geographic diversity, we have also collected previously-cultured viruses and IMCs for inclusion in the panel. Source specimens underwent an initial characterization to help select samples for culture. Testing included viral load, Fiebig staging, sterility testing, and full-length sequencing. Viral load was used to select plasma samples that would likely grow in culture; greater than 1×10^4 cp/mL was preferred as lower concentrations have a much lower success rate in culture (Manak et al., 2012). Previously cultured virus supernatants were tested for sterility to ensure they were free of bacterial and mycoplasma contamination to assure that contamination was not introduced into the BSL-3 culture facility and to allow the material to be used for the development of unique culture-based bioassays. Near full-length genome sequences were obtained by population sequencing for all source specimens prior to culture to determine the HIV subtype and if they contained more than one genotypes. All of this characterization data was compiled and used to select which samples would contribute to the overall genetic and geographic diversity of the panel.

3.3. Virus isolation and expansion to high-titer/high-volume

Viruses were cultured in a two-step culture process (see Fig. 1). In the first culture, virus isolated from plasma or culture supernatant from a previous HIV culture were mixed

Table 1

Summary of viral source specimens for EQAPOL Viral Diversity program.

Country of origin	# of samples
Brazil	3
Cameroon	20
China	10
France	5
Germany	1
Japan	11
Kenya	8
Russia	4
Rwanda	4
South Africa	148
Spain	48
Uganda	34
USA	53
Zambia	21
Total	370

Listing of countries and number of received from EQAPOL collaborators at the following institutions: Blood Systems Research Institute (BSRI), International AID Vaccine Initiative (IAVI), Instituto de Salud Carlos III, First Affiliated Hospital, China Medical University, SeraCare (collected as part of pilot panel), University of Pennsylvania, Food and Drug Administration, and Fraunhofer Institut für Biomedizinisch Technik (IBMT). Source specimens include plasma, PBMCs, previously cultured HIV and infectious molecular clones.

with activated, CD8-depleted, cryopreserved PBMCs pooled. This culture is termed the small-scale culture because the total culture volume is about 40 mL. Viral propagation was monitored by testing the culture supernatant for viral load every two to three days during the culture with culture supernatant collected and frozen at each timepoint. Once the culture viral load peaks, the culture was terminated, and the culture supernatant harvested from the peak was aliquoted. The product of the first culture was termed the “master lot”.

Of the 370 viral source specimens, an initial high-titer (small-scale) culture has been started with 148 source specimens, and 122 have been successfully cultured to high-titer (82.4% success rate). The success rate for isolation

of the viruses from plasma samples was 69.7% (53 out of 76). The success rate with plasma is similar to rates previously reported for plasma samples with greater than 1×10^4 cp/mL (Manak et al., 2012). The average culture time for the small-scale culture was 8.87 days, and the average peak VL was 8.9×10^9 cp/mL. Fig. 2A shows example growth curves for four viruses: DEMB09CN002 (Supernatant source, peaked at day 4), DEURF11UG004 (Plasma source, peaked at day 7), DEURF09UG002 (Plasma source, peaked at day 11), and DEMB09US002 (Plasma source, peaked at day 11). Master lot samples were all named according to the sample naming convention.

The second step of the culture process is termed the large-scale culture, which is initiated using an aliquot of the master lot. For the large-scale culture the total culture volume was 250 mL. Once the culture was at high-titer, the culture was terminated and stored as 1 mL aliquots. Some culture supernatant was reserved to spike into defibrinated plasma. All final products were aliquoted without any freeze/thaw cycles to preserve the infectivity of the virus. Since the inception of the EQAPOL program, a total of 101 viruses have been taken through both the small-scale and large-scale culture procedures, and the average culture time for the large-scale culture was 6.7 days.

3.4. Characterization of expanded viruses

As viruses were cultured to high-titer/high-volume, they were fully characterized including final viral load, p24, TCID, sterility testing, full length sequencing, and coreceptor analysis. A summary of the current EQAPOL panel is provided in Table 2. Viral load was quantified using the Roche COBAS Taqman Version 2.0 assay, which can quantify a broad range of subtypes, including Group O (De Bel et al., 2010). The average culture supernatant final product concentration was 4.20×10^9 cp/mL, and the average p24 was 206.59 ng/mL (Table 3). Infectivity was measured using a TZM-bl-based TCID assay, and the average TCID for the panel was 2.22×10^6 (Table 3). Sterility testing was performed on culture supernatant samples to ensure that they

Fig. 2. Example growth curves for small-scale and large-scale cultures. A) Example growth curves from four small-scale cultures as measured by viral load (VL). Culture DEMB09CN002 (Triangle) was initiated with previously cultured virus, whereas cultures DEURF11UG004 (Diamond), DEURF09UG002 (Square) and DEMB09US002 (X) were started with plasma. For cultures started with plasma, the VL of the source plasma is indicated as day 0. Cultures were terminated after they had peaked (peak marked as * on graph) and an aliquot of virus from the peak day was used to start the large-scale culture. B) Example growth curves from four large-scale cultures as measured by viral load.

Table 2

Characterization of expanded viruses in EQAPOL Viral Diversity panel.

Sample name	Fiebig stage	Donation year	Country of origin	Source GenBank	HIV subtype	Coreceptor usage	GenBank
DEMA106ES002	ND	2006	Spain ^a	FJ670523	A1	CCR5	JX140651
DEMA105TZ001	ND (CHI)	2005	Tanzania ^a	FJ670519	A1	CXCR4	JX140650
DEMA07UG005	IV	2007	Uganda ^b	JX236676	A1	CCR5	KC596070
DEMA03RW001	III	2003	Rwanda ^b	JX236678	A1	CCR5	KF716499
DEMA03UG002	IV	2003	Uganda ^b	JX236669	A1	CCR5	KF716500
DEMA11KE001	IV	2011	Kenya ^c	KC018749	A1	CCR5	KC018749
DEMA110UG001	VI	2010	Uganda ^c	KC019075	A1	CCR5	KF859745
DEMB09BO001	ND (AHI)	2009	Bolivia ^a	KC112996, KC112997	B	CCR5	JX140656
DEMB10CN002	ND (CHI)	2010	China ^d	None	B	CCR5	JX140658
DEMB05FR001	II/III	2005	France ^e	None	B	CCR5	JX140652
DEMB08FR002	II/III	2008	France ^f	None	B	CCR5	JX140654
DEMBXXDE001	I/II	UNK	Germany ^e	None	B	CCR5	KC596067
DEMB03JP004	I/I	2003	Japan ^f	None	B	CCR5	KC473846
DEMBXXPL001	II	UNK	Poland ^e	JN687774, JN687691	B	CCR5	KC596069
DEMB08ES001	ND (AHI)	2008	Spain ^a	FJ670531	B	CCR5	JX140653
DEMB08UY001	ND (AHI)	2008	Uruguay ^a	GU362886	B	CCR5	JX140655
DEMB09US002	I/II	2009	USA ^f	None	B	CCR5	JX140657
DEMB10VE001	ND (AHI)	2010	Venezuela ^a	KC113011, KC113010	B	CCR5	JX140659
DEMB10ES002	ND (AHI)	2010	Spain ^a	KC113004	B	CCR5	KC473842
DEMB09CN002	ND (CHI)	2009	China ^d	None	B	CCR5	KC596066
DEMB10US001	I/II	2010	USA ^f	None	B	CCR5	KC473825
DEMB11US006	I/II	2011	USA ^f	None	B	CCR5	KC473833
DEMB10US007	I/II	2010	USA ^f	None	B	CCR5	KC473828
DEMB10US011	VI	2010	USA ^f	None	B	CCR5	KC473830
DEMB11US002	VI	2011	USA ^f	None	B	CCR5	KC473831
DEMB10ES003	ND (AHI)	2010	Spain ^a	KC113005	B	CCR5	KC473843
DEMB09ES007	ND (AHI)	2009	Spain ^a	KC112998, KC112999	B	CCR5	KC473841
DEMB10US003	VI	2010	USA ^f	None	B	CCR5	KC473826
DEMB11US015	VI	2011	USA ^f	None	B	CCR5	KC473835
DEMB10US004	VI	2010	USA ^f	None	B	CCR5	KC473827
DEMB11US004	VI	2011	USA ^f	None	B	CCR5	KC473832
DEMB11US011	V	2011	USA ^f	None	B	CCR5	KC473834
DEMB10US009	VI	2010	USA ^f	None	B	CCR5	KC473829
DEMB09US003	I/II	2009	USA ^f	None	B	CCR5	KC473824
DEMB11FR001	I/II	2011	France ^f	None	B	CCR5	KF716496
DEMC07A0001	ND	2007	Angola ^a	EU884500	C	CCR5	JX140662
DEMC07BR003	VI	2007	Brazil ^e	JN687737, JN687655	C	CCR5	JX140663
DEMC08NG001	ND	2008	Nigeria ^a	EU786681	C	CCR5	JX140665
DEMC07ZA011	II	2007	South Africa ^e	JN687618–JN687627, JN687694–JN687703, JN687778–JN687798	C	CCR5	JX140664
DEMC08ZA011	II	2008	South Africa ^e	JN687724, JN687641	C	CCR5	JX140666
DEMC09ZA008	IND	2009	South Africa ^f	None	C	CCR5	JX140667
DEMC09ZA009	I/II	2009	South Africa ^f	None	C	CCR5	JX140668
DEMC10ZA001	I/II	2010	South Africa ^f	None	C	CCR5	JX140669
DEMC06ES003	ND (AHI)	2006	Spain ^a	EU786673	C	CCR5	KC473844
DE00210CM013	ND	2010	Cameroon ^g	JN864054	CRF02_AG and URF_01A1G	CCR5	KF859739
DE00109CN003	IV	2009	China ^d	JX960615	CRF01_AE	CCR5	KF859740
DE00109CN004	IV	2009	China ^d	JX960618	CRF01_AE	CCR5	KC596061
DE00110CN001	IV	2010	China ^d	JX960610	CRF01_AE	CCR5	KC596062
DE00111CN003	ND (CHI)	2011	China ^d	JX960600	CRF01_AE	CCR5	KC596063
DE00111CN002	IV	2011	China ^d	None	CRF01_AE	CCR5	KC596065
DE00206A0001	ND (RHI)	2006	Angola ^a	EU884501	CRF02_AG	CCR5	KC596064
DE00208CM004	VI	2008	Cameroon ^g	None	CRF02_AG	CCR5	JX140645
DE00208CM001	VI	2008	Cameroon ^g	None	CRF02_AG	CCR5	JX140647
DE00400GR002	ND	2000	Greece ^e	JN687744, JN687745, JN687660, JN687661	CRF02_AG	CCR5	JX140646
DE01405BR001	ND	2005	Brazil ^a	FJ670522	CRF04_CPX	CCR5/CXCR4	JX140648
DE01405ES002	ND (CHI)	2005	Spain ^a	FJ670528	CRF14_BG	CCR5/CXCR4	JX140649
DE02210CM011	ND	2010	Cameroon ^g	JN864051	CRF14_BG	CCR5/CXCR4	KC473837
DE02210CM012	ND	2010	Cameroon ^g	JN864058	CRF22_01A1	CCR5	KF716461
DE02210CM014	ND	2010	Cameroon ^g	JN864059	CRF22_01A1	CCR5/CXCR4	KF716462
DE02210CM010	ND	2010	Cameroon ^g	JN864050	CRF22_01A1	CCR5	KF716463
DE02408ES002	ND (RHI)	2008	Spain ^a	FJ670526	CRF22_01A1	CCR5	KF716460
DE04708ES004	ND (AHI)	2008	Spain ^a	FJ670529	CRF24_BG	CCR5	KC473838
DE04708ES003	ND (AHI)	2008	Spain ^a	GQ372987	CRF47_BF	CCR5	KC473840
DEMD10CM009	VI	2010	Cameroon ^g	None	CRF47_BF	CCR5/CXCR4	KC473839
DEMD07UG002	II	2007	Uganda ^b	JX236670	D	CCR5/CXCR4	JX140670
DEMD08UG001	I	2008	Uganda ^b	JX236672	D	CCR5	KC596071
DEMD07UG007	I	2007	Uganda ^b	JX236673	D	CCR5	KC596072
DEMD07UG001	IV	2007	Uganda ^b	JX236679	D	CCR5	KF716503
DEMD05UG001	II	2005	Uganda ^b	JX236668	D	Pending	KF716502
DEMD11KE003	IV	2011	Kenya ^c	KC018957	D	CCR5	KF716501
DEMD10UG004	VI	2010	Uganda ^c	KC018740	D	Pending	KF716476
					D	CCR5/CXCR4	KF716479

Table 2 (continued)

Sample name	Fiebig stage	Donation year	Country of origin	Source GenBank	HIV subtype	Coreceptor usage	GenBank
DEMD11UG003	VI	2011	Uganda ^c	KC018603	D	CCR5/CXCR4	KF716480
DEMF110ES001	ND (RHI)	2010	Spain ^a	JN010222, KC113009	F1	CCR5	JX140671
DEMF210CM001	VI	2010	Cameroon ^g	None	F2	CCR5	JX140672
DEMF210CM007	VI	2010	Cameroon ^g	None	F2	CCR5	JX140673
DEMG10CM008	VI	2010	Cameroon ^g	None	G	CCR5	JX140676
DEMG05ES001	ND (RHI)	2005	Spain ^a	EU786670	G	CCR5	JX140674
DEMG09ES002	ND (AHI)	2009	Spain ^a	GU362882	G	CCR5	JX140675
DEOXXDE004	ND	UNK	Germany ^g	None	Group O	CCR5	KF859742
DEOXXES001	ND	UNK	Spain ^g	None	Group O	CCR5	KF859743
DEOXXUS001	ND	UNK	USA ^g	None	Group O	CCR5	KF859744
DEURF09ES005	ND	2009	Spain ^a	KC113007	URF_A1B	CCR5	JX140678
DEURF10US008	I/II	2010	USA ^f	None	URF_A1B	CCR5	KC473836
DEURF07UG006	I	2007	Uganda ^b	JX236675	URF_A1D	CCR5	KC596073
DEURF08UG002	I	2008	Uganda ^b	JX236674	URF_A1D	CCR5	KF716504
DEURF10UG011	VI	2010	Uganda ^c	KC019010	URF_A1D	CCR5	KF716482
DEURF11UG004	VI	2011	Uganda ^c	KC019104	URF_A1D	CCR5	KF716484
DEURF09KE002	VI	2009	Kenya ^c	KC018751, KC018658, KC018750	URF_A1D	CCR5	KF716468
DEURF10UG005	VI	2010	Uganda ^c	KC018940	URF_A1D	CCR5	KF716487
DEURF09UG002	VI	2009	Uganda ^c	KC018893	URF_A1D	CCR5	KF859746
DEURF08CM005	ND	2008	Cameroon ^g	JN864047	URF_A1G	CCR5	KF716465
DEURF09GQ001	ND	2009	Equatorial Guinea ^a	KC113000, KC113001	URF_A1DG	CCR5	KC473845
DEURF07BR002	V	2007	Brazil ^e	None	URF_BC	CCR5	KC596068
DEMBF09ES003	ND	2009	Spain ^a	KC113006	URF_BF	CCR5	JX140660
DEMBF09ES006	ND (AHI)	2009	Spain ^a	KC113008	URF_BF	CCR5	JX140661
DEURF07ES002	ND (AHI)	2007	Spain ^a	GQ372986	URF_A1DGJK	CCR5	JX140677
DEURF10DZ001	ND	2010	Algeria ^a	KC113003, KC113002	URF_CRF02_AG/ CRF06_cpx	CCR5	JX140679
DEURF11RW001	VI	2011	Rwanda ^c	KC018537	URF_DC	CCR5	KF716473
DEURF06CM001	ND	2006	Cameroon ^g	FJ014688	URF_X	CCR5/CXCR4	KF716464
DEURF10KE002	VI	2010	Kenya ^c	KC018842	URF_X	CCR5	KF716470

Fiebig staging data provided by collaborating sites or when plasma was available performed by EQAPOL laboratory. HIV subtype is based on full-length sequencing, which was submitted to GenBank. Both Source specimen GenBank and Expanded virus GenBank numbers are provided. Coreceptor usage evaluated using a NP-2-cell-based assay.

ND, not determined; IND, Indeterminate (Negative EIA, Indeterminate WB); AHI, Acute HIV Infection; RHI, Recent HIV Infection; CHI, Chronic HIV Infection; UNK, Unknown; URF_X, Unique recombinant form with greater than three subtypes present.

^a Alvarez, Instituto de Salud Carlos III.

^b B. Hahn/G. Shaw, University of Pennsylvania.

^c International AIDS Vaccine Initiative.

^d H. Shang, First Affiliated Hospital, China Medical University.

^e SeraCare.

^f Blood Systems Research Institute.

^g I. Hewlett, Food and Drug Administration.

are free of bacterial any mycoplasma contamination. Thus far all sterility testing has been negative, except for one culture where the source mycoplasma contamination was traced to the original stock culture supernatant.

One near full-length genome was obtained by two overlapping half SGA amplicons for each isolate. The ambiguous bases were detected in 14 viruses, and the most of them contain only one ambiguous base. Thus, those rare ambiguous bases could not affect genetic subtyping of the viral isolates in the diversity panel. Although the diversity of the each expanded viral isolate was not investigated, one near full-length genome sequence from each isolate was sufficient to define the genotype of the isolate. Fig. 3 shows a phylogenetic tree for the 100 samples present in the EQAPOL panel. Thus far the EQAPOL Viral Diversity panel includes 6 subtypes (A, B, C, D, F, G), 2 sub-subtypes (F1 and F2), 7 CRFs (01, 02, 04, 14, 22, 24 and 47), 19 URFs and group O viruses from 22 countries (Table 4). The panel continues to grow with a focus on identifying samples to expand the overall genetic and geographic diversity of the panel.

Coreceptor usage was determined using a NP-2-cell-based assay (Shimizu et al., 2009; Jiang et al., 2011). Eighty eight

viruses were CCR5-tropic, one was CXCR4-tropic, and five were dual tropic (CCR5/CXCR4). Two viruses (DEMB07UG001 and DEMD11KE003) had a very low TCID. Their infectivity in NP-2 cells was too weak to be confirmed. Whether they use other alternative coreceptors is under investigation.

3.5. System for access to EQAPOL samples

Fully characterized samples are made available to order on the EQAPOL web-based system. The web-based system captures characterization data for HIV source specimens, intermediate products and final products, as well as details about the culture process. Additionally the web-based system displays a COA for each virus, which includes all of the characterization data and source specimen data. External sites, which are approved by NIAID, are able to access the web-based system and search through an inventory of viruses available to order. The web-based system provides a real-time inventory of available vials, and allows users to search/view characterization data to select samples of interest for order. The ordering process is captured in the web-based system and allows for tracking of samples from

Table 3

Viral load, p24 and infectivity of EQAPOL Viral Diversity panel.

	Viral load (cp/mL)	p24 (ng/mL)	TCID
Average	4.20E + 09	206.59	2.22E + 06
Max	1.75E + 10	738.00	3.01E + 07
Minimum	8.57E + 07	25.00	2.80E + 04

The average and range of viral load (Roche COBAS AmpliPrep/TaqMan HIV-1 v2.0), p24 (PerkinElmer HIV-1 p24 antigen capture assay) and TCID (TZM-bl-based cell assay) for the expanded viruses in the EQAPOL Viral Diversity panel.

order to receipt. Samples and panels are provided to qualified investigators, commercial assay and vaccine developers and regulators at no charge.

4. Discussion

For the EQAPOL Viral Diversity program, a comprehensive, well-controlled and standardized system to collect, propagate and characterize HIV was developed. Work by BSRI and other collaborators have yielded 370 viral specimens with an emphasis on samples from recently-acquired infections. Work is ongoing to acquire additional panel source specimens, in particular those from genetic subtypes and geographic locations not represented on the current panel, as well as from emerging recombinants. The goal is for the panel to represent the current trends observed in transmitted HIVs throughout world. As the panel expands, it may be able to identify viral diversity within geographic areas and be used to determine vaccine immunogens to include within candidate vaccines that may be required to confer protection.

One of the hallmarks for the EQAPOL program is the commitment to quality and standardization through the implementation of GCLP. This is of particular importance for the Viral Diversity Panel where products will be utilized in the development and assessment of a variety of assays. As discussed in [Todd et al. \(2014\)](#), the EQAPOL Viral Diversity program adheres to GCLP when available, in particular through the culture process and much of the characterization. Work is currently underway to bring all aspects of the characterization work to GCLP. The inclusion of a COA with all products allows investigators to have documentation attesting to the quality and characteristics of each sample.

In support of EQAPOL, we have developed a user-friendly web-based system where approved laboratories can search through an inventory of viral samples to select items for order. Users can search for items using criteria such as viral load, subtype, country of origin and year of donation. The system provides a link to the GenBank sequence so that sites can have access to the full-length sequence of each product. Once a user orders items, the web-based system is used to ensure the site has the appropriate compliance documents to receive

samples and to alert sites via email shipment tracking information. The web-based system, in combination with LDMS, allows for traceability of each vial from creation to shipment to a site.

Now that the panel has expanded to include a sizeable number of viruses, we are beginning to assemble the viruses into pre-made panels that will be made available to researchers for specific uses. Through collaboration with the Program for Appropriate Technology (PATH) we have created two panels using subtype B, C and D viruses to assist laboratories participating in the Gates Foundation Grand Challenges Point-of-Care Diagnostics program. One panel included a dilution series of HIV Spiked Plasma for the three subtypes. The other panel included a dilution series of isolated RNA for the three subtypes to be used by laboratories not equipped with the safety equipment required to work with live virus.

Globally diverse HIV strains may prove to be a challenge to current methods of genotyping and drug resistance testing. The Recipient Epidemiology and Donor Evaluation Study-III (REDS-III) program funded by the National Heart, Lung and Blood Institute was established to ensure safe and effective blood banking and transfusion medicine at domestic and international blood donation centers (<https://reds-iii.rti.org/REDSProgram.aspx>). The REDS program wanted to assess the molecular methods for accurately testing genotype and drug resistance in the international reference laboratories. A Genotype Diversity Panel was assembled from available viral isolates from the EQAPOL program; this contains 50 viral isolates representing globally diverse HIV genotypes and drug resistant phenotypes. It has been distributed to the international REDSIII sites located in South Africa, Brazil and China. Each of the laboratories will use standard procedures used for testing and this will determine accuracy or potential limitations in the current methods used. Both the PATH Panel and the Genetic Diversity Panel developed for the REDS program are available to interested laboratories. As the EQAPOL Viral Program continues to expand, additional panels will be constructed to meet the needs of the research community, with plans to develop diversity panels for evaluation of relative analytical sensitivity across HIV diagnostic assay platforms.

In summary, we have developed a HIV Subtype panel to capture the current genetic and geographic diversity observed as a continuation of a pilot viral diversity program ([Manak et al., 2012](#)). Combined with the pilot program, the Viral Subtype Diversity panel currently includes over 130 unique virus isolates and continues to grow each year. Laboratories interested in gaining access to the EQAPOL panel should contact the EQAPOL program via email (EQAPOL@duke.edu). Products are available free of charge to NIAID-approved laboratories. The EQAPOL Viral Diversity panel as an invaluable collection of well-characterized reagents are currently made available to the scientific community, including researchers, epidemiologists, and commercial manufacturers of diagnostics and pharmaceuticals to support HIV research.

Fig. 3. Phylogenetic analysis of newly characterized near full-length HIV-1 genome sequences. The phylogenetic tree was constructed with near full-length HIV-1 genome nucleotide sequences, using the neighbor-joining method. The tree was midpoint rooted. Horizontal branch lengths are drawn to scale (the scale bar represents 0.02 nucleotide substitution per site). Vertical separation is for clarity only. Asterisks at the nodes indicate the bootstraps in which the cluster to the right was supported by 80% and higher. Brackets at the right denote the major subtypes, CRFs and group Os. The reference sequences are indicated by italics. The unique recombinant form (URF) sequences are indicated by arrow.

0.02

Table 4
Genetic and Geographic Diversity Present in Viral Diversity Panel.

Country	Number of Samples	Genotype
Algeria	1	URF_CRF02_AG/CRF06_cpx
Angola	1	C
	1	CRF02_AG
Bolivia	1	B
Brazil	1	C
	1	CRF14_BG
	1	URF_BC
Cameroon	3	CRF02_AG
	4	CRF22_01A1
	1	D
	2	F2
	1	G
	1	URF_01A1G
	1	URF_A1G
China	2	B
	5	CRF01_AE
Equatorial Guinea	1	URF_ADG
France	3	B
Germany	1	B
	1	Group O
Greece	1	CRF04_CPX
Japan	1	B
Kenya	1	A1
	1	D
	1	URF_A1CD
	1	URF_A1D
Nigeria	1	C
Poland	1	B
Rwanda	1	A1
	1	URF_DC
South Africa	5	C
Spain	1	A1
	4	B
	1	C
	1	CRF14_BG
	1	CRF24_BG
	2	CRF47_BF
	1	F1
	2	G
	1	Group O
	1	URF_A1B
	2	URF_BF
	1	URF_CRF02_AG/A3
Tanzania	1	A1
Uganda	3	A1
	7	D
	6	URF_A1D
Uruguay	1	B
USA	13	B
	1	Group O
	1	URF_A1B
Venezuela	1	B
Total	100	

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