Streamlined Subpopulation, Subtype, and Recombination Analysis of HIV-1 Half-Genome Sequences Generated by High-Throughput Sequencing

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ABSTRACT  High-throughput sequencing (HTS) has been widely used to characterize HIV-1 genome sequences. There are no algorithms currently that can directly determine genotype and quasispecies population using short HTS reads generated from long genome sequences without additional software. To establish a robust subpopulation, subtype, and recombination analysis workflow, we amplified the HIV-1 3'-half genome from plasma samples of 65 HIV-1-infected individuals and sequenced the entire amplicon (~4,500 bp) by HTS. With direct analysis of raw reads using HIVE-hexahedron, we showed that 48% of samples harbored 2 to 13 subpopulations. We identified various subtypes (17 A1s, 4 Bs, 27 Cs, 6 CRF02_AGs, and 11 unique recombinant forms) and defined recombinant breakpoints of 10 recombinants. These results were validated with viral genome sequences generated by single genome sequencing (SGS) or the analysis of consensus sequence of the HTS reads. The HIVE-hexahedron workflow is more sensitive and accurate than just evaluating the consensus sequence and also more cost-effective than SGS.

IMPORTANCE The highly recombinogenic nature of human immunodeficiency virus type 1 (HIV-1) leads to recombination and emergence of quasispecies. It is important to reliably identify subpopulations to understand the complexity of a viral population for drug resistance surveillance and vaccine development. High-throughput sequencing (HTS) provides improved resolution over Sanger sequencing for the analysis of heterogeneous viral subpopulations. However, current methods of analysis of HTS reads are unable to fully address accurate population reconstruction. Hence, there is a dire need for a more sensitive, accurate, user-friendly, and cost-effective method to analyze viral quasispecies. For this purpose, we have improved the HIVE-hexahedron algorithm that we previously developed with in silico short sequences to analyze raw HTS short reads. The significance of this study is that our standalone algorithm enables a streamlined analysis of quasispecies, subtype, and recombination patterns from long HIV-1 genome regions without the need of additional sequence analysis tools. Distinct viral populations and recombination patterns identi-
fied by HIVE-hexahedron are further validated by comparison with sequences obtained by single genome sequencing (SGS).

**KEYWORDS** HIV-1, genetic recombination, quasispecies, sequencing

The genome of human immunodeficiency virus type 1 (HIV-1) is highly variable due to the error-prone reverse transcriptase (1, 2) and short life cycle during infection (3, 4). Genetic diversity of a viral population in an infected individual increases by ~1% annually during natural infection history (5). Thus, the viral population in a chronically infected individual is constituted of a swarm of genetically similar but distinct viruses, or quasispecies (6, 7). High genetic variability plays an important role in escape from host immune selection pressure and development of drug resistance (8–11). The complex quasispecies nature of HIV-1 makes it difficult to analyze the viral population in each infected individual. This genetic variability results in 10 subtypes among group M viruses (https://www.hiv.lanl.gov/) (12–14). Different subtypes can have significant impacts on pathogenesis, disease progression, and drug treatment efficacy (9–11). The amino acid difference in the envelope glycoprotein (Env) can vary up to 30% (15). This extraordinary level of genetic variation poses an obstacle for development of cross-reactive vaccines and challenges for genetics-based detection assays (9–11). Due to the highly recombinogenic nature of HIV-1, over 100 circulating recombinant forms (CRFs) and many unique recombinant forms (URFs) among different subtypes have been identified (13, 16). The recombinant viruses are often detected in regions where two or more distinct subtypes are present and account for ~20% of the global HIV-1 strains (11, 12). Since it is more difficult to detect recombinants among the viruses from the same subtype (17–19), their actual percentage in the world may be much higher.

Analysis of HIV-1 genome sequences is important for understanding viral evolution, subtype distribution, transmission, pathogenesis, transmission network, drug resistance surveillance, and vaccine development (9, 12, 15, 20–23). A large number of assays have been developed to study genetic diversity at the population and individual levels. Among them, the most widely used is the population sequencing method that directly sequences the bulk PCR products using Sanger sequencing (5, 6). While this method is simple and commonly used for subtyping and drug resistance surveillance, it only generates a consensus sequence of all viral populations in a PCR product from the sample. The final sequences often contain ambiguous bases because of complex quasispecies in the sample. When viral diversity is studied by sequencing multiple clones from a bulk PCR product, results can be seriously affected by resampling, artificial misincorporation errors, and recombinants generated during PCR (24, 25). To overcome these issues, a single genome sequencing (SGS) method was developed by PCR amplification of single viral genomes in individual reactions (26, 27). SGS can reliably study the viral populations and has played an important role in analysis of linked drug resistance mutations in the same viral genome (26) and determination of transmitted/founder viruses that establish clinical HIV-1 infection (27, 28). But it is labor-intensive, time-consuming, and costly.

High-throughput sequencing (HTS) that produces massively parallel sequences is now being used to study complex genome populations (29–33). HTS provides improved resolution over Sanger sequencing for identification and analysis of heterogeneous viral subpopulations (31, 34). Initially, the high cost of HTS was inhibitory for studying a quasispecies population in a sample. However, recently using sequence identifiers and tags, it provides unprecedented promise for large-scale sequencing of the viral population in a simpler, cost-effective, and time-efficient manner (35–38). Two kinds of HTS methods have been developed: long read (PacBio and Nanopore) and short read (Illumina, Ion Torrent, and 454). Though PacBio and Nanopore sequencing technologies have the advantage of longer read lengths (up to 900 kb), they have the disadvantage of lower throughput, higher error rate, and higher cost per base (39–41). Among HTS methods, Illumina is the most commonly used with an impressive increase in throughput and the lowest per-base cost (39–42). Currently, HTS has been
widely used to analyze viral quasispecies for small genomic regions (<500 bp). Minority variants present at ≥1% frequency in a sample can be characterized by HTS when sufficiently large numbers of templates are analyzed (33, 43). However, when long-range PCR products (>4,000 bp) are analyzed, it is difficult to reliably identify subpopulations to understand the complexity of a quasispecies because short sequence reads cannot be reliably assembled into full-length consensus sequences for each subpopulation in the sample.

Specialized sequence analysis is required to study the genetic diversity using HTS data. With advancement of analytical techniques, many algorithms have become available to analyze HTS short-read data, but accurate population reconstruction is still elusive. Despite significant improvements in analytical approaches, there has not been significant effort to address this question, and each of the current approaches has different limitations. For example, traditional algorithms like ShoRah (44) can align only a limited number of reads (up to tens of thousands) and call haplotypes based on the center of clusters. ViSpA (45) and QuRe (46) were designed to analyze reads generated by pyrosequencing and focus on identifying insertions and deletions as well as errors in homopolymeric regions. PredictHaplo (47) and HaploClique (48) can analyze data from methods other than pyrosequencing but still are limited in the number of reads that can be analyzed.

High-performance Integrated Virtual Environment (HIVE) is a robust cloud-based infrastructure designed to handle HTS data (49, 50). It provides secure web access to registered users to store, retrieve, annotate, and compute HTS data and visualize the outcome of computations on a user-friendly interface (49, 50). HIVE-hexahedron is a novel deterministic algorithm that can overcome the aforementioned limitations and process the HTS data at a much larger scale than before (32). This algorithm, when used with in silico reads, showed an improved performance in sequence reconstruction and identification of discrete populations in heterogeneous samples (51).

Targeted small regions (<600 bp) of HIV-1 genomes have been widely used to study viral populations in samples using short-read HTS methods (33, 43). However, because of the high levels of genetic variation and recombinogenic nature of HIV-1, such short sequences do not reveal the real quasispecies nature of HIV-1 in a given sample (52–54). When half or whole genomes are sequenced by HTS, the quasispecies cannot be determined with the current computational tools (44, 55–58). In addition, subtyping and recombination analyses of sequences generated by HTS from long sequences (>4,000 bp) need to be performed using additional conventional software (46, 47). To be able to simultaneously determine viral subpopulations, subtypes, and recombination patterns in a sample with HTS short reads on a single platform, we developed a new HIVE-hexahedron algorithm by incorporating a Nearest Neighbor pipeline and introduced advanced parameters in HIVE-hexagon (aligner). We tested the pipeline by analyzing HTS short reads from HIV-1 3'-half genome sequences (~4,500 bp) generated from 65 chronically infected individuals. More importantly, results from HIVE-hexahedron were validated by the consensus sequences generated by the commonly used Geneious software for all samples and multiple individual viral genome sequences generated by SGS from seven samples. Our results demonstrate that HIVE-hexahedron can simultaneously determine subpopulations, subtypes, coinfections, and recombination of a viral population by analyzing long-range sequences generated by short HTS reads without exporting sequences for additional analysis using other computational tools.

RESULTS

Polymorphic sites in a viral population are not resolvable in the consensus sequence generated by Geneious. The raw paired-end HTS reads from each sample were loaded to the Geneious assembler and mapped to 3'-half genome sequence from reference HXB2 using medium/fast sensitivity and five-time iterations of the assemblies with default parameters. After the reads were mapped to the reference, the majority of the samples (52 of 65) had <2% of unused reads while the rest had 2.22% to 21%
unused reads. The mean coverage was between 360 and 63,195 at any nucleotide position. The assembled reads resulted in one contig, and the consensus of the contig was generated. Phylogenetic analysis of the consensus sequences from 65 samples showed 17 subtype A1s (26.15%), 4 subtype Bs (6.15%), 27 subtype Cs (41.5%), 7 CRF02_AGs (10.76%), and 10 URFs (15.38%) (Table 1). Since the consensus sequence generated by the Geneious assembler is an aggregate of all variants at each site within the population, ambiguous bases were detected in 43 sequences (Table 1). Among them, 11 sequences had more than 50 ambiguous bases and as many as 217 ambiguous bases were found in one sequence (707010585), suggesting the presence of quasispecies in samples with many ambiguous bases.

Identification of distinct HIV-1 subpopulations in the same samples by HIVE-hexahedron. We previously developed a HIVE-hexahedron algorithm and determined subpopulations using simulated in silico sequence data sets (32). In order to investigate if the ambiguous bases in the consensus sequences generated by Geneious indicate the presence of viral populations in those samples, we analyzed the same sequence data sets using HIVE-hexahedron. Several modifications were made in the original algorithm to better define viral subpopulations in the real HTS data from 65 HIV-1 samples (see Materials and Methods). Five steps were followed to determine the subtype(s) and the number of subpopulations in the samples (Fig. 1a). First, the quality of HTS reads from each sample was inspected using quality control graphs for “Quality length counts” (Fig. 1b) and “Quality position count” (Fig. 1c) generated in HIVE. All the reads selected for subpopulation analysis had quality length count and quality position count higher than a 30 Phred score. The first alignment step was to select the nearest neighbor references (with \( \geq 10,000 \) reads per kilobase of transcript per million [RPKM]) for optimal assembling. Each read from a sample was mapped to the best-scored reference out of the reference set using the alignment tool HIVE-hexagon (59). The tool uses some advanced features for trimming loosely aligned ends. This is also a primary step to determine subtypes of the viral sequence. The second alignment was performed on the reads per sample against their nearest neighbor reference(s). If more than one nearest neighbor reference was identified in a sample, a multiple sequence alignment (MSA) of the nearest neighbor references was done using MAFFT (60). The results of the second alignment were remapped with a common coordinate system generated by MAFFT using HIVE-hexahedron. The multiple sequence alignments of the nearest neighbor references were not imperative to the HIVE-hexahedron. However, multiple references covering divergent HIV-1 sequences will provide a better platform for reference-assisted de novo assembly of HIV-1 populations and identify coinfections by different subtypes or recombinant viruses. The results were further improved by postprocessing steps (see Materials and Methods) and visualized using Sankey diagrams (Fig. 1d). HIVE-hexahedron displays subpopulation analysis results in the form of interactive Sankey diagrams, which exhibit the coverage per position, subtype(s) of each viral subpopulation, and number of variations that support each subpopulation. Each band in the Sankey diagram represents a separately reconstructed subpopulation, with vertical gray and red lines representing bifurcation and merging events, respectively (Fig. 2). The bifurcation and merging events depict where a subpopulation is detected and where it ends, respectively, relative to the coordinates of multiple sequence alignment (MSA) or the coordinates of resolved sequences. A population is considered major if it covers the entire length of the amplicon. Minor subpopulations are short contigs that bifurcate from the parent population and have at least five mutations in a 500-bp region (1%) compared to its parent. They have an average depth of coverage of 50 and minimum length of 500 bp. The Sankey plots provide a visualization of the reads that support the subpopulation detection. The algorithm resolves the subpopulation and calculates the depth of coverage for each population at each position. Thus, the width of each Sankey diagram is an indicator of the relative abundance at each position. As part of the interface, the user can postcomputationally retrieve the relative abundance of the inferred globally reconstructed sequences for all populations at any given positions.
TABLE 1  Diversity of the viral population in samples determined by HIVE and Geneious

| Sample ID  | Viral load (copies/ml) | Subtype     | No. of ambiguous bases detected by Geneious | No. of viral populations detected by HIVE |
|------------|------------------------|-------------|---------------------------------------------|------------------------------------------|
| 700010501  | 31,875                 | B           | 0                                           | 1                                        |
| 700010516  | 10,310                 | B           | 0                                           | 1                                        |
| 700010118  | 32,081                 | C           | 0                                           | 1                                        |
| 70010322   | 57,901                 | C           | 0                                           | 1                                        |
| 70010675   | 146,346                | C           | 0                                           | 1                                        |
| 70410566   | 26,700                 | C           | 0                                           | 1                                        |
| 70510303   | 26,833                 | C           | 0                                           | 1                                        |
| 70510661   | 31,081                 | C           | 0                                           | 1                                        |
| 70510801   | 4,704                  | C           | 0                                           | 1                                        |
| 70610391   | 271,000                | C           | 0                                           | 1                                        |
| 70710277   | 97,122                 | URF_A1C     | 0                                           | 1                                        |
| PK005      | 610                    | CRF02_AG    | 0                                           | 1                                        |
| PK010      | 1,250                  | C           | 0                                           | 1                                        |
| PK011      | 8,750                  | URF_A102    | 0                                           | 1                                        |
| PK021      | 93,500                 | A1          | 0                                           | 1                                        |
| PK025      | 26,900                 | CRF02_AG    | 0                                           | 1                                        |
| PK032      | 6,400                  | CRF02_AG    | 0                                           | 1                                        |
| PK034      | 4,665                  | A1          | 0                                           | 1                                        |
| PK016      | 13,000                 | A1          | 0                                           | 1                                        |
| PK026      | 5,350                  | A1          | 1                                           | 1                                        |
| 70010339   | 97,857                 | C           | 2                                           | 1                                        |
| 70311871   | 150,182                | C           | 3                                           | 1                                        |
| 70410715   | 319,000                | C           | 4                                           | 1                                        |
| 70310523   | 88,878                 | C           | 5                                           | 1                                        |
| PK036      | 96,500                 | A1          | 5                                           | 1                                        |
| PK039      | 60,000                 | CRF02_AG    | 6                                           | 2                                        |
| PK099      | 173,000                | C           | 7                                           | 1                                        |
| 70510381   | 21,352                 | C           | 8                                           | 2                                        |
| PK077      | 106,000                | A1          | 8                                           | 2                                        |
| 70010329   | 11,014                 | B           | 11                                          | 1                                        |
| PK004      | 464,000                | A1          | 11                                          | 4                                        |
| PK017      | 98,700                 | C           | 12                                          | 1                                        |
| PK027      | 111,500                | A1          | 15                                          | 4                                        |
| PK038      | 456,000                | CRF02_AG    | 16                                          | 3                                        |
| PK038      | 456,000                | CRF02_AG    | 16                                          | 3                                        |
| 707101138  | 17,213                 | A1          | 16                                          | 3                                        |
| 70310234   | 183,452                | C           | 17                                          | 1                                        |
| PK019      | 760,000                | CRF02_AG    | 18                                          | 3                                        |
| 70510474   | 40,497                 | C           | 20                                          | 1                                        |
| PK031      | 225,500                | A1          | 20                                          | 3                                        |
| PK001      | 70,000                 | A1          | 23                                          | 6                                        |
| PK020      | 208,000                | A1          | 24                                          | 5                                        |
| PK023      | 65,500                 | URF_A102    | 27                                          | 4                                        |
| PK035      | 461,500                | CRF02_AG    | 27                                          | 5                                        |
| PK002      | 488,000                | A1          | 34                                          | 9                                        |
| PK040      | 111,000                | A1          | 38                                          | 13                                       |
| 70010260   | 74,588                 | B           | 42                                          | 2                                        |
| PK015      | 300,000                | URF_A102    | 42                                          | 6                                        |
| 70410486   | 233,000                | C           | 48                                          | 3                                        |
| PK018      | 88,500                 | A1          | 57                                          | 6                                        |
| 70510499   | 46,057                 | C           | 61                                          | 2                                        |
| PK008      | 625,000                | URF_A102    | 61                                          | 5                                        |
| PK006      | 710,000                | URF_A102    | 67                                          | 8                                        |
| PK013      | 193,000                | A1          | 69                                          | 7                                        |
| PK033      | 425,500                | URF_A102    | 71                                          | 7                                        |
| PK017      | 119,000                | A1          | 80                                          | 5                                        |
| PK033      | 15,208                 | URF_CD      | 100                                         | 3                                        |
| PK003      | 15,208                 | URF_CD      | 166                                         | 3                                        |
| 70710585   | 79,367                 | URF_A1C     | 217                                         | 5                                        |
An example of the major and minor viral subpopulations observed in three samples using Sankey diagrams is shown in Fig. 2. Only one population was detected in PK034 (Fig. 2a), indicating a relatively homogenous viral population. The light green color of the Sankey diagram represents the subtype of the virus, in this case subtype A1. The width of the Sankey diagram represents the depth of coverage per position along the x axis. Three subpopulations were detected in 707010038: one major and two minor (Fig. 2b). All trajectories following the bifurcation and merging events are assemblies representing possible different subpopulations (32). The subpopulations 2 and 3 differed from the major population by 1.6% and 6.2%, respectively. The detection of three subpopulations in 707010038 also explains the unresolved 16 ambiguous bases detected by Geneious. In PK033, eight subpopulations were identified: one major and seven minor (Fig. 2c). In addition to multiple subpopulations, two nearest neighbor references were identified by HIVE-hexahedron, and they are shown in light green (CRF02_AG) and dark green (A1) in Sankey diagrams (Fig. 2c). The genetic differences among consensus sequences of all viral subpopulations were high (3.63% to 11.8%) and
this also explains why there were 71 ambiguous bases that could not be resolved by Geneious (Table 1).

Sequence analysis using HIVE-hexahedron showed that 47.69% (31/65) of samples harbored subpopulations. Twenty-four samples (36.92%) had 2 to 5 subpopulations, and six samples (9.23%) had 6 to 9 subpopulations, while one sample (1.53%) had up
to 13 subpopulations (Table 1; see also Fig. S1 in the supplemental material). Thirty-four samples that did not have detectable subpopulations by HIVE-hexahedron had 20 or fewer ambiguous bases detected by Geneious. All 22 samples with no ambiguous bases by Geneious and the majority (28/30; 93.33%) of the samples with ≥5 ambiguous bases had only one detectable subpopulation by HIVE-hexahedron. Conversely, the majority (29/35; 82.85%) of the samples with ≥5 ambiguous bases had more than one detectable subpopulation (Table 1 and Fig. S1). All 20 samples with >20 ambiguous bases had ≥2 subpopulations. The numbers of ambiguous bases identified by Geneious were significantly correlated with the numbers of subpopulations detected in samples by HIVE-hexahedron (nonparametric Spearman correlation $r = 0.8015; P < 0.0001$) (Fig. 3).

**Determination of HIV-1 subtypes.** One of the unique advantages of HIVE-hexahedron is that multiple sequences can be simultaneously used as references. This allows it to determine which HIV-1 subtype reference(s) the test sequences are most similar to and thus determine the subtypes of the viral sequences. Due to high variability among viral sequences in each subtype, we used subtype consensus sequences from the Los Alamos HIV sequence database to better define subtypes of the samples. This set of 20 consensus sequences contains eight subtypes (A1, A2, B, C, D, F1, G, and H) and 10 CRFs (which cover all subtypes detected in our samples by analyzing the Geneious-derived sequences) and two outlier controls (group O and SIVcpz). The raw read sequences from each sample were mapped to this set of 20 HIV-1 references using HIVE-hexahedron. The specific algorithm in HIVE-hexahedron was used to select the best-scoring reference for each read sequence in a sample. Thus, the first alignment step in HIVE-hexagon can determine subtype references that are the nearest neighbors for the reads (Fig. 1d). This is defined by the RPKM values greater than 10,000. When more than one nearest neighbor was identified by HIVE-hexahedron, an additional alignment step(s) of the reads was carried out with the other nearest neighbor(s). The analysis of the HTS data from all 65 samples by HIVE-hexahedron showed similar subtyping results as determined by Geneious (Table 1 and Fig. S1). Phylogenetic tree analysis of the major contig consensus sequences generated from HIVE-hexahedron and Geneious-derived consensus sequences from the same sample showed that both sequences from the same sample always clustered together (Fig. 4). While the differences were generally small between the sequences generated by HIVE and Geneious from the same viruses, larger genetic distances (>0.4%) were observed for 15 viruses (707010585, 707010134, 704010486, 705010699, 702010133, 706010375, PK002, PK003, PK006, PK008, PK013, PK017, PK018, PK033, and PK040). Examination of these sequences showed that there were many ambiguous bases in the sequences generated by Geneious in all but one sample (706010375). This suggested that higher genetic differences between sequences generated by HIVE and Geneious were caused by more divergent viral populations.
Detection of different viruses in the same sample. More than one virus can be frequently detected in an infected individual (27, 28, 61, 62). This can often result in generation of recombinants between the different subtype viruses in such infected individuals. Thus, it is important to know how frequently individuals are infected by two distinct viruses. Although analysis of 65 viruses by HIVE-hexahedron did not identify coinfections with two or more subtypes that differ from each other across the entire 3’-half genome (Fig. S1), regions that were clearly distinct between A1 and CRF02_AG were observed in two viruses, PK033 and PK006 (Fig. 2c and Fig. S1). The distributions of raw reads from CRF02_AG (light green) and subtype A1 (dark green) varied significantly among different contigs (Fig. S2 and S3), suggesting that viruses at these regions were derived from different subtypes. Phylogenetic tree analysis of these region sequences demonstrated that they clustered with CRF02_AG and subtype A1 separately (Fig. S2 and S3). These results showed that distinct viral populations were present in these samples. Although they were different from each other at part of the 3’-half genome, different viral populations were not identified by Geneious.

Detection of recombinant sequences. When the HTS read sequences were from different subtypes or CRFs, they could be correctly defined by aligning to different
nearest neighbor references in HIVE-hexahedron (Fig. 2 and Fig. S1). This should allow detection of recombinant genomes and define recombination breakpoints between different subtypes and/or CRFs using HIVE-hexahedron. For example, both subtype A1 and C read sequences were detected in 707010585 and aligned to corresponding reference sequences, shown in the Sankey diagram. The recombination pattern of 707010585 was visualized by the Sankey diagram (Fig. 5a). It contained three recombinant regions: subtype C (vif-tat-rev-vpu-env beginning; nucleotide [nt] 4906 to 6542 [based on positions in HXB2]), subtype A1 (middle part of env; nt 6543 to 8081), and subtype C (end of env-tat-rev-nef; nt 8082 to 9531), as schematically shown in Fig. 5b. Analysis of the 707010585 consensus sequence generated by Geneious, using jPHHM and Simplot, showed similar recombination breakpoints (Fig. 5c). Analysis of all consensus sequences generated by Geneious identified 10 URF viruses (6 CRF02_AG sequences were excluded [Table 1]). Analysis of the same 10 sequences by HIVE-hexahedron confirmed recombinant genomes (Table 1 and Fig. S1). The recombination
breakpoints in the major contig sequences identified by HIVE-hexahedron were very similar to those determined by analyzing the consensus sequences generated by Geneious in nine viruses (Table 2). However, recombination breakpoints between the major population determined by HIVE-hexahedron and the Geneious-derived sequences were different in PK006. In the last part of the 3'-half genome of PK006, the Geneious-derived sequence showed a recombination pattern as CRF02-A1-CRF02, while the HIVE-hexahedron-derived major population sequence had a CRF02-A1-CRF02 pattern (Fig. S3). When other partial minor subpopulation sequences were included for analysis, the recombination pattern at the first part of this region was shared between Geneious-derived sequences and HIVE-hexahedron-derived sequences for minor subpopulations 5 and 6 (Fig. S3b). Phylogenetic tree analysis of the region among all overlapping HIVE-derived contig sequences showed

| Virus    | HIVE-hexahedron | Geneious         |
|----------|-----------------|------------------|
|          | Region Subtype  | Region Subtype   |
| 707010585 | 4906–6541 C     | 4906–6425 C      |
|          | 6542–8080 A1    | 6426–8059 A1     |
|          | 8081–9531 C     | 8060–9411 C      |
| 707010134 | 4906–8321 C     | 4906–8274 C      |
|          | 8322–8864 D     | 8275–8764 D      |
|          | 8865–9621 C     | 8765–9392 C      |
| PK-011   | 4906–8777 CRF02_AG | 4906–8760 CRF02_AG |
|          | 8778–9591 A1    | 8761–9603 A1     |
| PK003    | 4932–8920 CRF02_AG | 4906–8784 CRF02_AG |
|          | 8921–9624 A1    | 8785–9606 A1     |
| PK033    | 4906–6037 CRF02_AG | 4906–6162 CRF02_AG |
|          | 6038–9608 A1    | 6163–9606 A1     |
| PK008    | 4906–6033 A1    | 4906–6056 A1     |
|          | 6034–7398 CRF02_AG | 6056–7680 CRF02_AG |
|          | 7399–8267 A1    | 7681–7929 A1     |
|          | 8068–8388 CRF02_AG | 7930–8330 CRF02_AG |
|          | 8389–9238 A1    | 8331–9279 A1     |
|          | 9239–9624 CRF02_AG | 9280–9604 CRF02_AG |
| PK015    | 4904–6243 CRF02_AG | 4906–6127 CRF02_AG |
|          | 6244–9611 A1    | 6128–9606 A1     |
| 707010277 | 4906–7025 C     | 4906–7072 C      |
|          | 7026–8457 A1    | 7073–8365 A1     |
|          | 8458–8776 C     | 8366–8672 C      |
|          | 8777–9587 A1    | 8673–9526 A1     |
| PK023    | 4907–6256 CRF02_AG | 4906–6154 CRF02_AG |
|          | 6257–6473 A1    | 6155–6553 A1     |
|          | 6474–8846 CRF02_AG | 6554–8801 CRF02_AG |
|          | 8847–9624 A1    | 8802–9605 A1     |
| PK006    | 4905–6252 CRF02_AG | 4906–6192 CRF02_AG |
|          | 6253–7577 A1    | 6193–7516 A1     |
|          | 7578–8474 CRF02_AG | 7577–7876 CRF02_AG |
|          | 8475–8852 A1    | 7877–8176 A1     |
|          | 8853–9624 CRF02_AG | 8177–8965 CRF02_AG |
|          | 8966–9606 A1    | 8966–9606 A1     |

*The shaded cells represent conflicting recombination patterns between Geneious and HIVE-derived sequences (Fig. S3).*
that sequences from contigs 5, 6, 7, and 8 clustered together with the Geneious-derived sequence, while the major contig 1 sequence branched out separately for the two regions (Fig. S3d). These results demonstrate that different viral populations that contain different recombinant patterns in a sample can be reliably detected by HIVE-hexahedron while the Geneious-derived consensus sequences of the overall viral population can detect only one of two or more different subpopulations in the sample.

**Subpopulation sequences identified by HIVE-hexahedron are confirmed by single genome sequencing.** One key question is whether subpopulation sequences determined by HIVE-hexahedron are indicative of viral subpopulations in real samples. To accurately characterize the viral population in a sample, we obtained complete 3'-half genome sequences by SGS using the same PCR primers used for HTS. Since the viral genomes are individually amplified and the amplicons are directly sequenced in bulk, the SGS-derived sequences are not affected by PCR-mediated misincorporation, recombination, and resampling errors (25–27). Thus, the viral population in a sample can be accurately characterized by the SGS method. Thirty-nine complete 3'-half genome sequences were obtained from PK006 by SGS (Table 3). Phylogenetic tree and Highlighter plot analyses showed that the virus had two subpopulations (A and B) and various recombinants between them (Fig. 6a).

Recombinant sequences could be further classified into two groups; A-like recombinants and B-like recombinants. HIVE-hexahedron analysis identified one major complete population (contig 1) and eight minor short subpopulations (contigs 2 to 8 [Fig. 6b]). To investigate if those subpopulation sequences represented the actual viral populations in the sample, the consensus sequences of all subpopulations determined by HIVE-hexahedron were exported and aligned together with all SGS sequences (Fig. 6a). We then performed phylogenetic tree and Highlighter plot analyses for each overlapping region to determine the genetic relationship between HIVE-hexahedron-derived subpopulation sequences and the SGS sequences. A longer subpopulation consensus sequence(s) was included whenever the overlapping regions covered the entire short subpopulation consensus sequence.

When the major complete consensus sequence (4,719 bp) was analyzed, it clustered with subpopulation B and B-like recombinant sequences but was different from those sequences (Fig. 6c). Thus, the major population sequence represented only subpopulation B and B-like recombinant sequences (about half of the viral population). At the regions where subpopulations 2 and 3 overlapped the major population 1, some of the SGS (A’ and B’) sequences formed a distinct cluster in the middle of the trees (Fig. 6d and e). Subpopulations 1 and 2 or 1 and 3 clustered with this group of sequences more closely than other sequences. At a region where resolution among all sequences was limited, subpopulation sequence 4 was highly similar to two of the subpopulation A sequences while subpopulation 1 and 3 sequences clustered within a clade consisting of population B, A’, and B’ sequences (Fig. 6f). Subpopulation 5 sequence clustered tightly with the population A’ sequences while the major population 1 sequence clustered within a clade consisting of population B and B’ sequences (Fig. 6g). At the region where subpopulation 5 and 6 sequences overlapped, SGS sequences formed two main clades. Interestingly, all three subpopulation sequences (1, 5, and 6) together

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**TABLE 3 Number of subpopulations identified in each sample by HIVE and SGS methods**

| Sample       | SGS No. of sequences | HIVE No. of populations |
|--------------|----------------------|-------------------------|
| PK006        | 39                   | 3                       | 8                       |
| 707010134    | 54                   | 3                       | 3                       |
| 707010038    | 21                   | 1                       | 1                       |
| PK038        | 34                   | 2                       | 3                       |
| 707010585    | 26                   | 4                       | 5                       |
| PK018        | 53                   | 3                       | 5                       |
| PK013        | 59                   | 2                       | 7                       |

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Distinct viral populations determined by HIVE are validated by analysis of SGS-derived sequences. (a) Phylogenetic tree analysis and Highlighter plot of SGS-derived sequences from PK006 identified two main viral populations (A and B; solid circles) and recombinant sequences that are either more A-like (A') or B-like (B') (open circles). Phylogenetic trees were constructed using the neighbor-joining method and Kimura 2-parameter model. Highlighter plots show nucleotide substitutions compared to the SGS-derived sequence at the top and color-coded nucleotide substitutions. Each SGS-derived sequence is denoted by a symbol and corresponds to the horizontal lines on the Highlighter plot. The contig consensus sequences from HIVE-hexahedron analysis are included in (Continued on next page)
with one of the SGS sequences represented distinct recombinant sequences between two main populations (Fig. 6h). At the end of the 3′-half genome, four subpopulations (1, 5, 7, and 8) overlapped each other (Fig. 6b). In this region, the SGS sequences formed two clades (A/A’ and B/B’). While HIVE contig 1 sequence clustered with B/B’ sequences, HIVE contig 5, 7, and 8 sequences clustered with A/A’ sequences (Fig. 6i). In fact, these A/A’ sequences and HIVE contig 5, 7, and 8 sequences were subtype A1 while the B/B’ sequences and HIVE contig 1 sequence were CRF02_AG (Table 4 and Fig. S3d). The detection of partial subtype A1 or CRF02_AG sequences at these regions by SGS sequences also unequivocally confirmed that HIVE-hexahedron can reliably detect distinct viral populations that are missed by population-based consensus sequence methods.

**FIG 6 Legend (Continued)**

the Highlighter plot for comparison. Two regions that are similar among all compared sequences (red boxes) were represented only in the main contig 1. (b) The Sankey diagram showed eight contigs: one major contig spanning the entire length of the 3′-half genome (contig 1) and seven smaller contigs (contigs 2 to 8). (c to i) Phylogenetic tree and Highlighter plot analysis of complete 3′-half genome sequences (c) and partial sequences (d to i). The relationship between HIVE-hexahedron contig sequences (red arrows) and SGS-derived sequences that overlap each other was determined individually by phylogenetic trees and Highlighter plots.
Results similar to those observed in PK006 were obtained by analyzing an additional six viruses (Fig. S4 to S9). Multiple SGS sequences (21 to 59) were obtained from these six samples, and 1 to 7 viral populations were identified (Table 3). The complete major contig consensus sequence in 707010585 was very close to two highly similar SGS sequences (Fig. S4c), most likely due to the high similarity within the viral populations that allowed HIVE-hexahedron to easily assemble them together. Taken together, analysis of HTS reads from a long genome (>4,000 bp) by HIVE-hexahedron can identify distinct viral subpopulations when genetic diversity levels are high enough. These results show that all subpopulation sequences determined by HIVE-hexahedron represent either the viral populations (major or minor) at the region or the unique recombinant sequences, demonstrating that HIVE-hexahedron-derived sequences can represent actual viral sequences in the samples.

Ambiguous bases can be resolved by parsing a quasispecies into distinct subpopulations using HIVE-hexahedron. Since HIVE-hexahedron can parse the quasispecies into distinct subpopulations, each subpopulation consensus would not be expected to have ambiguous bases. We next sought to determine how subpopulations identified by HIVE-hexahedron were able to resolve ambiguous bases in consensus sequences generated by Geneious using the SGS sequences generated for the same samples. For example, in a 93-bp region (nt 902 to 995) in the vpr/tat gene of PK006, the consensus sequence obtained by de novo assembly of raw reads using Geneious had seven ambiguous bases, indicating multiple bases were present at each of those sites (Fig. 7).

HIVE-hexahedron analysis of sequences in the same region identified three distinct viral populations (A, B, and C), with no ambiguous bases present in any consensus sequences for all populations. The analysis of 39 SGS sequences showed three subpopulations. All 12 SGS sequences, except one with an A/T mutation in subpopulations A’, were identical to the HIVE subpopulation A sequence. All 25 SGS sequences in subpopulation B’ were identical to the HIVE subpopulation B sequence. The other two SGS sequences constituted the last subpopulation C’, with the same bases at three sites and different bases at four other sites. Neither of them was the same as any HIVE subpopulation sequences. Thus, HIVE subpopulation C was not represented by the SGS sequences. This is most likely due to the limited SGS sequences available for analysis. Moreover, those two SGS sequences represent rare viral variants in the sample. These results demonstrate that HIVE-hexahedron can accurately detect different viral populations separately and generate consensus sequences without ambiguous bases for those subpopulations of HIV-1 quasispecies in a sample.

DISCUSSION

Half (>4,000-bp) and complete (>9,000-bp) HIV-1 genomes have been increasingly analyzed by HTS. However, there are no computational algorithms that can reliably identify distinct subpopulation sequences generated by HTS from samples with quasispecies. Moreover, subsequent subtyping and recombination analyses require sequences to be exported and determined using additional softwares. To streamline all analyses in a single environment, we developed a new HIVE-hexahedron tool (32, 49, 50). After uploading the HTS raw reads in the HIVE platform, HIVE-hexahedron can identify different subpopulations, subtype all subpopulations, detect recombinant genomes, and determine recombinant breakpoints without exporting the sequences for further analysis.

When the short HTS raw reads from a sample with a complex HIV-1 quasispecies are analyzed, HIVE-hexahedron uses advanced algorithms to progressively align each read to multiple reference sequences. It will generate one major complete contig which represents either overall consensus of the viral population, clonally expanded viral population, or one of the viral subpopulations, while partial genomes where genetic diversity levels are higher than the preset threshold (1%) will be represented as subpopulations. Thus, the quasispecies in a sample will be parsed by subpopulations. The algorithm allows a user to select the threshold of the minimum accepted depth of
coverage. In our study, we have selected the threshold of at least 50 reads. Therefore, the minimum sequencing depth required could be lower, but the user can select the threshold for confidence levels. Other tools, such as QuRe, ShoRah, and PredictHaplo, do not provide the granular option of selecting minimum coverage. However, due to the nature of the hexahedron algorithm, the main limiting factor in identifying a subpopulation is the length of the aligned read combined with the depth of coverage. Higher coverages (>1,000) and longer reads (>300 bp) will increase the likelihood to detect subpopulations. Thus, the detection of subpopulations in a sample will depend on both the depth of the sequence coverage and the sensitivity of the computer software algorithm (51). By comparing to SGS sequences, which characterize individual 3′-half genome sequences in one complete piece, we found that subpopulation sequences determined by HIVE-hexahedron were representative of distinct viral populations at the full 3′-half genome level or partial genome levels. Although the resolution of quasispecies determined by HIVE-hexahedron is not as high as that by SGS, the detection of subpopulations is a good indication of the presence of distinct viral subpopulations in a sample. Importantly, HIVE-hexahedron is much more cost-effective and faster than SGS. Moreover, each subpopulation sequence is
clearly defined since a quasispecies is parsed into distinct subpopulations by HIVE-hexahedron. This may be particularly important when a drug-resistant quasispecies is analyzed since this can reveal if drug-resistant viruses were generated from the same or different subpopulations. These results demonstrate that HIVE-hexahedron is a powerful tool that can identify subpopulations in samples analyzed by short-read HTS. To our knowledge, this is the only algorithm with this capability and confidence level.

Unlike other computational algorithms, HIVE-hexahedron progressively aligns each read to multiple reference sequences. Thus, the HIVE-hexahedron pipeline allows us to rapidly determine HIV-1 subtypes by identifying the nearest neighbors (32, 49, 50) without the prior knowledge of the HIV-1 classification. The results are visualized as Sankey diagrams. The subtyping results of complete major population consensus sequences from 65 samples were confirmed by conventional subtype analysis of consensus sequences obtained by Geneious using standard HIV-1 subtyping tools. Importantly, the sequences in the reference set can be any number and exchanged for optimal alignment to identify the best-matched reference sequence. Thus, one of the unique features of HIVE-hexahedron is that it can automatically determine the subtype of each subpopulations.

Since subtypes of tested HIV-1 sequences can be easily determined using HIVE-hexahedron, the pipeline was optimized to detect recombinant sequences among different subtypes and define the recombination breakpoints. Using this pipeline, we found 10 of 65 viruses were recombinants, which were the same as those detected by Geneious-derived sequences. In eight samples, the recombination breakpoints determined by HIVE-hexahedron were similar to those determined by analyzing Geneious-derived sequences using the jpHHM and Simplot tools. In the last part of the 3'-half genomes of PK023 and PK006, the recombination breakpoints were different between the results from analysis of major population sequences generated by HIVE-hexahedron and Geneious. Analysis of subpopulation HIVE-hexahedron sequences showed that all recombination patterns identified by Geneious-derived sequences can be found in HIVE-hexahedron subpopulation sequences. This demonstrates that HIVE-hexahedron can accurately detect not only different coinfected HIV-1 strains but also distinct recombinant viral populations in the samples than the population sequencing-based methods like Geneious. The analysis of 39 SGS sequences from PK006 unequivocally confirmed the presence of distinct recombinant viral sequences. These results demonstrate that HIVE-hexahedron is powerful and accurate to determine coinfections and recombination patterns of a quasispecies.

Some HIVE subpopulations did not cluster with any major groups. They showed mosaic patterns among different subpopulations and could represent recombinant sequences. This might be because these recombinant populations identified by HIVE were unique and are not represented by limited SGS sequences. It is cautioned that recombination may be artificially generated during assembling of reads by HIVE-hexahedron because sequence diversity at some parts of the viral genome is too low and different subpopulations are genetically indistinguishable from each other. When HIVE-hexahedron progressively assembles the reads into contigs, it may switch between different viral subpopulations and generate artificial recombinant sequences. However, it is also possible that these recombinant sequences may be generated during PCR, which can result in high levels of PCR-mediated recombination (25, 63). Based on our previous study results (63), we used relatively low template input in each PCR to minimize the likelihood to generate PCR-mediated recombinants. However, this will not affect the ability of HIVE-hexahedron to detect different subpopulations at highly divergent subgenome regions. When HIVE-hexahedron was compared against other Quasispecies Spectrum Reconstruction (QSR) algorithms including QuRe, ShoRah, and PredictHaplo, it was found to either match or outperform the rest. In the in silico studies, type 2 error was less than the other QSR algorithms and in fact it was the only algorithm that, given enough depth
of coverage and adequate length of the reads, was able to accurately reconstruct all subpopulations (51). HIVE-hexahedron should be further optimized to ensure that artificial recombination is minimized during assembly of short HTS reads. We plan to introduce a postcomputational step for statistical inference of potential artificial recombinants in HIVE-hexahedron to ensure that recombination is minimized in processing and assembly of short HTS reads.

The HIVE-hexahedron algorithm is powerful for streamline analysis of viral diversity, subtype, and recombination of short HTS data. It has a broad application by providing analysis of any quasispecies. In addition, HIVE as a platform can also be used for storing data and archiving results. This pipeline can be adapted readily to other less diverse RNA viruses (32).

MATERIALS AND METHODS

**Plasma samples.** Plasma samples obtained from 33 HIV-1-infected individuals in a chronic infection cohort supported by the Centre for HIV/AIDS Vaccine Immunology (CHAVI) and from 32 HIV-1-infected individuals from a chronic infection cohort in Pakistan. All individuals were not treated with antiretroviral drugs. Written informed consent forms were obtained from all participants, and the studies were approved by the Duke University Institutional Review Board and the ethics committee of Bridge Consultants Foundation. Viral loads in these 65 individuals ranged from 610 to 760,000 copies per ml.

**Amplification of 3’-half HIV-1 genome.** Viral RNA was extracted from 400 μl of each plasma sample using EZ1 virus mini kit v2.0 (Qiagen, Valencia, CA) and subjected to cDNA synthesis using Superscript III reverse transcriptase (Invitrogen, Carlbad, CA) with primer 1.R3.B3R (5’-ACTACTTGACCTCAAGGCAAGCTTATTG-3’; HXB2, nt 9611 to 9642). The 3’-half genomes were amplified with undiluted cDNA from each sample in triplicate using Platinum Taq high-fidelity DNA polymerase (Life Technologies, Carlsbad, CA) as previously described (64). The primers for the first-round amplification were 07For7 (5’-CAAATTAYAAAATCTAATTCTGGAAGGTTATACAG-3’; nt 4875 to 4912) and 2.R3.B6R (5’-GAAGCCTCAAGGCAAGCTTATTGAGGC-3’; nt 9607 to 9636), and the primers for the second-round amplification were VIF1 (5’-GGTTTATACAGGGGACAGCAGG-3’; nt 4900 to 4923) and Low2C (5’-GAAGCTTAAGCAGTGGGTTC-3’; nt 9591 to 9612). The PCR thermocycling conditions were as follows: one cycle at 94°C for 2 minutes; 35 cycles of a denaturing step at 94°C for 15 seconds, an annealing step at 58°C for 30 seconds, and an extension step at 68°C for 60 seconds. A final extension was performed at 68°C for 10 minutes. Positive amplification was confirmed on a 0.8% agarose gel, and amplicons were subsequently purified using the AMPure XP beads (Beckman Coulter, Indianapolis, IN).

**High-throughput sequencing.** The purified PCR product (1 ng) from each sample was indexed to make the sequencing library (5 μl) using the Nextera XT kit (Illumina, San Diego, CA). The normalized libraries were pooled and further quantified by quantitative PCR (qPCR) using the KAPA library quantification kit (Kapa Biosystems, Woburn, MA). The libraries were sequenced using the MiSeq reagent kit v3 (2 × 150 cycles) on an Illumina MiSeq. Raw sequence reads were filtered for Q-scores above 30. The filtered high-quality sequences from each sample were parsed based on the unique sequence tags using “BaseSpace Sequence Hub” (Illumina, San Diego, CA). The sequences from both ends for the same cluster were paired and exported as Fastq files for subsequent analyses.

**Sequence assembling by Geneious.** Paired sequence reads from each sample were assembled using Geneious, a suite of the HTS analysis tools (Biomatters, Auckland, New Zealand) (65). A “map to reference” assembly was performed for each read pool using the Geneious assembler. The algorithm used is a seed and expand style mapper followed by an optional fine-tuning step to better align reads around indels to each other rather than the reference sequence. HXB2 was used as the reference sequence with reads per kilobase of transcript per million (RPKM) mapped value of 10,000 or more were selected as nearest neighbors for that sequence. To resolve the conflict for selection of one good reference among many closely related references, the alignment parameters used in our previous study (59)
were modified. In addition, a specific algorithm that identified the nearest neighbor reference(s) to the raw reads from a pool of closely related references was implemented to HIVE-hexagon (59). The second alignment was done by aligning the raw reads from each sample to their nearest neighbor reference(s). The identified nearest neighbors were also indicative of the subtype of the sequence. If only one reference was the nearest neighbor, the sequence would represent the subtype of the sample. If there were more than one nearest neighbor, the individual was infected with different viruses or a recombinant between these nearest neighbors. For each sample with more than one identified nearest neighbor reference, multiple sequence alignments (MSAs) of nearest neighbor references were done using Multiple Alignment Fast Fourier Transform (MAFFT) (60) that was integrated in HIVE. The MSA of nearest neighbor references is used to create a common coordinate system for comparing the alignments of short reads from different references.

The final part of the pipeline is HIVE-hexahedron, a specialized tool in HIVE meant for reference-assisted de novo assembly of viral quasispecies (32). The input for HIVE-hexahedron is an alignment of sequences from a sample against its nearest neighbor reference sequence(s), and a multiple sequence alignment of the nearest neighbor reference sequences, in case there is more than one nearest neighbor. The output of HIVE-hexahedron is a Sankey diagram that uniquely shows if there are one or more viral populations in the sample and if recombinant genomes are present. It scans all variations above a given threshold in all available sequences and detects correlated groups of single nucleotide variation (SNV) patterns within each specific viral subpopulation. The subtypes and the recombination patterns are visualized using the interactive Sankey diagrams. Reads mapped against different sequences are re-mapped to a common coordinate system defined by the MSA (60) so they are piled up together. The consensuses of all subpopulation sequences can be exported in the FASTA format for further analysis. To improve the quality of the results, novel features were introduced in the tool that allow some postprocessing, which involves filtering out the small viral subpopulations with very low coverages. The following filters were used to define a subpopulation: at least 500 bp long, average depth of coverage of more than 50, and at least five SNVs.

**Sequence analysis.** The consensus sequences of viral subpopulations spanning the 3'-half genome (~4,500 bp) of all samples were exported from Geneious and HIVE-hexahedron. The sequences were aligned together with references from the HIV Sequence Database using CLUSTAL W (66), and manual adjustments for optimal alignments were done using SEAVIEW. Phylogenetic trees were constructed using the neighbor-joining (NJ) method with the Kimura two-parameter model (67, 68), and the reliability of topologies was estimated by bootstrap analysis with 1,000 replicates. Recombination patterns were initially analyzed by REGA HIV-1 & 2 Automated Subtyping Tool (version 3.41; http://krisp.ukzn.ac.za/app/typingtool/virus/) and the jumping profile Hidden Markov Model (jPHMM) at the GOBICS website (http://jphmm.gobics.de/) (69). Highlighter plots were generated using the Highlighter tool at the Los Alamos HIV sequence database (https://www.hiv.lanl.gov/content/sequence/HIGHLIGHT/highlighter_top.html). The recombination breakpoints were confirmed by BootScan implemented in Simplot version 3.5.1 (70). The recombination pattern map for each viral subpopulation was generated using RecDraw (71).

**Single genome sequencing.** The cDNA was made by reverse transcription of viral RNA using primer 1.R3.B3R as described previously (64). The sequences of individual 3'-half genomes were obtained by single genome sequencing (SGS) from seven samples (26, 64). The first-round PCR primers were 2.R3.B6R and 07For7, and the second-round PCR primers were VIF1 and Low2C. The PCR products amplified from individual 3'-half genomes were purified and directly sequenced by HTS on an Illumina MiSeq.

**Accession number(s).** The GenBank accession numbers for sequences are MT395382 to MT395511 and MT419970 to MT420255.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, PDF file, 2.1 MB.

**FIG S2**, PDF file, 0.2 MB.

**FIG S3**, PDF file, 0.3 MB.

**FIG S4**, PDF file, 1 MB.

**FIG S5**, PDF file, 1.2 MB.

**FIG S6**, PDF file, 0.5 MB.

**FIG S7**, PDF file, 1.3 MB.

**FIG S8**, PDF file, 1.1 MB.

**FIG S9**, PDF file, 0.6 MB.

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We declare no conflict of interest.
REFERENCES

1. Gao F, Chen Y, Levy DN, Conway JA, Kepler TB, Hui H. 2004. Unselected mutations in the human immunodeficiency virus type 1 genome are mostly nonsynonymous and often deleterious. J Virol 78:2426–2433. 10.1128/JVI.78.7.2426-2433.2004.

2. Mansky LM, Temin HM. 1995. Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase. J Virol 69:5087–5094. https://doi.org/10.1128/JVI.69.12.5087-5094.1995.

3. Coffin JM. 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science 267:483–489. 10.1126/science.2842947.

4. Perelson AS, Neumann AU, Markowitz M, Leonard JM, Ho DD. 1996. HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time. Science 271:1582–1586. https://doi.org/10.1126/science.271.5255.1582.

5. Shankarappa R, Margolick JB, Gange SJ, Rodriguez AG, Upchurch D, Farzadegan H, Gupta P, Rinaldo CR, Learn GH, He X, Huang XL, Mullins JI. 1999. Consensus-based HIV-1 genetic assignment of sequencing data from individual patients: a consensual approach to estimate diversity. J Virol 73:1049–1052. https://doi.org/10.1128/JVI.73.3.1049-1052.1999.

6. Meyerhans A, Cheynier R, Albert J, Seth M, Kwok S, Sninsky J, Morfeldt-Leanderson J, Li Y, Parks EC, Shaw GM. 1998. Extensive variation of human immunodeficiency virus type 1 in vivo. Nature 334:440–444. https://doi.org/10.1038/334440a0.

7. Beerenwinkel N, Hesper K, Gerbusher D, Jahn P, Weese J, Hellmuth M, Schaefer J, Stadler PF, hoyt D, Schuster SC. 2010. Conserved and variable parts of the evolution of HIV-1. Journal of Mathematical Biology 61:657–675. https://doi.org/10.1007/s00285-010-0348-0.

8. Hora et al. 2018. Human Immunodeficiency Virus Type 1 Subtype C Reveals a Non-Poisson Distribution of Transmitted Variants. J Virol 82:2815–2825. https://doi.org/10.1128/JVI.00344-18.

9. Surawicz CR, Talan DA, Holmes M, Seaton D, DeLong JL, et al. 2013. An assessment of the clinical utility of next-generation sequencing for diagnosis and management of Clostridium difficile infection. J Clin Microbiol 51:431–436. https://doi.org/10.1128/JCM.02604-12.

10. Mocroft A, Weber R, Jit M, Wierzbicki A, Gumbert K, et al. 2007. Time trends in survival after start of anti-human immunodeficiency virus therapy: results from the European Cost of AIDS cohort study. Clin Infect Dis 45:559–566. https://doi.org/10.1086/519935.

11. Hannett NM, Zambon PN, Hackett SG,_inflections and transmission in treatment-experienced patients. J Clin Virol 43:406–413. https://doi.org/10.1016/j.jcv.2010.05.001.

12. Mocroft A, Weber R, Jit M, Wierzbicki A, Gumbert K, et al. 2007. Time trends in survival after start of anti-human immunodeficiency virus therapy: results from the European Cost of AIDS cohort study. Clin Infect Dis 45:559–566. https://doi.org/10.1086/519935.

13. Hannett NM, Zambon PN, Hackett SG,_inflections and transmission in treatment-experienced patients. J Clin Virol 43:406–413. https://doi.org/10.1016/j.jcv.2010.05.001.

14. Mocroft A, Weber R, Jit M, Wierzbicki A, Gumbert K, et al. 2007. Time trends in survival after start of anti-human immunodeficiency virus therapy: results from the European Cost of AIDS cohort study. Clin Infect Dis 45:559–566. https://doi.org/10.1086/519935.

15. Hannett NM, Zambon PN, Hackett SG,_inflections and transmission in treatment-experienced patients. J Clin Virol 43:406–413. https://doi.org/10.1016/j.jcv.2010.05.001.

16. Mocroft A, Weber R, Jit M, Wierzbicki A, Gumbert K, et al. 2007. Time trends in survival after start of anti-human immunodeficiency virus therapy: results from the European Cost of AIDS cohort study. Clin Infect Dis 45:559–566. https://doi.org/10.1086/519935.

17. Hannett NM, Zambon PN, Hackett SG,_inflections and transmission in treatment-experienced patients. J Clin Virol 43:406–413. https://doi.org/10.1016/j.jcv.2010.05.001.

18. Mocroft A, Weber R, Jit M, Wierzbicki A, Gumbert K, et al. 2007. Time trends in survival after start of anti-human immunodeficiency virus therapy: results from the European Cost of AIDS cohort study. Clin Infect Dis 45:559–566. https://doi.org/10.1086/519935.

19. Hannett NM, Zambon PN, Hackett SG,_inflections and transmission in treatment-experienced patients. J Clin Virol 43:406–413. https://doi.org/10.1016/j.jcv.2010.05.001.

20. Mocroft A, Weber R, Jit M, Wierzbicki A, Gumbert K, et al. 2007. Time trends in survival after start of anti-human immunodeficiency virus therapy: results from the European Cost of AIDS cohort study. Clin Infect Dis 45:559–566. https://doi.org/10.1086/519935.
42. Bansal V, Mazumder R. 2014. High-performance integrated virtual environment (HIVE) tools and applications for big data analysis. Genes (Basel) 5:957–981. https://doi.org/10.3390/genes5040957.

50. Simonyan V, Mazumder R. 2014. High-performance integrated virtual environment (HIVE) tools and applications for big data analysis. Genes (Basel) 5:957–981. https://doi.org/10.3390/genes5040957.

51. Karagiannis K. 2019.Separation and assembly of RNA virus high throughput sequencing data into discrete full length sub-population genomes. ProQuest Dissertations Publishing, Ann Arbor, MI.

52. Hora B, Keating SM, Chen Y, Sanchez AM, Sabino E, Hunt G, Ledwaba J, Hackett J, Swanson P, Hewlett I, Ragupathy V, Vikram Vemula S, Zeng P, Tee K-K, Chow WZ, Ji H, Sandstrom P, Denny TN, Busch MP, Gao F, REDS-I11 and EQAPOL programs. 2016. Genetic characterization of a panel of diverse HIV-1 isolates at seven international sites. PloS One 11:e0157340. https://doi.org/10.1371/journal.pone.0157340.

53. Chen Y, Hora B, DeMarco T, Shah SA, Ahmed M, Sanchez AM, Su C, Carter M, Stone M, Hasan R, Hasan Z, Busch MP, Denny TN, Gao F. 2016. Fast dissemination of new HIV-1 CRF02/A1 recombinants in Pakistan. PloS One 11:e0167839. https://doi.org/10.1371/journal.pone.0167839.

54. Song H, Giorgi E, Ganusov VV, Caif, Athreya G, Yoon H, Carja O, Hora B, Habrer P, Romero-Severson E, Jiang C, Li X, Wang S, Li H, Salazar- Gonzalez JL, IF, Salazar-Negron E, NI, Keefe BF, Montefiori DC, Cohen MS, Shaw GM, Hahn BH, McMichael AJ, Haynes BF, Korber B, Bhattacharya T, Gao F. 2018. Tracking HIV-1 recombination to resolve its contribution to HIV-1 evolution in natural infection. Nat Commun 9:1928. https://doi.org/10.1038/s41467-018-04217-5.

55. Prosperi MC, Yin L, Nolan DJ, Lowe AD, Goodenow MM, Salemi M. 2013. Empirical validation of viral quasispecies assembly algorithms: state-of-the-art and challenges. Sci Rep 3:2837. https://doi.org/10.1038/srep02837.

56. Yang X, Charlebois P, Macalad A, Henn MR, Zody MC. 2013. V-Phaser 2: variant inference for viral populations. BMC Genomics 14:674. https://doi.org/10.1186/1471-2164-14-674.

57. Mangul S, Wu NC, Mancuso N, Zelikovsky A, Sun R, Eskin E. 2014. Accurate viral population assembly from ultra-deep sequencing data. Bioinformatics 30:i329–i337. https://doi.org/10.1093/bioinformatics/btu295.

58. Schirmer M, Sloan WT, Quince C. 2014. Benchmarking of viral haplotype reconstruction programmes: an overview of the capacities and limitations of currently available programmes. Brief Bioinform 15:431–442. https://doi.org/10.1093/bib/bbt208.

59. Santana-Quintero L, Dingerdissen H, Thierry-Mieg J, Mazumder R, Simonetty V. 2014. HIV-HEX-Hexagon: high-performance, parallelized sequence alignment for next-generation sequencing data analysis. PLoS One 9:e106658. https://doi.org/10.1371/journal.pone.0106658.

60. Wagner GA, Palcado ME, Kosakovskiy Pond SL, Caballerio G, Chaillon A, Rudolph AE, Morris SR, Little SJ, Richmond DD, Smith DM. 2004. Incidence of HIV superinfection following primary infection. JAMA 292:1177–1178. https://doi.org/10.1001/jama.292.10.1177.

61. Wagner GA, Palcado ME, Kosakovskiy Pond SL, Caballerio G, Chaillon A, Rudolph AE, Morris SR, Little SJ, Richmond DD, Smith DM. 2004. Incidence of HIV superinfection following primary infection. JAMA 292:1177–1178. https://doi.org/10.1001/jama.292.10.1177.

62. Sanchez AM, DeMarco CT, Hora B, Keating SM, Chen Y, Sanchez AM, Sabino E, Hunt G, Ledwaba J, Hackett J, Swanson P, Hewlett I, Ragupathy V, Vikram Vemula S, Zeng P, Tee K-K, Chow WZ, Ji H, Sandstrom P, Denny TN, Busch MP, Gao F, REDS-I11 and EQAPOL programs. 2016. Genetic characterization of a panel of diverse HIV-1 isolates at seven international sites. PloS One 11:e0157340. https://doi.org/10.1371/journal.pone.0157340.

63. Chen Y, Hora B, DeMarco T, Shah SA, Ahmed M, Sanchez AM, Su C, Carter M, Stone M, Hasan R, Hasan Z, Busch MP, Denny TN, Gao F. 2016. Fast dissemination of new HIV-1 CRF02/A1 recombinants in Pakistan. PloS One 11:e0167839. https://doi.org/10.1371/journal.pone.0167839.

64. Sanchez AM, DeMarco CT, Hora B, Keating SM, Chen Y, Sanchez AM, Sabino E, Hunt G, Ledwaba J, Hackett J, Swanson P, Hewlett I, Ragupathy V, Vikram Vemula S, Zeng P, Tee K-K, Chow WZ, Ji H, Sandstrom P, Denny TN, Busch MP, Gao F, REDS-I11 and EQAPOL programs. 2016. Genetic characterization of a panel of diverse HIV-1 isolates at seven international sites. PloS One 11:e0157340. https://doi.org/10.1371/journal.pone.0157340.

65. Karagiannis K. 2019. Separation and assembly of RNA virus high throughput sequencing data into discrete full length sub-population genomes. ProQuest Dissertations Publishing, Ann Arbor, MI.

66. Sanchez AM, DeMarco CT, Hora B, Keating SM, Chen Y, Sanchez AM, Sabino E, Hunt G, Ledwaba J, Hackett J, Swanson P, Hewlett I, Ragupathy V, Vikram Vemula S, Zeng P, Tee K-K, Chow WZ, Ji H, Sandstrom P, Denny TN, Busch MP, Gao F, REDS-I11 and EQAPOL programs. 2016. Genetic characterization of a panel of diverse HIV-1 isolates at seven international sites. PloS One 11:e0157340. https://doi.org/10.1371/journal.pone.0157340.
sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. https://doi.org/10.1093/nar/22.22.4673.

67. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.

68. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. https://doi.org/10.1007/BF01731581.

69. Schultz AK, Zhang M, Leitner T, Kuiken C, Korber B, Morgenstern B, Stanke M. 2006. A jumping profile Hidden Markov Model and applications to recombination sites in HIV and HCV genomes. BMC Bioinformatics 7:265. https://doi.org/10.1186/1471-2105-7-265.

70. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, Ingersoll R, Sheppard HW, Ray SC. 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. J Virol 73:152–160. https://doi.org/10.1128/JVI.73.1.152-160.1999.

71. Kijak GH, Tovanabutra S, Beyrer C, Sanders-Buell EE, Arroyo MA, Robb ML, Michael NL, McCutchan FE, Kim JH. 2010. RecDraw: a software package for the representation of HIV-1 recombinant structures. AIDS Res Hum Retroviruses 26:1317–1321. https://doi.org/10.1089/aid.2010.0127.