DETECTION OF VIRAL QUASISPECIES: A CASE FOR SINGLE GENOME AMPLIFICATION

***KEMEBRADIKUMO PONDEI[1] AND MIEEBI WANKASI[2]**
[1]Department of Medical Microbiology and Parasitology [2]Department of Medical Laboratory Sciences
Faculty of Basic Medical Sciences, College of Health Sciences, Niger Delta University, Amassoma, Wilberforce Island, Bayelsa State, Nigeria.

ABSTRACT
Sampling of viral quasispecies can be very challenging. Most molecular studies involve the amplification of DNA and RNA using the Polymerase Chain Reaction (PCR). However, differences and difficulties exist in how the amplification is done. We investigated the difference in using two popular methods by amplifying blood-derived DNA from a patient using both methods. The first method commonly called the Bulk PCR/Clonal sequencing method involves amplification of un-quantified DNA/RNA, whilst the Single-genome/Endpoint dilution method involves amplification of a single molecule of DNA/RNA. We observed greater heterogeneity and greater genetic distances between sequences generated using the Bulk PCR method compared with the Single-genome method. Also, these sequences were represented by longer terminal branches in phylogenetic trees compared with those from the single genome method. We conclude that the Single-genome method, even though more laborious, is more accurate in sampling viral quasispecies, and should be used.

Keywords: *Polymerase chain reaction, Amplification, Recombination, viral quasispecies*

BACKGROUND

Viruses are believed to exist as quasispecies, defined as a swarm of genetically related but distinct variants [1, 2]. It becomes imperative in the analysis of these quasispecies for variants which are representative of the virus population to be sampled. Sampling methods which unduly favour certain variant populations over others would tilt the outcome of such studies.

The polymerase chain reaction (PCR) is commonly used in the detection of viral quasispecies and has become established as an important tool for the processing of genetic material. Despite the known pitfalls of PCR, which include introduction of errors [3], recombination [4] and selective amplification, it is still indispensable in present day molecular studies. Optimizing the PCR conditions (buffer constituents, dNTP concentration, primer design and concentration and the cycling parameters), and using template with a high degree of integrity, have also significantly improved the quality of the PCR product [5]. Extensive PCR-induced errors have the capability of diminishing the usefulness of sequence analysis. *Taq* polymerase is known to introduce approximately one error per 9000 nucleotides per cycle [6], and for the full-length HIV-1 genome after 25 cycles of PCR, 13 introduced substitutions were observed out of 9000 positions [7], corresponding to 0.6×10^{-4}/nucleotide/cycle (reviewed in [8]). These *Taq*-induced errors have been reduced by the introduction of proof-reading thermostable polymerases [5, 9].

The commonest method of sampling viral quasispecies, known as bulk sampling or clonal sequencing method, involves PCR amplification of the target DNA, cloning of the PCR product and then sequencing. Whilst easy to use, certain drawbacks have been associated with this method. These include re-sampling and the possibility of PCR-induced recombination. Another method used, involves the amplification at endpoint dilution of a single copy of the target DNA, and is known as the Single genome method. This method is believed to be laborious and expensive, but is believed to avoid the pitfalls associated with the Bulk sampling method.

Here both methods have been compared by the amplification of DNA obtained from an HIV-1 infected patient. The aim is to ascertain which of these methods produced sequences representative of the viral population.

METHODS

DNA extraction

Whole blood was obtained from an HIV-1 positive patient. DNA was extracted from the sample using the Stratagene® kit according to a previously described, modified version of the manufacturer's protocol [10]. Extracted DNA was

quantified by Nanodrop spectrophotometry.

PCR Amplification

Nested PCR was used to amplify the V1-V3 region of the HIV-1 envelope from the extracted DNA.

Bulk PCR method

1µl of DNA template was amplified in 25µl PCR reactions containing 2.5µl of 10X PCR buffer (Contained 100mM Tris-HCl, 500mM KCl, (NH4)2SO4,15 mM MgCl2; pH 8.7 (20°C)), 1µl of dNTP (2.5mM of each dNTP), 1µl each of sense and antisense primers (5pmol/µl), 1 unit (5units/µl) of Hotstar Taq® DNA Polymerase (Qiagen), and sterile distilled water making up to 25µl final volume.

The amplification cycle consisted of activation at 95°C for 15min, followed by 34 cycles of denaturing at 94°C for 45s, annealing at 55°C for 45s, then elongation at 72°C for 6min, before cooling at 4ºC.

1µl of the first round reaction was used as template in a second round reaction, under the same conditions but with primers nested within those used in the first round.

Amplified products were visualised after electrophoresis on a 2% agarose gel, containing ethidium bromide.

Single genome Amplification

In order to amplify a single proviral DNA molecule, the endpoint dilution of the DNA sample was determined by the amplification of replicates of serially diluted target DNA. The endpoint being defined here, as the highest dilution of DNA at which amplified products can be detected, and at which only one in three reaction tubes was positive. The Poisson distribution predicts that when no more than 30% of reaction tubes are positive, almost 90% of positive reactions will contain one proviral DNA molecule [11]. It was assumed that at endpoint, only one copy of DNA was present in 1µl of diluted DNA.

1µl of two-fold serially diluted DNA was used as template in a 25µl PCR reaction containing 2.5µl of 10X PCR buffer (Contained 20 mM Tris-HCl, 100 mM KCl, 15 mM MgCl2; pH 7.5 (25°C)), 1µl of dNTP (2.5 mM of each dNTP), 1µl each of sense and antisense primers (5pmol/µl), 1 unit (5units/µl) of Hotstar Taq® DNA Polymerase (Qiagen), and sterile distilled water making up to 25µl final volume. Three reaction tubes were set up for each dilution of DNA.

To determine the validity of this end-point and to generate products for sequence analysis, multiple replicates of PCR reactions (10 tubes) were prepared, with the same composition and cycling parameters as used for the end-point determination. 1µl of DNA at endpoint dilution was used as template for each of the first round reactions, and then 1µl of the first round product was used as template for the second round. The aim was to achieve 30% positive reactions i.e 3 in 10 will be positive, because when 30% of reactions are positive, almost 90% of positive reactions will contain one template [11].

The PCR products were purified using Exonuclease (EXO-SAP-IT®)(GE Healthcare). The purified products were then quantified by spectophotometry.

Cloning

The purified products obtained from bulk-PCR method, were then cloned using the pcDNA 3.1/V5-His© TOPO® TA Expression kit (Invitrogen) according to manufacturer's instructions.

250µl of transformation reaction was spread on a pre-warmed Luria-Bertani (LB) agar plate containing 100µg/ml of Ampicillin and incubated overnight at 37°C to generate single colonies.

To check for the presence of the correct insert, a 20µl PCR reaction was prepared with 1µl each, of primers T7 (5′ TAA TAC GAC TCA CTA TAG GG 3′) and BGH (5′ TAGAAGGCACAGTCGAGG 3′); 2µl of PCR buffer (10X); 0.2µl of dNTP (100mM); 1 unit of Hotstar Taq® DNA Polymerase (Qiagen) and sterile distilled water making up the final volume to 20µl.

Single colonies were picked with sterile pipette tips and dipped in the PCR mix, and the reaction was incubated for 10 minutes at 94ºC to lyse the cells and inactivate nucleases. The same cycling conditions listed above were used. Colonies with the expected insert appear as clear bands on 2% agarose gel stained with ethidium bromide.

To screen for orientation of the insert, similar PCR reactions were used except with the antisense primer Env 307, instead of BGH.

Colonies containing the expected insert in the correct orientation, were each inoculated into 3ml of LB broth, containing 1ìg/ml of ampicillin, and incubated overnight at 37ºC with shaking. The cultures were then purified using the QIAprep® mini-prep kit (Qiagen) according to manufacturer's guidelines.

Sequencing

DNA sequencing was performed using the ABI Big Dye Capillary Sequencing protocol (Applied Biosystems Inc., Foster City, CA). The resulting chromatograms were visualized using Chromas 2.31 (http://www.technelysium.com.au), and sequence ambiguities resolved by manual editing.

Sequence analysis

The sequences generated by each sampling method were assembled into two datasets: the single genome amplification datset and the bulk PCR sampling dataset. To evaluate the relationship among the viral quasispecies in each dataset, the mean intrasample and intersample distances were calculated as the number of nucleotide substitutions per site, by the Kimura 2 - parameter model [12], which corrects for multiple hits whilst taking into account transitional and transversional substitution rates as implemented in MEGA 4® [13]. Pairwise distances were estimated for each sequence in a sample, and the values in the pairwise matrix generated were then plotted in a graph using the Graphpad Prism version 4® (Graphpad software, San Diego, CA).

Phylogenetic trees were constructed using maximum-likelihood methods. Firstly, gap-stripping of the sequences was done to remove segments which were ambiguous and not informative. Each dataset was subjected to 56 models of nucleotide substitution using PAUP® 10 [14], and each model was scored for a dataset. The model scores were run in a hierarchical likelihood ratio test, implemented in Modeltest® 3.7 [15] to test for a statistically justified model of DNA substitution, and the best-fit model for each dataset was chosen. The parameters of the chosen model were then used in branch-swapping heuristc algorithms in PAUP® 10, to generate a maximum-likelihood tree. In order to assess support for each node in the tree, bootstrap resampling of 1000 replicates was done using the neighbour-joining method [16].

Ethical consideration

Ethical clearance was obtained from the Nottingham University Hospitals Ethical committee

RESULTS

In order to further assess the diversity and the relationship between sequences derived by each method, intra-sample and inter-sample genetic distances were measured using the Kimura 2 - parameter model implemented in MEGA® 3. A mean pair-wise intra-sample distance of 0.012 nucleotide substitutions per site was estimated for the sequences obtained by the single-molecule method, compared with 0.032 nucleotide substitutions per site for the clonal sequences, suggesting a significant amount of nucleotide substitutions existing between both samples (Table 1). The large difference between them was statistically significant ($p<0.0001$, Mann-Whitney test) (Figure 1). The inter-sample distance of 0.056 nucleotide substitutions per site was more than the intra-sample distances for both sampling datasets.

To further discriminate between the two methods, the phylogenetic relationships of the sequences from both datasets were inferred. A maximum-likelihood tree was constructed using all the sequences. Bootstrap re-samplings of 500 replicates were done using the neighbour-joining method to confirm robustness of the tree.

There were three distinct clusters of sequences in the tree, each supported by high bootstrap values (Figure 2). The first two clusters contained sequences from the clonal-sequencing method, whilst the third cluster contained only sequences from the single-genome method.

The tree also showed that the clonal-sequencing-derived sequences had longer terminal branches for some of the sequences, thus supporting the fact that some of the clonal sequences may have had artifactual nucleotide substitutions.

DISCUSSION

To assess methods used for evaluating the genetic composition of quasispecies in a sample, we examined the use of either a single proviral DNA molecule or un-quantified/multiple proviral DNA molecules as template for PCR amplification. Having amplified the templates using the same conditions, they were processed in different ways prior to sequencing. The products from the single DNA molecule amplification (hereafter referred to as the single-genome method) were sequenced directly, whilst the products from the un-quantified/multiple proviral DNA molecules (referred to as the bulk PCR/clonal-sequencing method) were cloned before sequencing.

Many studies on HIV-1 quasispecies, especially compartmentalization of the virus and detection of selective pressures, have relied on the clonal-sequencing method [17-27], which is less laborious and the cloned products can be easily used for phenotypic assays since they are already

in expression vectors. However, this method has been associated with PCR-induced recombination [4, 28, 29], artifactual substitutions [30] and product re-sampling [11, 31]. The single-molecule method has also been used in many studies of quasispecies [1, 10, 19, 32-34], and is believed to eliminate PCR-induced recombination [35] and re-sampling [36], although it is labour-intensive. Also, PCR products derived with this method have to be cloned before they can be used for phenotypic assays. We compared both methods to know if they equally produced sequences which accurately represented the viral quasispecies.

Our results revealed significant differences in intra-sample distance estimated for each dataset, with more heterogeneous populations identified using the clonal-sequencing method. The observed heterogeneity was probably from artifactual nucleotide substitutions. Also, the observation of longer terminal branches in the phylogenetic tree for the clonal sequences was indicative of greater nucleotide substitutions. These observations are supported by the fact that artifactual errors produced during PCR amplification can be retained amongst plasmid clones of the amplification product (reviewed in [8]).

The presence of artifactual substitutions is a grave issue because any inferences from the analysis of such sequences will be entirely misleading. For example if the substitutions involved basic amino acids at certain positions in V3, an otherwise R5 virus might be predicted to be X4. Also, such substitutions might affect analysis of selective pressure and identification of sites under positive selection.

PCR-induced recombination, which is a common feature associated with clonal amplification [4], was not detected in this study, but is still an important issue since it is difficult to differentiate in vivo recombination from iatrogenic recombination. Also recombination has effects on phylogenetic analysis, reducing genetic distances, skewing the topology of trees and produces false positive estimates of selection pressure [37].

The clonal-sequencing method was used to generate most of the sequences analyzed to determine the HIV-1 group M subtypes [38-41], so the probability of iatrogenic recombination and artifactual substitutions cannot be ruled out.

A previous study comparing both methods observed that the sequences obtained by the clonal-sequencing method encoded for stop codons and had a higher degree of segregation of sequences than those obtained by the single-genome method, which were more interspersed [36]. The higher degree of segregation might be due to template re-sampling. Another study comparing both methods has obtained findings which echo part of our results [42]. Their major findings were long terminal branches in the trees and recombinants among the clonal sequences. However, an earlier study found similar distribution of sequences using both methods, although it looked at only distribution of divergences [19].

Selective amplification of some variants has the potential to under-represent or over-represent some variants from the quasispecies population. There is a high probability of resampling when the DNA copy number in a sample is low [43], as obtains in brain tissue which contains relatively lower proviral DNA copy numbers (36). Sampling bias and non-proportional representation of target sequences can be overcome by the endpoint dilution method [11, 30, 31].

In conclusion, the single-genome method even though more laborious, has the advantage of faithfully reproducing DNA with no artifactual substitutions and no recombinations, and is recommended for use in the study of quasispecies [44].

Table 1. Genetic distances. Distances between the sequences in each dataset and that between the different methods were estimated using the Kimura two-parameter model.

Sampling	Intra-sample distance	Inter-sample distance
Single genome amplification	0.012	0.056
Bulk PCR	0.032	

Figure 1. Comparison of pairwise distances. Each point represents the average pairwise distance of each sequence to all other sequences in a sampling dataset. The difference between datasets was significant ($p < 0.01$). The bars denote the median value of the dataset.

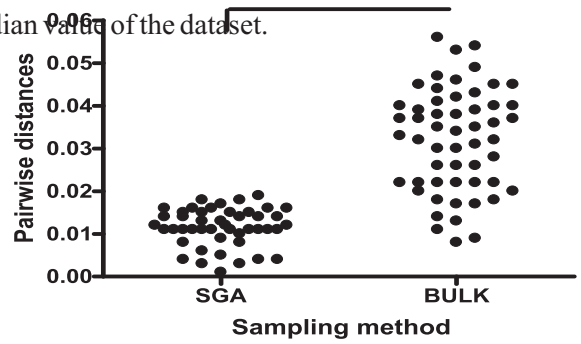
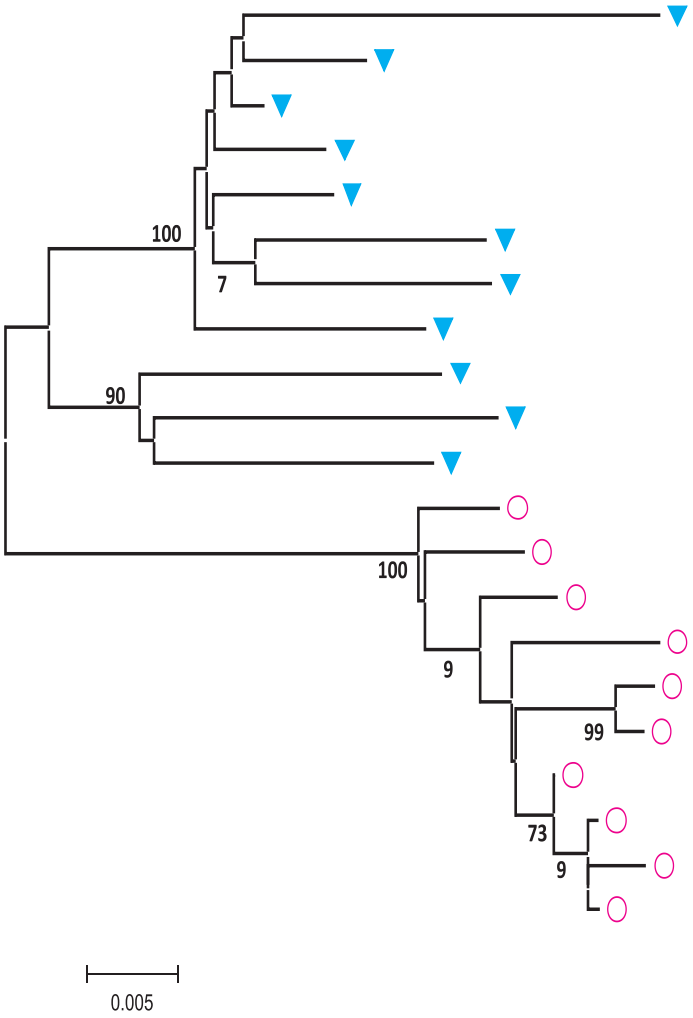


Figure 2. Phylogenetic relationship between sequences. Maximum-likelihood tree constructed with all sequences derived using both methods. Sequences generated by the clonal-sequencing method are indicated with inverted triangles whilst open circles are used to represent sequences from the single genome method. The clonal sequences had longer terminal branches. Scale bar represents 0.005 nucleotide substitutions per site. Bootstrap re-sampling was



ACKNOWLEDGEMENTS

The laboratory work was done in the School of Molecular Medical Sciences, University of Nottingham, Nottingham, United Kingdom. Thanks to the Bayelsa State Government of Nigeria and the Niger Delta University, Amassoma, Bayelsa State, Nigeria for funding.

REFERENCES

1. Curran R, Ball JK. Concordance between semen-derived HIV-1 proviral DNA and viral RNA hypervariable region 3 (V3) envelope sequences in cases where semen populations are distinct from those present in blood. J.Med. Virol. 2002 May; 67(1):9-19.
2. Domingo E, MenendezArias L, Holland JJ. RNA virus fitness. Rev. Med. Virol. 1997 Jul; 7(2):87-96.
3. Eckert KA, Kunkel TA. DNA polymerase fidelity and the polymerase chain reaction. PCR Methods Appl. 1991 August 1, 1991; 1(1):17-24.
4. Meyerhans A, Vartanian J-P, Wain-Hobson S. DNA recombination during PCR. Nucl. Acids Res. 1990; 18(7):1687-91.
5. Cline J, Braman JC, Hogrefe HH. PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. Nucl. Acids Res. 1996; 24(18):3546-51.
6. Tindall KR, Kunkel TA. Fidelity of DNA synthesis by the Thermus aquaticus DNA polymerase. Biochemistry. 1988; 27(16):6008-13.
7. Salminen MO, Koch C, Sandersbuell E, Ehrenberg PK, Michael NL, Carr JK, et al. Recovery of Virtually Full-Length Hiv-1 Provirus of Diverse Subtypes from Primary Virus Cultures Using the Polymerase Chain-Reaction. Virology. 1995; 213(1):80-6.
8. Smith DB, McAllister J, Casino C, Simmonds P. Virus 'quasispecies': making a mountain out of a molehill? J. Gen. Virol. 1997;78(7):1511-9.
9. Andre P, Kim A, Khrapko K, Thilly WG. Fidelity and Mutational Spectrum of Pfu DNA Polymerase on a Human Mitochondrial DNA Sequence. Genome Res. 1997, 1997; 7(8):843-52.
10. Ball JK, Holmes EC, Whitwell H, Desselberger U. Genomic variation of human immunodeficiency virus type 1 (HIV-1): molecular analyses of HIV-1 in sequential blood samples and various organs obtained at autopsy. J. Gen. Virol. 1994; 75(4):67-79.
11. Simmonds P, Balfe P, Peutherer JF, Ludlam CA, Bishop JO, Brown AJ. Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers. J. Virol. 1990; 64(2):864-72.
12. Kimura M. A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide-Sequences. J. Mol. Evol. 1980; 16(2):111-20.
13. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Mol. Biol. Evol. 2007; 24(8):1596-9.
14. Swofford DL. PAUP*: Phylogenetic Analysis Using Parsinomy (*and other methods), version 4.

Sunderland. MA: Sinauer Associates.; 2003.
15. Posada D, Crandall KA. MODELTEST: testing the model of DNA substitution. Bioinformatics. 1998; 14(9):817-8.
16. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 1987; 4(4):406-25.
17. Choudhury B, Pillay D, Taylor S, Cane PA. Analysis of HIV-1 variation in blood and semen during treatment and treatment interruption. J. Med. Virol. 2002; 68(4):467-72.
18. Daniels RS, Wilson P, Patel D, Longhurst H, Patterson S. Analysis of Full-Length HIV Type 1 env Genes Indicates Differences between the Virus Infecting T Cells and Dendritic Cells in Peripheral Blood of Infected Patients. AIDS Res. Hum. Retroviruses. 2004; 20(4):409-13.
19. Delwart EL, Mullins JI, Gupta P, Learn GH, Holodniy M, Katzenstein D, et al. Human immunodeficiency virus type 1 populations in blood and semen. J. Virol. 1998; 72(1):617-23.
20. Devereux HL, Burke A, Lee CA, Johnson MA. In vivo HIV-1 compartmentalisation: Drug resistance-associated mutation distribution. J. Med. Virol. 2002; 66(1):8-12.
21. Gupta P, Leroux C, Patterson BK, Kingsley L, Rinaldo C, Ding M, et al. Human immunodeficiency virus type 1 shedding pattern in semen correlates with the compartmentalization of viral quasi species between blood and semen. J. Infect. Diseases. 2000; 182(1):79-87.
22. Mayer KH, Boswell S, Goldstein R, Lo W, Xu C, Tucker L, et al. Persistence of human immunodeficiency virus in semen after adding indinavir to combination antiretroviral therapy. Clin Infect Diseases. 1999; 28(6):1252-9.
23. Paranjpe S, Craigo J, Patterson B, Ding M, Barroso P, Harrison L, et al. Subcompartmentalization of HIV-1 Quasispecies between Seminal Cells and Seminal Plasma Indicates Their Origin in Distinct Genital Tissues. AIDS Res. Hum. Retroviruses. 2002; 18(17):1271-80.
24. Tirado G, Jove G, Kumar R, Noel RJ, Reyes E, Sepulveda G, et al. Compartmentalization of Drug Resistance-Associated Mutations in a Treatment-Naive HIV-Infected Female. AIDS. Res. Hum. Retroviruses. 2004; 20(6):684-6.
25. van't Wout AB, Ran LJ, Kuiken CL, Kootstra NA, Pals ST, Schuitemaker H. Analysis of the Temporal Relationship between Human Immunodeficiency Virus Type 1 Quasispecies in Sequential Blood Samples and Various Organs Obtained at Autopsy. J. Virol. 1998; 72(1):488-96.
26. Wong JK, Ignacio CC, Torriani F, Havlir D, Fitch NJS, Richman DD. In vivo compartmentalization of human immunodeficiency virus: Evidence from the examination of pol sequences from autopsy tissues. J. Virol. 1997; 71(3): 2059-71.
27. Zhu TF, Wang N, Carr A, Nam DS, MoorJankowski R, Cooper DA, et al. Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: Evidence for viral compartmentalization and selection during sexual transmission. J Virol. 1996; 70(5):3098-107.
28. Fang G, Zhu G, Burger H, Keithly JS, Weiser B. Minimizing DNA recombination during long RT-PCR. J. Virol. Methods. 1998; 76(1-2):139-48.
29. Rousseau CM, Birditt BA, McKay AR, Stoddard JN, Lee TC, McLaughlin S, et al. Large-scale amplification, cloning and sequencing of near full-length HIV-1 subtype C genomes. J. Virol. Methods. 2006;136(1-2):118-25.
30. Palmer S, Kearney M, Maldarelli F, Halvas EK, Bixby CJ, Bazmi H, et al. Multiple, Linked Human Immunodeficiency Virus Type 1 Drug Resistance Mutations in Treatment-Experienced Patients Are Missed by Standard Genotype Analysis. J.Clin. Microbiol. 2005; 43(1):406-13.
31. Simmonds P, Balfe P, Ludlam CA, Bishop JO, Brown AJ. Analysis of sequence diversity in hypervariable regions of the external glycoprotein of human immunodeficiency virus type 1. J. Virol. 1990;64(12):5840-50.
32. Fulcher JA, Hwangbo Y, Zioni R, Nickle D, Lin XD, Heath L, et al. Compartmentalization of human immunodeficiency virus type 1 between blood monocytes and CD4(+) T cells during infection. J Virol. 2004; 78(15):7883-93.
33. Philpott S, Burger H, Tsoukas C, Foley B, Anastos K, Kitchen C, et al. Human immunodeficiency virus type 1 genomic RNA sequences in the female genital tract and blood: Compartmentalization and intrapatient recombination. J Virol. 2005; 79(1):353-63.
34. Kemal KS, Foley B, Burger H, Anastos K, Minkoff H, Kitchen C, et al. HIV-1 in genital tract and plasma of women: Compartmentalization of viral sequences, coreceptor usage, and glycosylation. Proc Natl Acad Sci. 2003. 28; 100(22): 12972-7.
35. Shriner D, Rodrigo AG, Nickle DC, Mullins JI.

Pervasive Genomic Recombination of HIV-1 in Vivo. Genetics. 2004;167(4):1573-83.

36. McCrossan M, Marsden M, Carnie FW, Minnis S, Hansoti B, Anthony IC, et al. An immune control model for viral replication in the CNS during presymptomatic HIV infection. Brain. 2006;129(2):503-16.
37. Shriner D, Nickle DC, Jensen MA, Mullins JI. Potential impact of recombination on sitewise approaches for detecting positive natural selection. Genetical Research. 2003; 81(2):115-21.
38. Gao F, Morrison SG, Robertson DL, Thornton CL, Craig S, Karlsson G, et al. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. J. Virol. 1996; 70(3):1651-67.
39. Gao F, Robertson DL, Carruthers CD, Morrison SG, Jian BX, Chen YL, et al. A comprehensive panel of near-full-length clones and reference sequences for non-subtype B isolates of human immunodeficiency virus type 1. J. Virol. 1998; 72(7):5680-98.
40. Gao F, Robertson DL, Morrison SG, Hui H, Craig S, Decker J, et al. The heterosexual human immunodeficiency virus type 1 epidemic in Thailand is caused by an intersubtype (A/E) recombinant of African origin. J Virol. 1996;70(10):7013-29.
41. Laukkanen T, Carr JK, Janssens W, Liitsola K, Gotte D, McCutchan FE, et al. Virtually full-length subtype F and F/D recombinant HIV-1 from Africa and South America. Virology. 2000; 269(1):95-104.
42. Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, et al. Deciphering Human Immunodeficiency Virus Type 1 Transmission and Early Envelope Diversification by Single Genome Amplification and Sequencing. J Virol. 2008; 82(8):3952-70.
43. Liu S-L, Rodrigo AG, Shankarappa R, Learn GH, Hsu L, Davidov O, et al. HIV Quasispecies and Resampling. Science. 1996; 273(5274):413c-7.
44. Bull ME, Learn GH, Mullins JI, Frenkel LM. Methods used to examine compartmentalization of viral populations between the genital tract and peripheral blood. J Infect Diseases. 2007;196(3):493-4.