Current HIV Research, 2018, *16*, 345-353

RESEARCH ARTICLE

Near Full-length Genomic Sequencing and Molecular Analysis of HIV-Infected Individuals in a Network-based Intervention (TRIP) in Athens, Greece: Evidence that Transmissions Occur More Frequently from those with High HIV-RNA

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ARTICLE HISTORY

Received: December 02, 2018
Revised: January 21, 2019
Accepted: January 27, 2019

DOI:
10.2174/1570162X17666190130120757

Abstract: ***Background*:** TRIP (Transmission Reduction Intervention Project) was a network-based, contact tracing approach to locate and link to care, mostly people who inject drugs (PWID) with recent HIV infection.

***Objective*:** We investigated whether sequences from HIV-infected participants with high viral load cluster together more frequently than what is expected by chance.

***Methods*:** Paired end reads were generated for 104 samples using Illumina MiSeq next-generation sequencing.

***Results*:** 63 sequences belonged to previously identified local transmission networks of PWID (LTNs) of an HIV outbreak in Athens, Greece. For two HIV-RNA cut-offs (10^5 and 10^6 IU/mL), HIV transmissions were more likely between PWID with similar levels of HIV-RNA ($p<0.001$). 10 of the 14 sequences (71.4%) from PWID with HIV-RNA >10^6 IU/mL were clustered in 5 pairs. For 4 of these clusters (80%), there was in each one of them at least one sequence from a recently HIV-infected PWID.

***Conclusion*:** We showed that transmissions are more likely among PWID with high viremia.

Keywords: HIV, recent infection, HIV transmission, PWID, HIV-RNA, TRIP.

1. INTRODUCTION

Approximately 37 million people were living with HIV (PLHIV) by the end of 2017 (http://www.unaids.org/). A lot of different approaches, both behavioral and biomedical, have been used to reduce HIV transmission [1] but still around 2 million people are infected every year. The global community is now committed to end the HIV epidemic as a public health threat by 2030. UNAIDS has set the 90-90-90 target: 90% of all PLHIV to be aware of their HIV status (first 90); 90% of the HIV-diagnosed to receive antiretroviral treatment (ART) (second 90); and 90% of those on ART to achieve virological suppression (third 90).

Social network-based interventions to prevent HIV transmission have shown promising results [2-9]. The Transmission Reduction Intervention Project (TRIP) was a recently implemented network-based intervention to detect people who had acquired HIV in the past 6 months [10]. Identifying people with recent HIV infection is very

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1873-4251/18 $58.00+.00

Table 1. Demographics, epidemiological and clinical characteristics of: i) HIV-infected participants (N=149) of the Transmission Reduction Intervention Project (TRIP) and ii) a subset of the HIV positives (N=63 of 149) who were in local transmission networks of PWID in Athens, Greece.

Characteristics		HIV Positives in TRIP	HIV Positives in PWID-LTNs
Total [N (%)]		149 (100)	63 (100)
Gender [N (%)]	Males	113 (75.8)	49 (77.8)
	Females	36 (24.2)	14 (22.2)
Median age [years (IQR)]		34 (30-40)	34 (30-39)
Education level [N (%)]	Up to high school	131 (87.9)	59 (93.7)
	Above high school	18 (12.1)	4 (6.3)
Accommodation status in the past 6 months [N (%)]	Non-homeless	97 (65.1)	42 (66.7)
	Homeless	52 (34.9)	21 (33.3)
Drug injection in last 6 months [N (%)][a, b]	Non- PWID	5 (3.4)	2 (3.2)
	PWID	141 (96.6)	60 (96.8)
Duration of injection [years (IQR)][c, d]		13 (7-17)	12.5 (7-17)
HIV-RNA [log IU/ml (IQR)][e]		5.4 (4.5-6.1)	5.5 (5.0-6.1)

IQR, interquartile range; PWID, people who inject drugs; [a]N=146 (of 149 HIV positives); [b]N=62 (of 63 HIV positives); [c]N= 140 (of 149 HIV positives); [d]N=60 (of 63 HIV positives); [e]N= 126 (of 149 HIV positives).

important. Increased HIV-RNA levels have been associated with heightened risk of HIV transmission [11-17]. Substantial reductions in HIV transmission or incidence have been observed or expected in settings with declines in individual or community viral load, respectively [12, 18-27]. Given that infectivity has been positively correlated with viral load, HIV transmission during the period of early infection (when viral load becomes very high) is extremely likely [28-41]. Phylogenetic studies estimate that almost half of the transmissions attributable to an HIV-infected person occur during the recent phase of his/her HIV infection [42-54].

Given the epidemiological associations described above, we aimed to investigate phylogenetically, using near full-length HIV sequences, whether HIV transmissions occur more frequently among persons with high viral load, which is a salient characteristic of recent HIV infection.

2. MATERIALS AND METHOD

2.1. Project Description

TRIP was a multi-site (Athens, Greece; Chicago, United States; and Odessa, Ukraine), network-based contact-tracing intervention to detect individuals with recent and/or undiagnosed HIV infection and link them to care. Details regarding the design and implementation of TRIP in Athens have been described elsewhere [10]. TRIP, Athens recruited 356 individuals (90.2% people who inject drugs - PWID). Of these, 149 participants (46.4%) tested HIV positive [10]. Demographics, epidemiological and clinical characteristics of the HIV positive participants in TRIP are presented in Table **1**.

2.2. HIV-1 Testing

Blood samples were tested for HIV antibodies by Ax-SYM HIV-1/2 gO (Abbott) and confirmed by Western Blot (MP Diagnostics). Recent HIV-1 infection was determined using the Limiting Antigen Avidity assay [55]. HIV-RNA in plasma was quantified using Artus HI Virus-1 RT-PCR (Qiagen), according to the manufacturer's recommendations.

2.3. HIV-1 Sequencing and Subtyping

104 near full-length genomic sequences were generated using methods developed under the Infection Response through Virus Genomics (ICONIC) project [56, 57]. Nucleotide sequences were aligned using the HIVAlign tool available on the Los Alamos HIV sequence database (http://hiv.lanl.gov/).

HIV-1 subtypes were identified for 104 sequences using the online automated HIV-1 subtyping tool COMET v.0.2 (COntext-based Modeling for Expeditious Typing) (http://comet.retrovirology.lu/) and the REGA HIV-1 subtyping tool. Subtyping results were further confirmed by using references to perform phylogenetic analysis.

These references were representative of all known HIV-1 subtypes and of most of the Circulating Recombinant Forms (CRFs), and were available on the Los Alamos HIV-1 sequence database. The presence of recombination was tested by bootscanning analysis as implemented in Simplot v3.5.1 [58] using pure subtypes and CRFs as references. Bootscanning analysis was run for a sliding window of 400 bps moving in steps of 50 bps. Putative recombinants were confirmed by phylogenetic analysis in separate genomic fragments with discordant phylogenetic clustering. Tree

visualization and annotation were done using the FigTree v1.4 program (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Hypothesis Testing

We performed phylogenetic analyses to examine whether transmissions occur more often among persons with high plasma HIV-RNA levels (10^6 or 10^5 IU/mL) [59], a marker of recent HIV infection. Phylogenetic analysis was performed using the maximum likelihood method (ML) under the Generalized Time Reversible (GTR) model of nucleotide substitution including a Gamma-distributed rate of heterogeneity among sites as implemented in RAxMLv8.0 [60] and FastTree v2.1 [61]. HIV-1 sequences that were found outside the local transmission networks (LTNs) of PWID [62] or the Unique Recombinant Forms (URFs) were excluded from the analysis. Short length sequences were also excluded (< 4,000 bps). Phylogenetic trees were derived from non-recombinant near full-length genome alignments that belonged to the 4 PWID LTNs (CRF14_BG/B/CRF14_BG, CRF35_AD/A/CRF35_AD, subtypes A and B).

We performed additional analysis to test whether transmissions among PWID with high HIV-RNA are frequent. The hypothesis that transmissions occur frequently among persons with high plasma HIV-RNA values (10^6 or 10^5 IU/mL) was investigated by reconstructing ancestral states assigned at the tips (high or low HIV-RNA values), and using the criterion of parsimony to estimate the total number of character changes across the phylogeny. In this case, character changes correspond to transmissions between persons with different levels of HIV-RNA ("low" and "high"). The analysis was performed on 300 bootstrap-reconstructed trees as estimated in RAxML version 8.0, using Mesquite program version 3.5 [63]. The estimated number of events corresponds to the "observed number of transmissions among persons with different HIV-RNA values". The null hypothesis that corresponds to a random distribution of the characters states at the tips was simulated after a random reshuffling of the characters on the full set of bootstrap trees.

We also tested if HIV sequences from persons with high viral loads (>10^6 IU/mL) formed significant phylogenetic clusters receiving Shimodaira-Hasegawa (SH) value > 0.9 or bootstrap support > 75%.

2.5. Statistical Analysis

The non-parametric, one-sided Mann-Whitney test was used to compare the distribution of the total number of character changes (transmissions between different groups of HIV-RNA) between the original bootstrap trees and the trees that were randomly reshuffled at the tips (STATA 14 - StataCorp LP).

3. RESULTS

HIV-1 subtyping and recombination analysis showed that sequences from 63 individuals (60.6%) (Table 1) fell within the LTNs of PWID: CRF14_BG/B/CRF14_BG (n=35, 33.7%); CRF35_AD/A/CRF35_AD (n=17, 16.3%); subtype B (n=8, 7.7%); subtype A (n=3, 2.9%). The rest of the sequences were classified as subtypes A (n=4, 3.8%) and B (n=1, 1%) that did not belong to PWID-LTN, and as URFs (n=36, 34.6%). HIV-1 sequences available in protease (PR) and partial reverse transcriptase (RT) that were classified initially as CRF35_AD and CRF14_BG [62,64], were later identified as unique recombinants with identical patterns (CRF35_AD/A/CRF35_AD and CRF14_BG/B/CRF14_BG, respectively, in their complete genome).

Phylogenetic analysis using the 62 near-complete HIV-1 sequences that belonged to the 4 PWID-LTNs (CRF14_BG/B/CRF14_BG, CRF35_AD/A/CRF35_AD, subtypes A and B) was conducted to investigate whether transmissions occur more often among individuals with high HIV-RNA levels. One sequence was excluded due to its short length (< 4,000 bps). Phylogenetic trees that were inferred using the 62 sequences are shown in Fig. (**1**). The sequences from persons with high HIV-RNA (> 10^5 IU/mL or > 10^6 IU/mL) are highlighted in different colors (Figs. **1A** and **1B**).

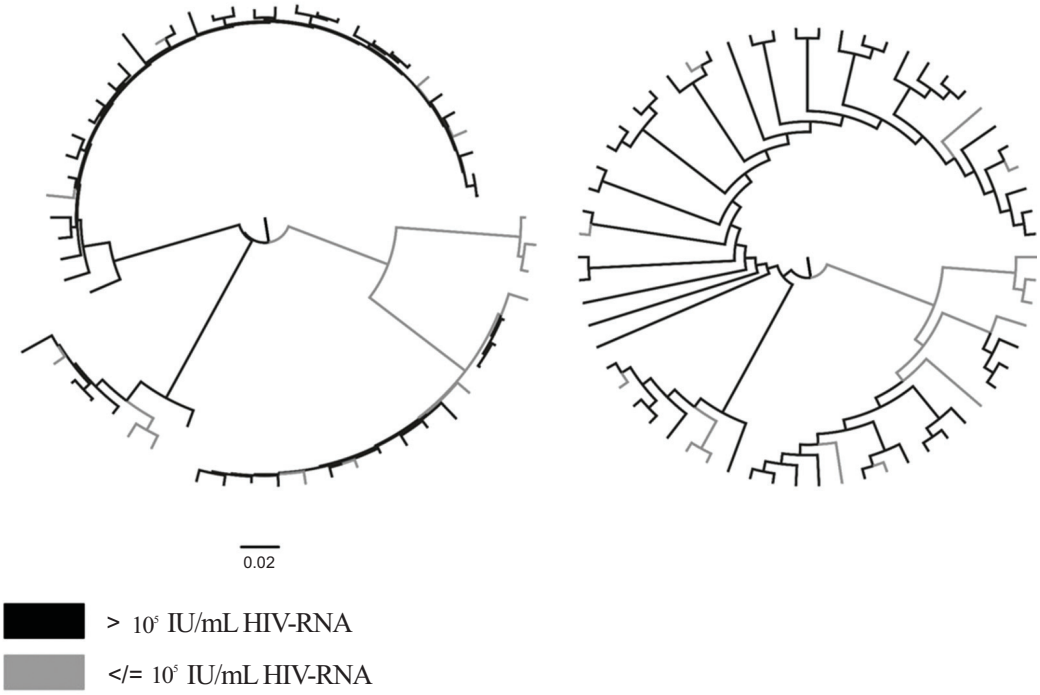
Additional analyses included the estimation of the total number of character changes between PWID with high and low HIV-RNA, which are indicative of transmissions between these two groups, and the examination of whether the number of transmissions is significantly lower than the number of changes expected by chance. For both HIV-RNA cutoffs (10^5 and 10^6 IU/mL), the number of transmissions between the two groups was significantly lower ($p<0.001$), suggesting that HIV transmissions occur more frequently between PWID with similar levels of HIV-RNA (Figs. **2A** and **2B**). Notably, most sequences from PWID with HIV-RNA > 10^6 IU/mL formed highly supported clusters (*i.e.*, they received SH-support > 0.9 or bootstrap support > 75%) (Fig. **1B**). For 14 of 18 PWID with HIV-RNA > 10^6 IU/mL, phylogenetic clustering with at least one sequence was highly supported. 10 of the 14 sequences (71.4%) from PWID with HIV-RNA > 10^6 IU/mL were clustered in 5 pairs (Fig. **1B**).

We also examined whether there were any PWID with documented recent infection (< 6 months) among the PWID with HIV-RNA > 10^6 IU/mL. For 4 of the 5 clusters (80%) of sequences (n=10) from PWID with high HIV- RNA, there was in each cluster at least one sequence from a recently HIV-infected PWID (Fig. **1C**). Specifically, 6 of 10 transmissions (60%) among PWID with HIV-RNA > 10^6 IU/mL in transmission pairs originated from people with recent HIV infection. For one cluster, both sequences were from recently HIV-infected individuals (Fig. **1C**).

4. DISCUSSION

This study examined, using molecular epidemiology methods, whether HIV transmissions occur more often among PWID with high HIV-RNA levels. Our analysis was based on near full-length genomic sequencing from HIV-infected PWID who participated in a network-based intervention (TRIP) in Athens, Greece. All analyses supported that transmissions among PWID with high HIV-RNA levels (> 10^5 IU/mL or > 10^6 IU/mL) occur at high rates. Given that very high HIV-RNA levels (> 10^6 IU/mL) are a marker for recent HIV infection, our results suggest that transmissions among people who acquired HIV recently are more frequent. The important role of recently HIV-infected individuals was further supported by our findings that in 80% of the clusters

A



B

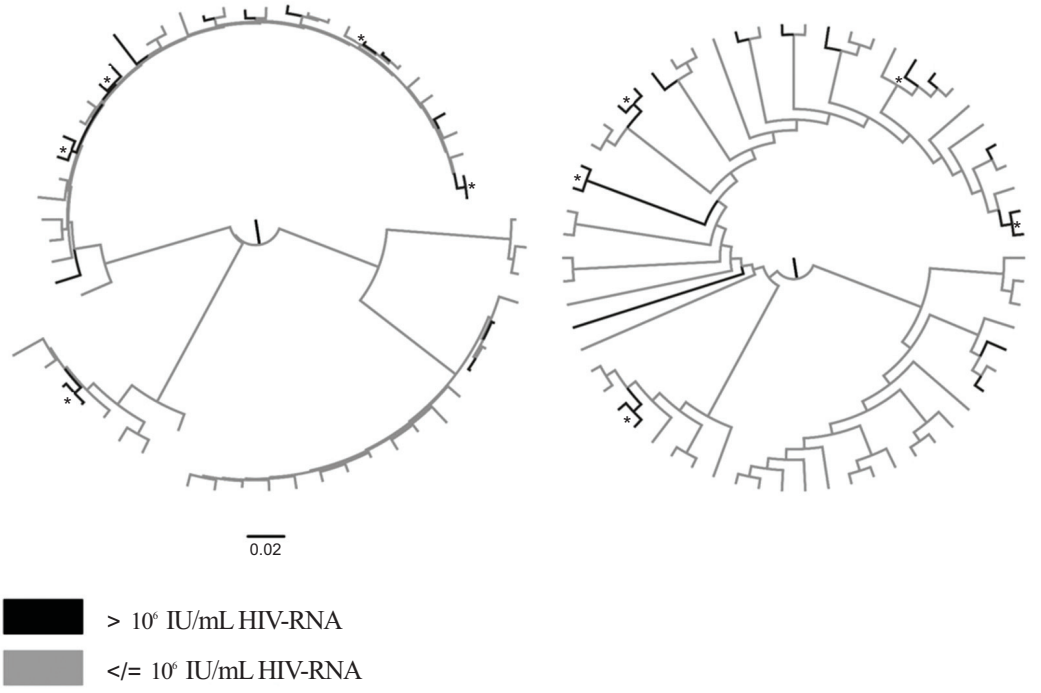


Fig. (1). contd….

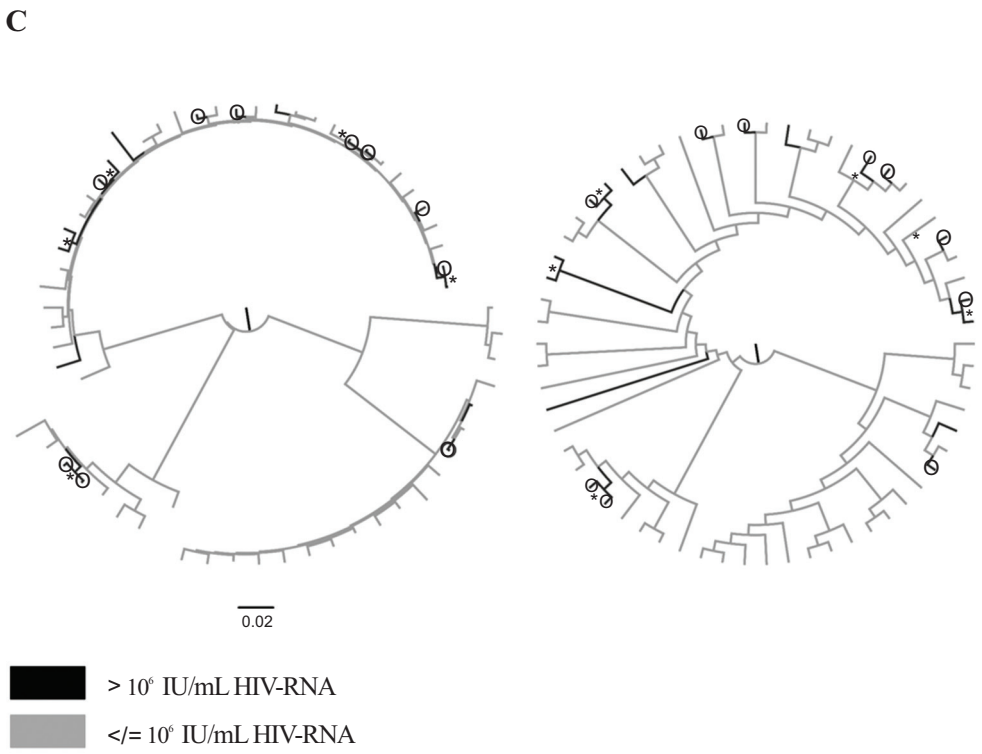


Fig. (1). Maximum likelihood phylogenetic trees of near full-length genomic sequencing found in local transmission networks (LTNs) of people who inject drugs (PWID) shown with branch lengths and without branch lengths. (**A**) PWID with HIV-RNA > 10^5 IU/mL are highlighted in black. (**B**) PWID with HIV-RNA > 10^6 IU/mL are highlighted in black. (**C**) PWID with HIV-RNA > 10^6 IU/mL are highlighted in black and PWID with HIV-RNA > 10^6 IU/mL and documented recent infection (< 6 months) are indicated by white circles with black outlines. Highly supported nodes (SH-support > 0.9 or bootstrap support > 75%) including viral sequences from PWID with HIV-RNA > 10^6 IU/mL are indicated by an asterisk.

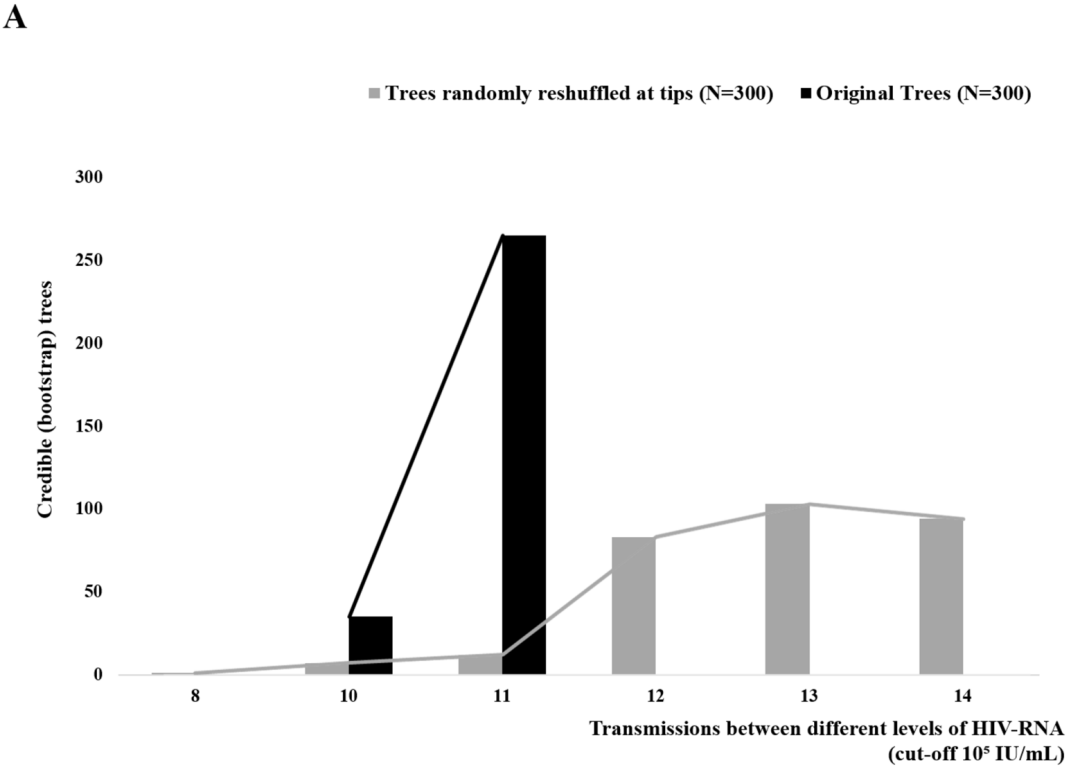


Fig. (2). contd….

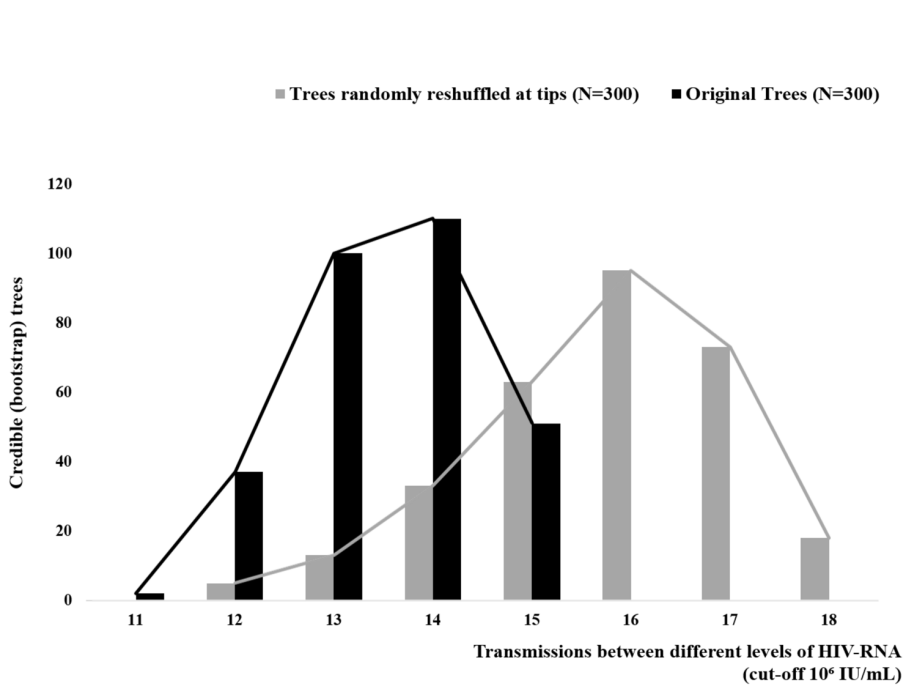


Fig. (2). Total number of character changes (transmissions between different levels of HIV-RNA) estimated between original bootstrap trees (black) and after a reshuffling at the tips (grey) (**A**) using a cut-off of 10^5 IU/mL and (**B**) using a cut-off of 10^6 IU/mL.

of PWID with HIV-RNA > 10^6 IU/mL, there was at least one PWID with documented recent infection. This suggests that those individuals who have recently been infected with HIV may be the source of HIV transmission within transmission pairs.

These findings are in accordance with those from other analyses in TRIP and also with evidence from previous studies. It has been shown in TRIP that the proportion of recently HIV-infected persons among the HIV positives in the networks of recently HIV-infected seeds was approximately 3 times the proportion of recently HIV-infected persons in the networks of seeds with long-term HIV infection [10]. Seeds refer to primary participants recruited by TRIP to start the network-based intervention. Brenner *et al.* have reported that almost 50% of viral sequences from primary infections in Quebec, Canada were clustered into groups of 2-17 sequences/cluster [43]. Pinkerton used mathematical modeling to show that the probability of HIV transmission during acute infection was approximately 42 times higher than that during chronic HIV infection [65]. The same analysis revealed that the acute phase was responsible for 89% of all transmission events in the first 20 months of follow-up [65]. Miller *et al.* also highlighted the important role of acute and early HIV infection (due to high levels of HIV-RNA during these stages), as opposed to chronic infection, in sexual transmission [66]. Similarly, several studies reported that during HIV outbreaks among PWID, a high number of individuals have been infected with genetically similar viruses [62, 64, 67-80] - a finding that supports the hypothesis of the involvement of recently HIV-infected people in HIV transmissions. The crucial role of the natural history of HIV-1 (high HIV-RNA) has also been investigated by simulation studies and was found to play a significant role (along with injection risk networks) during periods of high HIV prevalence among PWID [5].

CONCLUSION

In the current study, using molecular epidemiology methods, we generated additional evidence that transmissions were more likely to have occurred among PWID who had high HIV-RNA at the time the study was conducted. We also found that in 80% of the transmission pairs of PWID with very high viral load, there was at least one recent infection.

Given these findings, early diagnosis soon after infection and immediate treatment initiation to reduce viral load should receive attention as a major HIV prevention strategy.

LIST OF ABBREVIATIONS

ART = Antiretroviral treatment

CRFs = Circulating Recombinant Forms

GTR = Generalized Time Reversible

HIV = Human immunodeficiency virus

ICONIC = Infection Response through Virus Genomics

LTNs = Local transmission networks

PLHIV = People living with HIV

PR = Protease

PWID = People who inject drugs

RT = Reverse transcriptase

SH = Shimodaira-Hasegawa

TRIP = Transmission Reduction Intervention Project

URFs = Unique Recombinant Forms.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was approved by the Institutional Review Boards of the Hellenic Scientific Society for the study of AIDS and Sexually Transmitted Diseases (Athens, Greece), and of the National Development and Research Institutes (New York City, US).

HUMAN AND ANIMAL RIGHTS

No animals were used in the study. All humans research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10 ethics/10helsinki/).

CONSENT FOR PUBLICATION

Each participant of TRIP gave informed consent.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The study was supported by the United States (US) National Institute on Drug Abuse [DP1 DA034989], the Hellenic Scientific Society for the Study of AIDS and STDs, and the WT/DoH ICONIC grant. The funding agencies had no role in study design, collection, analysis or interpretation of data or in the decision to submit the article for publication.

AUTHOR CONTRIBUTIONS

Conceptualization, D.P., E.N., A.Hat., S.R.F. and G.K.N.

Methodology, D.P. and G.K.N. designed the study

D.F., B.F., J.R., P.G. and Z.K. generated the full-length sequencing data

E.P. conducted the interviews with TRIP participants and facilitated blood collection

Formal analysis, E.-G.K., K.P., A.Had. and L.D.W.

Writing-original draft preparation, E.-G.K., D.F. and D.P.

Writing-review and editing, G.K.N., A.Hat., A.Had., S.R.F., L.D.W., K.P., B.F., J.R., P.G., Z.K., E.N. and E.P.

Supervision, D.P., E.N. and G.K.N.

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