Infection, Genetics and Evolution 71 (2019) 1–6

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Inconsistent temporal patterns of genetic variation of HCV among high-risk subjects may impact inference of transmission networks

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ARTICLE INFO

Keywords:
Epidemiology
Clustering
Phylogeny
Evolutionary dynamics
Evolutionary rate

ABSTRACT

Hepatitis-C Virus (HCV) sequences are often used to establish networks of people who inject drugs (PWID). However, the degree to which within-host evolutionary dynamics affect those inferences has not been carefully studied. Here, we analyzed 702 longitudinally-sampled HCV E1 sequences from 88 HCV+ people who inject drugs (PWID) in the Baltimore Before and After Acute Study of Hepatitis (BBAASH) cohort. Individuals were tested for HCV RNA over multiple visits to the clinic, and the HCV E1 gene was sequenced for HCV+ samples. Genetic clustering was performed on the full set of sequences using a 3% genetic distance threshold to define epidemiological linkage. Maximum-likelihood (ML) phylogenies were inferred to assess evolutionary relationships. We found 22 clusters containing sequences sampled over five or more years (long-term clusters, LTC), of which 17 had > 1 subject. In six of the multi-subject LTC, one subject had a sequence sampled > 3 years earlier or later than the next-closest subject in the cluster (time-gap LTC). ML trees showed that, in three of the time-gap LTC, two subjects had identical sequences despite 7–10 years separating the sampling times. In four of the time-gap LTC for whom additional data were available, the subject with the later detected shared variant had both different variants and visits with no detectable HCV RNA (RNA-) prior to the appearance of the shared variant. In the subject with the earlier detection of the shared variant, different variants and RNA- visits were also detected in multiple cases subsequent to appearance of the shared variant. Complex patterns of shared viral variation among PWID reflect on-going re-infection, multiple transmission partners, and/or inconsistent detection of viral variants. Our results suggest that transmission events are currently underestimated by analysis of sequences at a single point in time.

1. Introduction

HCV continues to be a major health care burden, despite new classes of drugs with high efficacy (Thrift et al., 2017). HCV is also still a major cause of mortality throughout the world (Stanaway et al., 2016): in 2016, while up to 2 million persons were cured of HCV, 1.7 million new infections occurred (Hill et al., 2017). The vast majority (up to 80%) of new infections in high-income countries are among people who inject drugs (PWID) (Grebely et al., 2015), and an estimated 67% of PWID have been infected (Nelson et al., 2011). Especially troubling is the rising incidence of HCV infection due to opioid use in young people in the US (Zibbell et al., 2015).

Molecular epidemiological tools have been used to identify transmission networks among PWID (Aitken et al., 2004; Bretaña et al., 2015; Cochrane et al., 2002; Cunningham et al., 2015a; Jacka et al., 2014; Matthews et al., 2011; Pilon et al., 2011; Sacks-Davis et al., 2012). However, molecular epidemiological analysis of HCV in PWID is complicated by several factors. The actual onset of HCV infection is rarely known. Spontaneous clearance occurs in 25–35% of infected individuals, and some individuals who spontaneously clear infection can be reinfected (Osburn et al., 2010; Cox et al., 2005a; Villano et al., 1999). In addition, multiple viruses can establish the initial infection (coinfections) and those who develop chronic infection can acquire new viruses later (superinfection). A multitude of observational studies suggest that a substantial proportion of PWID carry mixed infections (Cunningham et al., 2015b). Furthermore, within-host evolutionary

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https://doi.org/10.1016/j.meegid.2019.02.025
Received 19 December 2018; Received in revised form 19 February 2019; Accepted 21 February 2019
Available online 22 February 2019
1567-1348/

dynamics are complex (Raghwani et al., 2016; Ho et al., 2017; Gray et al., 2012a; Gray et al., 2012b; Ray et al., 2005; Cox et al., 2005b; Timm et al., 2004; Raghwani et al., 2019). Multiple co-existing lineages persist over time and are not necessarily detected at every sampling time (Raghwani et al., 2016; Ho et al., 2017; Raghwani et al., 2019), even using high-throughput sequencing (Ho et al., 2017; Raghwani et al., 2019), likely resulting from viral population structure (Raghwani et al., 2016; Ho et al., 2017; Raghwani et al., 2019; Gray et al., 2012c). Lineages may exist for many years yet still show a relative lack of evolution (Raghwani et al., 2016; Ho et al., 2017; Raghwani et al., 2019; Gray et al., 2012c), and the evolutionary rate is highly variable over time and among lineages (Raghwani et al., 2016; Ho et al., 2017; Gray et al., 2012a; Gray et al., 2012b; Raghwani et al., 2019). Furthermore, among-host dynamics are complicated by the possible "reversion" of host-specific substitutions upon transmission (Ray et al., 2005; Gray et al., 2011; Liu et al., 2012). Thus, at any one point in time, the viruses detected may reflect a combination of reinfection, superinfection, and evolution (Cunningham et al., 2015b; Raghwani et al., 2016; Ho et al., 2017; Gray et al., 2012a; Gray et al., 2012b; Ray et al., 2005; Cox et al., 2005b; Timm et al., 2004; Raghwani et al., 2019; Gray et al., 2011; Liu et al., 2012).

Because these factors complicate cross-sectional analyses, we assessed longitudinal patterns of HCV in a highly-networked cohort of 88 PWID followed over ~20 years with known dates of seroconversion. We previously investigated patterns of intra-host variation in this cohort, and found that 1/3 of the subjects in this cohort had multiple distinct variants during the study period (Rose et al., 2017a). The most common pattern was that a subject had one particular viral variant early in infection, and then a second viral variant was subsequently detected at the remaining visits. Other more complex patterns included two different viral variants being detected simultaneously; the earlier variant "re-appearing" after detection of a different variant and/or and HCV RNA- visit; and a single subject having up to six viral variants (Rose et al., 2017a). In this report, we follow up on those results to investigate clustering patterns of shared viral variants over time among individuals and assess the impact on interpretation of epidemiological linkage.

2. Methods

2.1. Ethics statement

This study was approved by the Institutional Review Board of the Johns Hopkins University (IRB# NA_00046368, Title: CD8 + T cells and the Outcome of viral hepatitis- Monthly Blood Sampling). This study of stored samples and previously collected data and the parent cohort study was conducted according to the ethical standards set forth by the institutional review board of Johns Hopkins University and the Helsinki Declaration of the World Medical Association. All participants were adults who provided written informed consent.

2.2. Subjects

Subjects were enrolled in the "*Baltimore Before and After Acute Study of Hepatitis*" (BBAASH) cohort described previously, composed of PWID followed prospectively over a nearly 20 year period (1996–2016) (Cox et al., 2005a). The study followed hundreds of individuals who were frequently tested for HCV. All of the subjects either entered the study HCV seronegative, or had documented clinical evidence of a recent seroconversion. Enrolled subjects were tested and provided samples at regular intervals thereafter. For HCV RNA+ samples, sequencing was performed. The cohort included individuals who progressed to chronic infection, as well as individuals who appeared to clear infection and were subsequently re-infected.

2.3. Genetic sequence data

For this study, we used a subset of BBAASH subjects (n = 88) who had two or more sequences (range 2–31, average = 8 sequences/person) sampled over a 1–14-year period (average time span = 4.9 years) during the length of the study (1997–2016). We used E1 sequences (H77 nt 943–1288) from all 88 subjects, with a final alignment comprising 702 sequences. The generation of E1 sequence data was performed contemporaneously over two decades using methods previously described (Enomoto et al., 1990). Standard procedures to prevent and recognize cross-contamination have been in place for decades in the JHU Center for Viral Hepatitis Research laboratory. These build upon the standards articulated by Kwok and Higuchi (Kwok and Higuchi, 1989) and include: (i) physical separation of lab areas for blood handling, extraction/pre-amplification, post-amplification, and plasmid work, (ii) extensive use of negative controls and aerosol barrier tips, and (iii) immediate creation of multiple aliquots of each fresh sample prior to other manipulations, with segregation of aliquots for routine versus validation purposes. In addition to the precautions noted above, initial sequencing of the E1 amplicon was done within days of specimen collection, with approximately 8 positive reactions weekly.

2.4. Clustering/phylogenetics

We used genetic clustering to determine which sequences were likely to be epidemiologically closely related and define specific viral variants. We used HIV-TRACE to calculate the distance matrix and clusters (Kosakovsky Pond et al., 2018). We resolved ambiguities at 1.5% as suggested by the authors of the program (Kosakovsky Pond et al., 2018). In our previous study of E1-based sequence comparison (Rose et al., 2017a) we used a 3.9% genetic distance threshold based on an ROC analysis which assessed the distributions of intra-patient diversity and inter-subject diversity. In this study, we used a slightly lower threshold of 3% to maximize discrimination of clusters (since clusters grow larger as genetic distance increases with this method (Rose et al., 2017b)). Maximum likelihood (ML) trees were inferred using PhyML (Guindon et al., 2009) in the Geneious software platform (https://www.geneious.com/) with the GTR + gamma model of nucleotide substitution.

3. Results

3.1. Clusters

Among the full dataset of 702 sequences, 93 clusters were found using a genetic distance threshold of 3%. The median duration (i.e. time between earliest and latest sampled sequences) for all clusters was 2.3 years (Fig. 1). The number of clusters increased as total duration decreased (with total duration measured in one-year intervals), with a maximum of 23 clusters with < 1-year total duration, to a minimum of one cluster with 5–6 years total duration. This result was consistent with the expectation that genetic distance between sequences that share a common ancestor (i.e. at time of transmission) increases with time as sequences independently evolve. Interestingly, the number of clusters in each one-year interval remained relatively consistent from 5 to 14 years total duration, with an average of 2.5 clusters per interval. Altogether, 22 clusters contained sequences collected across an interval of five years or more (long-term clusters, "LTC"). Of the 22 LTC, 17 were comprised of sequences from > 1 subject.

We were interested in investigating further the characteristics of these LTC. First, we plotted the sampling time for each sequence in the cluster. Surprisingly, we found that in four of the LTC containing sequences from two subjects, the earliest sample from one subject was between 7.2 and 10.7 years later than the latest sample from the other subject (Fig. 2a–d). In the two LTC with multiple subjects, one subject had a sequence sampled 5.9 later (Fig. 2e) and 3.5 years earlier (Fig. 2f)

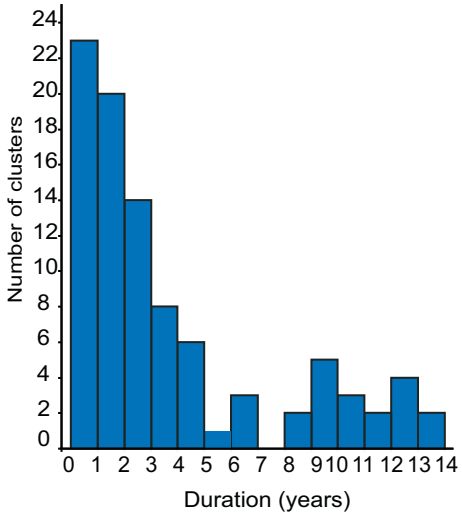

Fig. 1. Histogram of the time spanned (years) by the identified clusters. Each bar indicates the duration (x-axis) for 93 clusters (y-axis) found at the 3% genetic diversity threshold.

than the next-closest sample from another subject. Collectively, these six clusters were designated "time-gap LTC".

3.2. Phylogenetic trees

We then inferred ML trees for the four time-gap LTC with two subjects (Fig. 3a–d) and the two time-gap LTC with > 2 subjects (Fig. 3e–f). In three of the time-gap LTC trees, two subjects shared identical sequences, despite being sampled 7–10 years apart (Fig. 3b, d, f). In all three of these cases, the subject with the later sampled variant only had one sequence in that cluster. In one of these cases (Fig. 3f), the time-gap between identical sequences (8 years) was much larger than the time-gap initially observed above in Fig. 2f, because the later sampled individual (in 2011) shared an identical sequence with another subject sampled much earlier (in 2001–2003) rather than with the sequence from the next-closest sampled subject (in 2008). In two of the time-gap LTC, one sequence from the earlier sampled subject was closely related to sequences sampled 6–8 years later (Fig. 3c, e). In the remaining time-gap LTC tree, sequences from each subject were grouped on separate clades (Fig. 3a).

For comparison, we also inferred ML trees for the 16 non-time-gap LTC. In two clusters, identical sequences from the same subject were sampled up to four years apart (Fig. 4a, b). In one subject, nearly identical sequences were sampled 11 years apart (Fig. 4b). On the other hand, some trees showed more of a staircase-like pattern of accumulating mutations as would be expected under a more constant molecular clock (Fig. 4c).

3.3. History of time-gap LTC clusters

Most subjects in the time-gap LTC had additional sequences in other clusters and/or RNA- visits, in addition to the sequence(s) in the shared cluster. A longitudinal plot of viremia and all cluster assignments (i.e. "variants") was plotted for both subjects in each of three time-gap LTC (Fig. 5a, b, c) and for the two subjects with identical sequences in one of the larger time-gap LTC (Fig. 5d). Additional data was not available for one subject in remaining two time-gap LTC. The first cluster assignment represents the initial infecting virus around the time of seroconversion. In all four cases, the subject with later detection of the shared cluster previously had sequences in 1–5 other clusters and an RNA- visit (Fig. 5a–d). In three cases, the subject with the earlier detection of the shared variant subsequently had a sequence in a different cluster prior to detection of the shared variant in the other subject (Fig. 5a, b, d). In two cases, the subject with the earlier detection of the shared variant also had an RNA- visit prior to detection of the shared variant in the other subject (Fig. 5b, d).

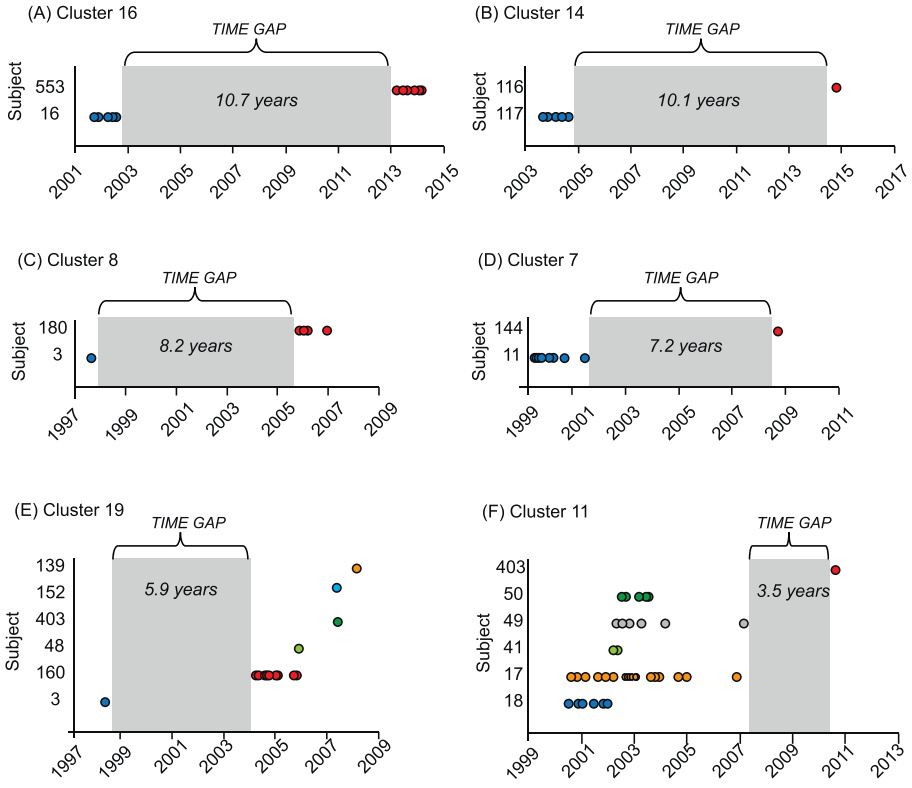

Fig. 2. Sampling dates for subjects' sequence in six time-gap long-term clusters (LTC). For each of the six clusters (A-F) for which a time-gap was identified, the sampling dates (x-axis) of all sequences are shown for the subjects in each cluster (subject ID on y-axis). Colors represent the sequences from a given subject. Non-overlapping sampling periods (i.e. "time-gap") are indicated by the grey box.

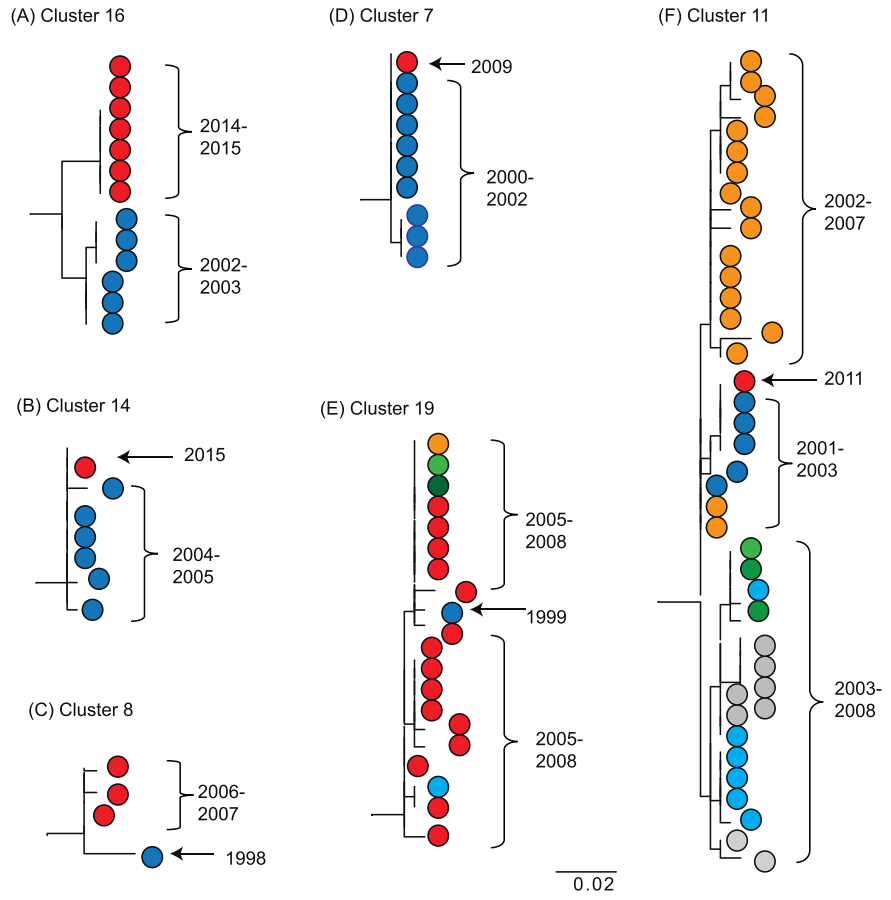

Fig. 3. ML trees for six time-gap LTC. Each ML tree (A-F) contains sequences from all subjects in that cluster. Circles represent tips of the branches (sequences). Colors represent different subjects. The sampling year is noted next to the tips. Branches are scaled according to the scale bar. For the purposes of maintaining the scale across all subjects for comparison, a single ML tree was inferred using sequences from all LTC, and the clades containing the subjects from each of the six clusters are shown here.

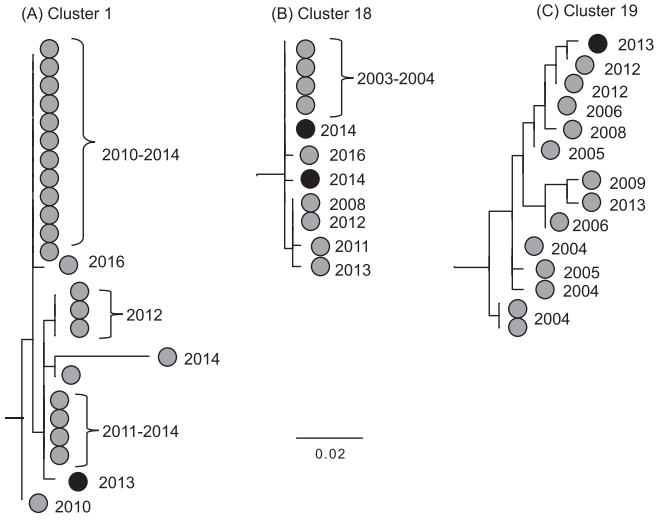

Fig. 4. ML trees for three representative non-time-gap LTC. Circles represent tips of the branches (sequences). Grey shades represent different subjects. The sampling year is noted next to the tips. Branches are scaled according to the scale bar. The tree was drawn in the same fashion as described in Fig. 3.

4. Discussion

In this study, we investigated the temporal patterns of clusters in a cohort of PWID whose initial infection was documented and who were then carefully followed through time. Our findings underscore the complexity of interpreting HCV sequences from PWID for epidemiological purposes.

We found 22 clusters containing sequences collected across an interval of five years or more (Fig. 1). In six of these clusters, the sequence sampling time for one subject did not overlap those for any other subject in that cluster (Fig. 2). In fact, an identical sequence was detected in three pairs despite the two subjects being sampled 7–10 years apart (Fig. 3). Furthermore, the subjects with the later shared variant had at least one different variant in another cluster and a visit with no HCV RNA prior to detection of the shared variant, pointing towards superinfection (Fig. 5). In two clusters, identical sequences in the same individual were sampled over a four-year period, while nearly identical sequences were sampled over an 11-year time period (Fig. 4).

There are multiple potential explanations for these patterns. Previous studies of intra-host evolution have detected extreme rate variation both within and among HCV+ subjects, as well as intermittent detection of persistent variants, consistent with a model of a highly structured within-host viral population (Raghwani et al., 2016; Ho et al., 2017; Gray et al., 2012a; Gray et al., 2012b; Raghwani et al., 2019; Liu et al., 2010). Putative population structure in the liver and/or extra-hepatic compartments may allow distinct viral sub-populations to persist while remaining undetectable in blood (Raghwani et al., 2016; Ho et al., 2017; Raghwani et al., 2019; Gray et al., 2012c). Thus, the shared variant may have been present concurrently in both subjects, but at an undetectable frequency in the blood. This explanation is consistent with studies using deep sequencing methods on blood samples which found similar patterns of alternating lineages and population structure (Ho et al., 2017; Raghwani et al., 2019), and suggests that using deep sequencing would not provide additional resolution. Alternatively, the virus could have continued to accumulate mutations in the first subject subsequent to its last detection, which were lost in the new host after transmission (Gray et al., 2011). While this could point to extremely strong selective pressure that maintains an early strain, or to

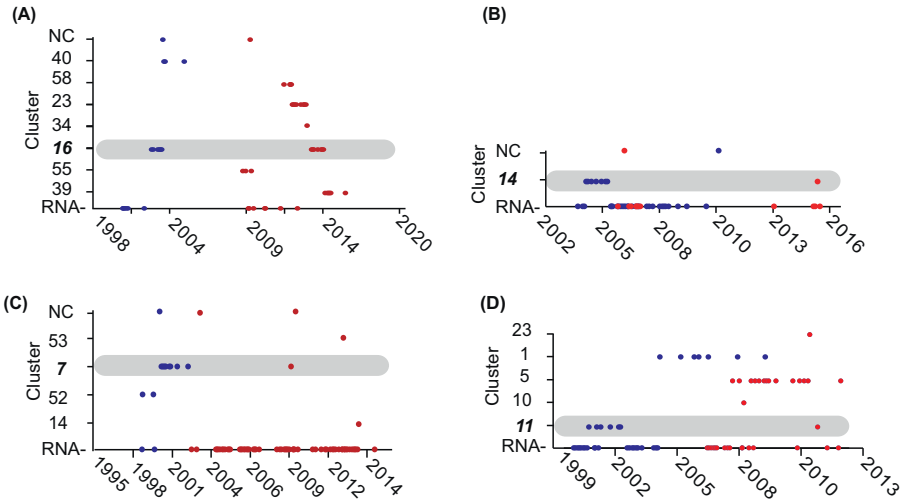

Fig. 5. Clusters and RNA- visits for subjects in four time-gap LTC. For each cluster, the two subjects (red and blue) with non-overlapping sampling dates are shown (A–D). Time is shown on the x-axis, and the cluster designation/RNA- visit is shown on the y-axis. Cluster numbers are categorical. The bolded, italicized cluster number and grey bar indicates the LTC. NC = no cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

a mechanism that allows for a reduced replication rate as seen for HIV (Vrancken et al., 2014; Raghwani et al., 2018) and HBV (Vrancken et al., 2017), the inconsistent differences in evolutionary rates at different scales across the genome rather point to a selection and constraint mechanism (Gray et al., 2011).

These findings have major implications for the interpretation of cross-sectional HCV sequence data. Typically, two or more individuals with genetically related variants (i.e. in the same genetic cluster) are interpreted as sharing a recent transmission event. In this study, however, multiple instances of shared variants were unrelated with time. Therefore, subjects sharing identical or near-identical variants are not necessarily indicative of a recent transmission. Additionally, genetic linkage among subjects could be underestimated in studies that use only one or a few time points, even if subjects are sequenced soon after seroconversion, as infection with multiple variants could be masked by the detection of only a single dominant variant. On the other hand, the observation that genetic relationships can be detected among viruses sampled a decade or more apart supports the use of networking studies for HCV, as genetic linkage may be retained for longer periods of time than for other viruses such as HIV for which a more gradual buildup of genetic differences over time will eventually exceed the genetic distance threshold used to establish transmission groups among subjects. We note that since this cohort of 88 subjects is a small portion of the entire Baltimore PWID population, one or more links in the transmission chain are likely unrepresented. Therefore, sharing closely related variants does not necessarily imply direct transmission, particularly in PWID where high-risk behavior is prevalent among multiple individuals.

Of course, the assignment of genetic linkage is predicated on the choice of genetic distance threshold. Clear rationale for choosing distance thresholds is important for any molecular epidemiological linkage study. Previously we used a ROC-based analysis to determine the point that maximized both sensitivity and specificity based on the intra- vs. inter-subject pairwise genetic distances (Rose et al., 2017a). We used a slightly lower threshold (3%) in this study as a conservative measure to avoid over-estimating linkage among sequences, since clusters tend to exponentially grow larger as genetic distance threshold increases using a hierarchical clustering method (Rose et al., 2017b). Given that we found identical or near-identical sequences over > 5-year time intervals, decreasing the threshold would not have greatly affected our results.

A possible explanation for observing the same sequence is PCR contamination. However, given the contemporaneous generation of sequence data and the quality control procedures in place this is highly unlikely to have generated the type of observations seen. The recovery of the same sequence from the same person at visits separated by months or years cannot be explained by carry-over contamination unless many other intervening specimens were also positive for this sequence, and we know from examining all E1 sequences that this was not the case.

Similar to many molecular epidemiological studies, we used bulk sequencing, which provides only one consensus sequence, thus masking genetic variation. Next-generation sequencing for full length and/or deeper sequencing may provide greater insight into the epidemiological connections within the population; however, we note the potential limitations of this approach above. On the other hand, more frequent sampling may provide greater opportunity to detect alternatively co-circulating lineages. Likely a combination of approaches will be required to fully explore which mechanism(s) account for our observations.

Funding sources

Support was provided by the Division of Intramural Research, NIAID. DT was funded by R37DA013806. SCR was funded by U19 AI088791. AC was funded by U19 AI088791.

Acknowledgements

We thank the participants and staff of BBAASH.

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