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Frequent HCV reinfection and superinfection in a cohort of injecting drug users in Amsterdam☆

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***Background/Aims*: This study investigates the occurrence of HCV reinfection and superinfection among HCV seroconverters participating in the Amsterdam Cohort Studies among drug users from 1985 through 2005.**

***Methods*: HCV seroconverters ($n = 59$) were tested for HCV RNA at five different time points: the last visit before seroconversion ($t = -1$), the first visit after seroconversion ($t = 1$), six months after ($t = 2$) and one year after ($t = 3$) seroconversion, and the last visit prior to November 2005 ($t = 4$). If HCV RNA was present, part of the NS5B region was amplified and sequenced. Additional phylogenetic analysis and cloning was performed to establish HCV reinfection and superinfection.**

***Results*: Multiple HCV infections were detected in 23/59 (39%) seroconverters; 7 had HCV reinfections, 14 were superinfected, and 2 had reinfection followed by superinfection. At the moment of HCV reinfection, 7/9 seroconverters were HIV-negative: persistent HCV reinfection developed in both HIV-positive cases but also in 4/7 HIV-negative cases. In total, we identified 93 different HCV infections, varying from 1 to 4 infections per seroconverter. Multiple HCV infections were observed in 10/24 seroconverters with spontaneous HCV clearance (11 reinfections, 3 superinfections) and in 13/35 seroconverters without viral clearance (20 superinfections).**

***Conclusions*: HCV reinfection and superinfection are common among actively injecting drug users. This might further complicate the development of an effective HCV vaccine.**

***Keywords*: HCV; Reinfection; Superinfection; Epidemiology; Phylogeny; Injecting drug use; Immune protection**

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Abbreviations: ACS, Amsterdam cohort studies; DU, drug users; HCV, hepatitis C virus; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; PY, person years.

1. Introduction

Injecting drug users (DU) are at high risk for hepatitis C virus (HCV) infection through the shared use of needles and injection equipment. The reported HCV seroprevalence among injecting DU ranges from 30 to 90% in Europe, North-America, and Australia [1–3]. Over decades, persistent HCV viremia can cause chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma

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[4]. Spontaneous resolution of the virus occurs in 15–40% of those infected [5]. However, both HCV reinfection and HCV superinfection have been documented among individuals with ongoing risk behaviour [6,7], suggesting that neither viral clearance nor ongoing HCV infection consistently protect against new HCV infection. Evidence for partial protective immunity against HCV infection is derived largely from chimpanzee studies. Chimpanzees that previously cleared HCV and were rechallenged with homologous HCV strains, generally showed lower levels of HCV viremia and self-limited infection [8,9]. Whether humans previously infected with HCV gain protection against a second HCV infection remains controversial [6,10–12].

The incidence rate ratio of naïve HCV infection versus HCV reinfection in a high-risk population, is an indirect but frequently used tool to gain insight into the presence or absence of partial HCV immunity [6,10–12]. Clearly, if HCV infection occurs at a lower rate in previously exposed individuals compared to naïve individuals it is reasonable to assume some level of immunity is being induced from past exposure to HCV. Alternatively, if HCV reinfection occurs at similar or higher rates, this means that at least some individuals despite having the immunological capacity to clear initial HCV infection, fail to elicit an immune response that provides sufficient protection against HCV reinfection [12]. Insight into HCV protective immunity after previous exposure contributes to vaccine development and also provides health guidance for preventive strategies in populations at risk. Therefore, we studied the occurrence of HCV reinfection and superinfection in a well-defined cohort of injecting DU from the moment of HCV seroconversion until the end of follow-up. This study uniquely combines longitudinal data from prospectively identified HCV seroconverters with an epidemiologic and phylogenetic approach.

2. Materials and methods

2.1. Study population and sample selection

This study comprises all DU participants ($n = 59$) who seroconverted for HCV during follow-up in the Amsterdam Cohort Studies (ACS) between December 1985 and November 2005 [13]. The ACS is an open, prospective cohort study of both injecting and non-injecting DU, initiated in December 1985 [14]. Recruitment is still ongoing and takes place via local methadone posts, sexually transmitted diseases clinics, and by word of mouth. At ACS visits every 4–6 months, participants complete a standardised questionnaire about personal health, risk behaviour and socio-economic situation; blood is drawn for HIV-testing and storage at −80 °C. The moment of HCV seroconversion was calculated as the midpoint between the last HCV-seronegative visit and the first HCV-seropositive visit.

For each HCV seroconverter, samples taken at five time points were selected, if available: the last visit before HCV antibody seroconversion ($t = -1$), the first visit with a positive HCV antibody test ($t = 1$), visits approximately six months later ($t = 2$) and one year later ($t = 3$), and the last ACS visit prior to November 2005 ($t = 4$). A subset of 19 HCV seroconverters from the ACS identified in an earlier study had more than five samples available [15].

2.2. Definitions: reinfection, coinfection and superinfection

Spontaneous viral clearance was assumed if two or more consecutive visits, at least 4 months apart, showed HCV antibodies without detectable HCV RNA. During our study period, none of the participating DU had received HCV antiviral treatment.

HCV reinfection refers to a situation in which a primary HCV infection is spontaneously cleared prior to subsequent infection with a HCV strain of a homologous or heterologous lineage. All subjects who had two or more consecutive anti-HCV positive visits without detectable HCV RNA, at least 4 months apart, prior to an HCV RNA-positive visit were defined as reinfected. This includes (i) HCV seroconverters that had HCV RNA positive visits, separated by at least two RNA negative visits, but also (ii) HCV seroconverters that remained HCV RNA negative at their first anti-HCV positive test ($t = 1$), then returned at least one more consecutive RNA negative visit prior to an RNA-positive visit.

HCV coinfection and HCV superinfection both refer to HCV dual infections. Coinfection is defined as infection with two or more heterologous HCV strains simultaneously or within a window period too narrow for the first HCV infection to have resulted in detectable HCV antibodies. Superinfection is defined as a subsequent infection with a heterologous HCV strain in the presence of a previous HCV strain and the antibodies that it has generated. All changes in HCV strains over time without a demonstrated spontaneous clearance in between were considered as superinfections.

2.3. HCV RNA quantification

HCV serum RNA was quantified by an in-house real-time PCR assay based on the 5′-UTR region of the HCV genome [15]. In brief, RNA extraction was performed using the Boom method [16], in which 200 μl of serum and 15 μl of internal control were added to 900 μl of lysis buffer and 20 μl of size-fractioned coarse silica particles. RNA was eluted in a volume of 100 μl and transcribed to cDNA as detailed elsewhere [17]. Real-time PCR mixes (25 μl total volume) contained 12.5 μl of 2× LC480 probes master, 0.6 μM of forward and reverse primers (HCV47F: 5′-GTGAGGAACTACTGTCTTCACG-3′, HCV312R: 5′-ACTCGC AAGCACCCTATCAGG-3′), and 0.2 μM of labeled HCV and IC taqman probes (HCV-P129: 5′-FAM-CTCCCGGGAGAGCCATAGTG GTCTGCG-MGB-NFQ-3′, HCV-IC: 5′-VIC-ATGGCCACAGCGCC GCGGTGTTAGTGC-MGB-NFQ-3′). Real-time PCR was performed on a Roche LC480 using the following cycling conditions: 2 min at 50 °C and 10 min at 95 °C followed by 50 cycles of: 20 s at 95 °C, 20 s at 55 °C, and 1 min at 72 °C. Quantification of viral RNA was performed by using standard curves which were produced by linear regression analysis of dilution series of plasmid DNA.

2.4. Dominant HCV variant detection: the NS5B PCR

HCV RNA-positive samples for each DU were selected to document changes in the dominant HCV viral variant in individuals over time. From each sample, 3 μl cDNA was used as input for a nested multiplex PCR which amplifies 449 nucleotides of the HCV NS5B region (nt 8546–8994). Except for some modifications made to use cDNA instead of RNA as PCR input, conditions and primers of the NS5B PCR were those we described earlier [18].

2.5. Minority HCV variant detection: the cloning PCR

The cloning PCR is a single round PCR targeting the NS5B region (337 bp, nt 8279–8615). In brief, 25 μl of cDNA was added to 25 μl reaction mixture containing PCR II Buffer (Applied Biosystems), 200 μmol/L of each dATP, dCTP and dGTP, plus 400 μmol/L dUTP (Applied Biosystems), 0.1 μg/μL bovine serum albumin (Roche Diagnostics), 0.9 μM sense primer 5′-TATGAYACCCGCTGYTTTGACTC-3′, 0.9 μM antisense primer 5′-TAYCTVGTCATAGCCTCCGTGAA-3′, 0.5 U Uracil N Glycolase (Applied Biosystems) and 2.5 U Amplitaq Gold (Applied

Biosystems). The final $MgCl_2$ concentration was 2.5 mmol/L. PCR cycling conditions were 2 min at 50 °C and 10 min at 95 °C followed by 45 cycles of: 20 s at 95;°C, 20 s at 55 °C and 60 s at 72 °C, with a final incubation of 5 min at 72 °C. The cloning PCR product was cut from the agarose gel, resolved in 900 μl lysis buffer and purified using a shortened Boom-isolation protocol [16]. Purified amplicons were ligated into PCR II vector using the TOPO TA Cloning kit (Invitrogen). The plasmid was used to transform competent *Escherichia coli* cells, which were plated on LB agar plates containing ampicillin (100 μg/ml) and Xgal (5-bromo-4-chloro-3-indolyl-B-galactoside). Based on Xgal selection, 50–100 white clones, containing the PCR insert, were picked for each time point and grown overnight at 37 °C on LB agar plates. For each clone, the PCR insert was amplified using M13 primers, according to the TOPO TA Cloning kit protocol, and then sequenced.

2.6. Sequencing and phylogenetic analysis

PCR products were sequenced using previously described methods [18]. Viral genotype was determined after phylogenetic analysis of the NS5B sequences obtained (GenBank Accession Nos. FJ024088 to FJ024273) along with established GenBank reference sequences [19]. A HCV phylogenetic tree was constructed by the neighbour-joining method in Mega version 4.0 [20], using the Tamura–Nei substitution model with γ-distribution ($\alpha = 0.40$). Bootstrap values ($n = 1000$) were calculated to analyze the robustness of tree topology.

3. Results

3.1. Study population

In the ACS, 456/1259 (36.2%) participating DU were HCV-antibody negative at entry. Their median age was 31 years (IQR, 28–36 years), 70% was male and 11/456 (2.4%) were HIV-positive. Only 132/456 (28.9%) HCV-antibody negative DU had ever injected, of whom 64 (14.0%) in the last 6 months [13,21]. We identified 59 DU who seroconverted for HCV during ACS follow-up: 58% was male and their median age at HCV seroconversion was 29 years (IQR, 25–34 years). The median

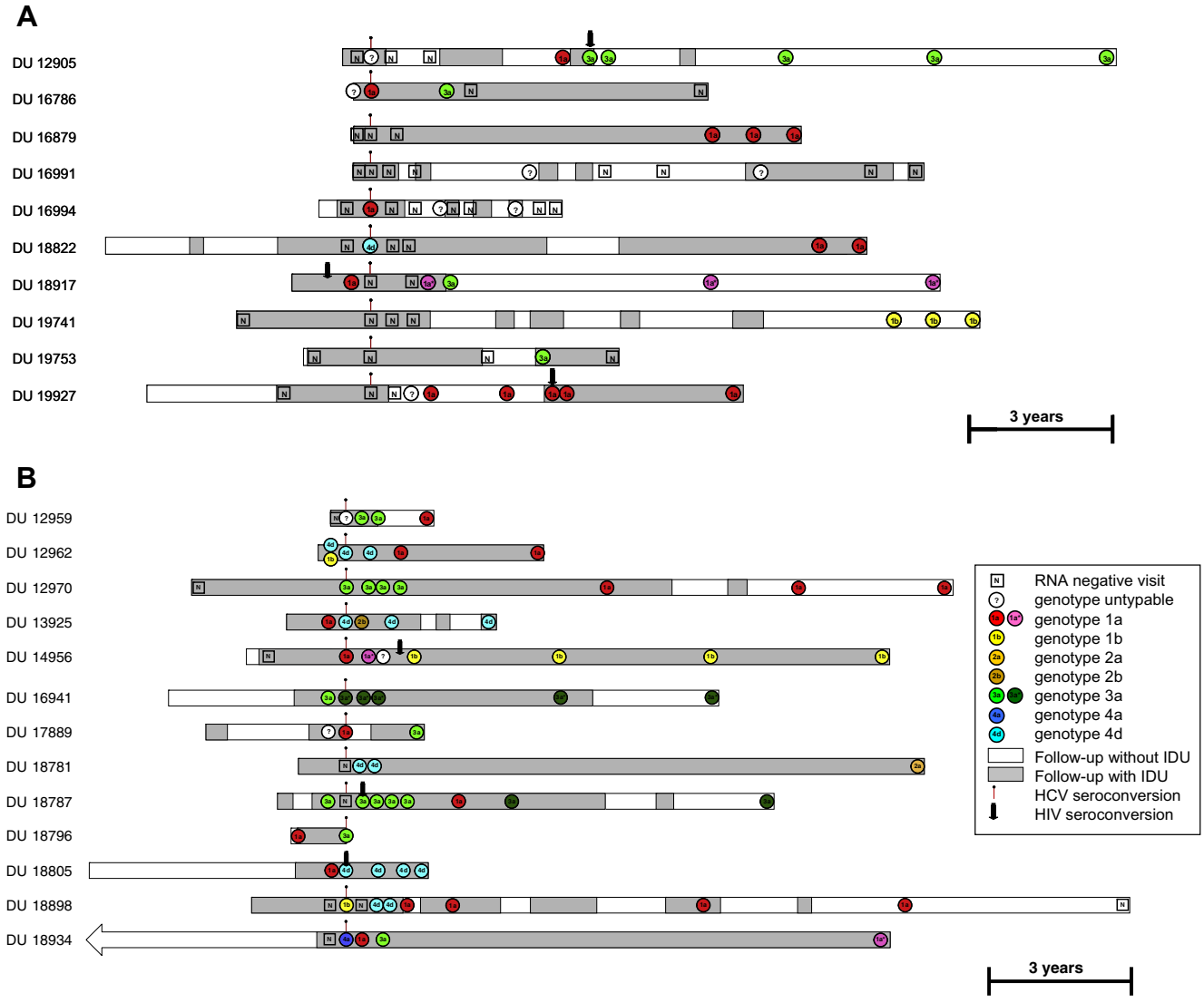

Fig. 1. (A) 10 HCV seroconverters with viral clearance and multiple HCV infections. (B) 13 HCV seroconverters without viral clearance and multiple HCV infections.

year of HCV seroconversion in the ACS was 1991 [IQR: 1989–1994], or 2.23 years (IQR: 0.93–6.49 years) after initiation of drug injection. The median ACS follow-up time since HCV seroconversion was 7.06 years [IQR, 2.81–12.1]. Only one HCV seroconverter denied ever injecting. HCV/HIV coinfection occurred in 13/59 (22%) HCV seroconverters; HCV infection preceded HIV infection in 6/13, 5 contracted both viruses during the same brief period, and two DU were HIV-positive before they acquired HCV.

3.2. Dominant HCV variant detection

The 59 HCV seroconverters were tested for HCV RNA on a total of 326 visits, varying from 2 to 10 visits per DU. At 211/326 (65%) of these visits, HCV RNA was detected using a real- time PCR based on the 5′-UTR; log HCV viral loads varied from 2 to 6.14 IU/ml. Amplification and sequencing of the HCV NS5B region succeeded in 170/177 (96%) samples with a viral load exceeding 1000 IU/ml and in 15/34 (44%) samples with a viral load below 1000 IU/ml. Sequencing analysis of HCV RNA-positive samples revealed that at least 23/59 (39%) HCV seroconverters had evidence for multiple HCV infections over time. According to our definitions, 7 HCV seroconverters had HCV reinfections, 13 were superinfected, 2 were reinfected and subsequently superinfected, and 1 was coinfected and subsequently superinfected. In total, 93 different HCV infections were identified: 59 initial infections, 11 reinfections, 22 superinfections and 1 coinfection. Genotyping and sequencing succeeded for 74/93 (80%) of HCV infections, finding a genotype distribution of 1a (47%), 3a (32%), 4d (8%), 1b (6%), 2a/b (6%) and 4a (1%).

3.3. HCV seroconverters with spontaneous HCV clearance

Spontaneous HCV viral clearance occurred in 24/59 (41%) HCV seroconverters including 2 DU who were HIV-positive at the moment of HCV seroconversion. Within this group of resolvers, 10/24 (42%) DU had evidence for multiple HCV infections over time; 7 DU had HCV reinfections, 2 DU had HCV reinfection followed by HCV superinfection, and 1 DU had HCV superinfection but spontaneously resolved both viral strains (Fig. 1a). In 2/9 DU with evidence for HCV reinfection we could confirm the presence of two distinct HCV strains over time (confirmed reinfection). In the other 7 DU, however, viral characterisation of either the primary ($n = 6$) or the reinfecting ($n = 1$) HCV strain failed due to rapid viral clearance of especially primary HCV viremia (probable reinfection).

In 6/9 (67%) seroconverters with confirmed or probable HCV reinfection, HCV reinfection persisted (Fig. 1a). It must be noted that 2/6 seroconverters were coinfected with HIV when they developed persistent HCV reinfection: DU 18917 already was HIV-positive before HCV seroconversion and DU 12905 developed persistent HCV re- and superinfection around HIV-seroconversion. The other four DU with persistent HCV reinfection were HIV-negative at the time; 1 DU (DU 19927) eventually acquired HIV years after the establishment of persistent HCV reinfection. None of the three HCV seroconverters that cleared a second or even third viremic episode were coinfected with HIV.

3.4. HCV seroconverters without spontaneous HCV clearance

Chronic HCV infection without evidence of previous viral clearance occurred in 35/59 (59%) HCV seroconverters. According to our definitions, 13/35 (37%) seroconverters had HCV superinfections over time (Fig. 1b); at least two clearly distinct HCV strains were detected in all 13 of them. From 6/13, we isolated three or even four distinct HCV viral strains. In DU 18934, the dominant viral strain switched from HCV genotype 4a to genotype 1a, subsequently to genotype 3a and eventually to a heterologous strain of genotype 1a (denoted as 1a*). DU 12962 had clear evidence of HCV coinfection. In the last visit predating HCV antibody presence, two distinct HCV strains were detected using the NS5B PCR, one was HCV subtype 1b and one was HCV subtype 4d. HCV subtype 4d became the dominant viral strain, however it seems to have submerged after superinfection with HCV genotype 1a. No particular pattern in the switch of HCV strains was observed; for example the dominant HCV strains switched from genotype 1a to 3a as often as from genotype 3a to 1a.

3.5. Sequencing and phylogenetic analysis

A phylogenetic tree was constructed of all 184 HCV sequences obtained from the 59 HCV seroconverters (Fig. 2). Minor genetic variations between HCV isolates obtained from one host, were classified as intrahost HCV evolution. However, 5 seroconverters had clear evidence of phylogenetically distinct HCV infections with strains of the same genotype (Fig. 2). HCV strains obtained from 2 other seroconverters were separated by bootstrap value >70, and genetic variation exceeded intrahost evolution observed in other participants (Fig. 2). Although reinfection with a very similar HCV strain for these 2 seroconverters is plausible, intrahost genetic evolution cannot be excluded.

3.6. Minority HCV variant detection

Two patients, DU 18898 and DU 18917, were selected for minority HCV variant detection. DU 18898 was selected because of two genotype switches

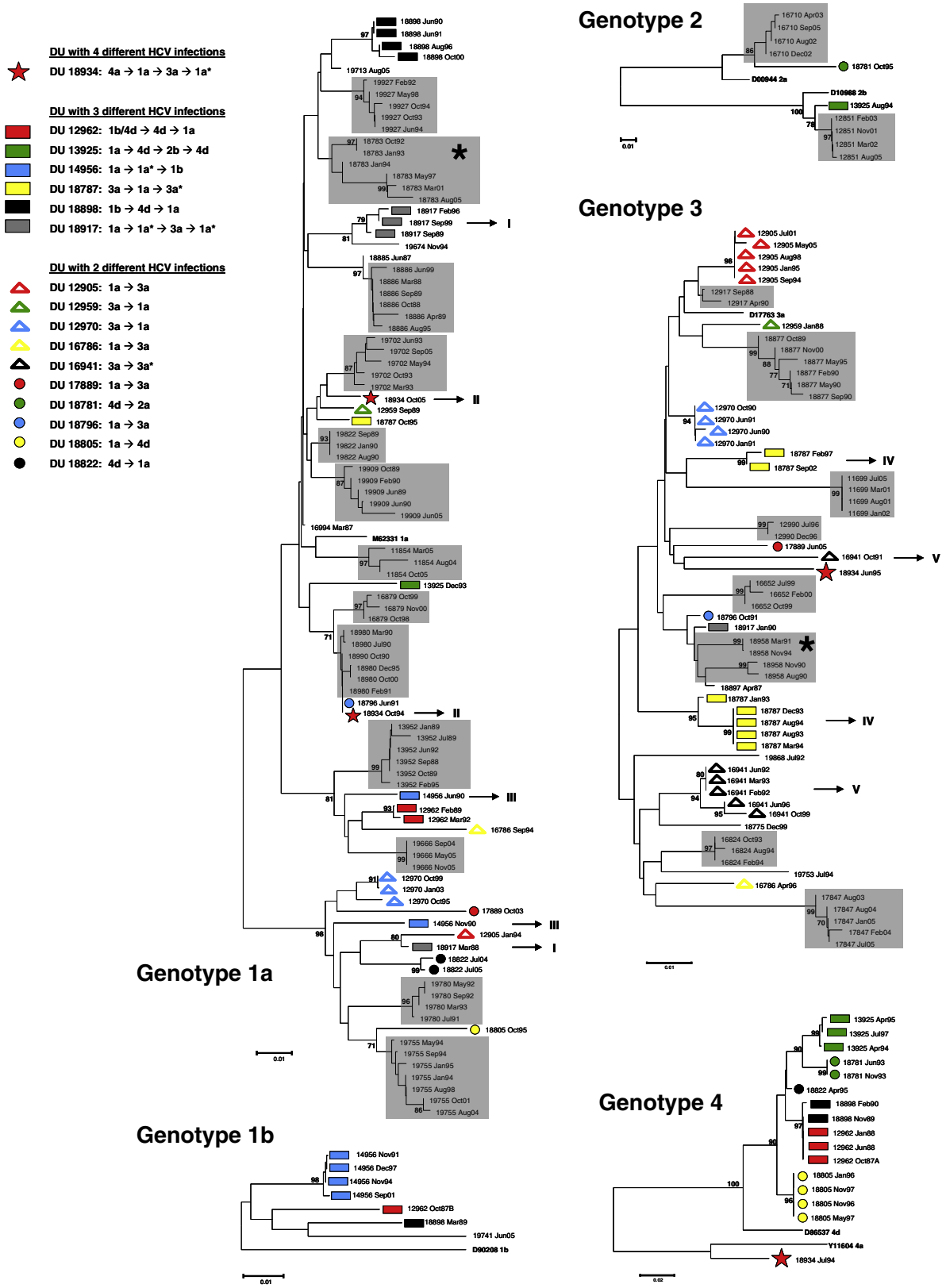

Fig. 2. HCV NS5B Phylogenetic tree comprising longitudinal samples from 59 drug users with documented HCV seroconversion. Coloured squares, triangles and circles represent HCV seroconverters with phylogenetically distinct HCV infections over time. Seroconverters with multiple infections of the same genotype are marked I–IV. Shaded boxes show intrahost HCV evolution among HCV seroconverters without evidence for HCV reinfection and superinfection. Shaded boxes with an asterisk represent seroconverters who might be superinfected with a very similar HCV strain.

(1b → 4d → 1a) within a period of 15 months. We selected, amplified, and sequenced 40–56 clones representing the four HCV RNA-positive visits during this interval (Fig. 1b). For each time point, all clones were quasispecies of one HCV strain consistent with the dominant genotype detected at that time point. Hence, no dual infections were detected (data not shown).

DU 18917 was selected because, after resolution of the primary HCV 1a infection, we observed reinfection with a different strain of HCV genotype 1a (1a*), subsequent superinfection with HCV 3a, and eventually a back-switch to the HCV 1a* strain, suggesting 3a/1a* superinfection (Fig. 1a). We selected, amplified, and sequenced 35 clones from the first and last HCV RNA sample. Except for variations in quasispecies, all clones belonged to the dominant subtype 1a and heterologous 1a*. For the three intermediate time points, 80–100 clones were sequenced. Evidence of superinfection was found only in the third intermediate sample; 98/100 clones were of subtype 1a* and 2/100 clones were subtype 3a (data not shown).

4. Discussion

In a large longitudinal cohort of DU in Amsterdam, we demonstrated that 39% of DU with a documented HCV seroconversion during follow-up, experience multiple HCV infections over time. Both HCV reinfection and superinfection were common in this high-risk population. Traditional HCV incidence calculations based on HCV antibody seroconversion, with the assumption that HCV seropositive individuals no longer are susceptible, therefore underestimate the true HCV incidence and do not properly reflect HCV transmission dynamics within a network of high-risk individuals [22].

As the occurrence of HCV reinfection after initial viral clearance has been previously described [6,10–12,23], there is no question that in humans HCV protective immunity is not even close to being complete. The existence of partial protective HCV immunity in humans, however, remains controversial. Previous studies among anti-HCV positive DU showed that HCV infection occurred at lower rate in previously exposed individuals than in naïve individuals [11,12,24]. Despite reinfection rates of 3–12%, this suggests that some level of immunity is being induced from past exposure. However, these studies were conducted in DU with prevalent HCV infection; the period of high-risk behaviour which led to anti-HCV seroconversion most likely preceded the study period by several years. As a result, previously HCV-infected DU were generally older (median age 41–47 years) and had less active drug use compared to HCV-uninfected DU [24]. In fact, 35–58% of participants in those studies had quit injecting before the actual start of the study period, being therefore at minimal risk of HCV reinfection. In contrast, studies performed in HCV seroconverter cohorts of young (<30 years) actively injecting DU implied absence of partial HCV protective immunity based on the fact that the incidence of HCV reinfection in these cohorts was similar [6] or even higher [10] than the incidence of initial infection, causing 46–50% of DU who previously cleared the virus to experience a second viremic episode.

The data in our study are concordant with those of other seroconverter cohorts of young actively injecting DU [6,10]; 42% of previously exposed DU experienced HCV reinfection. Also in our cohort, preliminary incidence calculations suggest that the incidence of HCV reinfection were at least similar to the incidence of naïve HCV infection. The incidence of HCV reinfection declined from 20.4/100 PY in the period 1985–1995 to 4.17/100 PY in the period 1995–2005, whereas the incidence of initial HCV infection in our cohort dropped from 27.5/100 PY in the late 1980s to approximately 2.0/100 PY in recent years [13]. Based on this incidence rate ratio it is tempting to imply that partial HCV protective immunity does not exist, but is this justified? As HCV reinfection might be associated with lower peak viremia and higher rates of spontaneous viral clearance, the real data that address this question are those regarding the outcome and nature of HCV reinfection. In contrast to long-lasting antibody seroconversion, HCV reinfection requires detection of new HCV viremia [24]. The probability of detecting transient HCV reinfection strongly depends on the frequency and interval of RNA testing [24]. In our study design the relatively long RNA testing intervals will support detection of all persistent HCV reinfections, but short episodes of resolved viremia are easily missed. Indeed 14/24 (58%) seroconverters resolved initial HCV viremia but lacked evidence for HCV reinfection, implying either rapid spontaneous viral clearance, protection against HCV reinfection or cessation of HCV risk behaviour. Of them, 7/14 (50%) were at minimal risk for HCV reinfection as they quit injection within 6 months after HCV seroconversion, the remaining 7 DU had no detectable HCV viremia despite ongoing risk behaviour. Additionally, 3/9 seroconverters with documented HCV reinfection resolved a second or even third viremic episode. Besides infrequent sampling, data on persistence of HCV reinfection are often skewed by HIV/HCV coinfection. HIV-coinfection is associated with increased rates of HCV persistence (up to 95%); lack of robust CD4+ T cell responses may contribute to the failure of early viral control of HCV [25,26]. HCV reinfection persisted in the two DU coinfected with HIV, but also in 4/7 DU without HIV.

Our study has several limitations. Despite our clear-cut definitions of HCV reinfection, coinfection and superinfection, the unpredictable course of HCV infection makes it difficult to distinguish them as such. Mosley et al. [27] showed that intercalating HCV RNA

positivity among those who eventually clear infection and intercalating HCV RNA negativity among those who eventually develop persistent infection, are common especially during the initial phase of HCV infection [27]. It cannot be excluded that in our study DU with 'probable reinfections' actually have fluctuating levels of low viremia, which would have led us to overestimate the rate of HCV reinfection. To minimise the risk of such misclassification, we defined viral clearance as two consecutive HCV RNA-negative visits at least 4 months apart. Underestimation of the rate of HCV reinfection was also very likely for three reasons. First, HCV reinfection in humans and chimpanzees has been associated with short episodes of low viremia [9,12], therefore reinfections might have been missed as a result of too low frequency of sampling and RNA levels below detection limit. Second, in the context of a steady injection partner, reinfection with a HCV strain almost identical to the initial strain, cannot be excluded. Third, superinfection requires detection of at least two divergent viral strains at the same time point, and our method did not exclude the possibility of rapid viral clearance of the initial strain followed by reinfection with a second strain. Although the latter would explain the difficulties we experienced with detecting minority variants in DU with superinfection, it seems more likely that the high viral turnover of HCV causes even the smallest fitness differences between two competing strains to result in rapid disappearance of the less fit strain over time [28]. Other laboratory methods involving strain-specific primers are currently being developed to specifically amplify minority variants.

In conclusion, both HCV reinfection and superinfection are common among actively injecting DU. At least some individuals despite having the immunological capacity to clear initial infection, fail to elicit an immune response that provides sufficient protection against HCV reinfection. Partial protective immunity, however, might result in lower peak viremia, increased rates of spontaneous viral clearance following reinfection, or protection against strains of the same HCV subtypes. Nevertheless, the results presented in this study further complicates vaccine development. Therefore, HCV harm reduction will remain dependent on precautionary measures preventing the further spread of HCV, and treatment of those who are chronically infected.

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