Molecular epidemiology of hepatitis C virus
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CHAPTER 2.1

Diversity and origin of hepatitis C virus infection among unpaid blood donors in the Netherlands

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Abstract

**Background:** To improve transfusion policy and to increase understanding of the spread of hepatitis C virus (HCV) in the general population, HCV infections among voluntary Dutch blood donors were examined using molecular epidemiological techniques.

**Study design and methods:** During six years, 1997 through 2002, confirmed anti-HCV-positive donors were interviewed on HCV-associated risk behavior using a standardized questionnaire. Additionally, HCV isolates were genotyped, partially sequenced, and compared to sequences obtained from Dutch injecting drug users (IDUs).

**Results:** HCV prevalence and incidence rates among Dutch donors were extremely low; the residual risk of transmitting HCV was calculated to be 1 in 30 million donations. Former IDUs (21%), transfusion recipients (30%) and immigrants (>12%) were identified as major HCV risk groups. Cryptogenic transmission caused 18% of infections among new donors and all infections among repeat donors. Compared to IDUs, genotype distribution among donors was highly diverse; major subtypes were 3a (27%), 1a (24%), 1b (24%), 2a/b (10%), and 4 (9%). Half of the donors were infected with IDU-related subtypes 1a and 3a, whereas subtype 1b mainly spread via blood transfusion and various other nosocomial modes of transmission in the past. HCV infections acquired in endemic countries could be clearly identified based on genotype.

**Conclusion:** Different modes of transmission are linked to infections with certain HCV subtypes, suggesting separate HCV epidemics, but spillover between different risk groups underlines the value of molecular epidemiological techniques to gain insight into the origin and dynamics of HCV infections on a population level.
Introduction

Transfusion-transmissible pathogens such as the hepatitis C virus (HCV) remain of major concern for the safety of blood supplies. In industrialized countries, introduction of increasingly sensitive and new laboratory tests have greatly improved blood safety over the last decades, as have indirect strategies such as no payment, donor education, call-back procedures and history-based donor selection [1,2]. Currently, residual contamination risk for HCV in the USA is estimated to be 1 per 800,000 donations for new donors and 1 per 2,000,000 donations for repeat donors [3].

HCV infection persists in 60 to 85% of persons infected and can, over decades, lead to serious liver-related illness and death [4,5]. Sharing of injection equipment among injecting drug users (IDUs) accounts for most HCV infections in industrialized countries [6]. Until the introduction of diagnostic screening in 1991, HCV could also be transmitted through blood, blood products, haemodialysis and organ transplantation [7]. In developing countries, unsafe injection practices and non-sterile medical procedures may still account for significant HCV transmission and serve as a bridge to the general population [8]; in developed countries, however, such nosocomial transmission is assumed to be restricted to incidental events [9,10]. In the Netherlands, prevalence rates are known for high-risk groups like IDUs (30%-90%) [11,12], persons with haemophilia (44%) [13], and dialysis patients (3.5%) [14], but little is known about seroprevalence and mode of transmission in the general population. Recent estimations suggest that HCV is carried by 0.1% of the Dutch population, of whom the majority (50%-70%) does not know that they are infected [12,15]. This lack of awareness clearly affects blood safety. Donor selection methods seem to be a cost-effective way to reduce the risk of transfusion-related HCV transmission, but they depend on donor awareness, recallability, and reliability of reporting past risk behavior [1,2].

The large genetic variability of the HCV genome has led to a proposed consensus of six genotypes and numerous closely related subtypes [16]. Genotype distribution differs by geographic region and by year and mode of transmission [17,18]. For HCV as well as other viruses, molecular epidemiology has proven a useful tool to identify risk groups and to distinguish different routes of transmission [19-21]. In western-Europe, HCV subtypes 1a and 3a predominate among IDU [22,23], whereas subtypes 1b and genotype 2 are mainly associated with, especially in older patients, contaminated blood transfusions and other types of nosocomial transmission [14,24-27].

To improve transfusion policy, it is essential to increase the understanding of HCV dispersal in the general population and to elucidate risk profiles of subjects still unaware of their positive HCV status. We therefore studied risk behavior of HCV-positive donors in the Netherlands during 1997-2002. To obtain more detailed information on the origin of HCV infection among Dutch donors and to investigate whether endemic HCV donor strains exist
in the Netherlands, HCV donor isolates were genotyped, sequenced and compared to HCV strains circulating among IDU.

**Materials and methods**

**Study population**

Participants (n=99) included all new and repeat blood and plasma donors in the Netherlands who tested HCV-positive during the 6-year period 1997 through 2002. Repeat donors were defined as those already registered in the Dutch blood banks who had at least one HCV-negative donation after HCV diagnostic screening was introduced in 1991. New donors were those who had either never donated in the Netherlands (candidate donors) or whose last HCV-negative donation pre-dated 1991 (irregular donors). For all participants, history-based donor selection had failed; none had been kept from donating based on a preceding questionnaire dealing with past risk behavior.

**Routine HCV screening in the Netherlands**

Donor plasma samples were tested with the screening and confirmation algorithm prescribed at the time of sampling by national guidelines concerning detection and notification of donors and recipients of HCV-infected blood. The college of blood transfusion of the Dutch Red Cross officially establishes these guidelines. During the period 1997 through 1999, routine HCV screening relied on the detection of HCV-antibodies only (Abbott PRISM, Abbott Park, IL); HCV-seropositive samples were confirmed with Immunoblot assays and polymerase chain reaction (PCR). Since 1999, in addition to HCV serologic screening, nucleic acid testing (NAT) minipool screening has been performed on all samples [28].

**HCV prevalence, incidence, and residual risk**

HCV prevalence among donors was calculated by dividing the number of confirmed HCV-positive new donors by the total number of new donors in that period. HCV incidence rate is the number of repeat donors that seroconverted during the study period divided by the number of person-years, that is, the time between the first and last donation of each donor during the study period. The residual risk of transfusion-transmitted HCV infection was estimated according to the incidence-rate/window-period model as described by Schreiber and co-workers [7]. Window periods used for HCV serologic screening (66 days) and HCV NAT screening (10 days) were those reported by Busch [29].
**Epidemiological data**

All new and repeat donors who tested HCV-positive were invited to the blood bank for medical counselling and repeat testing to exclude any possible identification errors. During medical counselling, donors were asked to complete a survey to clarify the possible route of HCV transmission. A questionnaire concordant to that of Orton and colleagues [30] was used, elaborately dealing with known and potential risk factors for acute and/or lifetime HCV transmission. Supplementary to the guidelines for hepatitis surveillance and case management (CDC, January 2005), additional questions regarding the donor’s demographic characteristics, health, sexual habits, travel behavior and history of other infectious diseases were included. No information on dental work/oral surgery and incarceration was obtained. Questionnaires were administered in a face-to-face interview, conducted by a transfusion physician. Based on the questionnaire the most plausible route of transmission was determined for each donor. Transmission routes were classified into five hierarchical categories: 1) injection drug use, 2) blood transfusion received before 1991, 3) endemic exposure (i.e., individuals born in countries with a HCV seroprevalence exceeding 2.0%)\(^{15}\), 4) parenteral and/or invasive exposure other than drug use or transfusion (needle-stick injuries, unhygienic injections, tattoos, piercing, acupuncture, surgery, endoscopy) and 5) cryptogenic exposure (e.g., nosocomial, occupational, sexual, intrafamilial). Donors reporting more than one possible mode of transmission were classified into the risk category of the highest hierarchical order. For donors who reported no risks (n=6) cryptogenic exposure was assumed.

**HCV RNA isolation and amplification**

Index samples from HCV RNA-positive new donors and the seroconversion samples of repeat donors were collected retrospectively. RNA isolation was performed on 100 µL of plasma with the TriPure method (Roche Diagnostics, Almere, the Netherlands) based on extraction once with phenol and guanidium thiocyanate solution, followed by chloroform extraction and an ethanol precipitation of the water phase. The pellet was washed and dissolved in 50 µL Tris-HCL buffer, pH 8.0. Samples had been stored at -80°C. Part of the HCV NS5B region was amplified with a nested multiplex reverse transcription (RT)-PCR method devised to amplify HCV RNA of genotypes 1-4 [22]. Primers were those described by Van de Laar and associates [11]. To extend the specificity of the NS5B PCR to a greater variety of strains, additional primer sets for genotype 5 and subtype 3k were designed (see Table 1). Strains of genotype 6 were detected with a nested multiplex RT-PCR based on the HCV core region [11].
Table 1: Primer sets targeting the NS5B region of HCV genotypes 5 and 3k*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Nucleotide position†</th>
<th>Specific for genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-OS</td>
<td>TTCACCACCTAGTATGGGCAA</td>
<td>8113-8132</td>
<td>5</td>
</tr>
<tr>
<td>5-OA1</td>
<td>CAAGCTTTGGAGCGGCACTA</td>
<td>8718-8737</td>
<td>5</td>
</tr>
<tr>
<td>5-OA2</td>
<td>CTCGGAGAAGGTATGAATGGA</td>
<td>8681-8699</td>
<td>5</td>
</tr>
<tr>
<td>5-IS</td>
<td>CCATGACGTGCTACATTAAGG</td>
<td>8135-8175</td>
<td>5</td>
</tr>
<tr>
<td>5-IA</td>
<td>TGAAAGCGGCTAAAGTCATCAG</td>
<td>8660-8679</td>
<td>5</td>
</tr>
<tr>
<td>3k-OS</td>
<td>AAAGCAAAAGGCAGGCACCCA</td>
<td>8152-8172</td>
<td>3k</td>
</tr>
<tr>
<td>F3a (OA)‡</td>
<td>TCTACTGGAGAGTAACTGTGGA</td>
<td>8681-8703</td>
<td>3k</td>
</tr>
<tr>
<td>3k-IS</td>
<td>TTGTCTGGCGAGACGACCTTGGT</td>
<td>8201-8222</td>
<td>3k</td>
</tr>
<tr>
<td>3k-IA</td>
<td>CCATGGAGTCGGTGCTTCAATGATGG</td>
<td>8642-8663</td>
<td>3k</td>
</tr>
</tbody>
</table>

*All primers are expected to show some degree of cross-hybridization to targets of other genotypes. A = antisense, I = Inner PCR, O = Outer PCR, S = Sense
† Numbered as in Choo et al. [47]
‡ Primer described by van Asten et al. [22] specific for genotype 3a

Genotyping and phylogenetic analysis

NS5B PCR products were ethanol precipitated. Sense and antisense strands were separately cycle-sequenced with a cycle sequencing system (BigDye Terminator system, version 1.1, Perkin Elmer, Monza, Italy). Sequence products were purified with spin kits (DyeEx, Qiagen, Venlo, the Netherlands) and analyzed on an automated sequencer (ABI-310, Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). Sequence alignment of the 420-bp NS5B fragment and translation into its 140 amino acids was performed with computer software packages (BioEdit [32] and GeneDoc [available from http://www.psc.edu]. Mega version 3.1; available from http://www.megasoftware.net) was used to construct a phylogenetic tree through its amino acid model, with Poisson correction. Bootstrap values (n=1000) were calculated to analyze the stability of tree topology. Viral genotype was identified after phylogenetic analysis of the NS5B sequences obtained (GenBank Accession Numbers DQ238625 to DQ238706) along with reference sequences of 18 confirmed HCV subtypes [16]. If subtyping failed due to the absence of a confirmed reference sequence, the preceding 237 bp of the amplified NS5B fragment were determined with a nested RT-PCR, as described by Cochrane and co-workers [23]. Subsequent subtyping was done using the NS5B reference sequences of 58 provisionally assigned HCV subtypes described by Simmonds and colleagues [16].

IDU database

Individuals who pass history-based donor selection were assumed to represent a very low to no-risk group. Their HCV sequences were compared to sequences in our IDU database. IDU were participants of the Amsterdam Cohorts Studies among drug users or visitors of the various drug health care institutions in Amsterdam [11]. HCV RNA-positive blood donors were matched to HCV RNA-positive IDUs, such that the distribution of birth year and sex were similar for both groups. IDUs were sampled between 1985 and 2004.
Statistical analysis

Differences in age and gender between individuals that were interviewed and those not interviewed, as well as differences in HCV prevalence among new donors over time, were tested by the chi-square-test. Differences in genotype distribution between donors and IDUs were tested by the Fisher-Exact test; genotypes were categorized into 6 subtypes: 1a, 1b, 2a/b, 3a, 4d and other genotypes. Two software packages (SPSS, SPSS Inc., Chicago, IL; and S-PLUS, Insightful Corp., Seattle, WA) were used.

Results

HCV prevalence, incidence and residual risk

From 1997 through 2002, 370,517 new donors registered at the Dutch blood banks, whereas repeat donors accounted for a total follow-up time of approximately 3,648,000 person years (Sanquin, Annual reports 1997-2002). In total, 99 donors were confirmed HCV positive in routine diagnostic screening: 94 new donors and 5 repeat donors. HCV prevalence significantly declined over time from 32.8 (1997-1999) to 18.4 (2000-2002) per 100,000 new donors (Table 2). HCV incidence rates did not change and remained low, approximately 0.14 per 100,000 repeat donor person-years. Owing to the introduction of HCV NAT screening in 1999, however, residual risk decreased almost 10-fold from 1:3,500,000 donations in 1997 through 1999 to 1:31,500,000 donations in 2000 through 2002 (Table 2).

Epidemiological data - new donors

Of 94 HCV-seropositive new donors, 73 (78%) returned to the blood bank for medical counselling and interviews regarding risk behavior. This high participation rate [25,30] and the fact that respondents were similar to non-respondents in median age and sex (Table 3) suggests that epidemiological data truly reflect the HCV-positive Dutch donor population. As to reported risk behavior, over 50% of respondents had either injected drugs or had received blood products before 1991 (Table 3). Remarkably, 48 of 73 (66%) respondents had lived outside the Netherlands for a period longer than 1 year; 41 of 73 (56%) lived abroad for more than 5 years (data not shown). Moreover, 18 new donors had donated blood before: 13 in foreign countries and 5 in the Netherlands (irregular donors). Risk behavior varied from injecting drug use (4x), blood transfusion before 1991 (6x), endemic exposure (1x), parenteral and/or invasive exposure excluding drug use and transfusion (4x) and cryptogenic exposure (3x). Most likely, at least 13 out of these 18 subjects donated HCV-contaminated blood at least once. All last donations predated 1991 (data not shown).
## Table 2: HCV prevalence, incidence rate and residual risk among Dutch donors

<table>
<thead>
<tr>
<th>Period</th>
<th>New Donors</th>
<th>Repeat Donors</th>
<th>Total number of new donors</th>
<th>Prevalance per 100.000 new donations (95% CI)</th>
<th>Number of HCV seroconversions*/total number of PY (95% CI)</th>
<th>Incidence per 100.000 repeat donor PY (95% CI)</th>
<th>Window period in days</th>
<th>Residual risk per 1.000.000 repeat donor PY (1:repeat donations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997-1999</td>
<td>56</td>
<td>3</td>
<td>179.817</td>
<td>32.8 (24.4-41.2)</td>
<td>3/1.919.000 (0.16 (0.12-0.21))</td>
<td>0.16 (0.12-0.21)</td>
<td>66</td>
<td>0.28 (1:3.500.000)</td>
</tr>
<tr>
<td>2000-2002</td>
<td>33</td>
<td>2</td>
<td>190.700</td>
<td>18.4 (12.3-24.4)</td>
<td>2/1.729.000 (0.12 (0.09-0.17))</td>
<td>0.12 (0.09-0.17)</td>
<td>10</td>
<td>0.03 (1:31.5000.000)</td>
</tr>
<tr>
<td>1997-2002</td>
<td>89</td>
<td>5</td>
<td>371.517</td>
<td>25.3 (20.2-30.5)</td>
<td>5/3.648.000 (0.14 (0.11-0.18))</td>
<td>0.14 (0.11-0.18)</td>
<td>66</td>
<td>0.28 (1:3.5000.000)</td>
</tr>
</tbody>
</table>

* Due to a stricter definition, the number of HCV positive repeat donors in this study (n=5) differs from the number noted in the annual reports of the blood bank (n=10). We categorized irregular donors whose last donation was from before the introduction of diagnostic screening in 1991 as new donors (prevalent cases), whereas they were classified as repeat donors (incident cases) in the annual reports. Classification of irregular donors as incident cases would have resulted in an HCV incidence rate of 0.27 (95% CI), 0.23-0.32) per 100,000 repeat PY [46].

## Table 3: General characteristics and risk behavior of HCV-positive new donors

<table>
<thead>
<tr>
<th>HCV-seropositive new donors</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non respondents (n=21)</td>
<td>Percent male, median age (years) 59%, 46 yrs (IQR 38-50)</td>
</tr>
<tr>
<td>Respondents (n=73)</td>
<td>Percent male, median age (years) 58%, 41 yrs (IQR 34-48)</td>
</tr>
<tr>
<td>Risk category*</td>
<td></td>
</tr>
<tr>
<td>1. Injecting drug use</td>
<td>15 (21)</td>
</tr>
<tr>
<td>2. Blood transfusion before 1991</td>
<td>22 (30)</td>
</tr>
<tr>
<td>3. Born in endemic countries</td>
<td>9 (12)</td>
</tr>
<tr>
<td>4. Other parenteral transmission</td>
<td>14 (19)</td>
</tr>
<tr>
<td>5. Cryptogenic transmission</td>
<td>13 (18)</td>
</tr>
</tbody>
</table>

* Risk categories are specified in the text (see Materials and methods).
Epidemiological data - repeat donors

Only five repeat donors seroconverted for HCV during the study period; they all returned to the blood bank for medical consultation and interviews regarding past risk behavior. All five were born in the Netherlands, four of five were female, and the median interdonation interval, the time between last HCV-negative and first HCV-positive donation, was 0.80 yrs (IQR 0.40-2.0) (Table 4). One repeat donor (Donor 2), a psychiatric nurse, had a relatively large interdonation interval of 3 years. She reported no needle-stick injuries but did report accidents involving biting and the spraying of blood. DON53, a 55-year old male, reported no risk behavior except for hospitalization in Belgium after a car crash. He received no blood products but, with severe cuts and bruising, he had stitches and was put on an intravenous drip. The remaining three women (Donors 56, 72, 93) initially appeared to lack any form of risk behavior. All three, however, reported a new heterosexual partner. On questioning, two of these partners (Partners 56 and 72) admitted to a history of injecting drug use. Both their corresponding donors (Donors 56 and 72) were infected with IDU-related genotype 3a. Blood of Partner 72 was investigated revealing that he and Donor 72 had 100 percent identical HCV nucleotide sequences. A history of shared injecting drug use seemed unlikely. Both Partner 56 and Partner 72 quit injecting (long) before they met Donors 56 and 72, respectively, and both donors genuinely denied being aware of the fact that their new partners were ex-IDUs. Sexual and/or household exposure, therefore, remained as the most plausible routes of HCV transmission.

Sequencing, genotyping and phylogenetic analysis

The samples of 86 of 99 HCV-positive donors were positive for the presence of HCV RNA. Of these, 4 were unavailable for study and in 1 sample, genotyping and sequencing failed for unknown reasons. Thus, 81 NS5B HCV sequences were obtained. Figure 1 represents a phylogenetic tree of 14 reference sequences and all 81 donor sequences. Donor sequences are shown with their assumed mode of transmission and allocated risk category. All donor strains belong to HCV genotypes 1 through 4; no strains of genotype 5 or 6 were observed. HCV isolates of Donor 80 and Donor 91 could only be subtyped by amplification and sequencing of a different part of the NS5B gene for which a complementary reference sequence was available; they were eventually subtyped 1g and 4k, respectively. HCV subtypes 3a (27%), 1a (24%), 1b (24%), 2a/b (10%), 4a (4%) and 4d (4%) were the most prevalent; the remaining six percent belonged to various other subtypes. Different modes of transmission were not randomly distributed among HCV subtypes. HCV subtypes 3a (50%) and 1a (31%) predominate among donors who reported injecting drug use or close contact with IDUs (Table 5). Donors infected with subtype 1b mainly reported a history of blood transfusion (44%) or other nosocomial modes of transmission (Figure 1). The appearance of foreign subtypes is associated with endemic exposure: subtype 1g from Sudan, subtype 4a from Egypt, subtype 4k from Rwanda, and subtypes 2e and 3k from Indonesia. The only
Table 4: General characteristics and risk behaviour of repeat donors who seroconverted for HCV

<table>
<thead>
<tr>
<th>Repeat donor</th>
<th>Donor Number</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Country of birth</th>
<th>Number of donations*</th>
<th>Seroconversion Year</th>
<th>IDI (yrs)</th>
<th>RNA</th>
<th>Geno</th>
<th>Risk behaviour (risk category)</th>
<th>Risk factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>F</td>
<td>35</td>
<td>NL</td>
<td>&gt;2†</td>
<td>1997</td>
<td>3</td>
<td>Y</td>
<td>1b</td>
<td>Occupational: health care (5)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>53</td>
<td>M</td>
<td>55</td>
<td>NL</td>
<td>77</td>
<td>1997</td>
<td>0.3</td>
<td>Y</td>
<td>NT</td>
<td>Nosocomial: hospitalization (5)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>56</td>
<td>F</td>
<td>42</td>
<td>NL</td>
<td>5</td>
<td>1997</td>
<td>0.9</td>
<td>Y</td>
<td>3a</td>
<td>Sexual/household: partner HCV-pos (5)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>F</td>
<td>56</td>
<td>NL</td>
<td>28</td>
<td>2002</td>
<td>0.5</td>
<td>Y</td>
<td>3a</td>
<td>Sexual/household: partner HCV-pos (5)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>93</td>
<td>F</td>
<td>40</td>
<td>NL</td>
<td>28</td>
<td>2002</td>
<td>0.8</td>
<td>N</td>
<td>-</td>
<td>Sexual/household: partner HCV-unk (5)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of donations prior to HCV seroconversion, † The exact number of donations before the last seronegative donation in 1994 could not be determined. IDI = interdonation interval; F = female; M = Male; NL = the Netherlands; Y = Yes; N = No; NT = not typable; unk = unknown

Table 5: HCV genotype distribution among Dutch blood and plasma donors according to mode of transmission

<table>
<thead>
<tr>
<th>Mode of transmission</th>
<th>Genotype</th>
<th>RNA negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1a</td>
<td>1b</td>
<td>2a/b</td>
</tr>
<tr>
<td>IDU-related †</td>
<td>5‡</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Transfusion</td>
<td>5‡</td>
<td>8‡</td>
<td>1</td>
</tr>
<tr>
<td>Endemic</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Other parenteral</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Nonparenteral</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>No data</td>
<td>5‡</td>
<td>2</td>
<td>5‡</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>20</td>
<td>8</td>
</tr>
</tbody>
</table>

* Other subtypes comprise HCV subtypes 1g, 2c, 2e, 3k and 4k
† IDU-related refers to donors that injected drugs in the past (n=15) as well as donors that contracted HCV from an IDU by sexual or occupational contact (n=6).
‡ For each genotype, the transmission category with the highest number of isolates
§ Actually 99 donors were HCV positive but four samples were not available for genotyping, and one sample could not be genotyped.
foreign strain not related to endemic transmission (subtype 2c) was isolated from an Italian woman who had acquired a tattoo under unhygienic conditions in Switzerland. Indeed, subtype 2c is highly prevalent in the Mediterranean area, especially in Italy. Remarkably, five of the eight subjects infected with genotypes 2a or 2b did not return to the blood bank and hence were not interviewed. The others reported transmission through tattooing in the United Kingdom (2b), hospitalization in Germany (2b), and a blood transfusion in the United Arab Emirates (2a).

The genotype distribution among blood donors is significantly different (p<0.001) from that of a matched population of IDUs (Figure 2a). Among donors, genotype distribution is more diverse; 12 percent of strains belong to subtypes absent among IDUs. Among donors subtypes 1a (25% vs. 51%) and 3a (28% vs. 38%) are less abundant than among IDUs, and subtype 1b (25% vs. 5%) is more abundant. Separate phylogenetic trees were made of IDU-related HCV subtypes 1a and 3a to investigate whether separate clustering would distinguish HCV strains from IDUs, donors who reported injecting drug use or close contact with IDUs (IDU-related risks), and donors who denied IDU-related risks (Figure 2b). Sequences of donors that denied IDU-related risks were phylogenetically interspersed with IDU sequences suggesting mixing of the virus between the different risk groups. For different routes of transmission, however, an intermediate level of clustering was observed within these subtrees. For subtype 3a, the upper part of the tree (Cluster I) is dominated by IDUs; including 6 out of 8 donors who reported IDU-related risks. In fact, Cluster I comprises only 2 donors that denied IDU-related risks. One of them reported no risk at all (Donor 30), but was infected with a strain identical to that of 2 IDUs (IDUs 21 and 95). In contrast, the lower part of the tree (Cluster II) is dominated by donors who denied IDU-related risks. For subtype 1a, the distinction between IDU and donors who denied IDU-related risks, is less pronounced. Nonetheless, 8 of 20 donor isolates were identical (Cluster III); Cluster III, however, comprises both donors that reported injecting drug use and donors that denied IDU-related risks. Cluster IV comprises 8 identical isolates from IDUs. Strikingly, one isolate (Donor 86) of a donor that denied injecting drug use but did report a blood transfusion, was also identical.

**Discussion**

HCV prevalence rates among blood and plasma donors in the Netherlands are among the lowest ever reported in the world and still declining. HCV incidence rates have stabilized over time, 0.14 per 100,000 donor years in the period 1997 through 2002, but were lower than, for example, in Spain (3.70) [33], the USA (1.89) [3], Germany (1.50) [34], Canada (1.35) [35], France (0.34) [36,37] and the UK (0.23) [38]. Despite unchanged incidence rates, the introduction of NAT-screening in 1999 has drastically decreased the residual risk
Figure 1: HCV phylogenetic tree (NSSB) of Dutch blood and plasma donors (1997-2002). Neighbour-joining tree based on amino acid model with Poission correction (amino-acid 2736-2875). Risk category and risk behaviour are listed opposite each sequence. Strains from donors lacking epidemiological data were labelled “no data”.

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of transmitting HCV through contaminated blood products. Based on data from the entire Dutch donor population, this risk is now 1 in approximately 31,500,000 repeat donations. Underestimation due to laboratory error, new or rare viral variants not being recognized by the assay, and the fact that estimated residual risk of new donors exceeds that of repeat donors cannot be entirely excluded [37,38]. In contrast, the incidence-rate/window-period model used to calculate the residual risk assumes infectivity of the virus throughout the window period, an assumption that, if invalid could result in overestimation [7].

Donor-selection methods were invented to minimize the risk of viral transmission, especially during the window-period of infection. Unfortunately, not all risky donors are filtered out [1,2]. Three major risk-profiles were identified: contaminated blood transfusions received in the past, a history of injecting drug use, and immigration and/or travelling. As in France [25], injecting drug use and contaminated blood transfusions accounted for more than 50 percent of HCV infections among new donors. To prevent possible contamination with prions causing variant Creutzfeld-Jacob Disease, recipients of blood after 1980 have been excluded from donation only since April 2005. A further decrease of the number of HCV-positive new donors passing donor-selection will be an indirect result of this measure. Current or former injecting drug use, however, has always been a donor exclusion criterion, yet 21 percent of HCV-positive new donors were former IDUs. Except for one donor who had injected drugs until one year before HCV screening, median time since last injection was 20 years (IQR, 13-26 years). Given this length of time and the alteration of lifestyle, lack of identification with the “drug addict” stereotype, and the absence of health complaints, former IDUs might be incapable of self-reference with regard to the risks they took [30,39]. Deliberately concealed HCV risk behavior, however, should not be ruled out. General education programs on HCV have increased the awareness of risks associated with former drug use, perhaps causing ex-IDUs to register as donors primarily to obtain free and anonymous HCV-testing [40].

The third risk profile was immigration and long-time residence abroad. Among HCV-positive new donors, 56 percent had lived in foreign countries for more than 5 years. Of these, 40 percent had lived in HCV-endemic countries and another 30 percent in countries with HCV prevalences exceeding 1 percent. Import of HCV from countries with intermediate to high HCV prevalences should not be underestimated, especially because migration to European countries continues to increase. Recent estimates suggest that first-generation immigrants are responsible for 56 percent of HCV infections in the Netherlands [12]. HCV prevalences in former Dutch colonies like Surinam (5.5%) and Indonesia (2.1%), as well as countries like Morocco (1.3%) and Turkey (1.5%) where large groups of immigrants originate, exceed those of the Netherlands by a factor 10 to 55 [15]. But also within the European Union, in countries like Italy and Greece, HCV high-endemic areas exist [26,41].
Figure 2: Comparing HCV strains from Dutch donors with HCV strains from a matched population of IDUs in the Netherlands. (A) HCV genotype distribution among Dutch IDUs and Dutch donors. (B) Neighbour joining phylogenetic trees of donors and IDUs of HCV subtypes 1a and 3a (NS5B region, amino acid 2736-2875, amino acid model with Poisson correction). The background of sequences from the IDU database are grey, sequences from donors are hatched grey (IDU-related transmission), outlined (non-IDU related transmission), or white (no data on transmission).
Although participants were stimulated to reflect upon past risk behavior by confronting them with a confirmed positive HCV test result, recall bias might have influenced the results. Indeed, 40 percent of HCV infections among new donors could not be explained by the three risk-profiles identified and were eventually classified as “other parenteral” or “cryptogenic”. For these donors, identification of the exact source of HCV infection was hampered by long acquisition times and the lack of HCV-negative donor controls [30].

Acquisition time was limited for HCV-positive repeat donors. Recent acquisition and hence the possibility of donation within the window-period of infection poses a serious threat to the blood supply. In the US, 35 to 40 percent of HCV-positive repeat donors admitted to recent injecting drug use or recent incarceration (a potential marker for injecting drug use) [30]. None of the five HCV seroconverting Dutch donors, however, admitted to the use of any drugs at all. HCV seroconverting Dutch as well as Italian donors [42] only reported cryptogenic exposure, risk behavior for which they cannot be excluded from donation. Heterosexual and/or household exposure was plausible for three out of five Dutch HCV-positive repeat donors. Although heterosexual HCV transmission is rare, in some countries sexual partners of HCV-positive subjects are excluded from blood donation. However, none of the three repeat donors that possibly contracted HCV through sex was aware of former risk behavior or the HCV status of their new sexual partner.

The HCV genotypic pattern among Dutch donors resembles the pattern of other western European countries in that HCV subtypes 1a, 1b and 3a account for 80 to 90 percent of total infections, whereas genotypes 2 and 4 each contribute 5 to 10 percent [6,25,26,43]. Donor populations in the United States (and Italy), however, typically show higher rates of HCV genotype 2 (up to 25%), and in the United States genotype 4 seems absent [24,30]. The genotypic pattern mainly depends on geographic origin, route of transmission, and year of infection [18]. The latter is clearly reflected within the three risk-profiles identified. In line with the recent introduction of genotype 4 into Europe by immigrants from Africa and the Middle East [22,44], the presence of rare HCV subtypes among Dutch donors was directly associated with immigration and travelling. With respect to the more prevalent genotypes, the majority of infections with subtype 1a and 3a were traced back to former injecting drug use, whereas subtype 1b was mainly contracted through blood transfusions and medical interventions [25,25,43]. The worldwide distribution of genotype 2, its wide genetic diversity within the Dutch population as well as the risk behavior reported suggest long-standing nosocomial transmission [14,26,41,45,46].

Although acquisition time might have skewed the observed genotype distribution, and natural virus evolution might have obscured the phylogenetic identification of transmission networks, the observed differences in epidemiology between the spread of subtypes 1a and 3a on one hand, and the spread of subtypes 1b and 2 on the other suggest two independently developing epidemics, one IDU-related and the other donor-related. The
introduction of donor screening in the early 1990s halted the expansion of transfusion-related strains (1b and 2) [6,27,43]. Indeed, history-based modelling shows that in many countries, transfusion-related strains initially had exponential growth followed by hampered growth, gradual decline, and finally replacement by strains linked to injecting drug use (1a and 3a) [20]. Our data, however, provide strong evidence that IDU-related subtypes 1a and 3a have spread beyond the boundaries of the IDU-scene via high-risk sexual behavior, occupational contact, and blood donations of former IDUs [46]. Incidental spillover from IDUs to the general population is confirmed by the observed levels of phylogenetic mixing between IDU- and donor-related HCV sequences within subtypes 1a and 3a (Figure 2) [46]. In contrast, introduction of new genotypes from low- to high-risk populations as a result of migration, can explode rapidly into new epidemics [11,17,22].

In conclusion, current donor-selection methods in the Netherlands appear highly effective for HCV. HCV prevalence, incidence, and residual risk among Dutch donors are extremely low. Among new donors, donor-selection incidentally misses HCV positive recipients of blood and blood products before 1991, former IDUs and first-generation immigrants. HCV-positive repeat donors, however, reported only low-risk cryptogenic modes of transmission which are impossible to detect by preliminary screening of risk-behavior. Although in the Netherlands different HCV subtypes have spread via different transmission networks, phylogenetic analysis proves that incidental spillover from high-risk groups to the general population does occur, mainly through cryptogenic modes of transmission.

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