Epidemiological trends of HIV-1 shown through phylogenetic trees

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Molecular epidemiology of HIV-1 among heterosexuals in the Netherlands
Multiple HIV-1 subtypes present amongst heterosexuals in Amsterdam 1988-1996: no evidence for spread of non-B subtypes

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HIV-1 subtype B predominates in Europe, where homosexual contact and injecting drug use account for the majority of HIV-1 infections. Recently, several epidemiological studies were published in which the presence of non-B subtypes in heterosexuals in Europe was described.¹³ We studied HIV-1 subtypes amongst asymptomatic heterosexual infected individuals in Amsterdam in 1988-1996, in particular to determine whether there was evidence for spread of non-B subtype viruses.

HIV-1 sequences were obtained from samples of heterosexuals (no other HIV risk factors besides heterosexual contact) stored at the Regional Laboratory in Amsterdam. Samples were taken as part of various HIV studies. First was HIV surveillance among pregnant women, which started in 1988 in city hospitals, midwife practices, abortion and infertility clinics in the Amsterdam region. In 1988-1996, 31 770 pregnant women [excluding injecting drug users (IDU) and blood product recipients] were tested for HIV-1; 55 women were HIV-1-positive (28 samples available).⁴ Second was HIV surveillance among sexually transmitted disease (STD) clinic visitors started in 1991 at the Amsterdam STD clinics. In 1991-1996, eight cross-sectional studies were performed with approximately 1000 participants each. A total of 7582 heterosexuals were tested for HIV; 41 were HIV-positive (30 samples).⁵ Third was alternative screening, which was carried out at an HIV test facility at the Municipal Health Service for those who requested an HIV test on their own initiative. A total of 9656 heterosexuals were tested in 1988-1996 of whom 63 were HIV-seropositive (48 samples). Finally, other studies included three HIV-infected participants in a cohort amongst heterosexuals at the STD clinic⁶ and eight heterosexually infected women participating in a European study on HIV infection in women (nine samples).

Demographic information, including age, gender, residence, country of origin and risk factors of sexual partners [bisexual men, IDU, blood products recipients, partner from HIV endemic area, and commercial sex workers (CSW)] was collected. The sequencing procedure has been previously described.⁷ Phylogenetic analysis of the V3 region (270 base pairs, accession numbers AF032134-AF032225) with subtype A-H consensus sequences was performed by using the MEGA program.⁸⁹

Overall, 49 520 heterosexuals were tested in HIV studies in Amsterdam in 1988-1996, of whom 170 positive for HIV-1. A total of 115 out of 170 serum samples were available and 90 samples were PCR-positive for HIV-1. To study the possibility of selection bias we compared the demographic characteristics of individuals with PCR-positive samples and those with PCR-negative samples, and also between subjects with PCR-positive samples and subjects with no samples available. No major demographic difference were found, except that subjects with PCR-negative samples were significantly younger and more
often had a partner originating from sub-Saharan Africa than individuals with PCR-positive samples.

Phylogenetic analysis of the nucleotide sequences of the 90 subjects revealed six HIV-1 subtypes: 54 subtype B, 20 subtype A, six subtype C, one subtype E, four subtype F, three subtype G, and two unclassified, HIV-1 subtype distribution did not differ significantly between men and women (Table 1). Subtype B predominated in four source studies but relatively more non-B infections were observed among pregnant women (P=0.04). The variables 'origin of subject' and 'origin of partners' strongly correlated with HIV subtype (P<0.0001). Sixty-seven per cent of individuals with a non-B subtype originated from sub-Saharan Africa and 70% had a partner from this area, whereas these percentages were 0 and 4%, respectively, among persons with subtype B. The variables 'drug use among partners' and 'visiting CSW' were also associated with subtype B. Men with subtype B visited CSW more often than men with a non-B infection (52 versus 9%). Thirty-one per cent of the subjects with subtype B reported a drug-using partner

Table 1. Characteristics of HIV-1 subtype in heterosexualls (n=90).

<table>
<thead>
<tr>
<th></th>
<th>Subtype B (n=54)</th>
<th>Non-B subtype (n=36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex [n (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>26 (48)</td>
<td>12 (33)</td>
</tr>
<tr>
<td>Female</td>
<td>28 (52)</td>
<td>24 (67)</td>
</tr>
<tr>
<td>Mean ± SD age (years)</td>
<td>31 ± 7.2</td>
<td>30 ± 8.1</td>
</tr>
<tr>
<td>Study population [n (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant women</td>
<td>8 (15)</td>
<td>13 (36)</td>
</tr>
<tr>
<td>Sexually transmitted disease clinic</td>
<td>17 (15)</td>
<td>6 (17)</td>
</tr>
<tr>
<td>Alternative screening</td>
<td>23 (43)</td>
<td>16 (44)</td>
</tr>
<tr>
<td>European women study</td>
<td>4 (7)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>HIV heterosexuals</td>
<td>2 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Years of sampling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1993-1996</td>
<td>33 (61)</td>
<td>27 (75)</td>
</tr>
</tbody>
</table>

compared with 4% of subjects in the non-B group. No significant changes were observed in the proportion B/non-B subtypes before 1993 versus 1993-1996 in the two surveillance studies.

To further study the possible spread of non-B viruses, subjects were divided in three categories: group 1, individuals originating from HIV endemic areas; group 2, individuals of European origin who reported one or more sexual partners from an HIV endemic area; group 3, individuals of European origin with no known sexual partners from HIV endemic areas. In group 1, 30 (60%) out of 50 individuals had a non-B infection. In group 2, this proportion was 56%, and in group 3 it was 0% (Table 2).

Multiple HIV-1 subtypes (A,B,C,E,F,G) were identified amongst heterosexually infected individuals in Amsterdam, in contrast to Dutch homosexual men and IDU, among whom
only subtype B viruses were found. Similar observations have been reported in other European studies where mainly non-B subtypes have been identified, as in this study, in subjects originating from HIV endemic areas and, less frequently, in European individuals with a history of sexual contact with individuals from these areas. An important result was that in 26 subjects originating from European countries, with no known sexual partners from HIV endemic areas, only subtype B viruses were found. Because our study includes a large group of heterosexuals, and given that non-B subtypes have been present in Europe for more than a decade, this observation suggests that no substantial spread of non-B subtypes beyond those with an epidemiological link with an HIV endemic region. The majority of viruses in our study group belonged to subtype B. Because the early epidemics of HIV-1 amongst homosexual men and IDU were associated with subtype B, there are far more subtype B viruses circulating in the Netherlands than non-B viruses. The chance of being exposed to B viruses for heterosexuals at risk is therefore larger than non-B viruses, which makes the predominance of B viruses amongst this heterosexual population explicable.

Table 2. Distribution of HIV-1 subtypes amongst heterosexuals in Amsterdam (n=90), according to origin of the subjects and their sexual partners.

<table>
<thead>
<tr>
<th>Subtype (n)</th>
<th>B</th>
<th>A</th>
<th>C</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>Unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: subject from HIV-endemic area</td>
<td>54</td>
<td>20</td>
<td>6</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>sub-Saharan Africa</td>
<td>18</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asia</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>South/Central America</td>
<td>16</td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Group 2: subject from Europe with partner(s) from HIV-endemic area</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3: subject from Europe with no sexual partner(s) from HIV-endemic area</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>HIV-risk partner(s) unknown</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IDU using partner(s) or partner(s) visits CSW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subject originating from other country</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Origin of subject unknown</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

1. 18A: Ghana (8) Senegal (1) Benin (1) Uganda (1) kenya (1) Guinea Bissau (1) Côte d'Ivoire (2) sub-Saharan Africa (3); 3C: Ethiopia (2) sub-Saharan Africa (1); 1F: Zaire; 1G: Liberia, 1 unknown: Ethiopia.
2. 4B: Thailand (1) India (1) Indonesia (1) China (1); 1E: Thailand (1).
3. 16B: Surinam/Dutch Antilles (9) Curacao (1) Dominican Republic (4) Argentina (1) South/Central America (1); 2A: Zambia (1) sub-Saharan Africa (1); 2C: sub-Saharan Africa (1) Malaysia (1); 1 unknown: sub-Saharan Africa.
4. 26B: The Netherlands (16) Belgium (1) France (1) Germany (1) UK (1) Spain (1) Turkey (4).
5. 4B: Pakistan (1) Russia (1) Morocco (2).
6. IDU, injecting drug user.
7. CSW, commercial sex worker.

The predominance of subtype B amongst heterosexuals is an observation that is not in favour of possible differences in heterosexual transmissibility between B and non-B
subtypes. Therefore, risk group-associated differences in subtypes might be a result of historical and sexual network factors, rather than a result of phenotypic differences. In conclusion, this study shows the presence of multiple HIV-1 subtypes in the Netherlands, but we found no evidence of spread of non-B infections amongst heterosexuals with no epidemiological link with HIV endemic regions. Because these patterns might change in the future, we will continue to monitor the introduction and spread of HIV-1 subtypes.

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References

HIV-1 strains specific for Dutch injecting drug users in heterosexually infected individuals in The Netherlands

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Objective: To study the molecular epidemiology of HIV-1 subtype B amongst heterosexually infected individuals in The Netherlands.

Design: The study population comprised 54 individuals infected by subtype B viruses through heterosexual contacts. Serum samples were collected between 1988 and 1996.

Methods: Sequences of the gp120 V3 region were obtained from serum samples and analysed by using the signature pattern and phylogenetic methods.

Results: In 22 (41%) out of 54 subtype B sequences from heterosexually infected individuals, the synonymous nucleotide substitution in the second glycine codon at the tip of the V3 loop (the GGC pattern), previously identified as specific for Dutch injecting drug users (IDU), was found. The other previously described IDU sequence patterns were observed significantly more often among GGC- than among non-GGC-containing sequences. In addition, we identified another amino-acid change specific for the GGC sequences. In the phylogenetic and principal coordinate analyses, the GGC sequences from heterosexually infected individuals clustered separately from the non-GGC sequences and together with the IDU consensus sequence. Both the nonsynonymous and particularly the synonymous distances amongst the GGC sequences were significantly lower than amongst the non-GGC sequences.

Conclusions: Our data provide evidence for a common origin of the viruses in Dutch IDU and the GGC viruses in heterosexuals. We suggest that a considerable proportion of the viruses in heterosexually infected individuals in The Netherlands may have originated from Dutch IDU.

Keywords: HIV-1, sequence analysis, heterogeneity, molecular epidemiology, drug users, sexual transmission, The Netherlands

Introduction

Homosexual and bisexual contacts, injecting drug use, and heterosexual contacts are the main routes of HIV-1 transmission in The Netherlands. Together they account for the majority of AIDS cases (3789 out of 4020; 94%) diagnosed in The Netherlands between 1982 and 1995 [1]. The relative proportion of heterosexually infected individuals among newly diagnosed AIDS patients has increased over the last few years [1], a trend which has also been observed in other European countries and the United States [2]. Recent higher incidence of AIDS in heterosexual individuals with Dutch nationality appears to be associated with a more recent spread of viruses in this risk group than in homosexual men and injecting drug users (IDU).

Subtype B is the only HIV-1 subtype found so far amongst homosexual men and IDU in The Netherlands. From the *Department of Human Retrovirology, Academic Medical Centre, University of Amsterdam, and the †Department of Public Health and Environment, Municipal Health Service, Amsterdam, The Netherlands. Sponsorship: The study was supported by the Ziekenfondsraad as part of the Stimulation Programme on AIDS Research of the Dutch Programme Committee for AIDS Research. Requests for reprints to: Vladimir V. Lukashov, Department of Human Retrovirology, Academic Medical Centre, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Date of receipt: 17 October 1997; revised: 12 December 1997; accepted: 19 December 1997.
Netherlands [3–5], whereas other HIV-1 subtypes (A, C, D, E, F, and G) have been present in The Netherlands for more than a decade amongst individuals originating from AIDS-endemic regions [6]. It has been shown that subtype B viruses from IDU differ from the viruses of homosexual men in several HIV-1 genomic regions [7,8]. In the envelope gp120 V3 region, viruses from IDU are distinguishable from viruses of homosexual men based on two synonymous nucleotide substitutions and one amino-acid change, of which the most conserved is a synonymous substitution in the second glycine codon at the tip of the V3 loop (the GGC pattern) [4,7,9,10]. The GGC pattern has not been found in the V3 sequences obtained from homosexual men in The Netherlands (sequences from more than 100 individuals have been analysed) or other European countries and the United States [7,11–14], but is present in more than 80% of the sequences obtained from Dutch IDU. GGC viruses have also been found in IDU in Scotland and Germany [7–9,13], but not in Norway [12], Italy or Spain [7,8,14], although the molecular epidemiological data from these countries were often limited. Besides the GGC pattern, two other distinctions have been described in the IDU V3 sequences: a synonymous nucleotide substitution at HIV-1,env position 837 (the TCC pattern), and the non-T pattern (an amino-acid other than threonine) at HIV-1,env codon 288 [7,9]. Several nucleotide substitutions in Dutch IDU sequences, compared with the sequences from homosexual men, have also been found in the vpr and vpu genomic regions of HIV-1 [8]. Phylogenetic clustering of the sequences according to the risk group has been observed for all three genomic regions [4,5,8,9]. To explain the risk group-associated differences of virus strains, two hypotheses were originally proposed: specific adaptation of viruses to a certain risk group (transmission route) and a more likely founder effect [7,8]. In a later study [9], we provided evidence that the risk group-associated distinctions between viruses in The Netherlands is likely to be the result of a founder effect. We suggested that the possible uniform origin of the HIV-1 epidemic among Dutch IDU was as a result of HIV-1 transmission(s) from US IDU, and not from Dutch homosexual men [9].

Although several molecular epidemiological studies have been carried out in The Netherlands [4,6–9,13], no sequence information has been obtained for individuals who became HIV-1-infected through heterosexual contacts. In the present study, we obtained the gp120 V3 sequences from 54 individuals infected with HIV-1 subtype B viruses as a result of heterosexual contacts. We used HIV-1 V3 sequence patterns of Dutch IDU as molecular markers to study the origin of HIV-1 strains in heterosexually infected individuals in The Netherlands.

Materials and methods

Study population

Between 1988 and 1996, about 50,000 heterosexual individuals were tested for HIV-1 antibodies in Amsterdam, and 170 individuals were found to be HIV-1-seropositive. Sequences of the V3 region were obtained from serum samples of 90 (53%) of these individuals. The serum samples were obtained as part of several HIV studies, including HIV surveillance among pregnant women, HIV surveillance among sexually transmitted disease clinic visitors, the alternative screening, the European Women’s Study, and the HIV Heterosexuals Project [16]. All individuals were AIDS-free at the time of sampling. By using the phylogenetic analysis, we characterized 54 (60%) out of 90 individuals as infected by subtype B viruses. For the individuals involved in the study, the following demographic and epidemiological information was collected: age, gender, nationality, country of origin, risk factors for HIV-1 infection, and information about sexual partners. All individuals with non-B viruses originated or had a partner from HIV-endemic regions [16]. None of the 54 individuals with subtype B viruses originated from sub-Saharan Africa.

Sequencing and sequence analysis

The procedures of viral isolation, reverse transcription, amplification and sequencing have been described previously in detail [17]. Briefly, RNA was isolated from 100 μl serum, and viral RNA was transcribed into cDNA using the 3'-V3-NOT primer. The cDNA obtained was subjected to nested polymerase chain reaction (PCR). The outer primers used for the first PCR were 5'-V3-NOT and 3'-V3-NOT, the inner primers used for the second PCR were SP6-5'-ksi and T7-3'-ksi. Nested PCR resulted in the amplification of a sequence of approximately 270 base pairs in length. Double-stranded sequencing was performed on an automatic sequencer (Model 373A, Applied Biosystems, Foster City, California, USA) using the Taq polymerase dye primer sequencing kit (Applied Biosystems). The sequences have been deposited in the GenBank (accession numbers AF032134–AF032225).

Nucleotide sequences were aligned manually. The consensus sequences for Dutch homosexual men and IDU were calculated based on previously published sequences [3–5,15] and used in the analyses, as well as the global subtype B consensus sequence [18]. All positions with an alignment gap at least one sequence were excluded from any pairwise sequence comparison. Synonymous and nonsynonymous nucleotide p-distances (the proportion of synonymous and nonsynonymous differences between two sequences; Ds and Dn, respectively) were calculated by using the MEGA program [19]. Phylogenetic analysis was performed by using the MEGA program (neigh-
hour-joining method), and distance matrices were generated by using the Kimura two-parameter distance estimation method [20] as well as the synonymous p-distances. Bootstrap analysis was performed by generating 100 trees on the whole set of sequences. In addition, bootstrap analysis was performed for the sequence sets, in which all but one GGC sequence were excluded. This was performed for each of the GGC sequences.

Multivariate principal coordinate analysis was performed by using the PCOORD software [21]. Signature pattern analysis was performed according to Korber and Myers [10]. The Pearson correlation coefficients were calculated to evaluate the association between sequence similarity and group membership (GGC group versus non-GGC group). To assess the correlation that one can expect to find accidentally when no real association is present, a simulation method was used [8] in which all sequences were randomly divided into two groups 100 times, and on each occasion the correlation coefficient was calculated for all positions. This accidental correlation never rose above 0.33 (P > 0.1). Statistics were calculated by using the SPSS/PC+ software (version 5.0, SPSS Inc., Chicago, Illinois, USA). The Mann–Whitney test was used to compare groups.

**Results**

HIV-1 sequences obtained from 54 individuals infected by subtype B viruses through heterosexual contacts are shown in Fig. 1. Signature pattern analysis revealed that 22 (41%) individuals had the GGC pattern specific for Dutch IDU. The other previously described IDU sequence patterns (TCC and non-T) were significantly more often found amongst the GGC sequences than amongst the non-GGC sequences from heterosexuals (Fig. 1, pattern). The TCC pattern was found in 17 (77%) out of 22 GGC sequences and one (3%) out of 32 non-GGC sequences (P < 0.001), and the non-T pattern was seen in 19 (86%) GGC sequences and seven (22%) non-GGC sequences (P < 0.001: Fig. 1).

In addition to previous reports from our group [7–9], we identified another amino-acid change (the presence of isoleucine, the I-pattern, Fig. 1), which was present in 16 (73%) out of 22 GGC sequences, significantly more often than in the non-GGC sequences (five out of 32 sequences, 16%; P < 0.001). There were significant correlations between the presence of the GGC pattern and each of the other three sequence patterns (Fig. 1). Twenty (91%) out of 22 GGC sequences contained two or three other sequence patterns, compared with one (3%) out of 31 non-GGC sequences (P < 0.001; Fig. 1).

Phylogenetic analysis revealed clustering according to the sequence patterns described above (Fig. 2). Because of the lower rate of synonymous compared with non-synonymous substitutions in the V3 region [3,5] and the possible functional saturation at non-synonymous positions [22], we performed phylogenetic analysis by using the Kimura two-parameter distances (for all nucleotide positions) as well as the synonymous p-distances. Phylogenetic difference between the GGC- and non-GGC sequences was more pronounced at the synonymous level (Fig. 2b). All GGC sequences from heterosexually infected individuals, with a single exception, clustered together with the consensus sequence of Dutch IDU, whereas all non-GGC sequences clustered separately from the GGC sequences (again with a single exception) and together with the global subtype B consensus (Fig. 2b). Similar to our earlier observations for the sequences from homosexual men and IDU [9], the bootstrap values for the GGC and non-GGC clusters were low (data not shown), probably reflecting the high variation of the V3 loop, of which only a very limited part is risk-group-specific. Another possible reason could be related to the peculiarities of the bootstrap analysis, which assigns the values for the whole cluster but not for any two sequences within this cluster. Therefore, a higher number of closely related sequences in the cluster could result in a lower bootstrap value for this cluster. To test this possibility, we performed bootstrap resampling for the sequence sets, in which all but one GGC sequences were excluded but all non-GGC sequences as well as the consensus sequence of Dutch IDU were present. This analysis was performed for every GGC sequence. We observed that each GGC sequence clustered separately from the non-GGC sequences and together with the Dutch IDU consensus sequence with a mean bootstrap value of 73 (range, 60–96; Fig. 1). The non-GGC sequence that exceptionally belonged to the GGC cluster when all sequences were included in the phylogenetic analysis (sequence TUR9340) did not cluster with the IDU consensus when all GGC sequences were excluded from the analysis. Similar to the phylogenetic analysis, a separation between the GGC and non-GGC sequences was also observed in the principal coordinate analysis (PCOORD; data not shown).

Subsequently, we analysed the evolutionary distances amongst the sequences with and without the GGC pattern. Each sequence within a group was compared with every other sequence within the same group, and the mean distance for the group was calculated as the mean of pairwise sequence distances. Both Ds and especially Dr were significantly higher amongst the sequences without the GGC pattern than amongst the GGC sequences: Ds, 0.128 ± 0.027 (SD) versus 0.093 ± 0.020 (P < 0.001); Dr, 0.145 ± 0.047 versus 0.069 ± 0.030 (P < 0.001) for the non-GGC and GGC sequences.
Discussion

In this study, we obtained and analysed the V3 sequences from 54 individuals from The Netherlands who became infected through heterosexual contacts. We observed that (i) 22 (41%) out of 54 sequences had the GGC sequence pattern, previously identified as specific for Dutch IDU; (ii) the other IDU sequence
patterns were observed significantly more often amongst the GGC sequences; (iii) the GGC sequences from heterosexually infected individuals clustered separately from the non-GGC sequences and together with the IDU consensus sequence in the phylogenetic and principal coordinate analyses; and (iv) the GGC group of sequences was significantly more homogeneous.

These findings point to the likely common origin of the viruses in Dutch IDU and the GGC viruses in
heterosexuals. Taking into account the more recent spread of HIV-1 amongst heterosexuals than amongst IDU [1,23], it is likely that a considerable proportion of the GGC viruses in heterosexually infected individuals in The Netherlands may have originated from Dutch IDU. At the same time, these findings provide additional support to our previous conclusion, that the difference in viruses between homosexual men and IDU in The Netherlands is a result of a founder effect, rather than risk-group-associated adaptation [7–9].

Similarly, the close relation of viruses in IDU and heterosexuals has been previously observed in Edinburgh, Scotland [13], where sequences from three out of three heterosexually infected individuals clustered with the IDU sequences. Based on these data, the common origin of the viruses in IDU and heterosexuals in Scotland has been proposed [13]. Sequence similarity between the viruses from IDU and heterosexuals has also been reported in Norway [12], but in that study all four heterosexually infected individuals were known to be sexual partners of IDU. Based on the epidemiological data collected for all individuals, we were unable to determine whether the presence of GGC viruses in heterosexuals could be the result of direct transmission from IDU or via intermediate heterosexual transmission(s). Two (18%) out of 11 individuals with the GGC viruses, for whom this information was available, reported sexual contacts with IDU. Ten (56%) out of 18 individuals infected by the GGC viruses, for whom this information was available, reported sexual contacts with multiple partners (five or more partners in the preceding 6 months or 10 or more partners in the last 5 years), some of whom may have been IDU. In the whole study population of 54 individuals, we identified 15 individuals who were most likely to have been infected in The Netherlands (those of Dutch origin who did not report contacts with individuals from AIDS-endemic regions). For the rest of the individuals (39 out of 54), the likely country of infection is uncertain because of the absence of data (Fig. 1). Amongst the 15 individuals most likely infected in The Netherlands, eight (53%) had GGC viruses. The proportion of individuals with Dutch nationality was twice as high in the GGC group (11 out of 22, 50%) as in the non-GGC group (eight out of 32, 25%; P = 0.06).

Based on molecular and epidemiological data, we suggest that about one-half of subtype B-infected heterosexuals in Amsterdam are likely to have been infected (directly or indirectly) by IDU.

The mean sequence diversity amongst the GGC sequences in heterosexuals was considerably lower than the mean sequence diversity reported for the countries with a relatively long (more than 10 years) history of HIV-1 epidemics, such as the United States or many European countries, in which the mean sequence diversity of the V3 region is above 10% [9,12,18,22,24,25]. On the other hand, relatively low levels of sequence diversity have been reported for populations with a short history of HIV-1 epidemics that started after a single virus introduction [9,13,15,22,24–30]. Higher heterogeneity of the non-GGC sequences compared with the GGC sequences observed in our study suggests an older or, more likely, less uniform origin of HIV-1 infections in individuals with non-GGC viruses [9,13,15,26,27,31]. These viruses may originate from other risk groups in The Netherlands as well as from abroad.

In summary, while the early HIV-1 epidemic in Western Europe and the United States has been mainly restricted to homosexual men and IDU, recent studies have showed increasing HIV-1 incidence associated with heterosexual transmission. Our study demonstrated that a substantial number of HIV-1 infections among heterosexual infected individuals may have originated from IDU.

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HIV STRAINS OF IDU IN HETEROSEXUALS

References


E.L.M. Op de Coul¹, R.A. Coutinho¹,², A. Van der Schoot², G.J.J. van Doornum¹,², V.V. Lukashov ², J. Goudsmit ², and M. Cornelissen ² for the Dutch HIV-1 Subtype Surveillance ⁴.

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ABSTRACT

Nationwide serosurveillance was conducted among 21 HIV/AIDS centres to study epidemiological factors influencing the distribution and spread of HIV-1 subtypes among heterosexuals in the Netherlands. Blood samples were collected from persons diagnosed HIV-1 positive from 1997 to 1999, for whom the mode of HIV transmission was heterosexual contact or unknown. HIV-1 subtypes were determined by phylogenetic analysis of envelope V3 sequences and correlated with sociodemographic characteristics of the subjects and their sexual partners if identified.

Among the 200 subjects, 60% (n=121) were infected with HIV-1 subtype B. Non-B subtypes identified were: A (n=31), C (n=24), D (n=10), E (n=6), F (n=4), G (n=3), and unclassified (n=1). In four of the six regions, the proportion of subtype B viruses was about 60%, but in the Northwest and Southwest regions these proportions were 76% and 46%, respectively. The Surinamese and Antilleans, large immigrant groups in the study population, were all infected with subtype B. So were almost all individuals who did not know how they became infected.

In Amsterdam, where HIV-1 subtypes were studied among samples obtained from 1988 onward, the proportions of non-B viruses did not change significantly over time, but a shift in the various subtype B strains was observed, suggesting introductions of new subtype B strains in Amsterdam.

To date, HIV-1 non-B subtypes in the Netherlands are still found predominantly among heterosexual individuals with an epidemiological link with sub-Saharan Africa. Despite continuing introductions of non-B subtypes, the B/non-B distribution has been stable over time, most likely due to simultaneous introductions of subtype B strains from Caribbean and South American countries.

INTRODUCTION

As in most Western European countries, the epidemic of the human immunodeficiency virus (HIV) in the Netherlands started in the early 1980s, primarily affecting homosexual men and injecting drug users (IDUs).¹,² Currently, the total number of people living with
HIV/AIDS in the Netherlands is estimated at 15,000.\(^3\) The absolute number of heterosexually infected individuals as well as the number of imported HIV infections is unknown, but the relative impact of heterosexual transmission among the AIDS cases is increasing.\(^4\) For several heterosexual subpopulations in Amsterdam including persons attending sexually transmitted disease (STD) clinics, blood donors, and pregnant women, the HIV prevalence is relatively low (<1-2%) and stable over time.\(^7\)\(^10\)

The molecular epidemiological studies on HIV-1 conducted so far in the Netherlands have mainly focused on various HIV-1 risk groups in the city of Amsterdam. Among homosexual men and IDUs, these studies found almost exclusively subtype B viruses,\(^11\)\(^13\) but in the heterosexual population, they identified the env HIV-1 subtypes A-G and K, as well as recombinant strains, all of which originated largely from sub-Saharan Africa except for subtype B strains which are found predominantly in people from Western countries.\(^14\)\(^17\) This finding raised the public health concern that non-B subtypes from developing countries, where the predominant route of transmission is heterosexual intercourse, might spread among the indigenous Dutch heterosexual population. To date, there is no nationwide systematic data regarding the distribution and spread of various HIV-1 subtypes and related sociodemographic characteristics among heterosexuals in the Netherlands. The proportions of subtypes in different parts of the country could be expected to vary due to risk group and ethnic variation in the HIV-1 infected population. To study the geographic distribution and heterosexual transmission patterns of the genetic subtypes of HIV-1 nationwide, the Municipal Health Service (MHS) in Amsterdam started in April 1996 surveillance for HIV-1 subtypes. In this surveillance, blood samples and epidemiological data were collected from diagnosed HIV-1 positive heterosexuals between 1997 and 1999. The frequency of subtypes was studied in relation to country of origin, presumed country of infection and the risk group of sexual partners.

**MATERIALS AND METHODS**

**Organization of the surveillance system**

In 1996, 25 HIV/AIDS centres across the Netherlands were invited to participate in the Dutch HIV-1 Subtype Surveillance designed to track HIV-1 subtypes among heterosexually infected individuals who tested HIV-1 positive between 1997 and 1999. Of these centres, 21 participated as follows (sample collection 1). For each HIV-1 positive test, a questionnaire was sent to the patient's physician. For individuals, who most likely acquired the HIV-1 infection by heterosexual contact as determined by other HIV risk factors such as homosexual contacts, injecting drug use, and haemophilia, and those for whom the source of infection was unknown, serum or plasma samples were collected. In each case, the physician collected information regarding the patient's gender, age, city of residence, nationality, country of birth, ethnicity, presumed country where the HIV infection was acquired, presumed year of infection (according to the patient's statement and/or dates of the last HIV-negative and first HIV-positive test) and year of AIDS diagnosis. Information was also collected concerning the risk factors of sexual partners (IDU, homosexual contacts, recipient of HIV contaminated blood (products), and origin from AIDS-endemic area). The samples were transported to the department of Human Retrovirology of the Academic Medical Center (AMC) in Amsterdam, where the HIV-1 strains were sequenced. Epidemiological data was collected at the MHS. The HIV-1
subtype results were reported back to the participating physicians and added to the database at the MHS.

For Amsterdam, additional surveillance data was augmented by samples and epidemiological information collected from recently diagnosed HIV-1 positive heterosexuals through three HIV surveys conducted in that city (sample collection 2). The survey sites included two Amsterdam hospitals, one midwife practice where pregnant women were tested for HIV, an STD clinic which twice a year conducts a cross-sectional study among 1000 visitors, and the MHS facility for persons requesting an HIV test on their own initiative. Samples and epidemiological information collected through these HIV surveys covered approximately one decade.7–9 The HIV-1 subtypes circulating among heterosexuals who became infected between 1988 and 1996 (n=90) are described in two previous studies.15–18 In the present study, we compare those strains with the strains identified between 1997 and 1999 to reveal possible changes in HIV-1 distribution over time in Amsterdam.

Sample collection, sequencing and phylogenetic analysis

In total, serum or plasma samples were collected for 320 individuals diagnosed HIV-1 positive at 21 Surveillance sites in the Netherlands or three survey sites in Amsterdam. The individuals acquired the HIV-1 infection either by heterosexual intercourse or an unknown source.

Using methods described previously,19 we isolated and directly amplified the HIV-1 sequence in each case, by using PCR primers that enclose the V3 region of the HIV-1 envelope (env) gene (276 nucleotides). Of the 320 specimens, three were from HIV-2 infected patients and were therefore excluded from this study. HIV-1 RNA from 215 of the 317 remaining specimens was successfully amplified, and sequences were obtained from 200 samples, which comprised the ultimate study population. PCR failure showed no association with study site, gender, age, or AIDS diagnosis (chi-square test, p>0.05), but individuals from Africa showed a PCR negative result more often than individuals from the Netherlands, albeit with a borderline significance (chi-square test, p=0.05).

Of the 200 samples, 173 were included via the Dutch HIV-1 Subtype Surveillance (sample collection 1). The remaining 27 were asymptomatic individuals included via the three HIV surveys conducted in Amsterdam (collection 2). The majority of the study population was diagnosed HIV-1 positive at university hospitals (70%), whereas the remaining individuals were diagnosed at MHS sites, regional laboratories or their physician's office. Of the study population, 61% had not been tested previously for HIV. Of the individuals that were tested previously, 70% had a previous HIV-1 positive test result of which the majority was tested in 1995-1996.

Among the 200 samples that were sequenced, 11 were from the North, 73 from Northwest, 42 from Southwest, 30 from the East, 17 from the South, and 27 from the Central region in the Netherlands. The samples were classified according to the subject's place of residence or, if this information was not available, the city where the HIV test was conducted.

The V3 sequences were phylogenetically analysed by using the Neighbour-joining method (MEGA program).20 To evaluate the consistency of the phylogenetic clustering, the sequences were subjected to bootstrap analysis. Bootstrap values above 75 were
considered definitive for significant clustering. The phylogenetic trees were based on Kimura-2-parameter distances or synonymous p-distances, since the latter method is sometimes more powerful in showing risk-group-related differences. Reference strains from each subtype were included in the trees.

**Signature pattern analysis**

Previous Amsterdam studies have shown that subtype B viruses from different risk groups can be distinguished on the basis of mutations in various genomic regions. Among the majority of Amsterdam IDUs, a synonymous nucleotide substitution (GGC) was observed in the second glycine codon (position 312) at the tip of the V3 loop (GPGR), while among homosexual men the GGG (or GGA) variant was observed. Heterosexuals in Amsterdam carry both variants, and a relatively large proportion of the subtype B viruses found in heterosexuals harboured the GGC mutation, suggesting transmission of HIV from IDUs to the non-drug using heterosexual population. Since the GGC mutation is stable during intrahost evolution, a change in the distribution of these GGC/non-GGC subtype B viruses most likely represents new virus introductions in a population. To study the possibility of new subtype B virus introductions in Amsterdam, we examined all subtype B sequences - from reported heterosexuals including individuals with unknown risk factors - for these signatures.

**Statistical analysis**

To identify variables that were associated with HIV-1 subtype the chi-square test, Fisher's exact test and the Mann-Whitney test were used to evaluate differences in proportions and means (SPSS, version 9.0). The outcome variable was dichotomous (subtype B versus non-B subtypes, or PCR-positive versus PCR-negative); gender, age, country of origin, and city of residence were the independent variables. To test changes over time in proportions of subtype B strains with the GGC or non-GGC codons (as an indication of new virus introductions in Amsterdam), the chi-square test for linear trend was used (Epi-info, version 6.0). A p value of <0.05 was considered significant.

**RESULTS**

*HIV-1 heterogeneity among heterosexuals in the Netherlands*

Figure 1A shows the phylogenetic tree for all the sequences classified as subtype B (60% of our sample), while figure 1B shows all the non-B sequences: A (n=31), C (n=24), D (n=10), E (n=6), F (n=4), G (n=3) and unclassified (n=1). The subtype A cluster is not statistically supported by bootstrap analysis, which illustrates the heterogeneity of this cluster (fig 1B). The subtype F cluster includes the subgroups F1 and F2 of which F1 was highly significant (bootstrap value: 100%).
Fig 1A. Phylogenetic tree of HIV-1 subtype B sequences from heterosexuals in the Netherlands
Fig 1B. Phylogenetic tree of HIV-1 non-B sequences from heterosexuals in the Netherlands
The F1 subgroup includes strains from Africa, Brazil, and Romania, with strain 416 from Sudan being classified as subgroup F2. The subtype C cluster showed, as subtype A, a highly diverse structure and we observed the presence of viruses in the Netherlands belonging to C subclusters described previously. One unclassified strain (303) was placed interspersed in the phylogenetic tree. When examined in more detail by bootscan analysis, as implemented in the Simplot program, it appeared to represent a highly diverse variant of subtype A or H, or a recombinant of those two subtypes (data not shown).

Fig 2 shows the HIV-1 subtype distribution in the Netherlands. In four of the six geographic regions, the proportion of subtype B viruses is about 60%. In the regions Northwest (Amsterdam and vicinity) and Southwest (Rotterdam, The Hague, Delft and Leiden), the proportions were: 76% and 46%, respectively. In the Southwest area, the variety of genetic subtypes was the highest compared to other regions in the Netherlands.

Table 1A summarizes the sociodemographic characteristics of the study population, in relation to subtype B and non-B strains. Statistical analysis of the epidemiological data revealed that the variables 'origin of subject' and 'heterosexual risk during a stay in an AIDS-endemic area' were strongly associated with the genetic subtype (p<0.0001). The variables 'gender', 'year of the HIV-positive test result', 'previously tested for HIV', 'previous test result', 'year of the previous test result' and 'having an AIDS diagnosis', were not related with the genetic subtype (p>0.05) (data not shown).

More than half of our subjects are of non-Dutch origin, but the majority (96%) lives in the Netherlands. The sub-Saharan Africans formed the largest immigrant group; they originated from 21 different countries (table 1A). 93% of the sub-Saharan Africans had a non-B infection. Of the indigenous Dutch population with a non-B infection, 53% most likely acquired the infection while travelling in sub-Saharan Africa, and 33% reported sexual risk in the Netherlands with a partner from sub-Saharan Africa. When a non-B infection could not be linked with an AIDS-endemic region, it was usually because various sexual partners were reported, but sometimes simply no further information had been collected by the patient's physicians.

Among persons with a subtype B infection (n=121), the largest group included people indigenous to the Netherlands and other European countries (n=71). The second largest group (n=34), originated either from the Caribbean (the Netherlands Antilles and the Dominican Republic) or from South America (Surinam, Brazil, and Ecuador). Of the second group, 56% reported that they most likely acquired the HIV-1 infection in these areas; either when living there in the past or travelling there; 44% reported sexual contact in the Netherlands with a partner originating from these areas. In a third group, six individuals with subtype B originated from North Africa (Morocco n=3, Algiers n=1, Egypt n=1 and the Cape Verde Islands n=1) and two from Asia (Singapore and Indonesia).

Sex with a drug using partner accounted for 9% (11/121) of the subtype B infections, while five individuals had sexual contact with a homo- or bisexual partner. These five were all transsexuals (male to female), with a history of homosexual contacts, and four
carried a virus with a non-GGC mutation (GGG or GGA), the virus that largely infects the non-drug using homosexual population in the Netherlands.11-13

Table 1A. Subject characteristics and HIV-1 subtypes among heterosexuals in the Netherlands.4

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
<th>Subtype B (%)</th>
<th>non-B subtypes (%)</th>
<th>ρ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (n=195)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>90 (46.2)</td>
<td>59 (65.6)</td>
<td>31 (34.4)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Female</td>
<td>100 (51.3)</td>
<td>54 (54.0)</td>
<td>46 (46.0)</td>
<td></td>
</tr>
<tr>
<td>Transsexual</td>
<td>5 (2.6)</td>
<td>5 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Median age 2</td>
<td>35.0</td>
<td>37.0</td>
<td>32.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Residence in the Netherlands (n=185)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes (total)</td>
<td>178 (96.2)</td>
<td>102 (57.3)</td>
<td>76 (42.7)</td>
<td></td>
</tr>
<tr>
<td>North</td>
<td>11 (6.2)</td>
<td>6 (54.5)</td>
<td>5 (45.4)</td>
<td></td>
</tr>
<tr>
<td>South</td>
<td>15 (8.4)</td>
<td>11 (73.3)</td>
<td>4 (26.7)</td>
<td></td>
</tr>
<tr>
<td>Central</td>
<td>24 (13.5)</td>
<td>14 (58.3)</td>
<td>10 (41.7)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Northwest</td>
<td>68 (38.2)</td>
<td>50 (73.5)</td>
<td>18 (26.5)</td>
<td></td>
</tr>
<tr>
<td>Southwest</td>
<td>28 (15.7)</td>
<td>14 (50.0)</td>
<td>14 (50.0)</td>
<td></td>
</tr>
<tr>
<td>East</td>
<td>27 (15.2)</td>
<td>13 (48.1)</td>
<td>14 (51.9)</td>
<td></td>
</tr>
<tr>
<td>No 3</td>
<td>7 (3.8)</td>
<td>2 (28.6)</td>
<td>5 (71.4)</td>
<td></td>
</tr>
<tr>
<td>Origin of subject (n=195)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>the Netherlands</td>
<td>86 (44.1)</td>
<td>61 (70.9)</td>
<td>25 (29.1)</td>
<td></td>
</tr>
<tr>
<td>Other European countries 4</td>
<td>12 (6.2)</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
<td></td>
</tr>
<tr>
<td>North Africa 5</td>
<td>7 (3.6)</td>
<td>6 (85.7)</td>
<td>1 (14.3)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sub-Saharan Africa 6</td>
<td>46 (23.6)</td>
<td>3 (6.5)</td>
<td>43 (93.5)</td>
<td></td>
</tr>
<tr>
<td>Caribbean/ South America 7</td>
<td>35 (18.2)</td>
<td>34 (97.1)</td>
<td>1 (2.9)</td>
<td></td>
</tr>
<tr>
<td>Asia 8</td>
<td>7 (3.6)</td>
<td>2 (28.6)</td>
<td>5 (71.4)</td>
<td></td>
</tr>
<tr>
<td>Other 9</td>
<td>2 (1.0)</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Heterosexual risk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partner HIV positive/AIDS (total)</td>
<td>19 (9.5)</td>
<td>16 (84.2)</td>
<td>3 (15.8)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>- IDU</td>
<td>4 (2.0)</td>
<td>3 (75.0)</td>
<td>1 (25.0)</td>
<td></td>
</tr>
<tr>
<td>- Partner heterosexual risk</td>
<td>3 (1.5)</td>
<td>2 (66.7)</td>
<td>1 (33.3)</td>
<td></td>
</tr>
<tr>
<td>- Risk partner unknown</td>
<td>12 (7.1)</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
<td></td>
</tr>
<tr>
<td>During stay in HIV endemic area</td>
<td>71 (35.7)</td>
<td>11 (15.5)</td>
<td>60 (84.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>During stay in other European country (Greece,Turkey)</td>
<td>2 (1.0)</td>
<td>2 (100)</td>
<td>0 (0)</td>
<td>n.t. 12</td>
</tr>
<tr>
<td>Partner who was IDU</td>
<td>10 (5.0)</td>
<td>8 (80.0)</td>
<td>2 (20.0)</td>
<td>n.s. 13</td>
</tr>
<tr>
<td>Various sexual partners</td>
<td>15 (7.5)</td>
<td>14 (93.3)</td>
<td>1 (6.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Sexual contact with CSW 10 in the Netherlands</td>
<td>6 (3.0)</td>
<td>6 (100)</td>
<td>0 (0)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Subject is/was CSW</td>
<td>1 (0.5)</td>
<td>1 (100)</td>
<td>0 (0)</td>
<td>n.t.</td>
</tr>
<tr>
<td>Heterosexual contact, no extra information obtained</td>
<td>24 (12.1)</td>
<td>23 (95.8)</td>
<td>1 (4.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Other or unknown risks</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transsexual, no drug use</td>
<td>5 (2.5)</td>
<td>5 (100)</td>
<td>0 (0)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Other risks 11</td>
<td>5 (2.5)</td>
<td>3 (66.7)</td>
<td>2 (33.3)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Subject has no idea how he/she became infected</td>
<td>24 (12.1)</td>
<td>22 (91.7)</td>
<td>2 (8.3)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clinical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AID S diagnosis (n=121)</td>
<td></td>
<td></td>
<td></td>
<td>n.s.</td>
</tr>
<tr>
<td>Yes</td>
<td>40 (33.1)</td>
<td>26 (65.0)</td>
<td>14 (35.0)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>81 (66.9)</td>
<td>46 (56.8)</td>
<td>35 (43.2)</td>
<td></td>
</tr>
</tbody>
</table>

1 Totals may vary due to missing values; 2 age at moment of filling out the questionnaire; 3 residence in: Germany, Spain, Malawi, Rwanda, US; 4 Germany, Spain, Denmark, Greece, Portugal, Romania, Ireland; 5 Morocco, Egypt, Algeria; 6 Angola, Cameroon, Cape Verde Islands, Djibouti, Ethiopia, Gambia, Ghana, Guinea, Kenya, Liberia, Malawi, Nigeria, Rwanda, Somaliland, South Africa, Sudan, Togo, Uganda, Zambia, Zimbabwe, Democratic Republic of Congo; 7 Curacau, Surinam, Dominican Republic and Dutch Antilles not specified; 8 Thailand, Indonesia, Singapore, India; 9 USA, Russia; 10 CSW = commercial sex worker; 11 rape or blood contamination; 12 n.t. = not tested; 13 n.s. = not significant
Fig 2. Distribution of HIV-1 env subtypes in the Netherlands
Of the study population, information on heterosexual risk was not received for 16% (32/200), and a similar proportion did not know how he/she became infected (n=24). Interestingly, 94% of these were infected with subtype B (table 1A and 1B). An infection with subtype B was observed also for the majority of subjects whose reported a history of heterosexual promiscuity or visits to commercial sex workers. Of the 24 individuals who did not know how they became infected, the majority (68%) harbored a subtype B strain with a non-GGC mutation.

Table 1B. Transmission factors and HIV-1 subtypes (%) among heterosexuals in the Netherlands.

<table>
<thead>
<tr>
<th>HIV-1 subtype</th>
<th>A (n=31)</th>
<th>B (n=121)</th>
<th>C (n=24)</th>
<th>D (n=10)</th>
<th>E (n=6)</th>
<th>F (n=4)</th>
<th>G (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stay in HIV-endemic area</td>
<td>20 (64.5)</td>
<td>11 (9.1)</td>
<td>19 (79.2)</td>
<td>10 (100)</td>
<td>5 (83.3)</td>
<td>4 (100)</td>
<td>2 (66.7)</td>
</tr>
<tr>
<td>Partner from HIV-endemic area</td>
<td>3 (9.7)</td>
<td>10 (8.3)</td>
<td>2 (8.3)</td>
<td>0 (0)</td>
<td>1 (16.7)</td>
<td>0 (0)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Partner who was IDU</td>
<td>2 (6.5)</td>
<td>8 (6.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Various sexual partners</td>
<td>1 (3.2)</td>
<td>14 (11.6)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Heterosexual contact, no further information</td>
<td>2 (6.5)</td>
<td>23 (19.0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Subject has no idea how he/she became infected</td>
<td>1 (3.2)</td>
<td>22 (18.2)</td>
<td>1 (4.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Subject is transsexual</td>
<td>0 (0)</td>
<td>5 (4.1)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Other risks or risk unknown</td>
<td>2 (6.4)</td>
<td>28 (23.1)</td>
<td>2 (8.3)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

1: HIV positive partner, Sex in exchange for money

Influx of subtype B strains in the Netherlands

For Amsterdam, we studied the HIV-1 subtype distribution among heterosexuals over time, since samples and epidemiological information were collected through three surveys for more than a decade (1988-1999). For this analysis, we included the 90 sequences from a previous study and the 27 recently obtained sequences. Two sequences showed high similarity with two other sequences and most likely were epidemiologically linked, and were therefore excluded from the analysis. All 115 subjects were surveyed at the MHS in Amsterdam (see materials and methods). In fig 3A it was shown that there was no increase over time in the proportion of non-B viruses compared to subtype B viruses, present among the 115 heterosexually HIV-1 infected in this eleven-year study period. Since non-B HIV-1 subtypes are continually introduced in Amsterdam, we hypothesized that the ratio of B/non-B viruses remains stable over time due to simultaneous introductions of new subtype B viruses. To test this hypothesis, we examined the 75 subtype B strains for risk-group-specific nucleotide mutations.

Fig 3B shows that the percentage of viruses with a non-GGC mutation in Amsterdam increased significantly from 46% in '88-'92 to 77% in '97-'99 (p<0.05), suggesting influx of new subtype B viruses over time in the population of HIV-1 infected heterosexuals. Analysis of the epidemiological information showed that a relative large proportion of the individuals having subtype B viruses with a non-GGC codon originated from South America and the Caribbean countries (Surinam, the Netherlands Antilles, and the
Dominican Republic), suggesting that the newly introduced subtype B strains in the Dutch heterosexual population originates from these countries. The percentage of individuals with a non-GGC subtype B strain diagnosed HIV-positive in other parts of the country was 73% in the period '97-'99 and did not differ significantly from Amsterdam.

**DISCUSSION**

We studied HIV-1 subtypes and heterosexual transmission patterns among heterosexuals diagnosed from 1997 to 1999 in different regions in the Netherlands through prospective surveillance. Phylogenetic analysis of 200 env V3 sequences revealed that seven different HIV-1 subtypes circulate among heterosexuals in the Netherlands, of which subtype B is the most common subtype. For Amsterdam, we studied the HIV-1 subtype distribution among heterosexuals over a longer period: 1988 to 1999. Although non-B viruses are regularly introduced in Amsterdam, we found no significant increase of non-B viruses over time, probably due to simultaneous introductions of subtype B strains from the Caribbean countries and Surinam; which have a colonial history with the Netherlands. Half of the heterosexually HIV-1 infected persons in the Netherlands come from a foreign country. Individuals from Surinam and the Netherlands Antilles, where subtype B predominates, comprise the second most important immigrant group in our HIV sequence study. A report on the HIV epidemics in the Caribbean countries and Surinam suggests that AIDS in that area is evolving from an epidemic that began in 1983 among homosexual men to one in which infections are increasingly caused by heterosexual contact. Although the estimated number of HIV/AIDS cases is lower in Surinam than in the Netherlands, the relative contribution of heterosexuals to the AIDS cases in Surinam is 56%, compared to 15% in the Netherlands. The Caribbean region has one of the worst HIV-1 epidemics beyond Africa with an estimated adult HIV seroprevalence of 2.3% and a wide variation among countries.

The proportional increase of subtype B viruses with the non-GGC codon in Amsterdam and the relatively large group of Surinamese and Caribbean people among those who carry these viruses, suggests new virus introductions from that area, since most of these individuals reported sexual risk in their homeland. The higher percentage of subtype B viruses observed for the Northwest (Amsterdam and vicinity) compared to the Southwest (Rotterdam and other cities) can be explained by the relatively high number of Surinamese and Caribbean people living in Amsterdam; 42% of the total number of Surinamese live in Amsterdam, while 16% lives in Rotterdam. In 33% of our subjects, HIV infection had progressed to AIDS: 25% for the Dutch indigenous population and 40% for individuals from AIDS-endemic areas. Since the majority of these persons had not been tested previously, they were probably unaware of their HIV status while being infected for a relatively long period of time. Possibly, heterosexuals in the Netherlands do not consider themselves being at risk for HIV. Among the individuals who had no idea of how they became infected (n=24), 92% had subtype B viruses, mainly the non-GGC type, suggesting that individuals travelling in African countries are aware of the risk for HIV, in contrast to those having unprotected sex either in the Netherlands or in Caribbean countries.
Fig 3A. Distribution of HIV-1 env subtypes over two time-periods in Amsterdam.

Fig 3B. Distribution of subtype B GGC and non-GGC viruses over time in Amsterdam.
The increase of non-GG\textsubscript{C} strains over time could also reflect exchange of viruses between the heterosexual and homosexual population, since this type of virus also circulates among homosexual men in the Netherlands; however, only the five transsexuals included in this study reported a homo/bisexual partner. Obviously, this group of 24 individuals could include a few men unwilling to acknowledge homosexual contacts.

Among the Caribbean and Surinamese individuals, only subtype B viruses were identified. This observation confirms the results from a previous study on sexual risk behaviour and sexual mixing patterns among immigrant groups in Amsterdam.\textsuperscript{30} It showed that compared to the Dutch indigenous population, the Surinamese, Antilleans, and sub-Saharan Africans more frequently had multiple partners. Also they often had sexual relations in the country of origin during a visit. Interestingly, there was a considerable degree of sexual mixing between the Dutch indigenous population and both sub-Saharan African and Caribbean individuals, but not between the African and Caribbean people. This finding might explain the absence of non-B viruses among the Caribbean and Surinamese people in our study.

Regarding limitations to the present study, we attempted to include all important HIV testing sites in the Netherlands. However, four did not participate in the Dutch HIV-1 Subtype Surveillance, and might have influenced the HIV-1 subtype distribution in the country. Furthermore, 120/320 (38\%) of the samples were PCR-negative despite use of a PCR-system capable of amplifying the \textit{env} subtypes A-K. Since there was a slightly higher PCR- failure for samples from Africans compared to Dutch people, the proportion of non-B subtypes that we found in the Netherlands could be an underestimation.

In conclusion, the HIV-1 molecular diversity in the Netherlands is to a large extent determined by the immigrant population structure, as in other Western European countries. In those countries, the majority of the non-B infections are subtype A and C infections,\textsuperscript{31} but subtype distribution varies from one to another. In a recent study, discussing the relative prevalence of HIV-1 subtypes in the United Kingdom,\textsuperscript{32} the majority of infections among heterosexuals were with non-B subtypes. A study among French blood donors showed that the prevalence of non-B strains increased between 1985 (4\%) and 1995 (20\%).\textsuperscript{33}

Although non-B viruses have been introduced in the Netherlands, their percentage did not increase over time. However, the shift in subtype B viruses (from a majority of GG\textsubscript{C} viruses to a majority of non-GG\textsubscript{C} viruses) shows that the distribution of HIV-1 subtypes in the Netherlands is not stabilised but rather seems to be a dynamic process, which is influenced by the influx of subtype B strains from countries with a Dutch colonial past. The number of ethnic minorities is currently still increasing, but since such movements relate to many factors, it is difficult to predict whether the subtype distribution might change, with the proportion of non-B infections increasing in the future. Africans are relatively ‘new’ immigrant groups compared to Antilleans and Surinamese, who have a much longer history with the Netherlands.\textsuperscript{30} The occurrence of disassortative mixing (sexual contact between persons with distinct ethnicity) raises the potentiality for heterosexual spread of non-B subtypes of HIV in the country. The role of immigrants in the current HIV epidemic among heterosexuals in the Netherlands requires surveillance that addresses the heterosexual transmission of HIV, with specific attention for primary
infections. Surveillance should cover immigrant populations from AIDS-endemic areas and areas with increasing numbers of HIV infections, such as the African, Caribbean, South American and Asian countries.

Appendix:

The study was conducted under joint authorship: dr. Cruys ('De Weverziekenhuis', Heerlen), M. Buimer (MHS, Amsterdam, currently working at IMB b.v., Utrecht), J. Bax, A. van den Hoek (MHS, Amsterdam), dr. Claessen, dr. E.A.N.M. Mooi-Kokenberg (VU-hospital, Amsterdam, currently working at the Groene Hart Hospital, Gouda), H. Paap (Slotervaart-hospital, Amsterdam), dr. H. Weigel, dr. Frissen, drs K. Hoeksema (OLVG Hospital, Amsterdam), dr. M. van de Ende, prof A. Osterhaus (Dijkzigt Hospital, Rotterdam), dr. A. Lampe (Westeinde Hospital, The Hague), dr. R. Kauffmann (Leyenburg Hospital, The Hague), dr. A.C.M. Kroeze, dr. F. P. Kroon, (LUMC, Leiden), Dr. R. W. Vrede, (Reinier de Graafgroep, Delft), dr. Galama, M. Fluitsma, (Radboud Hospital, Nijmegen), dr. A. van Griethuysen (Canisius Wilhelmina Hospital, Nijmegen), dr. A.C.A.P. Leenders, dr. G. Weers-Pothoff (Bosch Medical Center, Den Bosch), Regional laboratory (Goes), dr. Leemhuis, A. Ketzer (MCL, Leeuwarden), dr. M. Schneider (UMC, Utrecht), Dr. L. Sabbe and dr. J. Goudswaard (Regional Public Health Laboratory Zeeland), Dr. F.G.C. Heilmann (Stichting Medische Microbiologie, Deventer)

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Legends

Fig 1. Unrooted neighbour-joining tree of HIV-1 subtype B env V3 sequences (1A) and non-B sequences (1B) from HIV-1-positive heterosexuals, collected from 1997 to 1999 in the Netherlands. The sequences were aligned with reference sequences from subtypes A-J (underlined).23 The values at the nodes indicates the percentage of bootstraps in which the cluster was found, using 100 replicates. The two trees include a few transmission cases, indicated with *.
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