Impact of antiretroviral therapy on HIV-1 transmission dynamics

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Declining trend in transmission of drug-resistant HIV-1 in Amsterdam

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Objective: Symptomatic primary HIV infections are over-represented in the mainly hospital-based studies on transmission of resistant HIV-1. We examined a more general population for the prevalence of resistant HIV-1 strains among primary infections.

Design: From 1994 to 2002 primary infections were identified within the Amsterdam Cohort Studies (ACS) among homosexual men and drug users, and at the Academic Medical Center (AMC). Whereas primary HIV-1-infected AMC patients, often presented with symptoms of acute retroviral syndrome, ACS participants largely seroconverted during follow-up and thus brought also asymptomatic primary infections to our study.

Methods: Reverse transcriptase (RT) and protease sequences were obtained by population-based nucleotide sequence analysis of the first HIV RNA-positive sample available. Subtypes were identified by phylogenetic analysis. Mutations were identified based on the IAS–USA resistance table.

Results: A total of 100 primary HIV-1 infections were identified (32 AMC and 68 ACS). Transmission of drug-resistant strains decreased over calendar time, with 20% [95% confidence interval (CI), 10–34%] of infections bearing drug-resistant mutations before 1998 versus only 6% (95% CI, 1–17%) after 1998. No multi-drug resistance pattern was observed. The median plasma HIV-1 RNA level of the first RNA positive sample was significantly lower for the individuals infected with a resistant strain versus those infected with wild-type, suggesting a fitness-cost to resistance. Four of seven non-B subtypes corresponded with the prevalent subtype in the presumed country of infection, and none showed resistance mutations.

Conclusions: The transmission of drug-resistant HIV-1 strains in Amsterdam has decreased over time. Monitoring should be continued as this trend might change.

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Keywords: HIV-1, transmission, resistance associated mutations, therapy, cohort

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Introduction

Reports from several countries [1–7] show that in 10–30% of primary human immunodeficiency virus type 1 (HIV-1) infections, the virus bears mutations conferring drug-resistance, indicating quite frequent transmission of such strains. The reported mutations conferred resistance to all three classes of antiretroviral therapy (ART): nucleoside reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (nNRTI), and protease inhibitors (PI). As resistant HIV strains limit therapy options [8], it is important to continue monitoring their frequency of transmission. However, investigation of longitudinal trends must focus on individuals shortly after infection. Newly diagnosed patients without a known duration of infection cannot indicate the calendar year of transmission. In the literature, primary HIV-1 infections are mostly identified among people that seek health care because they suffer from acute symptoms. Consequently, symptomatic primary HIV infections are over-represented in these mainly hospital-based studies. Mutations conferring resistance tend to cause a lower viral fitness in the absence of therapy, resulting in lower viral load [9,10] and possibly less severe primary HIV infection. Thus hospital-based studies may be biased against HIV-1 variants with resistance mutations, and it is important to also study primary infections among persons not suffering from acute symptoms.

The ‘prospective Amsterdam Cohort Studies (ACS) on HIV infection and AIDS’ enables study of HIV transmission among participants who seroconvert during follow-up. Any selection bias with respect to a symptomatic acute HIV infection is therefore eliminated. Here we report the prevalence of resistant HIV-1 strains among primary infections within the ACS of men having sex with men (MSM) and drug users (DU). As resistant HIV strains limit therapy options [8], it is important to continue monitoring their frequency of transmission. Newly diagnosed patients without a known duration of infection cannot indicate the calendar year of transmission. In the literature, primary HIV-1 infections are mostly identified among people that seek health care because they suffer from acute symptoms. Consequently, symptomatic primary HIV infections are over-represented in these mainly hospital-based studies. Mutations conferring resistance tend to cause a lower viral fitness in the absence of therapy, resulting in lower viral load [9,10] and possibly less severe primary HIV infection. Thus hospital-based studies may be biased against HIV-1 variants with resistance mutations, and it is important to also study primary infections among persons not suffering from acute symptoms.

The date of infection was defined in the following order of importance: (1) the date of the last seronegative but RNA-positive sample; (2) the date of an indeterminate result on the western blot; (3) the midpoint between the last seronegative and the first seropositive sample; (4) 100 days before testing negative by the LS-EIA. Report of clinical symptoms was obtained prospectively within the ACS and retrospectively for the AMC patients.

Material and methods

Study population

Individuals infected with HIV-1 between January 1994 and January 2003 were identified within the ACS and at the AMC.

The ACS started in 1984 among MSM [11] and in 1985 among DU [12]. Only young individuals, aged <30 years have entered the MSM cohort since 1995 and the DU cohort since 2000. The participants return for follow-up every 4 to 6 months. At entry and follow-up, questionnaires on risk behaviour are filled in, and blood samples are taken for virological and immunological testing. All serum samples are stored at −70°C. When a sample from a previously HIV-negative participant is found to be HIV-1 positive by enzyme immunoassay (EIA; using HIV-1/2 EIA tests from Abbott Laboratories, Abbott Park, IL, USA, and from bioMérieux, Boxtel, The Netherlands) and confirmed by a positive Western blot (Genelabs Diagnostics, Singapore), the last antibody-negative sample is tested for the presence of HIV-1 RNA. For our study primary infections were defined by a seroconversion interval smaller than 18 months. For seroconverters whose interval was larger than 18 months, a less sensitive enzyme immunoassay (LS-EIA) [13] was used to identify whether the person was recently infected. The LS-EIA was also used to identify primary HIV-1 infections among ACS participants who entered the ACS unaware of their HIV-positive status. The optical density cut-off value of this assay was set at 0.5 to select primary infections at the individual level with high specificity (B. Parekh, pers. comm.).

At the AMC primary HIV-1 infections were identified among people attending the HIV clinic. Diagnosis of primary HIV-1 infection was defined as detectable HIV-1 RNA load and/or detectable serum p24 antigen in plasma combined with one of the following test results: (1) HIV-1 specific antibody negative; (2) HIV-1 specific antibody-positive with a negative, incomplete or indeterminate western blot; (3) HIV-1 specific antibody positive and positive western blot, but with a negative HIV-1 ELISA documented in the preceding 180 days.

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Sequence analysis of drug resistance and subtypes

Population-based nucleotide sequence analysis of the HIV-1 polymerase (pol) gene was performed using the first HIV-1 RNA-positive sample available. The 5’ half of the reverse transcriptase (RT) gene was sequenced for all samples; and for those collected since 1996, when the protease inhibitors became commonly available, codons 1–99 of the protease (PR) gene were sequenced as well. From 1996 until 1999, a home-brew assay was used, described earlier by de Jong et al. [14]. From 1999 until mid 2002 a second home-brew assay was used as described by Boom et al. [15]: viral RNA was isolated from either 200 µl or 1 ml plasma. The plasma was prepared from blood samples collected in
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Evacuated tubes containing ethylenediaminetetraacetic acid. The choice of volume was based on the HIV-1 load: of samples with high loads (>50,000 HIV-1 RNA molecules/ml), 200 μl was used as input. Purified RNA was reverse-transcribed using MMLV-RT (Invitrogen, Breda, The Netherlands) with 4 ng of reverse transcription primer (3'-RT-OUT: 5'-TCTACTTT GTCCATGATGCCCTC-3' pos HIV-1-BRU: 3974). The entire RT mixture was added to the first PCR mixture containing 100 ng of primer 5'Prot-I (5'-AGGCTATTTTTTATTGGAAGCATCTGGCCTTCC-3' pos HIV-1-BRU: 1624) and 100 ng of primer 3'ET21 (5'-AGCTGCTACTATTTTCTTTGGCTATCAGGTTG-3' pos HIV-1-BRU: 3930). A 35 cycle PCR was performed. A nested PCR of 25 cycles contained 5 μl of the first PCR. 100 ng of primer 5'Prot-II (5'-TCAGACGAGACCGAGCCCAACAGC-3' pos HIV-1-BRU: 1718) and 3'RT20 primer (5'-CTGCCAGCTTACCTGCTGCTCCTC-3' pos HIV-1-BRU: 3020). Ten microlitres of the PCR products was analysed by agarose gel electrophoresis. The PCR products were subsequently sequenced with internal primers and the BigDye terminator reagent. Electrophoresis and data collection was performed on an ABI 377 instrument. Protease and RT sequences were assembled using the AutoAssembler DNA sequence Assembly software version 2.0 (Applied Biosystems, Foster City, California, USA). With samples collected since mid-2002, the Viroseq HIV-1 genotyping kit version 2 was used (Abbott Laboratories). Electrophoresis and data collection have performed on an ABI 3730 instrument since mid-2003.

Resistance-conferring mutations were screened for at the amino acid sites described by the International AIDS Society–USA [16]. Alternative substitutions at position 215 (T215S/C/D/E/N/I/V), which represent transitional forms between wild-type and the resistance-conferring mutations Y and F, were included as major drug-resistance mutations [9,17–19]. Subtypes were identified by phylogenetic analysis of RT and protease sequences, using reference sequences from the Los Alamos database [20] and our own database. One thousand bootstrap replicates were performed under the Kimura 2-parameter model [21] using the neighbour-joining method [22] in MEGA [23]. For bootstrap values under 85, SimPlot [24] software was used to determine the subtype.

**Plasma HIV-1 RNA and CD4 cell count**

HIV-1 RNA levels in the first HIV-1 RNA-positive sample available were measured using commercially available assays (NASBA HIV-1 RNA QT and NucliSens; bioMérieux and Versant HIV-1 RNA 3.0, Bayer, New York, USA) according to the manufacturer’s instructions. Absolute CD4 cell counts were measured with a FACS flow cytometer in all blood samples used for sequencing from the AMC patients, and in the first-available HIV-seropositive sample from the ACS patients.

**Statistical methods**

The AMC versus the ACS participants, the infections with resistance mutations versus those without, and the subtype-B versus non-B infections, were compared with respect to baseline characteristics using the Mann–Whitney U test, the Pearson chi-square test with a continuity correction, the Fisher’s exact test and a linear regression model. A logistic regression model was used to test for trends in resistance over time and to perform multivariate analysis. P < 0.05 was considered statistically significant.

**Results**

**Study population**

During the period 1994–2002, 74 ACS participants acquired an HIV-1 infection during follow-up, of whom five had a seroconversion interval longer than 18 months. Using the LS-EIA, one of these five participants (20%) was identified as a primary infection. In addition, 106 participants entered the ACS unaware of their HIV-1 positive status, of whom four (4%) were identified as primary infections by LS-EIA. Sequencing was successfully performed for 68 (92%) of in total 74 (69 + 1 + 4) primary infections. For three seroconverters the last HIV-1 antibody-negative but RNA-positive sample was used for sequencing; whereas for the others the first HIV-1 antibody-positive sample was used. At the AMC 32 primary infections were identified, and all had the HIV-1 polymerase gene sequenced.

Table 1 shows characteristics of the 100 (68 + 32) participants of our study. The 68 ACS participants were mainly of Dutch origin, and the median age at seroconversion was 32 years; 38 were MSM and 30 DU, of whom eight were female. Of the 30 seroconverters from the DU cohort, three had never injected drugs. In the first RNA-positive sample, the median HIV-1 RNA load was 4.7 log10 copies/ml, and the median CD4 cell count was 580 × 106 cells/l. For the 63 seroconverters in the ACS, the median interval between the last HIV antibody-negative samples and the samples from which an RT-sequence was obtained was 5.7 months; this interval was 6.0 months for the protease-sequences. The 32 AMC participants were mainly of Dutch origin, and their median age at seroconversion was 39 years; 23 were MSM, and nine were heterosexual, of whom three were female. Six patients had an RNA-positive but antibody-negative sample, 24 had an incomplete western blot, and two had an antibody-negative test less than 180 days prior to the antibody-positive test. At the first RNA-positive
sample, the median HIV-1 RNA load was 5.5 log_{10} copies/ml, which was significantly higher than in the ACS cases (P < 0.01). This most likely reflects the acute phase of infection of the AMC participants, which is characterized by a high viral load. The median CD4 cell count was 520 \times 10^6 cells/l, which did not differ significantly from the ACS participants. Fever has been described as a symptom of primary infection that predicts HIV-1 disease progression [25]. Of the MSM participating in the ACS, 45% (17 of 38) reported fever in the 6 months prior to their first antibody-positive visit, whereas among AMC patients, 82% (23 of 32) reported a recent fever episode. DU were left out of this analysis as drugs can both enhance and diminish symptoms.

Genotypic analysis of drug resistance and subtypes
Sequences of the RT and protease genes were obtained for, respectively, 100 and 83 participants. In 2002, a MSM and a male DU were found to be infected with strains having identical pol genes. As later samples of both patients still showed a very high similarity, erroneous mixing of samples can be ruled out. The DU was known to have sexual contacts with men. Strains that were non-B for both RT and protease genes were found in only one MSM (subtype CRF01_AE) from the ACS and five of the AMC patients. The latter were Dutch heterosexuals of whom four reported a non-Dutch partner from, respectively, Uganda (subtype A), Thailand (subtype CRF01_AE), Russia (subtype A), and Italy (subtype C). In the first three cases, the subtype found corresponds with the prevalent subtype in the respective country. The individual that did not report a non-Dutch partner was infected with subtype CRF02_AG. In addition, the strain in one infection from the AMC was a recombinant having a subtype B RT sequence and subtype CRF02_AG protease. This heterosexual patient’s origin and country of infection appeared to be Ghana, where subtype CRF02_AG is the prevalent subtype and recombinants are common.

Resistance to any ART was found in 13 of 100 primary infections [13%; 95% confidence interval (CI), 7–21%]. These included three of 32 patients from the AMC (9%; 95% CI, 2–25%), and eight of 63 seroconverters from the ACS (13%; 95% CI, 6–24%); and two of five patients identified by LS-EIA (40%; 95% CI, 5–85%). No significant difference was found in the prevalence of resistant infections between the ACS and the AMC. No resistance mutations were detected in the six non-B subtypes or the recombinant subtype. The respective resistance-conferring mutations are shown in Table 2. There were two infections with strains bearing mutations associated with resistance to nNRTIs (2%; 95% CI, 0–7%), one with a strain resistant to PIs (1%; 95% CI, 0–5%), and ten with a strain resistant to NNRTIs (10%; 95% CI, 5–18%).
There were no infections with multi-drug-resistant strains.

The proportion of resistant infections per calendar year are shown in Figure 1. In the years 1998, 2000 and 2002, none of the primary infections showed mutations conferring resistance to any ART. Leaving out the non-B subtypes did not substantially change our results. The proportion of primary HIV-1 infections with drug-resistant strains decreased over calendar time. Before 1998, 20% (95% CI, 10–34%) of 50 primary infections involved a virus bearing drug-resistant mutations versus only 6% (95% CI, 1–17%) of 50 primary infections after 1998 ($P = 0.074$). A linear logistic regression model with calendar time as a continuous variable showed a significantly declining trend in the proportion of transmitted resistance (odds ratio $= 0.75$ per year; 95% CI, 0.58–0.96). After adjustment for risk group, gender, age, or route of recruitment the odds ratio became 0.68 per year (95% CI, 0.50–0.94). These factors did also not change significantly over time. However, the declining trend was not significant for the ACS alone (adjusted odds ratio $= 0.78$ per year; 95% CI, 0.55–1.13). Addition of a quadratic term did not significantly improve the fit of the models.

The median plasma HIV-1 RNA level in the first RNA positive-sample was significantly ($P = 0.036$) lower for the individuals infected with a resistant strain [4.4 (IQR, 3–5.1) log$_{10}$ copies/ml, tested for 11 of 13 infections] versus the individuals infected with a non-resistant strain [5.0 (IQR, 4.3–5.5) log$_{10}$ copies/ml, tested for 85 of 87 infections]. Using linear regression this significant relation was robust to adjustment for potential confounders such as the interval between estimated date of infection and the first HIV RNA-positive sample (within the ACS), the type of viral load assay, age, sex, or risk group. For those participants with a (pre-treatment) follow-up sample available (median time since estimated infection-date was 7.6 (IQR, 6.4–9.8) months, the viral load was comparable for both groups in univariate and multivariate analysis. Individuals bearing resistant versus non-resistant strains did not significantly differ by CD4 cell count, presence of fever at the time of acute infection, sex, risk-group, or definition of primary infection.

**Discussion**

In this study we assessed the prevalence of drug-resistant strains among primary HIV-1 infections in Amsterdam.
Amsterdam. During the observation period, from 1994 to 2002, the overall prevalence was 13%. However, the longitudinal pattern shows considerable variation. The first strain that carried a mutation (L100I) conferring resistance to nNRTIs was transmitted to an ACS drug user in 1994. Since this mutation was never seen among HIV-infected cohort participants before the first trials of nNRTIs, it most probably arose during mono-therapy trials with nevirapine or efavirenz in the early 1990s. As drug users were not involved in those trials, a two-step transmission seems likely, although a natural polymorphism cannot be excluded. The only transmission of a virus conferring resistance to PIs was found in 1998; this mutation probably arose during PI-mono-therapy trials in 1994–1995, prior to the introduction of highly active antiretroviral therapy (HAART).

Adherence to ART is likely to be relatively poor among DU, but we did not find this risk group to be related to the proportion of resistant HIV-1 transmission. An explanation might be that HAART is more frequently used among MSM than in DU [26]. HAART is widely used in Amsterdam since it became generally available in 1996. For the HIV-positive participants of the ACS between 1996 and 2002: NRTIs were used in 37% of MSM and in 39% of DU, PIs in 51% of MSM and in 22% of DU, and nNRTIs in 33% of MSM and in 11% of DU.

We found that the overall proportion of resistant HIV transmission in Amsterdam decreased after the introduction of HAART in 1996. Furthermore, the number and type of resistant mutations per resistant strain decreased, and could result from earlier transmissions by people on zidovudine mono-therapy [27]. Our results differ from reports from the USA [1–3] and the UK [4], where transmission of drug-resistant HIV increased in recent years, and transmission of multi-drug-resistant strains were reported. This geographical variation might be explained by differences in study design, such as sample size, study population and the resistance table used. Other important differences may relate to social–cultural factors, such as the physicians readiness to prescribe therapy, access to therapy, the history of mono-therapy among the treated HIV-1 population, adherence and risk behavior. Our study was not designed to evaluate such factors. However, the fact that in 1990 the Dutch government nominated a few specialist hospitals to concentrate HIV care and the prescription of ART by specialists might have played a role.

The conditions for transmission of drug-resistant strains are present in Amsterdam, and may increase over time. Of patients on HAART in the Netherlands, 37% experienced virological failure during follow-up [28]. In 64% of the patients from whom a sequence was obtained after virological failure, drug-resistant mutations were detected [29]. In addition, unsafe sex is increasing among both the HIV-uninfected and -infected MSM population in Amsterdam [30], which may result in a rise in transmission of resistant strains. Fortunately, people on failing therapy probably switch to another therapy that keeps HIV RNA levels low.

Although numbers are small, none of the seven participants infected with a non-B subtype harboured a resistant strain. This finding is consistent with another study in the Netherlands among 41 non-B mainly recently diagnosed patients [29]. The HIV epidemic among MSM and DU still seems uninfluenced by non-B subtypes. In accordance with a previous report [31], most non-B subtypes in our study were introduced through heterosexual transmission by people from countries where these subtypes are prevalent. However, it is possible that non-B subtypes do spread in networks that remained unidentified within our study among primary infections.

In six cases no sample was sequenced due to there being a viral load that was too-low, which could be a result of resistance mutations that reduce the viral fitness [32]. Indeed, we found a significantly lower viral load in initial samples from patients infected with a resistant strain versus infections by wild-type virus. No difference in viral load was found in follow-up samples, which were taken after the viral peak at the beginning of an infection. In the short term, resistance may be associated with a loss in viral fitness, but this loss may be counteracted over time by subsequent evolution [33]. We did not find a significant difference in symptomatic fever between infections with resistant and non-resistant HIV strains. We found no significant difference in frequency of resistant infections between the ACS and the AMC, and thus can not certify if hospital-based studies are indeed biased against resistant strains. One of the limitations of our study is that the number of individuals included in our study is relatively small, and more research is needed to gain insight into this hypothesis. Nevertheless, our results are unique because within the ACS it was possible to study transmission without any bias due to symptomatic infections.

In conclusion, we found the transmission of drug-resistant HIV-1 in Amsterdam to be decreased since the introduction of HAART. Yet the conditions for their transmission are present, and argue strongly for continued monitoring.

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References


