HIV-1 superinfection in homosexual men
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Chapter 4

**Low incidence of HIV-1 superinfection even after episodes of higher-risk sexual behavior of homosexual men in the Amsterdam Cohort Studies on HIV Infection and AIDS**

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**Abstract**

**Background** HIV-1 superinfection is infection of an already HIV-1 seropositive individual with another HIV-1 strain. The rate at which HIV-1 superinfection occurs might be influenced by sexual behavior. This suggests that superinfection might be detected more often by analyzing longitudinal samples collected from time periods during which HIV-1 positive individuals reported unsafe sexual behavior.

**Material and methods** Longitudinal serum samples that were obtained around self-reported sexual risk periods from 15 homosexual therapy-naïve men participating in the Amsterdam Cohort Studies on HIV Infection and AIDS were investigated for HIV-1 superinfection by sequencing multiple env C2-C4 and gag PCR products from viral RNA in serum. Maximum likelihood (ML) phylogenetic analysis was performed to determine whether HIV-1 superinfection had occurred upon higher-risk sexual behavior.

**Results** We studied a total of 24 serum samples from the 15 study participants with a median of 8 samples and a median of 5.8 person-years (PY) of follow-up per patient. ML phylogenetic analysis on 907 C2-C4 env and 672 gag sequences revealed one potential case of HIV-1 superinfection, resulting in a superinfection incidence rate of 1.1% per 100 PY.

**Conclusions** Based on the detection of only one potential case of HIV-1 superinfection, we conclude that incidence of HIV-1 superinfection is low in this subgroup of homosexual men who reported higher-risk sexual behavior during their natural course of infection. This may point to a combination of host and external risk factors involved in absence of HIV-1 superinfection. Further studies are required to determine and estimate the impact of each factor necessary to establish HIV-1 superinfection.

**Introduction**

Detection of HIV-1 superinfection is commonly based on highly laborious investigations, which has probably contributed to the relatively low number of close to 50 HIV-1 superinfection cases that have been documented worldwide [1-23]. Detection of HIV-1 superinfection is critically dependent on the sampling frequency and the time period of sampling as superinfecting strains may be present only transiently. Therefore, approaches taken thus far with cross-sectional cohort screenings or analysis with limited follow-up time may have resulted in an underestimation of HIV-1 superinfection incidence rates and to the statement that HIV-1 superinfection occurs at substantial lower rates compared to initial HIV-1 infection. Additionally, HIV-1 superinfection was suggested to cause an accelerated HIV-1 disease progression [1, 3-6, 8-10, 15]. However, without methods and study approaches ensuring high sensitivity of detecting HIV-1 superinfection, any conclusions on clinical consequences of HIV-1 superinfection remain speculative.
HIV-1 superinfection incidence rates differ between reports, from absent HIV-1 superinfection [24, 25, Rachinger et al, in press] to equal rates of HIV-1 superinfection and initial HIV-1 infection [9, 13, 18, 20]. The rate at which HIV-1 superinfection occurs might presumably depend on sexual risk behavior and on HIV-1 prevalence within sexual networks. Hence, differences in reported HIV-1 superinfection incidence rates might depend on a combination of detection sensitivity, sampling time and frequency and factors influencing occurrence of HIV-1 superinfection.

More recent studies showed that analysing two viral genome parts compared to one and investigation of more than one time point increased the number of detected cases of HIV-1 superinfection [18, 20, 22]. Risk in sexual behavior, such as higher number of sexual partners, receptive anal intercourse and no condom use are known factors independently enhancing the risk for HIV-1 initial infection [26, 27]; these factors might prove to be involved in acquisition of HIV-1 superinfection as well.

In this study, longitudinal samples isolated before, during and after self-reported periods of higher-risk sexual behavior from 15 therapy-naïve homosexual men participating in the Amsterdam Cohort Studies on HIV Infection and AIDS (ACS) were studied for evidence of HIV-1 superinfection.

**Patients and Methods**

*Patient population*

Enrolment for a prospective study on the prevalence and incidence of HIV-1 infection and risk factors for AIDS among homosexual men started in October 1984. 961 asymptomatic men who were living in the Amsterdam area and who reported having had at least two homosexual contacts in the preceding six months were enrolled [28]. In the first serum sample taken, 728 men tested negative for HIV-1 antibodies; 141 of these men subsequently seroconverted during active follow-up between 1984 and 1997. Every three months, clinical data were collected and serum and peripheral blood mononuclear cells were cryopreserved. Written informed consent is obtained from every participant. The Amsterdam Cohort Studies on HIV Infection and AIDS are conducted in accordance with the ethical principles set out in the Declaration of Helsinki and were approved by the Medical Ethics Committee of the Academic Medical Center. Behavioral data were collected from structured questionnaires conducted at 6-monthly intervals, representing behavior in the preceding six months.
RNA isolation, RT-PCR and PCR amplification

Viral RNA was isolated from 140 μl serum (Qiamp Viral Mini Kit, Qiagen, Hilden, Germany) and eluted in 50 μl from which 10 μl RNA was reverse transcribed into cDNA (SuperScript™ First strand synthesis system, Invitrogen, Carlsbad, CA) using the env-specific primer Seq2 (5’-TCCTCCATATCTCCTCCTCCAGGTC-3’) and the gag-specific primer gag outer rev (5’-GCCTGTCTCTCAGTAC-3’), respectively. For generating the env C2-C4 fragment primer couples Seq2 - Seq3 (5’-TATGGGATCAAAGCCTAAAGCATG-3’) and Seq5 (5’-GTCAACTCAAACGTGCTTAAATGGC-3’) - Seq6 (5’-ATCTAATTGTCCACTGATGGGAGG-3’) were used for the first- and second-round PCR, respectively (GoTaq Flexi DNA Polymerase, Promega, Madison, WI). For amplification of gag fragments, the primer couples gag fw (5’-CGACGCAGGACTCGGCTTGCTG-3’) - gag outer rev (5’-GCCTGTCTCTCAGTAC-3’) and BssHIIfw (5’-TGCTGAAGCGCCGCACGGGC-3’) - p17rev (5’-CAAAACCTCTGTGCTTATGG-3’) were used in the first- and second-round PCR, respectively (GoTaq Flexi DNA Polymerase, Promega, Madison, WI). For both fragments 5 μl cDNA input was used for the first-round PCR, and 2 μl first-round PCR product as input in the second-round PCR. Both, first- and second-round PCR reactions were carried out in a total volume of 25 μl and the amplification for both PCR was as follows: one cycle at 94°C for 5 minutes, 35 cycles at 94, 50 and 70°C of respectively 45, 30 and 90 sec, followed by a final elongation step of 10 min at 70°C resulting in the final PCR products env C2-V3-C3-V4-C4 (549 nucleotides, HXB2 positions 7012-7560) and gag (1175 nucleotides, HXB2 positions 704-1878) respectively.

Molecular cloning and sequencing of C2-C4 env and gag

From each env and gag PCR-positive serum sample a median of three second-round PCR products was cloned (range, 1-4 (env) and 2-4 (gag)) into the pGEM T Easy Vector System (Promega, Madison, WI), transformed into competent DH5α E.coli (Invitrogen, Carlsbad, CA) and plated on LB agar using blue/white screening. White colonies were picked at random (2-16 colonies per plate). The vector primers T7 (5’-TAATACGACTCTATAGGG-3’) and SP6 (5’-GATTAGGTTGACACTATAG-3’) were used to amplify the cloned env and gag PCR products with the above described PCR program. After purification (ExoSAP-IT, USB, Cleveland, OH), PCR products were sequenced with T7 and SP6 (env) and T7, SP6, p17fw (5’-GCTAAACCGTAGGCTGGGACCATC-3’) and p24_5I (gag) (Big Dye terminator v1.1 cycle sequencing kit, Applied Biosystems, Foster City, CA). Two to 16 colonies per cloning reaction were sequenced. Sequences were determined with an automated DNA sequencer (Applied Biosystems, Foster City, CA).
Phylogenetic analysis

Clonal sequences were aligned using the ClustalW algorithm [29] and alignments were manually edited in BioEdit (BioEdit, version 7.0.5.3; Ibis Biosciences). Reference panels consisting of published and unpublished sequences generated from homosexual participants of the ACS were added to the merged patients’ env and gag alignments. The best-fitting nucleotide substitution models for each region were chosen with Modeltest v3.7 [30] using the hierarchical likelihood tests (hLRTs). Subsequently, phylogenetic analyses were performed using PAUP*4.0 [31]. Neighbour-Joining (NJ) trees were inferred for env and for gag, followed by a heuristic search for Maximum Likelihood (ML) trees with the best-fit substitution models and subtree-pruning-regrafting (SPR) as the branch-swapping algorithm. Statistical support for nodes was generated with bootstrapping on the NJ trees (1000 repeats). The ML trees were rooted with the earliest sequence derived from ACS participants and displayed with Dendroscope [32].

Results

Selection of HIV-1 seroconverters with higher-risk sexual behavior

Questionnaires from therapy-naïve seroconverters of the ACS who seroconverted during active follow-up in the cohort studies between March 1986 and June 1997 (n=141) were screened for indicators of higher-risk sexual behavior defined as self-reported unprotected anal intercourse (UAI) with two or more partners, and/or self-reported syphilis, and/or self-reported gonorrhoea. A risk period was defined as a time period in which higher-risk sexual behavior was reported. If a time period between questionnaires reporting higher-risk sexual behavior was less than 9 months, this was considered as one risk period, if it was more than 9 months, this was considered as two risk periods. In total, 51 seroconverters of the ACS reported 93 risk periods with higher-risk sexual behavior. In 90 periods, UAI or syphilis or gonorrhoea was reported, in two periods a combination of syphilis and gonorrhoea was reported, and in one period a combination of UAI and syphilis and gonorrhoea was reported. Of these 51 individuals, 26 reported one risk period, 13 two risk periods, seven three risk periods and five seroconverters reported four risk periods during their course of HIV-1 infection. For analysis we selected seroconverters with a more pronounced risk behavior: we selected five seroconverters with four risk periods, five seroconverters with three risk periods, and from the group of seroconverters reporting one or two risk periods, we selected 5 seroconverters with the highest numbers of self-reported higher-risk sexual behavior (3 to 5) in a single risk period (Figure 1, Table 1). Around these risk periods (optimally before, during and after), serum samples were selected for analysis (Figure 2).
Figure 1: Approach for patient selection and identification of HIV-1 superinfection in a subset of 15 homosexual men of the Amsterdam Cohort Studies on HIV Infection and AIDS (ACS) exhibiting higher-risk sexual behavior. Seroconverters were screened for indicators of higher-risk sexual behavior: unprotected anal intercourse with two or more partners, and/or self-reported syphilis and/or self-reported gonorrhea. 5 seroconverters reporting 4 risk periods, 5 reporting 3 risk periods and 5 reporting 1 or 2 risk period(s) with ≥ 3 questionnaires reporting unprotected anal intercourse with ≥ 2 partners, and/or self-reported syphilis, and/or gonorrhea were selected for analysis. SC: seroconverters.
Table 1: Patients’ characteristics, samples, number of risk periods and risk(s), sequences and putative cases of HIV-1 superinfection

<table>
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<tr>
<th>Patient</th>
<th>Date of SC</th>
<th>Median VL in investigated samples</th>
<th>Number of risk periods</th>
<th>Number of questionnaires with UAI, and/or self-reported syphilis or gonorrhea per risk period *</th>
<th>Number of investigated samples</th>
<th>Total follow-up time [years] *</th>
<th>env sequences</th>
<th>gag sequences</th>
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<td>8.2*10^4</td>
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<td>C</td>
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<td>8</td>
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<td>37</td>
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<td>12</td>
<td>5.3</td>
<td>73</td>
<td>58</td>
<td></td>
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* Unprotected anal intercourse (UAI) with two or more partners in the previous six months and/or self-reported syphilis and/or self-reported gonorrhea were used as indicators of higher-risk sexual behavior. SC: Seroconversion. VL: Viral load. Median VL in copies/mL plasma. * Total follow-up time: time between 1st and last time point analyzed. S: Syphilis. G: Gonorrhea.
Figure 2: Behavioral risk periods, number of risks per risk period and selected serum samples of patients A to O over time from seroconversion onwards. Displayed are self-reported risk periods by the 15 study participants (patients A to O), minimum number of risks per risk period and selected serum samples around risk periods. Risk periods are boxed in gray. Number of risks (unprotected anal intercourse with ≥ 2 partners), and self-reported syphilis or gonorrhea is displayed within the respective risk period. SC: seroconversion. *: investigated serum sample. Samples of interest but with viral loads <1,000 copies/mL were not chosen for analysis. For further information on patients and/or risk see table 1.
**Serum samples**

We studied cryopreserved serum samples with detectable viral load (VL>1,000 copies/mL plasma) that were obtained from 15 therapy-naïve homosexual participants (Table 1). In total, 124 serum samples, with a median of eight samples per patient (range, 4-12, Table 1) were analyzed from time points around (optimally before, during and after) the behavioral risk periods (Figure 2). A sample selected following one risk period could have served as the sample before the next risk period. If viral load at one time point of interest was undetectable, the respective sample was not investigated. Total follow-up time between 1st time point and last time point analyzed was 88.3 person-years (PY) with median time periods of 5.8 PY per patient (range, 3.7-8.8). Median viral load (VL) in the serum samples used for analysis varied per patient from $7.7\times10^3$ – $8.2\times10^4$ copies/mL (median VL of $2.8\times10^4$ copies/mL, Table 1). 119 of the 124 serum samples were analyzed in env (96%), and 110 of the 124 serum samples were analyzed in gag (88%, Table 1), due to negative PCR in env or gag for single serum samples.

**Phylogenetic analysis of C2-C4 env and gag reveals one potential case of HIV-1 superinfection**

A total of 907 HIV-1 env sequences and 672 HIV-1 gag sequences were included in the phylogenetic analysis with a median of six env (range, 4-15) and six gag (range, 3-12) sequences per time point. These sequences were combined with env and gag reference sets comprising published and unpublished local sequences. The best-fit nucleotide substitution models (env: K81uf+I+G, gag: TVM+I+G) were applied to a ML topology for each dataset, applying the subtree-pruning-regrafting (SPR) heuristic search algorithm. The derived env ML tree displayed monophyletic clustering of 14 of the 15 patients, consistent with absence of HIV-1 superinfection in these patients. Sequences derived from patient “I” were retrieved in two distinct clusters, suggesting HIV-1 co- or superinfection (Figure 3). The gag ML tree showed monophyletic clusters of sequences derived from all 15 patients. In disagreement with the env tree topology, gag sequences from patient “I” were forming a monophyletic cluster (Figure 4).
Figure 3: Maximum likelihood tree of env reference sequences and sequences generated from serum samples derived from 15 homosexual men of the Amsterdam Cohort Studies. The tree topology supports HIV-1 superinfection in one of the 15 patients with sequences of 14 patients clustering monophyletically (circled in gray) and sequences of the potentially superinfected patient "I" being retrieved in two distinct clusters (two clusters of sequences derived from patient "I" circled in black ("initial" and "superinfection")). The tree is rooted with the earliest sequence derived from ACS participants. All patient clusters are supported by bootstrap values above 70%, based on 1000 replicates of NJ calculations. Scale is represented as nucleotide changes per site.
Figure 4: Maximum likelihood tree of gag reference sequences and sequences generated from serum samples derived from 15 homosexual men of the Amsterdam Cohort Studies. The tree topology supports absence of HIV-1 superinfection in all 15 patients with sequences clustering monophyletically per patient (patient “I” sequences cluster circled in black, clusters from all other patients’ sequences circled in gray). The tree is rooted with the earliest sequence derived from ACS participants. All patient clusters are supported by bootstrap values above 70%, based on 1000 replicates of NJ calculations. Scale is represented as nucleotide changes per site.
HIV-1 superinfection of patient "I" after self-reported risk period

In patient "I", HIV-1 superinfection was concluded based on the detection of two distinct sequence clusters in the env ML tree with cluster 1 containing all sequences derived at time point 8 and cluster 2 containing all sequences generated from time points 27, 38, 41, 42, 43 and 44 which argued for occurrence of HIV-1 superinfection after time point 8 in November 1986, at least five months after seroconversion. The superinfecting strain was first detected at time point 27 in August 1991, five years after seroconversion and following a period of 3.1 years in which the patient repressed viral replication to undetectable levels (Figure 5). Patient "I" reported a behavioral risk period from December 1986 until June 1988, one month after the initial strain was detected for the last time in this patient. In disagreement with the env phylogeny, all gag sequences from this patient clustered together, pointing to a recombination event after superinfection. As gag and env sequences at each time point were derived from the same serum samples, HIV-1 superinfection is more likely than sample switch to account for the presence of two distinct viral strains in this patient over time. However, further analysis is required to confirm presence and timing of HIV-1 superinfection in this patient.

Figure 5: Viral load (log RNA copies/mL plasma) versus time when a blood sample was obtained for HIV-1 superinfected patient "I" and self-reported risk episodes. Arrows indicate the time points when the superinfecting strain was detected in env. The number of sequences of the initial and the superinfecting strain is indicated below the viral load graph. TP: time point. n.a.: not available (negative PCR). Filled circles: samples investigated. Non-filled circles: samples not investigated.
Discussion

With one putative case of HIV-1 superinfection detected in 15 individuals over a total of 88.3 PY, we observed a low incidence rate of HIV-1 superinfection (incidence rate: 1.1% per 100 PY, 95% CI: 0.06-5.59) upon self-reported episodes of higher-risk sexual behaviour by HIV-1 positive homosexual men in the Amsterdam Cohort Studies between 1986 and 1997. To our knowledge, this is the first investigation that searched longitudinally before, during and after time periods with self-reported sexual risk behaviour in two viral genome parts for the presence of HIV-1 superinfection. The one putative case of HIV-1 superinfection was solely detected in env, pointing to a recombination event following superinfection, with the initial strain detectable in env at one time point at five months after seroconversion, and the superinfecting strain first detected in env at five years after seroconversion. This patient reported a behavioral risk period of 1.5 years from the time onwards when the initial viral strain was last detectable. In this patient, superinfection would have been missed if only gag would have been investigated underscoring the importance of multiple genome region analysis for increasing chances to detect HIV-1 superinfection. Depending on when exactly the superinfection has occurred, superinfection was either followed by a period of three years of undetectable plasma viral load or might have resulted in increased viral loads upon superinfection. Further in-depth analysis of specimen isolated from the patient is warranted to confirm HIV-1 superinfection in this patient and to be able to draw conclusions on the date of superinfection and possible effects on disease progression.

While we tried to increase odds of detecting HIV-1 superinfection in this study by studying longitudinal samples covering time periods of higher sexual risk behavior, small minority strains or strains present only transiently at untested time points may have remained undetected. In addition, our selection of 51 patients who reported periods with unsafe sexual behaviour may have been sub-optimal. Sexual risk behavior was defined as having had unprotected anal intercourse with two or more partners within one risk period and/or self-reported syphilis and/or gonorrhea. However, we did not select study participants based on the actual number of sexual partners. Moreover, at the time, questionnaires did not include the number of sex acts with each partner, the HIV status of a partner nor the viral load of an HIV-1 positive partner at the time of sexual intercourse. We did include self-reported syphilis and gonorrhea as indicators for unsafe sexual encounters, both factors known to increase susceptibility for HIV-1 infection (reviewed in [33]), possibly also involved in acquisition of HIV-1 superinfection. Sexually transmitted infections (STI) might follow an asymptomatic disease course remaining unnoticed to the patient (reviewed in [33]) leading to underestimation of the presence of STI in this cohort (Lambers et al, unpublished data) and to omission of patients with increased risk for
HIV-1 superinfection. However, neither have we selected study patients solely for STI, nor were the patients who reported syphilis and/or gonorrhea identified as HIV-1 superinfected.

In conclusion, analysis of longitudinal serum samples isolated from 15 individuals reporting periods with higher-risk sexual behavior resulted in detection of one possible case of HIV-1 superinfection. This low incidence of HIV-1 superinfection despite relatively high-risk behavior may point to a multitude of factors involved in acquisition of HIV-1 superinfection. Although research has provided contradicting evidence for the impact of adaptive immunity on resistance against HIV-1 superinfection [2, 34-36], other yet unknown host factors might play a role in establishment of HIV-1 superinfection and being also involved in the low superinfection incidence rate in this subgroup of patients selected for higher-risk sexual behavior. Willberg et al have reported on a correlation between exposure to HIV-1 during receptive anal intercourse and adaptive immunity [37]. In our study, we have selected patients with the most pronounced self-reported risk behavior in the ACS increasing the likelihood of having included patients who might have had developed stronger adaptive immune responses at the time when serum samples were analysed. Additionally, the dimensions of unsafe sexual behavior necessary to establish systemic HIV-1 superinfection have not been addressed yet. It is possible that the sexual risk behavior of homosexual men in the Amsterdam Cohort Studies during the early period of the HIV-1 epidemic was too low to result in more cases of HIV-1 superinfection and that the higher-risk sexual behavior for which we have selected in this study, might still not be high enough to sufficiently promote HIV-1 superinfection, maybe in combination with other factors. This might be supported by the majority of superinfection cases being detected in cohorts of female sex worker [7, 10, 13, 14, 18, 20] reporting a 10-30 fold higher number of sexual partners [18, 10] than homosexual men of the ACS in which HIV-1 superinfection was absent at 1 year after seroconversion (Rachinger et al, in press). In conclusion, the low incidence of HIV-1 superinfection in this study may point to a combination of host and external factors involved in absence of HIV-1 superinfection. Additional longitudinal studies are needed to estimate the impact of each factor necessary to establish HIV-1 superinfection.
Acknowledgements

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