Impact of antiretroviral therapy on HIV-1 transmission dynamics

Bezemer, D.O.

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Transmission networks of HIV-1 among men having sex with men in the Netherlands

Daniela Bezemer¹, Ard van Sighem¹, Vladimir V. Lukashov², Lia van der Hoek², Nicole Back², Rob Schuurman³, Charles A.B. Boucher³,⁴, Eric C.J. Claas⁵, Maarten C. Boerlijst⁶, Roel A. Coutinho⁷,⁸, Frank de Wolf⁹,¹⁰ for the ATHENA observational cohort

Abstract

Objective: To obtain insight in the HIV-1 transmission networks among men having sex with men in the Netherlands.

Design: A phylogenetic tree was constructed from polymerase sequences isolated from 2877 HIV-1 subtype B infected patients monitored in one of the 24 HIV treatment centres in the Netherlands as part of the ATHENA national observational cohort.

Methods: For men having sex with men with a known date of infection, the most similar sequences were selected as potential transmission pairs when they clustered with bootstrap value ≥ 99%. Time from infection to onward transmission was estimated as the median time between dates of infection for each transmission pair. The source of infections with a resistant strain was traced using the entire phylogenetic tree.

Results: Of sequences from 403 men having sex with men with a known date of infection between 1987 and 2007, 175 (43%) formed 63 clusters. Median time to onward transmission was 1.4 years (IQR 0.6-2.7). Twenty-four (6%) men having sex with men carried a virus with resistance-related mutations, 13 of these were in 8 clusters together with sequences from 28 other patients in the entire phylogenetic tree. Six clusters contained sequences obtained from 29 men all presenting the same resistance-related mutations.

Conclusions: Onward transmission of HIV-1 from infected men having sex with men happens both during and after primary infection. Transmission of resistant strains from the antiretroviral-therapy-treated population is limited, but strains with resistance-related mutations have formed sub-epidemics.

¹HIV Monitoring Foundation, Amsterdam, The Netherlands; ²Department of Medical Microbiology, Academic Medical Centre, Amsterdam, The Netherlands; ³Department of Virology, University Hospital Utrecht, The Netherlands; ⁴Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands; ⁵Department of Medical Microbiology, Leiden University Medical Center, The Netherlands; ⁶Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, The Netherlands; ⁷Department of Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands; ⁸Center for Infectious Disease Control, National Institute of Public Health and the Environment, Bilthoven, The Netherlands; ⁹Department of Infectious Disease Epidemiology, Imperial College London, United Kingdom
Impact of antiretroviral therapy on HIV-1 transmission dynamics

Introduction

Despite the success of combination antiretroviral treatment (cART) in reducing viral load, HIV-1 transmission continues among men having sex with men (MSM) in industrialised countries, including the Netherlands [1, 2]. Using a mathematical model to describe trends in the transmission dynamics of HIV-1 amongst MSM in the Netherlands, we recently estimated that 90% of onward transmission in this risk group takes place from the undiagnosed group. The average time from infection to diagnosis was 2.7 years [1]. However, the impact of primary infection on onward transmission remains unclear. As usually no diagnostic tests are performed during this initial phase of the infection when the viral load peaks and infectiousness is high [3, 4], the rate of partner change may be crucial for epidemic spread [5].

Despite discrepancies, HIV sequence analysis can reveal information on contact networks [6]. Based on the phylogenetic clustering of HIV-1 polymerase (pol) sequences obtained from primary infections, previous studies suggested that 25% to 50% of transmissions among MSM take place during primary infection [7-9]. However, clustering of sequences obtained from primary infections does not necessarily represent transmission during the period of primary infection [10]. Lewis et al applied a Bayesian Monte Carlo Markov Chain method on sequences obtained from 402 patients without a known date of infection, and estimated that 25% of transmissions took place within the first 6 months of infection [11]. But in this study assumptions needed to be made to estimate the dates of infection. We estimated the median time between infection and onward transmission for potential transmission pairs selected from a phylogenetic tree of HIV-1 subtype B pol sequences obtained from 403 MSM shortly after their known date of infection.

Further insight in transmission dynamics of HIV-1 can be obtained by investigating the transmission networks of strains with resistance-related mutations [12]. Transmission soon after infection facilitates transmission of resistant HIV-1 strains, which would revert to the original wild type within a few months in absence of antiretroviral therapy [8, 13-15]. Certain resistance-related mutations revert to a new wild type, a process that is well documented for amino acid position 215 in reverse transcriptase (RT) [13, 16]. In previous studies, we found that 6% of new infections presented resistance-associated mutations, and that 23% of ART-naive patients failing cART were infected with a resistant strain [17, 18]. Several studies reported phylogenetic clustering of resistant strains obtained from ART-naive patients [9, 15, 19]. We used the set of HIV-1 subtype B pol sequences obtained from 403 MSM with a known date of infection to monitor the transmission of resistant strains over calendar time, and linked these resistant strains to the source of infection in a phylogenetic tree of all 4090 HIV-1 subtype B pol gene sequences obtained from 2877 infected patients, both before and after starting ART. All patients were monitored in one of the 24 HIV treatment centres in the Netherlands as part of the ATHENA national observational cohort.
Methods

Database
The ATHENA cohort encompasses all patients infected with HIV-1 followed longitudinally in one of the 24 HIV treatment centres in the Netherlands [20]. Demographic data were collected at entry in the cohort. At each follow-up visit, clinical, virological, and immunological data were collected, as well as data on the use of cART [1, 18, 21]. HIV-1 pol gene sequences were obtained as part of the screening for resistance to antiretroviral drugs, both before and during treatment with cART [22-24].

Sequences
Patients were eligible for this study if at least one pol sequence was available containing at least the first 251 amino acids of the RT gene. Population-based nucleotide sequencing of the HIV-1 pol gene was performed as described in detail previously [17]. Sample contamination was checked for at the respective sequencing sites. Multiple sequence alignment was done by hand and using the default parameters of the ClustalX 1.83 program. Subtype B was identified by phylogenetic analysis of combined RT and protease sequences, using reference sequences from the Los Alamos database [25] and our own database. The percentage of ambiguous sites was estimated for all sequences. Sequences were screened for major resistance-conferring mutations at the amino acid positions described by the International AIDS Society-USA, including alternative substitutions at position 215 [26].

New HIV-1 infections
For this study, new HIV-1 infections were defined as those infections with either a seroconversion interval of ≤18 months between the last negative and the first positive HIV-1 serology test, or documented evidence of a primary infection. A diagnosis of primary HIV-1 infection was defined as detectable HIV-1 RNA and/or detectable serum p24 antigen in plasma combined with either a negative HIV-1 antibody-test or a positive HIV-1 antibody-test with a negative, incomplete, or indeterminate HIV-1 Western Blot. The estimated date of infection was defined as the midpoint between the last seronegative and the first seropositive sample, or the date of the last seronegative but RNA-positive sample, or the date of an indeterminate result on the Western blot [17]. Sequences corresponding to new infections were obtained from a sample taken ≤18 months after the estimated date of seroconversion.

Distance method
The percentage pairwise sequence distances between all available entire protease sequences and RT sequences cut to equal lengths of 251 amino acids were calculated taking into account ambiguous sites according to a mixed weighted distance method [27]. To study the level of mixing of MSM HIV-1 transmission networks with transmission via other routes, we used a
pairwise sequence distance ≤1.5% between RT sequences as a selection criterion for potential transmission pairs [18, 28]. Transmission clusters were defined as groups of linked potential transmission pairs.

**Phylogenetic analysis**

Phylogenetic trees were constructed of pol gene RNA sequences that included at least the first 251 amino acids of the RT gene. Trees were constructed with the Neighbor-Joining method [29, 30] within the MEGA program [31], and ambiguous sites were ignored. To prevent false clustering due to convergent evolution, 36 amino acid sites associated with major drug-resistance were excluded [26]. When available, multiple sequences per person were included. All trees were rooted against an HIV-1 subtype K sequence (Los Alamos Database accession number AJ249235).

**Potential transmission pairs**

The first phylogenetic tree included all HIV-1 subtype B pol sequences obtained from therapy-naive MSM within 18 months after their estimated date of infection. The Maximum Composite Likelihood method was used to compute evolutionary distances, and a bootstrap analysis with 1000 replications was performed. From this tree, a selection was made of patients in clusters with a bootstrap value ≥99% [18, 28, 32]. Each patient in this selection was combined with the other patient in the same cluster with the smallest pairwise sequence distance to form the most likely transmission pair. The time between infection and onward transmission was estimated as the median time between dates of infection of all these potential transmission pairs. The correlation between the pairwise sequence distance and time between the dates of infection of transmission pairs was estimated from linear regressions.

**Tracing the source of resistant strains**

In order to trace the infecting source of the transmitted resistant strains with a known date of infection, a second phylogenetic tree was made that contained all subtype B sequences available in the ATHENA database. For the construction of this tree, the Kimura 2-parameter model was used [33], and a bootstrap analysis with 100 replications was performed. The clusters observed were confirmed in a smaller tree of all sequences that clustered with a resistant strain with a known date of infection. Evolutionary distances in this smaller tree were computed using the Maximum Composite Likelihood method with a gamma distribution of the substitution rate with shape parameter 1.0 [34], and 1000 bootstrap replications were performed. The clusters were studied with the aim to identify whether patients on treatment transmitted the resistant strain.
Results

Patient characteristics
By June 2007, 12,951 persons infected with HIV-1 were included in the ATHENA national observational cohort; 6845 (53%) were reported to have been infected by MSM contact. In total, 4090 HIV-1 subtype B \textit{pol} gene sequences were obtained from 2877 persons, of whom 2022 (70%) were MSM, 486 (17%) were infected via heterosexual contact, 167 (6%) via drug injection, and 202 (7%) via other or unknown transmission routes. The first sequence of each patient was obtained between 1987 and 1996 for 101 (3.5%) patients, between 1996 and 2000 for 503 (17.5%), between 2001 and 2004 for 1114 (38.7%), and after 2004 for 1159 (40.3%) patients. Of 832 patients who had a non-B HIV-1 infection and a sequence available, only 68 (8%) were MSM.

Transmission of HIV-1 between MSM and other risk groups
HIV-1 subtype B RT sequences from 817 (28%) out of 2877 persons clustered within a sequence distance of 1.5% from another person’s RT sequence. Of these 817 patients, 603 (74%) were MSM, 91 (11%) were heterosexuals, 51 (6%) were infected via injection drug use, and 72 (9%) were infected via other or unknown transmission routes. Only 8% of MSM had an infection with HIV-1 that was most similar to that found in patients from other risk-groups.

Table 1. Characteristics of 403 MSM identified with an HIV-1 subtype B infection with a known date of infection in the period 1987 – 2007.

<table>
<thead>
<tr>
<th>N</th>
<th>403</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age at estimated time of infection, in years</td>
<td>34.8 (IQR 29.9 – 41.7) (range 21.0 – 61.9)</td>
</tr>
<tr>
<td>Percentage born in The Netherlands</td>
<td>81</td>
</tr>
<tr>
<td>Number of acute infections</td>
<td>107</td>
</tr>
<tr>
<td>Median interval between the last antibody-negative and the first RNA positive visit, in months</td>
<td>6.4 (IQR 4.9 – 9.9), for 296 seroconverters</td>
</tr>
<tr>
<td>Median interval between the estimated date of seroconversion and the sequenced sample, in months</td>
<td>3.3 (IQR 1.3 – 6.2)</td>
</tr>
<tr>
<td>Median interval between the first RNA-positive visit and the sequenced sample, in months</td>
<td>0.4 (IQR 0.0 – 1.4)</td>
</tr>
<tr>
<td>Median plasma HIV-1 RNA concentration at first RNA positive sample (copies/ml)</td>
<td>78495 (IQR 16976 – 250729)</td>
</tr>
<tr>
<td>Median CD4 count at diagnosis (10^6 cells/l)</td>
<td>0.49 (IQR 0.36 – 0.67)</td>
</tr>
<tr>
<td>Percentage ambiguous sites</td>
<td>0.08 (IQR 0.0 – 0.4)</td>
</tr>
<tr>
<td>Median percentage pairwise nucleotide sequence distance of 403 new infections’ pol sequences</td>
<td>5.5% (IQR 4.9 – 6.1; range 0.0 – 9.7)</td>
</tr>
</tbody>
</table>
New infections
From 403 (20%) of the 2022 MSM with a subtype B pol sequence, the approximate date of HIV-1 antibody seroconversion was known (Table 1). The number of new infections by year of seroconversion is shown in Figure 1A. For 393 MSM, the pol gene was sequenced including both protease and RT, whereas for 10 MSM only RT was available. The median percentage of ambiguous sites among the 403 sequences was low (0.08%, interquartile range [IQR] 0.0 – 0.4), which allowed for calculation of pairwise sequence distances either including or excluding these sites. Of the 403 MSM, 292 (72%) were monitored in an HIV treatment centre in Amsterdam, and 294 (73%) reported that they were most likely infected in the Netherlands. Of the 42 MSM infected before 1996, 64% were prospectively identified within the Amsterdam Cohort Studies [17].

Clustering among new HIV-1 subtype B infections
We constructed a phylogenetic tree of 499 available pol sequences from 403 MSM with at least one RT sequence within 18 months of their estimated date of infection. The tree showed 63 transmission clusters with a bootstrap value ≥99%, including 175 (43%) patients (Figure 2A). Both HIV-1 protease and RT sequences were obtained for all 175 patients. The clusters were confirmed in a separate phylogenetic tree containing sequences of the 393 MSM with both protease and RT sequenced. The size of the clusters varied from 2 to 8 patients (median 3, IQR 2 – 4). The median minimum number of nucleotide substitutions per site per person within each cluster was low (0.0035, IQR 0.0014 – 0.0065; range 0 – 0.0158), indicating clustering of potential transmission pairs [28]. The time between the two most distant dates of infection in each cluster ranged from 0.05 to 9.7 years (median 2.0, IQR 0.9-4.2). The median difference in time between the dates of infection of the most likely transmission pair for all patients in a cluster was 1.4 years (IQR 0.6–2.7, range 0.03 – 9.05). The corresponding median pairwise sequence distance was 0.9% (IQR 0.4 – 1.5), and increased by 0.33% (95% confidence interval [CI] 0.28-0.38; p<0.0001) per year of separation in time. The distribution of the median difference in time between the dates of infection of the most likely transmission pair for all patients in a cluster is shown in Figure 2B. Constraining the analysis to transmission pairs with a pairwise sequence distance ≤1.5% or a synonymous sequence distance ≤4.5%, showed that the median difference in time varied between 1.1 and 1.4 years (Table 2, #2-7). Undetected transmissions related to individuals with unknown date of infection do not impact these estimates (Table 2, #9).

Transmission of resistant HIV-1 subtype B strains
Figure 1A shows the annual percentage of infections with a resistant virus strain among all 403 MSM with a new HIV-1 subtype B infection. In total, 24 patients (6.0%, 95% CI 3.8 – 8.7%) were infected with an antiretroviral drug-resistant strain. Of these 24 patients, 18 (4.5%) had one or more mutations associated with resistance to nucleoside RT inhibitors, 2 (0.5%) patients were resistant to non-nucleoside RT inhibitors, 2 (0.5%) to a protease inhibitor, and 2 (0.5%) to two or three drug classes. Fourteen of 24 resistant strains (58%) had a mutation at position
215 in RT. Figure 1B shows the annual percentage of infections with HIV-1 strains with a resistance-conferring mutation at RT position 215, separated into 215 resistant mutations (215 Y or F) and 215 revertant mutations (215 C, D, E, or S). After 1996, only revertant mutations were found at position 215.

**Figure 1.** Annual proportion of sequences with resistance-associated mutations among 403 MSM with a new HIV-1 subtype B infection.  
A. Percentage of patients resistant to antiretroviral drugs from each drug class. NRT: nucleoside RT inhibitor, NNRTI: non-nucleoside RT inhibitor, PI: protease inhibitor, MD: Multidrug. The 95% confidence intervals are given above each bar. The annual number of infections is denoted below the calendar year on the horizontal axis. Eight of 10 patients with only a RT sequence were infected in 1994 and earlier; the other two were infected in 1997 and 2002, thus only in years when no resistant mutations were found in the available protease sequences of new infections. Thus, the percentages in Figure 2 are correct, even though the corresponding number of new infections (n) in these years does not refer to the number of people with a complete corresponding pol sequence.  
B. Percentage of patients infected with a strain harbouring a resistance-associated mutation (m215) or a revertant mutation (r215) at position 215 in RT.
Figure 2. Transmission clusters of HIV-1 strains obtained from MSM with a known date of infection

A. Phylogenetic tree of HIV-1 subtype B polymerase sequences belonging to 403 MSM patients with an identified new HIV-1 subtype B infection. The significant transmission clusters with bootstrap values ≥99 are shown in red. Blue denotes the reference subtype K sequence. Phylogenetic analyses were conducted in MEGA4.

B. The distribution in time between the dates of infections of the most likely transmission pair for all patients in a significant cluster. Light grey bars represent the percentage per category of 3 months, and dark grey bars per 6 months.
Networks of transmitted resistant HIV-1 subtype B strains

Based on a phylogenetic analysis of all 4090 HIV-1 subtype B pol sequences in the ATHENA database, we selected sequences from 88 persons that clustered with the sequences obtained from the 24 MSM who were newly infected with a resistant HIV-1 strain. Phylogenetic analysis of this subset of 152 sequences of 112 patients showed 8 significant clusters (Figure 3). The 8 clusters contained sequences of 13 (54%) of the 24 newly infected patients who had a resistant strain, including 7 with a bootstrap value ≥ 99, and 1 with a bootstrap value of 95 but a pairwise sequence distance of the closest sequence pair of only 1.0%. The same clusters were also identified when clusters where based on connections between potential transmission pairs with a mixed weighted distance ≤ 1.5% at RT, except for 3 patients (M24, M31, and M54). Sequences from the other 11 patients infected with a resistant strain did not cluster significantly with other
sequences in the database. One of them (M30), appeared to be infected with an HIV-1 strain resistant to three drug classes and reported the possibility of having been infected by someone from outside the Netherlands. Both M33 (infected in 1994) and M34 (infected in 2002) might have been infected by someone on treatment as their HIV strains contain the RT mutations 70R and 184V, respectively, known for mutating back to wild type in the absence of antiviral therapy [13].

**Figure 3.** Transmission networks of HIV-1 strains with resistance-related mutations.
A phylogenetic tree constructed of HIV-1 subtype B polymerase sequences from 24 new resistant infections among MSM and 88 infections selected from all 2877 subtype B infections. Analyses were conducted in MEGA4. New infections are coded as M#-RCYEAR, which codifies respectively; M = MSM; # = unique number; R = resistant; C = the code to which phase the sequences belong: P = primary infection, S = seroconverter, N = therapy-naïve, T = during treatment, I = during treatment interruption; and YEAR = the estimated calendar year of infection. (N)NRT = (non-) nucleoside reverse transcriptase; PR = protease; µ = amino acid mutation conferring antiretroviral-treatment resistance. Undated infections are coded similarly but without YEAR. H instead of M represents heterosexual transmission. In the tree, the 24 new resistant infections from Figure 1 are represented in bold, and resistance-conferring mutations are given either in the table when part of significant cluster, or following the branch name in the tree. In the table, the dates (SEQUENCE) and mutations (MUTATION) of the clustering sequences are specified, as well as the dates of the first HIV-positive test, the dates of the first ART, and the dates of the first cART regimen of the patients. Patients selected from Figure 1 are indicated in yellow, and the dates of sequences corresponding to new infections are shown in yellow. Sequences labelled ‘RT only’ in the first column do not cluster, but they are not part of the phylogenetic tree presented.
Twenty-one persons in three clusters had sequences with a revertant mutation at position 215 in RT, specifically 215C in cluster 1 and 8, and 215S in cluster 5. The mutant strain with 215C and 219E at RT in cluster 1 might have been circulating for a period of 15 years, i.e. the period between the earliest and the latest date of diagnosis in the cluster. No pre-treatment HIV-1 RT sequence was available from the earliest diagnosed patient (M9) in this cluster. However, the sequence obtained from M9 at the time of therapy failure harboured the same 215 revertant mutant as the other sequences in the cluster. A pre-treatment RT sequence from patient M11 diagnosed in 1989 that was linked to cluster 1 with a bootstrap value of 80 did not show any resistant mutation. Cluster 2 was linked to one other antiretroviral-naive patient (M16) infected with a strain that harboured a 219Q mutation. In cluster 3, the resistance-associated mutation 33F in HIV-1 protease obtained from patient M22 was not present in any of the other 8 persons in the same cluster, and was possibly a natural polymorphism [35]. Cluster 4 was neither linked to other persons with a sequence with a 210W mutation in RT, nor to persons on cART at the time of infection. Clusters 6 and 7 possibly show a direct transmission from someone failing treatment with a 215Y mutation in cluster 6, and a 215F mutation in cluster 7. In cluster 6, the selection of a resistant strain in the potential initial source failing therapy (M52) was observed.

Discussion

Our study on transmission networks of HIV-1 subtype B among MSM in the Netherlands indicates that 25% of onward transmissions occur within 7 months after infection, half of transmissions within 17 months, and 75% within 2.7 years. This finding is compatible with our previous analysis of the dynamics of the HIV-1 epidemic among MSM in the Netherlands, in which we estimated that individuals who were unaware of their infection were the source of 90% of new infections, with an average of 2.7 years between infection and diagnosis for those infected after 2000 [1]. Our estimate of the median time between transmissions, for which we used only sequences corresponding to infections with an approximate known date of infection, is in agreement with a study by Lewis et al, who estimated the time between the nodes in a tree of sequences with an unknown date of infection [11]. They reported an estimated 25% of transmissions taking place within the first 6 months of infection and 50% within 14 months after infection. This might indicate that the HIV-1 transmission dynamics amongst MSM in the Netherlands and the United Kingdom are similar.

However, phylogenetic studies on transmission dynamics have shortcomings. Discrepancies have been demonstrated to arise when comparing the viral phylogeny with known sexual-contact networks [6]. Our study contained a subset of infections with an estimated date of infection that clustered and formed likely transmission pairs, although it cannot be excluded that there were intermediate transmissions. In addition, distinguishing people who infected more than
one person is not always feasible, and individuals that were infected by a common source within a short time period might cluster as a transmission pair. Furthermore, transmission dynamics are known to vary over calendar time [1], yet we estimated an average transmission rate. The search for new infections was intensified in the later years of our study, resulting in more available sequences, and thus the identification of more transmission pairs in these years. In addition, a significant proportion of patients identified as newly infected were familiar with their HIV-positive status after their first positive test, and thus soon after infection. Consequently, their behaviour may differ from those diagnosed at a later stage of infection.

In several countries, the overall transmission of resistant HIV-1 strains is reported to have decreased since the introduction of cART in 1996 [17, 36-38]. This has been explained by the efficacy of cART and the lower transmission potential of resistant HIV-1 strains [8, 9, 13, 39-41]. We found that 6% of HIV-1 subtype B strains in new infections among MSM had resistance-related mutations. Tracing the source of these resistant strains showed clusters with mainly transmission of HIV-1 with a revertant mutation at position 215 of RT. These observations are compatible with the estimate that the 215 RT revertant mutations have no significant fitness effect on the fitness of the virus [13, 16]. In contrast to an incidence of 20% among new transmissions in 1994, the 215 mutants were not found after the introduction of cART in 1996 until 2003, except for one case in 1999 [40]. However, from 2003 onwards, revertant mutants were present among new infections in all subsequent years. This could be due to the introduction of baseline sequencing around that time, but it might also be a result of changing transmission dynamics. Previously, we estimated that transmission decreased in the early cART era, but a resurgence of the epidemic occurred in later years [1]. Thus the reappearance of the revertant 215 RT mutation among recent transmissions may be a sentinel for changing transmission dynamics [42, 43]. Additionally, the HIV-1 incidence is increasing among older MSM, suggesting the possibility of a re-opened reservoir [44]. However, the initial resistant mutations at position 215 were only observed before the introduction of cART, which reflects the contribution of patients failing monotheapy.

Sampling methods might influence the monitoring of transmitted resistance since viruses from failing patients are often sequenced retrospectively. Contact tracing on an individual basis might also have an impact as recent partners of a person are identified. The cluster of sequences obtained that way could have an impact on the percentage of resistance found in the respective year of infection (whether or not with a resistant strain) that was larger than expected when sampling was random. We reduced these effects by only selecting infections with a known date of infection.

In conclusion, our study indicates that onward HIV-1 transmission from infected MSM takes place both during and after primary infection. Strains with resistance-related mutations have
formed sub-epidemics, and transmission of resistant strains from the antiretroviral-treated population is limited. However, with the current changes in risk behaviour among people using cART [45-47] transmission from the treated population might increase. Intensifying contact tracing and facilitating frequent testing could help to identify people earlier in their infection and prevent onward transmission.

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