Molecular epidemiology of hepatitis B in the Netherlands
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CHAPTER 3

HBV AMONG RISK GROUPS
Chapter 3.1

Ongoing transmission of a single hepatitis B strain among men having sex with men in Amsterdam

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Abstract

For the past decade, a specific hepatitis B virus (HBV) genotype A strain has been prevalent among men having sex with men (MSM) in Amsterdam, the Netherlands. At what point in time this strain was introduced in the MSM population, and why only this specific strain continues to be transmitted, remains unclear.

Between 1984 and 2003, sera of 1862 MSM were retrospectively screened for anti-HBc in the context of the Amsterdam Cohort studies. After 2003, most MSM participating in this study were vaccinated, making further testing less useful. HBV DNA (S-gen, 672 nt.) of the anti-HBc seroconverters was amplified and sequenced. Poisson regression was used to test for temporal trends in HBV and HIV incidence.

Of the 1042 MSM who were negative for anti-HBc at entry, 64 had seroconverted during follow-up at a median age of 32. At the moment of seroconversion, 31 MSM were HIV positive. HBV incidence declined dramatically in the first years and then remained stable throughout the study period. The HBV and HIV incidence ran almost parallel. With the exception of 3 MSM, all were infected with genotype A; 15 of those (41%) were infected with an identical genotype A strain.

For the past two decades, an identical genotype A strain has been circulating among MSM in the Netherlands. Although HBV is generally considered more infectious than HIV, this study shows that the trend and magnitude in HBV and HIV incidences among MSM are similar.
Introduction

In countries where HBV is highly endemic, most transmissions take place at birth or during early childhood. In most western countries where HBV endemicity is low, HBV transmission is mainly restricted to risk groups, such as drug users (DUs), commercial sex workers (CSWs), and men having sex with men (MSM). The Netherlands is such a low endemic country, where sexual transmission, especially between men, is the main route of HBV infection [1;2]. The overall HBsAg prevalence in the Netherlands is estimated to be 0.3-0.5%, whereas for MSM it is estimated to be up to ten times higher [3].

Previous studies have shown that in the past decade a single genotype A strain has been circulating among MSM in Amsterdam, despite the introduction of a nationwide vaccination program also targeted at MSM [4;5]. At what point in time this strain was introduced in the MSM population, remains unclear. As yet there is also no explanation regarding why only this specific genotype A strain continues to be transmitted.

To obtain more insight into the circulation of this genotype A strain among MSM, a retrospective study was undertaken of MSM who participated in the Amsterdam Cohort Studies (ACS). The ACS is an ongoing, prospective, open cohort of MSM initiated in 1984 [6]. To obtain better insight into the strains that are actually being transmitted, only strains from MSM who had seroconverted for anti-HBc during follow-up were included in the phylogenetic analysis. The S-gene (672 nt.) of those who had seroconverted for anti-HBc during follow-up was amplified and sequenced.

HBV incidence is often estimated or based on the reported number of acute HBV infections. The significant number of asymptomatic acutely infected patients makes it difficult to achieve a reliable estimation. The ACS gave us an opportunity to study the trend in HBV incidence among MSM over a 19-year period - including both symptomatic and asymptomatic infected individuals, by allowing us to retrospectively screen the entire cohort on anti-HBc. In addition, we compared HBV and HIV incidence among MSM in Amsterdam to determine whether HIV awareness in the early 1980s had the same decreasing effect on HBV incidence as it had on the HIV incidence.

Methods

Study population

The Amsterdam Cohort Studies recruits MSM at the Clinic for Sexually Transmitted Infections (STI) in Amsterdam, and by word-of-mouth. Participation is voluntary, and a written, informed consent is obtained at intake. Until 1995, healthy men of all age groups, who had had at least two male sexual partners in the previous 6 months, and lived in or around Amsterdam, were recruited. After June 1995, only men under 30 were recruited and in February 1996 the follow-up of men over 35 years was discontinued. Every 3 to 6 months, these MSM come to Amsterdam’s Public Health Service for a follow-up visit. At each visit, a standardized questionnaire about their risk behaviour, medical history, and socio-
demographic situation is administered by public health nurses and blood is collected for laboratory testing and storage. HIV positive MSM are referred to a general internist. MSM with at least 2 follow-up visits between 1984 and 2003 were included in our analyses. As of 2003, all participants in the Amsterdam Cohort Studies were systematically vaccinated against HBV, making the measurement of anti-HBc redundant after January 2003.

**Serology**

To study the HBV incidence, stored blood samples were retrospectively tested for anti-HBc (AxSYM Core, Abbott, Germany; and Hepanostika, Organon Technika, the Netherlands [7]). When MSM tested negative for anti-HBc on entering the ACS, the sample taken at their last visit was also tested. If this sample tested anti-HBc positive, samples between the first and last cohort visit were tested to identify the moment of seroconversion. ACS participants are routinely screened for HIV at each visit by means of an ELISA with western blot confirmation (HIV blot version 2.2, Genelab Diagnostics, USA).

**Isolation, amplification and sequence analysis**

The HBV genotypes of anti-HBc seroconverters were determined by means of sequence analysis. HBV DNA was isolated from 100 µl serum taken at every last anti-HBc negative and every first anti-HBc positive visit, using 600 µl lysis buffer and 700 µl isopropanol. The isolated DNA was amplified in a semi-nested PCR. PCR products were precipitated with 100% ethanol and subsequently sequenced in both directions, using the ABI BigDye Termination v1.1 kit. The sequencing products were purified using a DyeEx spin kit and analyzed in an ABI 310 genetic analyzer. The PCR and sequencing conditions, as well as the various primers used, have been described [4].

**Phylogenetic analysis**

A 672-nucleotide fragment of the pre-S2 and S region was used for sequence alignment, using the BioEdit 5.0.9 software [8]. Neighbor-joining phylogenetic analysis was carried out on the nucleotide alignments provided by the MEGA-3.1 software package [9]. Nucleotide distances were calculated according to the Kimura 2-parameter model. Phylogenetic reproducibility was estimated by means of a bootstrap analysis with 1000 replicates. The nucleotide sequence data have been deposited in the GenBank sequence database under accession numbers FJ561406-FJ561445.

A Bayesian skyline plot was used to detect trends in the transmission of HBV [10]. The plot-construction procedure was implemented in the BEAST software package [11]. Likelihoods were calculated using the HKY+I model for molecular evolution and a skyline prior of 5 steps. Two simulations were carried out and continued for $10^7$ updates, compared for convergence, and pooled after 10% had been discarded as burn-in.

**Statistical analyses**

The participant follow-up time was calculated from the date of study entry until the anti-HBc seroconversion, vaccination, or end of follow-up. The date of HBV seroconversion was
estimated as the midpoint between the last anti-HBc seronegative visit and the first anti-HBc seropositive visit. Those who did not seroconvert were censored on their last visit, the date of the third vaccination, or, ultimately, on 31 December 2002. A Poisson regression analysis was used to model the trend in HBV incidence over time. Both HBV and HIV incidence were allowed to change flexibly over time, using restricted cubic splines. The characteristics of the participants entering the cohort negative for anti-HBc were compared with those who entered the cohort positive for anti-HBc. The characteristics of participants of whom sera were available for sequencing were compared with those of participants of whom no sera were available by applying the Student’s t-test, Mann-Whitney U test, and the Chi-square test. All analyses were undertaken using SPSS 15.0 and the Design package of the R statistical program [12;13].

Results

General characteristics
Of the 2084 MSM entering the cohort between 1984 and 2003, 1862 had at least two follow-up visits and were included in this study. Their median age on entering the cohort was 30 years (Table 1). At entry, 820 (44%) were already anti-HBc positive; those who were anti-HBc positive were older than those susceptible to HBV (P<0.01). The proportion of participants with a Dutch nationality was respectively 77% and 86% for those entering the cohort positive and negative for anti-HBc (P<0.01). Injecting drug use was not commonly practiced by these MSM; only 6 MSM (0.5%) of the entire cohort had ever injected drugs. Drugs, not injected, that were used, were cannabis (43%), XTC (19%), cocaine (13%), amphetamine (6%), and nitrite (46%). MSM who were anti-HBc positive at entry, had a significantly higher intake of cannabis, XTC, cocaine, and nitrite (P<0.01). However, information on XTC usage was only available for a small part of the total study population. The majority of MSM practiced anal intercourse (82%). MSM who were anti-HBc positive when entering the cohort were more involved in sexual risk behaviour before entering the cohort than the MSM entering the cohort naïve for HBV. This is reflected by a significantly higher proportion of anti-HBc positive MSM practicing unprotected anal intercourse, reporting an STI during the past 5 years, and being HIV positive (all P<0.01). This is also reflected by anti-HBc positive MSM’s median reported number of sexual partners in the past six months compared to those of MSM susceptible for HBV, being respectively 11 and 5 (P<0.01). The most frequently reported STI was gonorrhoea – 46% overall. Overall HIV prevalence at entry was 29% (N=531).

Incidence
Of the 1042 MSM who were negative for anti-HBc at entry, 64 had seroconverted for HBV during their follow-up. The median age at HBV seroconversion was 32 years (IQR: 26-38). The median time between the last anti-HBc negative visit and the first anti-HBc positive visit was 3.0 months (IQR: 2.1-5.7 months). At the time of seroconversion, 31 (48%) MSM were
Table 1. General characteristics of MSM participating in the Amsterdam Cohort studies, who had more than one follow-up visit at entry, 1984-2002.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Total</th>
<th>Anti-HBc-negative</th>
<th>Anti-HBc-positive</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>1862</td>
<td>1862</td>
<td>1042 (56%)</td>
<td>820 (44%)</td>
<td></td>
</tr>
<tr>
<td>Median age (IQR)</td>
<td>1862</td>
<td>30 (25-36)</td>
<td>27 (23-32)</td>
<td>34 (29-40)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Dutch nationality (%)</td>
<td>1862</td>
<td>1498 (80.5)</td>
<td>883 (86.4)</td>
<td>615 (77.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Median duration of follow-up, years (IQR)</td>
<td>1862</td>
<td>6.1 (2.6-11.0)</td>
<td>5.8 (2.6-9.1)</td>
<td>6.4 (2.5-11.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Injecting drug use (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main drugs used in the past six months (not injected) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cannabis</td>
<td>1269</td>
<td>547 (43.1)</td>
<td>287 (37.9)</td>
<td>260 (50.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>XTC</td>
<td>544</td>
<td>101 (18.6)</td>
<td>74 (15.1)</td>
<td>27 (49.1)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1258</td>
<td>159 (12.6)</td>
<td>70 (9.3)</td>
<td>89 (17.6)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Amphetamine</td>
<td>1250</td>
<td>74 (5.9)</td>
<td>36 (4.8)</td>
<td>38 (7.6)</td>
<td>0.05</td>
</tr>
<tr>
<td>Nitrite (poppers)</td>
<td>1266</td>
<td>581 (45.9)</td>
<td>264 (35.0)</td>
<td>317 (62.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Anal intercourse (%)</td>
<td>1529</td>
<td>1246 (81.5)</td>
<td>691 (76.8)</td>
<td>555 (88.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Median number of partners in the past 6 months (IQR)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Unprotective anal intercourse (%)</td>
<td>1038</td>
<td>790 (76.1)</td>
<td>400 (66.9)</td>
<td>390 (88.6)</td>
<td>&lt;0.01</td>
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<tr>
<td>STI in the past 5 years (%)</td>
<td>1286</td>
<td>750 (58.3)</td>
<td>249 (47.1)</td>
<td>501 (66.2)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Syphilis in the past 5 years (%)</td>
<td>1216</td>
<td>301 (24.8)</td>
<td>66 (13.8)</td>
<td>235 (31.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Gonorrhoea in the past 5 years (%)</td>
<td>1215</td>
<td>562 (46.3)</td>
<td>166 (34.5)</td>
<td>396 (54.0)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Herpes in the past 5 years (%)</td>
<td>1815</td>
<td>197 (10.9)</td>
<td>90 (8.8)</td>
<td>107 (13.5)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HIV positive (%)</td>
<td>1862</td>
<td>531 (28.5)</td>
<td>179 (17.2)</td>
<td>352 (42.9)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HIV seroconversion during follow-up</td>
<td>173</td>
<td>87</td>
<td>96</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

positive for HIV. Two of these MSM had seroconverted for HIV and HBV during the same time interval. Specific risk factors for HBV seroconversion could not be identified, as there were too much missing data on risk behaviour at the moment of seroconversion, especially for HIV-positive men.

The HBV incidence declined strongly during the first few years of the cohort’s study period, remaining stable during the remaining period (Figure 1). In the early 1980s, the HIV incidence was somewhat higher; thereafter, the HBV and HIV incidence ran almost parallel.

The majority of HIV seroconversions was found among MSM who entered the cohort positive for anti-HBc (56%).

**Phylogeny**

Of the 64 seroconverters, the HBV DNA of 50 (78%) could be amplified, and the viral DNA of 40 of the 50 MSM could be sequenced. These participants’ age, year of HBV infection, and country of birth did not differ from those of whom the virus could not be sequenced (all P>0.05). Their HIV status differed significantly in that the proportion of HIV positive MSM was smaller in the group of participants of whom the virus could be sequenced compared to those of whom HBV could not be sequenced: 35% and 71%, respectively.

All, except 3 MSM of whom HBV could be sequenced, were infected with genotype A and 15 of those (38%) showed identical HBV S-gene sequences (figure 2). Infections with this
genotype A strain occurred throughout the study period and is still ongoing as is indicated by the Dutch MSM reference strains that were included in the phylogenetic tree. A second, smaller cluster could be identified within genotype A (N=5), however, the bootstrap value was lower than 70 and did therefore not differ significantly from the main strain. Other infections with genotype A seemed to be single introductions with no ongoing transmission. The Bayesian skyline plot was estimated from all sequences of MSM who had seroconverted for anti-HBc and who were infected with genotype A. During the study period, there was no indication of a change in the genetic diversity within genotype A of MSM, as there was no change in the trend and height of the Bayesian skyline plot. The phylogenetic signal in the data, which is used to estimate changes in genetic diversity over time, could have been weak due to the short genetic distance and because most seroconversions had occurred in the first decade of the study.

Two of three MSM infected with another genotype were infected with an identical genotype D strain, the other one being infected with a genotype F strain. The two genotype D strains could not be linked to other genotype D strains found among MSM or drug users in the Netherlands, but were identical to strains that were found among patients who could be linked to Morocco, as became evident from our national databank and from previous molecular epidemiological studies in Amsterdam (reference strains in figure 2) [1;4]. Both MSM infected with genotype D were born in the Netherlands, whereas the one infected with genotype F was born in Vietnam. This genotype F strain was identical to a strain that has
recently been shown to circulate in the Netherlands and probably has its origin in South America.

**Discussion**

In the mid-1980s, a steep decline in the HBV incidence could be observed among MSM, which also held for the HIV incidence. This decline reflects the change in sexual risk behaviour due to HIV/AIDS awareness. Between 1990 and 2003, the incidences of HBV and HIV ran parallel. Although HBV is generally considered to be more infectious than HIV, this study shows that for two decades the trends in HBV and HIV incidences among MSM were similar. In the early 1980s, the HIV incidence was even somewhat higher than the HBV incidence. At that time, the pool of susceptible MSM was smaller than it was for HIV, since HBV had been circulating among MSM for a much longer time than HIV. This resulted in a larger proportion of MSM who had been infected previously with HBV which was reflected by the high seroprevalence of anti-HBc among MSM. Another factor that may explain the incidences’ similar trend is that only a small proportion of MSM who contract HBV become chronically infected and, therefore, chronically contagious, whereas HIV-infected MSM all remain HIV positive and therefore a potential source of transmission, especially before the introduction of antiretroviral therapy. Furthermore, in the early 1980s, a HBV vaccine trial was undertaken among MSM in Amsterdam, during which over 1000 high-risk MSM were vaccinated, making the pool of susceptible MSM even smaller [14].

On the other hand, in the same time period, the HBV incidence among hard-drug users in Amsterdam was higher than the HIV incidence [15]. Furthermore, the decrease in the HBV incidence among drug users started much later in 1993, whereas the decrease in HIV incidence had already set in at the start of the ACS. In respect of the pool of susceptible and infectious MSM, HBV and HIV transmissions were at that time similar to those among drug users. A difference in the transmission route, for example, sexual versus blood borne, could explain the difference in the incidence’s trend. Another explanation might be that the reduction in sexual risk behaviour among drug users was only limited, unlike the reduction observed among MSM [16].

Our results demonstrate that a conserved genotype A strain has been circulating among MSM in Amsterdam for at least 25 years and probably much longer, since no change in genetic diversity has been observed. This particular strain has not only been demonstrated to circulate among MSM in Amsterdam, but also in the rest of the Netherlands [1,4]. The ongoing transmission of this particular strain among MSM is not restricted to the Netherlands, as it has also been found to circulate among MSM in Japan and is probably circulating among MSM in other western countries as well [17]. The transmission of this strain throughout other countries is likely, as specific strains of other viruses (hepatitis A and C) have been shown to circulate among MSM in Europe [18,19]. Other HBV genotypes and other genotype A strains were also introduced in the MSM population, but there seems to be no ongoing transmission of these strains. These were probably single, sporadic
Figure 2. Phylogenetic tree of the anti-HBc seroconverters among MSM participating in the Amsterdam Cohort Studies, 1985-2002 (shaded boxes). The last two digits of the participants' number indicate the year of HBV seroconversion. Only bootstrap values above 70 are depicted. Reference strains that indicate the different genotypes and strains from Dutch patients belonging to various risk groups are also included.
introductions from outside the main Dutch MSM population. Furthermore, the majority of MSM in the Netherlands with an active HBV infection are infected with this genotype A strain, making it less likely to get infected with another strain. Better fitness of this particular strain is unlikely to be the reason for the ongoing transmission among MSM, as other genotypes are able to maintain themselves equally well within the general population [1]. The genotype F strain with which one of the ACS participants was infected in 1998 has been observed to circulate in the Netherlands [1]. Although the majority of people acutely infected with this strain were MSM, it was not restricted to MSM, but was transmitted through various modes of transmission. This study, together with an earlier study in which two MSM were found to have been infected with this genotype F strain, demonstrates that this strain has been circulating in the Netherlands much longer than was previously assumed [4]. However, more research is needed to identify the exact source of this strain and the point in time that it was introduced in the Netherlands.

An advantage of screening a cohort of MSM retrospectively is that it enabled us to include both symptomatic and asymptomatic HBV-infected participants, resulting in a reliable calculation of the HBV incidence. A limitation of this study is that only the pre-S2 and S-gene were sequenced and used in the phylogenetic analysis. HBV is a conserved virus due to its overlapping open reading frames; consequently, analyzing only the S-region might not be conclusive enough, especially since genotype A is a common genotype in North-western Europe [20-22]. Sequencing a larger part or the entire genome, will probably give a more detailed impression on the circulation and variation of this particular strain [23].

Sexual contact, especially between men, is the most frequently reported mode of transmission in the Netherlands [1]. In contrast to findings in other countries, where MSM practice injecting drug use relatively more often, new HBV infections among MSM caused by injecting drug use are rare in the Netherlands [1;4,24]. This sexual transmission has resulted in circulation of an identical genotype A strain among MSM for more than two decades. Transmission of this strain did not terminate after the implementation of a nationwide vaccination program targeted at behavioural risk groups. This is not only true of Amsterdam, but also of the rest of the Netherlands and probably of other western countries as well. Collaboration is required between research groups throughout Europe to establish whether this strain is also circulating in other countries. Further transmission of this strain needs to be prevented by optimizing prevention strategies targeted specifically at MSM, as the current strategies fail to prevent HBV transmission among this group. However, in the long run universal HBV vaccination will probably solve this issue.

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**References**


Chapter 3.2

Two decades of hepatitis B infections among drug users in Amsterdam: are they still a high-risk group?

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Chapter 3

Abstract

In general, little is known about the incidence of hepatitis B virus (HBV) among drug users, especially among non-injecting drug users. Therefore, changes in incidence, risk factors, and circulating genotypes over time were determined among drug users in Amsterdam over an 18-year period (1985-2002).

Sera of 1268 drug users, both injecting and non-injecting, were screened for anti-HBc. HBV genotypes of the anti-HBc seroconverters were determined. Poisson regression was used to test for temporal trends in incidence and to identify risk factors for seroconversion.

Of the 598 participants who were anti-HBc negative at entry, 83 seroconverted for anti-HBc. The incidence of HBV declined from 5.9/100 Person Years up to 1993 to 0/100 Person Years in 2002. Of the drug users infected acutely, both injecting and non-injecting, 88% were infected with the same genotype D, serotype ayw3 strain. Multivariate analyses revealed current injecting, age, and calendar year of visit as independent risk factors.

The decline in the incidence of HBV among drug users in Amsterdam is probably caused by a decline in injecting behaviour. Injecting and non-injecting drug users were infected with the same strain, indicating that drug users infect one another, regardless of their risk behaviour. After 2000, no injecting drug users with an acute HBV infection were reported to the Public Health Service Amsterdam and the specific genotype D strain had disappeared. These findings suggest that drug users may no longer be a high-risk group for HBV infection in Amsterdam. However, trends in drug use need to be monitored.
Introduction

Of the estimated 2 billion people ever infected with the hepatitis B virus (HBV) worldwide, over 350 million are infected chronically. These chronic carriers are at high risk for developing cirrhosis and cancer of the liver, diseases that kill over a million people each year [1].

In countries where HBV is highly endemic, most transmissions take place at birth or during early childhood. In most western countries where endemicity of HBV is low, transmission of HBV is mainly restricted to certain risk groups, such as drug users, commercial sex workers, and men having sex with men. Hard drug users for whom drug use is problematic and not recreational - injecting drug users in particular - can easily contract and transmit HBV, due to their high-risk behaviour and the fact that the prevalence of HBV is much higher in this group than in the general population. Therefore drug use, injecting drug use in particular, is an important factor in the spread of HBV in low as well as in high-endemic countries, as drug use is not limited to industrialized countries.

In the Netherlands, the overall prevalence of HBsAg is estimated to be 0.2%, whereas for injecting drug users it is 4-6% [2,3]. However, injecting drug use has strongly decreased in the last decade, and so has the number of reported acute HBV infections among injecting drug users [4-6]. On the other hand, little is known about the transmission of HBV among non-injecting drug users, which seems to have become the largest group of drug users in the Netherlands [7]. Since most non-injecting drug users never come in contact with the healthcare system, tracking them is difficult, as is implementing prevention programs for this specific group.

In general, little is known about the incidence of HBV among both injecting and non-injecting drug users. Therefore, to see whether the incidence, risk factors, and circulating genotypes have changed over time among drug users in Amsterdam, a retrospective study of 18 years (1985-2002) was undertaken of participants of the Amsterdam Cohort Studies (N=1268). The cohort started in 1985 and is an ongoing, prospective, open cohort of hard-drug users including both injecting and non-injecting drug users [8].

This study was designed to give insight in the evolution of the transmission of HBV among drug users in Amsterdam over the past decades, and the extent to which drug users nowadays should be considered a high-risk group for an infection with HBV.

Methods

Study population

The Amsterdam Cohort Studies recruits drug users by means of local methadone outposts, sexually transmitted diseases clinics, and by word-of-mouth. Both injecting and non-injecting drug users, using hard drugs, e.g., heroin, cocaine, at least 3 times a week are included. Participation is voluntary, and a written informed consent is obtained at intake. Every 4 to 6 months, these drug users come to Amsterdam’s Public Health Service for a follow-up visit.
At each visit, a standardized questionnaire about their risk behaviour, health, and socio-demographic situation is administered by public health nurses and blood is collected for laboratory testing and storage. HIV positive drug users are examined by a physician. Drug users with at least 2 follow-up visits between 1985 and 2003 were included in the analyses. As of 2003, all participants of the Amsterdam Cohort Studies were vaccinated systematically against HBV, making the measurement of anti-HBc redundant after January 2003.

**Serology**

To study the HBV incidence, stored blood samples were tested retrospectively for anti-HBc (AxSYM Core, Abbott, Germany and Hepanostika; Organon Technika, the Netherlands [9]). When drug users tested negative for anti-HBc on entering the cohort, the sample taken at their last visit was also tested. If this sample tested anti-HBc positive, samples between the first and last cohort visit were tested to identify the moment of seroconversion. All participants were also screened retrospectively for anti-HCV (AxSYM HCV version 3.0, Abbott, Germany) [10]. There was no result for 10 participants. Participants of the ACS are routinely screened for HIV, using an ELISA with western blot confirmation (HIV blot version 2.2, Genelab Diagnostics, USA).

**Isolation, amplification and sequence analysis**

HBV genotypes of anti-HBc seroconverters were determined, by means of sequence analysis. HBV DNA was isolated from 100 µl serum taken at every last anti-HBc negative and every first anti-HBc positive visit, using 600 µl lysis-buffer and 700 µl isopropanol. The isolated DNA was amplified in a semi-nested PCR. PCR products were precipitated with 100% ethanol and sequenced in both directions, using the ABI BigDye Termination v1.1 kit. The sequencing products were purified using a DyeEx spin kit and analyzed in an ABI 310 genetic analyzer. The PCR and sequencing conditions, as well as the various primers used have been described [5].

**Phylogenetic analysis**

A 674-nucleotide fragment of the pre-S2 and S region was used for sequence alignment, using the BioEdit 5.0.9 software [11]. Neighbour-joining phylogenetic analysis was carried out on the nucleotide alignments as provided by the MEGA-3.1 software package [12]. Nucleotide distances were calculated according to the Kimura 2-parameter model. Phylogenetic reproducibility was estimated by bootstrap analysis with 1000 replicates. The nucleotide sequence data have been deposited in the GenBank sequence database under accession numbers EU581953-EU582017. The serotypes of the HBV-strains were deduced from amino acids at positions 101-180 [13].

**Statistical analyses**

The participant follow-up time was calculated from the date of study entry until anti-HBc seroconversion, vaccination, or end of follow-up. The date of HBV seroconversion was estimated as the midpoint between the last anti-HBc seronegative visit and the first anti-HBc
seropositive visit. Those who did not seroconvert were censored at their final visit, the date of the third vaccination, or ultimately at 31 December 2002. Incidence rates were calculated by person time methods. Poisson regression analysis was used to test for the trend in incidence of HBV over time and to identify risk factors for HBV infection. Cumulative incidence was calculated using the Kaplan Meier method. Variables related to drug use, sexual behaviour, and general characteristics were examined as potential determinants for seroconversion. The drug use variables included injecting, borrowing of needles, obtaining needles through a needle exchange program, and methadone dosage. For current injectors, frequency of injecting and the type of drugs injected were examined. Sexual behaviour included protected versus unprotected sex, the number of partners, and whether performing commercial sex work (women only). General characteristics included gender, calendar year of visit, nationality, ethnicity, age, housing situation, HIV status, and HCV status. Variables subject to change over time were treated as time-dependent covariates.

Multivariate models were built using backward-stepwise techniques, considering calendar time and the variables that had a p-value < 0.15 in the univariate analysis for entry in the final model. The final model was checked for interactions between the variables present in the multivariate model. A p-value < 0.05 was considered to be statistically significant. To see whether there was a shift in risk factors for HBV infection over time, the most likely route of HBV transmission was studied among those who seroconverted for anti-HBc, using logistic regression analysis to calculate the odds ratio (OR) for sexual contact versus injecting drug use as a transmission route, with calendar time as the covariate of interest (before and after 1993).

The basic demographic characteristics of patients of whom sera were available for sequencing were compared with patients of whom sera were unavailable, using Student’s t-test and Chi-square test. All analyses were performed using SPSS 15.0 and Stata Intercooled 9.2.

Results

General characteristics
Of the 1634 drug users entering the ACS between 1985 and 2003, 1268 had at least two follow-up visits and were included in this study. Their median age on entering the cohort was 31 years. Of these participants, 960 (76%) started injecting drugs before or during follow up and 308 (24%) stated to have never injected drugs (Table 1). Heroin and cocaine were the main drugs used by both injecting and non-injecting drug users. The majority of the drug users of the cohort had the Dutch nationality (75%). However, 49% of the never-injecting drug users had a non-western European ethnicity (mainly Surinamese and Moroccan); whereas 11% of the injecting drug users had a non-western ethnicity. At entry, 671 (53%) participants were already anti-HBc positive. The seroprevalence of anti-HBc on entry was respectively 62% and 26 % for injecting and non-injecting drug users.
Among drug users with a non-western European ethnicity, it was 46% overall and 65% and 28% for injecting and non-injecting drug users, respectively. The seroprevalence of anti-HCV at entry was higher (64%) than anti-HBc prevalence. For injecting and non-injecting drug users, it was 82% and 7% respectively. For HIV, 267 (21%) participants were already infected before entering the cohort, the majority being individuals who had ever injected drugs.

Table 1. General characteristics of drug users who had more than one follow-up visit on entry of the Amsterdam Cohort Studies, 1985-2002.

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Ever-injecting drug users</th>
<th>Never-injecting drug users</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>1268</td>
<td>960</td>
<td>308</td>
</tr>
<tr>
<td>Median age (IQR)</td>
<td>31 (27-36)</td>
<td>30 (26-35)</td>
<td>30 (26-36)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>811 (64.0)</td>
<td>594 (61.9)</td>
<td>217 (70.5)</td>
</tr>
<tr>
<td>Dutch nationality (%)</td>
<td>948 (74.8)</td>
<td>684 (71.3)</td>
<td>264 (85.7)</td>
</tr>
<tr>
<td>Median duration of follow-up, yrs (IQR)</td>
<td>5.9 (2.5-10.6)</td>
<td>6.7 (3.2-11.0)</td>
<td>3.8 (1.6-8.4)</td>
</tr>
<tr>
<td>Main drugs injected (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heroin</td>
<td>118</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cocaine</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heroin/cocaine</td>
<td>379</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Main drugs not injected (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heroin</td>
<td>299</td>
<td>175</td>
<td></td>
</tr>
<tr>
<td>Cocaine</td>
<td>239</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>Heroin/cocaine</td>
<td>56</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Frequency of injecting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No current injecting</td>
<td>277 (28.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monthly</td>
<td>61 (6.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weekly</td>
<td>295 (30.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Daily</td>
<td>323 (33.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-HBc positive at entry (%)</td>
<td>671 (52.9)</td>
<td>592 (61.7)</td>
<td>79 (25.6)</td>
</tr>
<tr>
<td>HBV seroconversion during follow-up (%)</td>
<td>83</td>
<td>76</td>
<td>7</td>
</tr>
<tr>
<td>Anti-HCV positive at entry (%)</td>
<td>803 (63.8)</td>
<td>783 (82.2)</td>
<td>20 (6.5)</td>
</tr>
<tr>
<td>HIV positive at entry (%)</td>
<td>267 (21.0)</td>
<td>256 (26.7)</td>
<td>14 (4.5)</td>
</tr>
</tbody>
</table>

* Injection of drugs before or during follow-up
b No HCV results for 10 patients
IQR: Inter-quartile range

Incidence

Of the 597 participants who were negative for anti-HBc on entry, 83 seroconverted for anti-HBc during follow-up. The median time between the last anti-HBc negative visit and the first anti-HBc positive visit was 4 months (IQR: 4-6 months). The incidence of HBV remained relatively stable at 5.9/100 Person Years (PY) until 1993, but had declined to 0/100 PY by 2002 (Figure 1). At the time of HBV seroconversion, 60 HBV seroconverters (72%) had recently injected drugs, 69 (83%) had antibodies against HCV, and 17 (21%) were HIV-
positive. The majority had the Dutch nationality (86%) and western European ethnicity (88%).

After 13 years of follow-up, the cumulative incidence of HBV was 34% among drug users who had ever injected and 8% among those who had never injected.

**Figure 1.** The incidence of HBV per 100 Person Years among drug users in Amsterdam per calendar year, 1985-2002. The measured incidence is the thin line; the fitted incidence is the thick line, and the shaded area is the 95% confidence interval.

**Molecular epidemiology**

Of the 83 seroconverters, the viral DNA of 65 (78%) could be amplified and sequenced. The basic demographic characteristics of those participants did not differ from the 18 whose viral DNA could not be sequenced (all p>0.05). The various strains circulating among drug users in Amsterdam and their calendar year of infection are depicted in a phylogenetic tree (Figure 2). HBV genotypes A and D were the only 2 genotypes circulating among drug users in Amsterdam, and genotype D was by far the most prevalent (N=62, 95%). Within genotype D, 3 subtypes could be distinguished: a subtype specific to injecting drug users [4,14,15], one isolated strain, and one subcluster of 4 strains that significantly differed from the other genotype D strains.

Of the 65 HBV seroconverters whose sequences could be obtained, 57 (88%) were infected with the genotype D strain specific to injecting drug users. This specific strain corresponded with serotype ayw3. The genetic distance between the various sequences in this subcluster was negligible. The subcluster included recent injecting drug users, drug users who had not injected recently, and participants who said that they had never injected drugs, both men and women, commercial sex workers, various ethnicities and nationalities (mainly Dutch).
Figure 2. Phylogenetic tree of the anti-HBc seroconverters of the Amsterdam Cohort Studies, 1985-2002. The year of seroconversion is indicated for each case. Bootstrap values above 70 are depicted.

- Genotype D, drug user type
- Genotype D, unknown type
- Genotype D, Moroccan type
- Genotype A, men having sex with men type
After comparison with other strains found in Amsterdam, the isolated strain within genotype D turned out to be a Moroccan strain [4,15], serotype ayw2. However, this person could not be epidemiologically linked to Morocco or to any other Mediterranean country. He had recently injected drugs.

The strains in the third genotype D subcluster (N=4) differed significantly from the other genotype D strains. Besides having recently injected drugs, having antibodies against HCV, and being infected in 1989 or 1990, no other characteristics linked the participants. One of them was a Surinamese commercial sex worker.

The 3 seroconverters infected with HBV genotype A, serotype adw2, were all Dutch females. Two of them said that they had been working as a commercial sex worker since their last visit, and one said that she had recently injected drugs. In Amsterdam, infections with HBV genotype A are mainly restricted to men having sex with men [4].

Risk factors

The determinants associated with an increased risk for seroconversion that were statistically significant in the univariate Poisson regression analysis were: recent injecting, a higher frequency of injecting, HIV co-infection, HCV co-infection, performing commercial sex work, a higher number of sexual partners, use of needles from a needle exchange program, and a higher methadone dosage. A decreased risk of HBV infection was associated with increases in age and alcohol consumption, and a more recent calendar year of visit.

Despite their univariate significance, the following variables were not considered for entry in the multivariate analyses as a result of multicollinearity: performing commercial sex work, number of sexual partners, using needles from a needle exchange program, methadone dosage, and frequency of injecting. All these excluded variables were strongly correlated with either sexual activity or injecting drug use.

Multivariate analyses revealed recent injecting, increasing age, and more recent calendar year of visit as independent risk factors (Table 2). Recent injecting increased the risk of HBV infection significantly; the incidence rate ratio (IRR) was 6.8 (95% CI: 3.2-14.5). Increasing age and more recent calendar year were associated with a decreased risk of HBV infection; the IRR was 0.96 (95% CI: 0.93-0.99) and 0.94 (95% CI: 0.89-0.99), respectively.

Due to missing data, 56 out of 83 HBV seroconverters were included in the analysis to check whether there was a shift in risk factors over time. Before 1993, 23 seroconversions were related to injecting drug use and 1 to unprotected sexual contact, whereas after 1993, these numbers were 24 and 8, respectively. After 1993, the unadjusted odds ratio was 7.7 (95% CI: 0.9-66.2) for HBV transmission through unprotected sexual contact, compared to the period before 1993.
Table 2. Univariate and multivariate analyses of risk factors for seroconversion of anti-HBc that had a P-value < 0.15 in univariate analysis and were not omitted from multivariate analyses due to multicollinearity.

<table>
<thead>
<tr>
<th></th>
<th>Univariate Analyses</th>
<th></th>
<th>Multivariate Analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HBV sc</td>
<td>IRR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Year of visit</td>
<td>83</td>
<td>0.88</td>
<td>0.83-0.93</td>
</tr>
<tr>
<td>Age</td>
<td>83</td>
<td>0.93</td>
<td>0.90-0.96</td>
</tr>
<tr>
<td>Alcohol consumption/day</td>
<td>55</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
<td>0.68</td>
<td>0.37-1.25</td>
</tr>
<tr>
<td>1-4</td>
<td>8</td>
<td>0.43</td>
<td>0.21-0.91</td>
</tr>
<tr>
<td>&gt;4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injecting</td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>8</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Recent(^a)</td>
<td>60</td>
<td>8.9</td>
<td>4.3-18.6</td>
</tr>
<tr>
<td>Ever</td>
<td>15</td>
<td>2.1</td>
<td>0.9-4.9</td>
</tr>
<tr>
<td>HCV status(^a)</td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>53</td>
<td>6.4</td>
<td>3.3-12.7</td>
</tr>
<tr>
<td>Seroconverter</td>
<td>19</td>
<td>8.4</td>
<td>3.9-18.1</td>
</tr>
<tr>
<td>HIV status</td>
<td></td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>59</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>8</td>
<td>1.6</td>
<td>0.8-3.4</td>
</tr>
<tr>
<td>Seroconverter</td>
<td>16</td>
<td>3.0</td>
<td>1.7-5.2</td>
</tr>
<tr>
<td>Sex(^b)</td>
<td></td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>No sex</td>
<td>36</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Safe sex</td>
<td>22</td>
<td>0.59</td>
<td>0.34-0.99</td>
</tr>
<tr>
<td>Unsafe sex</td>
<td>25</td>
<td>0.91</td>
<td>0.55-1.52</td>
</tr>
</tbody>
</table>

\(^a\) Although HCV co-infection was significant in both univariate and multivariate analysis, HCV co-infection was excluded from the final model, since it is not a risk factor but merely a marker for HBV infection. Furthermore, the IRR for recent injecting increased dramatically when HCV co-infection was excluded from the multivariate analysis.

\(^b\) In the previous six months

Sc: seroconverters

IRR: incidence rate ratio

CI: confidence interval

Discussion

Injecting drug use is still a widespread mode of HBV transmission worldwide. However, the injecting drug users population is decreasing in the Netherlands, given its high mortality rate and the low rate of starting injectors. Most drug users in the Netherlands do not inject drugs anymore and have found other ways to take their drugs, i.e., basing and smoking [7]. In general, little is known about the transmission of HBV among drug users, since there are only a few studies on changes in incidence, risk factors, and circulating genotypes over a long time period. Moreover, there is no information on the ever increasing non-injecting drug user population. The present study is among the first to study changes in incidence, risk
factors, and genotypes over a long time period among both injecting and non-injecting drug users.

The prevalence of anti-HBc among drug users at entry of the cohort was 53%, reflecting that drug users were already involved in high-risk behaviour before entering the cohort and that they were no longer susceptible for HBV. The relatively high prevalence of anti-HBc (25%) among the non-injecting drug users might be explained by their non-western European ethnicity (mainly Surinamese and Moroccan). They were probably infected with HBV at birth or during early childhood.

This study shows a decline in HBV incidence among drug users in Amsterdam from 5.9/100 PY before 1993 to 0/100 PY in 2002. A decline in incidence has likewise been demonstrated for HIV and HCV among injecting drug users and is probably caused by a decline in injecting behaviour [6,10]. The incidence of HBV among drug users who had never injected remained low throughout the study period. As was the case for a cohort study in Baltimore, USA [16]. Recent injecting was the predominant risk factor for HBV infection in this study. Sexual transmission became more important over time, although the majority of new HBV infections were due to injecting, even in the last years of the study. Therefore, recent injecting was and still is the main transmission route for HBV infection. Together with the low incidence of HBV, this indicates that sexual transmission of HBV among drug users plays a minor role, which is in agreement with recent findings in China [17]. The non-injecting drug users are probably infected with HBV via unsafe sexual contacts and sharing of crack-use equipment, as demonstrated by others [18].

The phylogenetic analysis shows that almost all drug users (88%) are infected with the same genotype D strain, serotype ayw3. This strain has not only been found in Amsterdam, but also in other parts of the western world and appeared to be specific to injecting drug users [4,14,15,19]. It is likely that this specific strain long ago entered the injecting drug user population and remained the primary strain circulating in this group. However, the genotype D subcluster in this study, included both recent injecting drug users and non-injecting drug users, indicating that hard drug users infect one other, whether by sharing needles (or other paraphernalia) or by sexual contact. Among drug users, ethnicity is not a determinant for a HBV infection with a specific genotype, as is the case within the general population [5].

A previous study reported spill-over of HBV from injecting drug users to the general population, via heterosexual clients of commercial sex workers who injected drugs [4]. The phylogenetic results indicate sporadic spill-over from the general population into the drug user population, again via injecting commercial sex workers, since genotype A is usually found among men having sex with men and sometimes among heterosexuals. However, these were single introductions, since the three commercial sex workers were infected at different times and they were not infected with identical strains. It appears that introductions from the general population into the drug user population can occur, but there is no sustained transmission and these introduced strains disappear soon after introduction, as was illustrated by the genotype D cluster containing four strains that was introduced in 1989 and 1990 and the three separate introductions of genotype A strains just mentioned.
The incidence of HBV among hard drug users in Amsterdam has strongly declined over time and, in general, injecting and non-injecting drug users become infected with the same genotype D strain. An earlier molecular epidemiological study in Amsterdam, showed that no drug users with an acute HBV infection were reported at the Public Health Service and that the specific genotype D strain had also disappeared after 2000 (Van. Although, the drug users in the Amsterdam Cohort Studies may not reflect the general drug user population, these findings suggest that drug users may no longer be a high-risk group for HBV infection. However, trends in drug use need to be monitored, because when injecting regains popularity in the Netherlands, drug users will become a high-risk group again.

Acknowledgements

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References


