Molecular epidemiology of hepatitis B in the Netherlands
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CHAPTER 2

MOLECULAR EPIDEMIOLOGY OF HBV IN THE NETHERLANDS
Molecular epidemiology of acute hepatitis B in the Netherlands in 2004: a nationwide survey

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Abstract

To gain insight into hepatitis B virus (HBV) transmission in the Netherlands, epidemiological data and sera were collected from reported cases of acute HBV infections in the Netherlands in 2004. Cases were classified according to mode of transmission. A fragment of the S-gene of HBV (648bp) was amplified, sequenced, and subjected to phylogenetic analysis.

Of the 291 acute HBV cases reported in 2004, 158 (54%) were available for genotyping. Phylogenetic analysis identified 6 genotypes: A (64%), B (3%), C (3%), D (21%), E (5%) and F (5%). Of HBV infected men having sex with men, 86% were infected with genotype A, accounting for 43% of all patients infected with this genotype. There were only 3 reported cases of injecting drug use of which 1 was available for sequencing (genotype A). Unlike the genotype A cluster, sequences within the genotype B-E clusters were heterogenic. Within genotype F, several isolates had identical sequences, but patients could not be epidemiologically linked.

Sexual transmission, particularly by men having sex with men was the most important transmission route for HBV. Injecting drug use plays a minor role. Genotype A is predominant in the Netherlands, especially among men having sex with men. In addition to imported strains, there seems to be a pool of related but non-identical strains circulating among chronic carriers in the migrant population, from which occasionally new patients are infected, primarily by heterosexual transmission.
Introduction

Of the estimated 2 billion people ever infected with the hepatitis B virus (HBV) worldwide, over 350 million are currently infected chronically. These chronically infected persons are at high risk for cirrhosis and cancer of the liver, diseases that kill over a million people each year [1]. Because HBV infection is preventable with an effective vaccine, the WHO advised worldwide universal vaccination in 1997.

The Netherlands is a low endemic country for HBV, with an estimated mean incidence of reported acute HBV infections between 1.4 and 2.0 per 100,000 inhabitants for the past decade [2]. This low incidence is one of the reasons that the Netherlands, like the UK, Ireland and the Scandinavian countries, adopted in 2002 a policy of vaccination targeted towards high-risk groups, rather than a policy of universal vaccination [3]. The high-risk groups that are vaccinated currently in the Netherlands are commercial sex workers, hard drug users, men having sex with men, heterosexuals with multiple sex partners, and newborns with at least one parent born in a country with an HBV prevalence over 2%. Before 2002, HBV prevention consisted mainly of prenatal screening and the vaccination of newborns with a mother infected chronically, certain patient groups, and healthcare workers.

Thus far, 8 different HBV genotypes, A-H, have been described worldwide [4-7]. Each has a distinct geographical distribution, with HBV genotype A being most prevalent in Northern Europe and North America [8]. Genotypes B and C are predominantly found in Asia and the Pacific. Genotype D is most prevalent in the Mediterranean area, the Middle East, and India. Genotype E is found mainly in Africa. Genotypes F and H are found primarily in South and Central America. The epidemiology of genotype G has yet to be determined. In the Netherlands, two studies have demonstrated the presence of genotype A-G in the capital Amsterdam [9-11].

To gain more precise insight into HBV transmission networks and the distribution of the various genotypes in the Netherlands, a nationwide molecular epidemiological survey was conducted in 2004. Only patients infected acutely with HBV were included, since they represent new infections and recent HBV transmissions. Increased insight into transmission networks will provide more information on whether our current vaccination efforts are sufficient and targeted towards the correct risk-groups.

Methods

Patients

In the Netherlands, it is mandatory that all cases of HBV infection are reported to the local public health service and subsequently to the Inspectorate of Health. Reporting criteria for acute HBV are: clinical signs and symptoms of acute hepatitis in combination with the presence of the hepatitis B surface antigen (HBsAg) in the serum. All patients reported are approached by public health nurses for active surveillance and for acquiring information on risk-behavior during the six months preceding the infection with HBV. Demographic data are
also collected, including age, gender, country of origin and travel history for the past six months. Epidemiological data and stored sera, if available, were obtained for the acute cases reported in 2004 (N=291). Of those from whom sera were available, anti-HBc IgM was determined for 117 (40%) patients; of those, 5 (4.3%) patients were considered negative for anti-HBc IgM and excluded from the study.

**Isolation, amplification and sequence analysis**

HBV DNA was sequenced in three different laboratories, the Public Health Laboratory in Amsterdam, the Erasmus Medical Center in Rotterdam, and the National Institute for Public Health and the Environment in Bilthoven. As detailed below, each used its own protocol and primers (Table 1), but all yielded PCR amplicons and sequencing data spanning the pre-S2 and S-region.

**Table 1.** Primers used in the various protocols.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>0S1</td>
<td>GCTGGTGGCAGTCCAGTCCAGGAAAC</td>
<td>59-82</td>
</tr>
<tr>
<td>0S2</td>
<td>GCTGGTGAGTCCAGTCCAGGAAAC</td>
<td>59-82</td>
</tr>
<tr>
<td>S3</td>
<td>TTGGTACAGCGGTATACACGG</td>
<td>786-806</td>
</tr>
<tr>
<td>FR1A</td>
<td>GTAAACCTGCTCGACTA</td>
<td>83-101</td>
</tr>
<tr>
<td>FR1B</td>
<td>GTCAGCTGGTACGAGTA</td>
<td>83-101</td>
</tr>
<tr>
<td>0AS3</td>
<td>TGTAACACAGCGGTATACAGG</td>
<td>782-805</td>
</tr>
<tr>
<td>ACPR</td>
<td>CCTGTGGTGAGTCCAGTCCGGGAACAGTA</td>
<td>56-85</td>
</tr>
<tr>
<td>YMDD-2</td>
<td>ACCCCATCTTTTTTTTGTGGGGT</td>
<td>838-861</td>
</tr>
<tr>
<td>YMDD-1</td>
<td>GGCACACTGAAACTGAGCCA</td>
<td>668-687</td>
</tr>
<tr>
<td>HBC2</td>
<td>CTGCTGGGCTCCAGTTTTGG</td>
<td>57-76</td>
</tr>
</tbody>
</table>

In Amsterdam, DNA was isolated from 100 µl serum with 600 µl lysisbuffer (bioMérieux, Boxtel, the Netherlands), 1.2 µl glycogen (20 mg/ml, Roche Diagnostics, Almere, the Netherlands) and 700 µl isopropanol (-20°C). The precipitate was then dissolved in 50 µl 10mM Tris buffer, pH 8.0. The DNA isolate was amplified in a semi-nested PCR, using a PTC 200 (Biozym, Landgraaf, the Netherlands) in a volume of 25 µl containing 0.5 U Silverstar Taq Polymerase (Eurogentec, Maastricht, the Netherlands), 50 µM of each dNTP, 3 pmol of each primer, and 5 µl of DNA extract. In the semi-nested PCR, 2 µl of the outer amplicon was used. Primers 0S1/0S2 and S3 were used in the outer PCR; FR1A/FR1B and 0AS3 were used in the semi-nested PCR. Cycling conditions were: denaturing at 94°C for 3 min, then 30 cycles of 93°C for 30 s, 55°C for 30 s and 72°C for 50 s, with a final extension at 72°C for 7 min. PCR products were precipitated with 100% ethanol and subsequently sequenced in both directions, using the ABI BigDye Termination v1.1 kit (Applied Biosystems, Nieuwerkerk a.d. IJssel, the Netherlands), using primers FR1A/B and 0AS3. Sequencing products were purified using a DyeEx spin kit (Qiagen, Venlo, the Netherlands) and analyzed in an ABI 310 genetic analyzer (Applied Biosystems).

In Rotterdam, HBV DNA was isolated from 200 µl serum using the Total Nucleic Acid isolation kit on a MagnaPure LC isolation station (Roche Diagnostics), using an external
lysis protocol. DNA was eluted in 100 µl buffer and amplified with the primers ACPR and YMDD-2, using the following cycling conditions: 10 min 94 °C, 40 cycles of 1 min 94°C, 1 min 52°C, and 1 min 72°C, and an elongation step of 10 min 72°C, using AmpliTaq Gold polymerase. The amplicon was sequenced with the same primers described above and an additional YMDD-1 primer; 2 µl amplicon was sequenced using the Big Dye terminator V3.1 cycle sequencing kit (Applied Biosystems). The PCR products were precipitated with 96% ethanol and resuspended in 20 µl High Dye Formamide (Applied Biosystems). Sequencing data were analyzed using Sequence Navigator software sequencer (Applied Biosystems).

In Bilthoven, DNA was isolated from 200 µl serum, by using either the QIAamp DNA blood mini kit (Qiagen), or by the LC Nucleic Acid isolation kit (MagnaPure, Roche). DNA was recovered in 50 µl elution buffer and amplified in a nested PCR, using a thermocycler (GeneAmp 9700), in a volume of 50 µl containing 0.5 U AmpliTaq Polymerase (Applied Biosystems), 50 µM of each dNTP, 10 pmol of each primer, and 5 µl of DNA extract. Primers HBC2 and S3 were used in the outer PCR. In the nested PCR, primers 0S1/0S2 and 0AS3 were used. The outer PCR was performed under the following conditions: denaturing at 94ºC for 60 s, then 35 cycles of 94ºC for 15 s, 55ºC for 15 s and 68ºC for 60 s, with a final extension at 68ºC for 60 s. The nested PCR was performed under the same conditions, for 25 cycles. PCR products were purified (Qiaprep spin-column) and sequenced in both directions, using the 3.1 version of the Big Dye terminator chemistry with oligo’s 0S1/0S2 and OAS3 and a 3700 DNA analyzer (Applied Biosystems).

Phylogenetic analysis
A 648-nucleotide fragment of the pre-S2 and S region present in all amplicons isolated in the three laboratories was used for sequence alignment, using the BioEdit 5.0.9 software [12]. Neighbor-Joining phylogenetic analysis was carried out on the nucleotide alignments as provided by the MEGA-3.1 software [13]. 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 DQ988364-DQ988521.

Statistical analyses
The basic demographic characteristics of patients from whom sera was available for sequencing were compared with patients from whom no serum was available, using the chi-square test. Median ages were compared among groups using the Mann-Whitney U-test. P-values lower than 0.05 were considered significant. All analyses were done in SPSS 14.0.

Results

Patients
In 2004, 291 patients infected acutely with HBV were reported in the Netherlands, equivalent to a mean reported incidence of 1.8 per 100,000 inhabitants. Based on the interviews
carried out by the public health nurses, patients were classified according to the most probable mode of transmission (Table 2). For both men and women, sexual intercourse was the most reported mode of HBV transmission in 2004 (63.2%). Sexual transmission among homosexual men, this also includes men having sex with men, accounted for the largest share of acute HBV infections (35.7%). Injecting drug use was a less important transmission route in 2004, since only three injecting drug users with an acute HBV infection were reported, all in the same region. The mode of transmission remained unclear for 72 (24.7%) patients, of whom 58 (80.6%) were male. The incidence of HBV was higher in urban areas, especially in cities like Amsterdam, Rotterdam and the Hague, where 96 (33.0%) patients were reported with an acute infection.

The median age of homosexual men infected acutely with HBV was significantly higher than that of the heterosexual population; it did not differ from the group with an unknown mode of transmission.

Of the 291 reported patients with an acute HBV infection, 46 (15.8%) were admitted to a hospital. Five patients who became infected with HBV reported during the interview that they had been fully vaccinated, 4 others reported that they had not completed their vaccination.

Table 2. Representation of all reported patients acutely infected with HBV in the Netherlands in 2004, according to most probable mode of transmission.

<table>
<thead>
<tr>
<th>Mode of Transmission</th>
<th>Reported cases (%)</th>
<th>Male (%)</th>
<th>Median age (quartile range)</th>
<th>Cases sequenced (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homosexual</td>
<td>104 (35.7)</td>
<td>104 (100)</td>
<td>38 (31-44)</td>
<td>61 (38.6)</td>
</tr>
<tr>
<td>Heterosexual</td>
<td>73 (25.1)</td>
<td>47 (64.4)</td>
<td>33 (23-40)</td>
<td>39 (24.7)</td>
</tr>
<tr>
<td>Sexual</td>
<td>7 (2.4)</td>
<td>7 (100)</td>
<td>45 (35-58)</td>
<td>4 (2.5)</td>
</tr>
<tr>
<td>Injecting drug use</td>
<td>3 (1.0)</td>
<td>0</td>
<td>33 (31-39)</td>
<td>1 (0.6)</td>
</tr>
<tr>
<td>Other</td>
<td>32 (11.0)</td>
<td>28 (87.5)</td>
<td>34 (23-47)</td>
<td>18 (11.4)</td>
</tr>
<tr>
<td>Unknown</td>
<td>72 (24.7)</td>
<td>58 (80.6)</td>
<td>39 (28-48)</td>
<td>35 (22.2)</td>
</tr>
<tr>
<td>Total</td>
<td>291 (100)</td>
<td>244 (83.8)</td>
<td>37 (28-44)</td>
<td>158 (100)</td>
</tr>
</tbody>
</table>

Molecular epidemiology

On the basis of the availability of 171 sera in the different laboratories in the Netherlands, the HBV DNA of 158 serum-samples could be amplified and sequenced. The basic demographic characteristics of patients whose sera were available for sequencing analysis did not differ from those whose serum was not available (all P > 0.05). The various strains circulating in the Netherlands in 2004 are, together with strains from other countries (GenBank), depicted in a phylogenetic tree (Figure 1). The genotypes were differently distributed among the various modes of transmission (Table 3). HBV genotypes A-F were circulating in the Netherlands in 2004, but the predominant genotypes were A and D. Most homosexual men (86.9%) were infected with HBV genotype A. The cases with an unknown
mode of transmission were distributed over the range of genotypes, with a majority found in genotypes A and D. The five patients who claimed to be fully vaccinated against HBV were all homosexual men, three were infected with HBV genotype A, one with genotype C and one with genotype D. They could not be linked to one another, either by their molecular or epidemiological data.

The larger part of the patients within the genotype A cluster were homosexual men (52%), and 16% of the cases were infected via heterosexual transmission. The cluster also contained 1 strain from a female injecting drug user. There were seven additional female patients infected with genotype A, five via heterosexual transmission, for a total of eight (7.9% of all genotype A cases). The sequences in the genotype A cluster were highly similar, especially among homosexual men, in whom 33 sequences were identical and 11 differed by only 1 nucleotide. These 44 men were living throughout the Netherlands. Also identical to this strain were 21 sequences derived from patients other than homosexual men. Of these, 18 were male, of whom 13 had an unknown or other mode of transmission; the other 5 claimed to be infected via heterosexual transmission. Although some subclusters within genotype A could be distinguished, they were not fully supported by the bootstrap analysis (values < 70). One of the subclusters consisted of 4 people from the same area in the Netherlands, who either were born in the Netherlands Antilles or had had heterosexual contacts with an Antillean (figure 1, subcluster 1). Another subcluster consisted of eight patients with various transmission routes (figure 1, subcluster 2). Finally, one patient, age 15, with a mother infected chronically from Croatia (unknown17-04), had a sequence that did not cluster with other genotype A sequences.

Patients infected with HBV genotype D were infected mainly via heterosexual transmission (42.4%), as opposed to genotype A. The sequences in the genotype D cluster were less similar to one another than those in the genotype A cluster. The genotype D cluster contained nine female patients (27.3%). Within this cluster, a Turkish subcluster could be distinguished in which five of the six cases were related to Turkey, either by Turkish ethnicity or heterosexual contact in Turkey (figure 1, subcluster 3). There was also a more diverse subcluster consisting of nine strains, all from patients with different ethnicities (Moroccan, Dutch, Netherlands Antilles, and Surinamese). Patients in this subcluster were also infected through different modes of transmission, namely: other, heterosexual, and homosexual.

Figure 1. Phylogenetic tree of sequences from patients with an acute HBV infection reported in the Netherlands in 2004, together with strains from different countries (GenBank), using the Neighbor-joining Kimura 2-parameter method with 1000 bootstrap replications. Only bootstrap-values above 70 are shown. The arrows point out the subclusters mentioned in our results and the patients who claimed to be fully vaccinated are marked with a v.  

<table>
<thead>
<tr>
<th>MSMB</th>
<th>Transmission via male homosexual contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hetero</td>
<td>Transmission via heterosexual contact</td>
</tr>
<tr>
<td>Sexual</td>
<td>Transmission via sexual contact, not known to be homo- or heterosexual</td>
</tr>
<tr>
<td>IDU</td>
<td>Transmission via injecting drug use</td>
</tr>
<tr>
<td>Other</td>
<td>Transmission via household contacts, blood-blood contacts, etc</td>
</tr>
</tbody>
</table>
Figure 1.
transmission (figure 1, subcluster 4). Finally, 3 strains from patients with an Indonesian ethnicity, a mother and her two sons (25 and 28 years old), all with an unknown mode of transmission, were very similar to a strain earlier found among injecting drug users [9,14,15]. An additional patient with this strain reported having sex with his Polish girl friend who has a history of injecting drug use. The remaining patients in the genotype D cluster were largely from the Middle East or Mediterranean area.

The genotype B and C clusters, associated with Asia [8], consisted mainly of patients with a Chinese ethnicity or patients who were connected to Asia in one way or another. However, the genotype C cluster additionally contained two homosexual men with a Dutch ethnicity who reported no traveling to a foreign country.

The cluster of genotype E, which is associated with Western Africa [8], consisted of eight patients, four of whom could be linked to countries in Western Africa through heterosexual contact, blood-blood contact, or ethnicity. Of the other four, three had similar strains, were of Dutch ethnicity and were living in the same part of the Netherlands (figure 1, subcluster 5).

The genotype F cluster included a patient with Dutch ethnicity who had a heterosexual contact in Brazil, plus a subcluster containing strains of six patients (figure 1, subcluster 6). Remarkably, these six strains were very similar (four were identical) to strain AB116552F from Venezuela. These six patients each had a different mode of transmission and originated from a different part of the Netherlands and could therefore not be linked directly to one another.

Patients infected with HBV genotype B, C, D, E, and F were infected primarily in the Netherlands (86%) and not abroad.

<table>
<thead>
<tr>
<th>Table 3. Distribution of HBV genotypes in the Netherlands in 2004.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Homosexual</td>
</tr>
<tr>
<td>Heterosexual</td>
</tr>
<tr>
<td>Sexual</td>
</tr>
<tr>
<td>Injecting drug use</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>Unknown</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Discussion

Sexual transmission, in particular by homosexual men, was the most reported transmission route for HBV in the Netherlands in 2004. Injecting drug use plays a minor role in HBV
transmission in the Netherlands, in contrast to Denmark and the UK [15,16]. A decline in injecting drug use in the Netherlands is probably the reason for the very low number (N = 3) of reported cases of acute HBV among injecting drug users [17]. Dutch injecting drug users are decreasing in number and getting older, making it more likely that they are either immune or chronically infected. Furthermore, one of the injecting drug users in our study carried genotype A, whereas in earlier studies, genotype D was typical among injecting drug users [9,14,15]. In Amsterdam, the injecting drug user cluster seen before 1999 seems to have disappeared [11]. Likewise, this appears to be the case in the rest of the Netherlands.

The total number of acute HBV patients reported in the Netherlands is an underestimation, since only about one third of patients acutely infected with HBV have symptoms, and not all patients are reported. Also, due to our reporting criteria, a few cases of chronic HBV with severe acute exacerbation might have been included. To check whether the inclusion criteria were sufficient, anti-HBc IgM was examined in a subgroup of patients; of these, 4.3% were considered negative for anti-HBc IgM, indicating that only a few chronically infected patients with acute exacerbations were included.

Although Dutch homosexual men have been targeted for vaccination for several years, homosexual men made up the relatively largest group with an acute infection with HBV. This finding is in accordance with an increase in sexually transmitted infections and sexual risk behavior among homosexual men in the Netherlands in recent years [18,19]. Genotype A was found to be predominant among homosexual men (86%). Studies in Amsterdam have shown that genotype A is restricted to homosexual men [9,11]. However, in this nationwide survey, only 52.4% of the cases with genotype A were homosexual men and there were also 11 (10.9%) heterosexual men and 8 (7.9%) females infected with this genotype.

Overall, genotype A was the predominant genotype (63.9%), and the genetic distances between the strains within this genotype were very small, indicating that they were closely related. In this cluster, 92.1% of the patients were male, and most of them were homosexual men. The high similarity between the various strains was found among all patients within genotype A and not only among homosexual men. The high proportion of homosexual men and male heterosexuals infected with similar strains, as well as 6 (5.9%) male bisexuals (data not shown), implies possible spill-over from the homosexual population to the heterosexual population. Admitting having homosexual contacts is sometimes problematic, especially in rural areas and therefore the proportion of homosexual men might even be higher than 52.4%. If this is the case, it would explain the similarity between the various strains found in men with seemingly different modes of transmission.

Genotype D is found primarily in the Mediterranean area, in countries like Turkey and Morocco. A substantial number of people with a Mediterranean ethnicity live in the Netherlands, and apparently most of the patients infected acutely with HBV genotype D were limited to this group, although there was some transmission into the general population. The genotype D cluster was more diverse than the genotype A cluster, implying less sustained transmission and more new introductions. In addition to imported strains, there seems to be a pool of related but non-identical HBV strains among chronic carriers in the Dutch population of Mediterranean ethnicity. From this pool, new patients are
occasionally infected, primarily via heterosexual transmission (42%). Probably, there are also pools of HBV-genotypes B, C, E, and F circulating in the Netherlands, since most patients with these genotypes were infected in the Netherlands and not abroad. However, we can only speculate about transmission networks, since there is no information on the source of the various infections.

Several acute infections with HBV genotype F were found, a genotype that is most prevalent in South America. Surprisingly, a cluster of six persons with sequences that were very similar (four were identical) to a South American strain was found. However, being infected by different routes of transmission and originating from different parts of the Netherlands, these people could not be epidemiologically linked to one another by any means.

A substantial group of patients infected acutely with HBV had an unknown mode of transmission (24.7%). These cases were distributed over the different genotypes, with a majority found in genotype A and D, as expected, since these genotypes are predominant. These cases were not located in a particular part of the Netherlands. It is likely that better source-contact tracing or registration could reduce the number of patients with an unknown mode of transmission.

In conclusion, the results show that in 2004, the most important factor in the spread of HBV in the Netherlands was sexual transmission, especially by homosexual men. Increased efforts in education and vaccination are necessary to prevent HBV in this risk group. This study also shows the value of molecular epidemiological surveillance in countries where the vaccination coverage of risk-groups is not yet optimal, as implied by Zollner [20].

Acknowledgements


References


