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Two Years’ Prospective Collection of Molecular and Epidemiological Data Shows Limited Spread of Hepatitis A Virus outside Risk Groups in Amsterdam, 2000–2002

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We performed a viral sequencing study on samples representing all reported primary cases of acute hepatitis A virus (HAV) infection reported for 2 years in Amsterdam. Two regions of HAV RNA were amplified, sequenced, and used for phylogenetic analysis. Of 156 cases, strains of 104 isolates (66.6%) clustered into 3 genotypes: 1A, 1B, and 3. Two separate transmission circles occurred, without mutual interrelation. In genotype 1A, 4 clusters occurred in men having sex with men (MSM), and the fifth cluster was related to a virus from Morocco. In genotype 1B, 6 small clusters were directly related to the Moroccan virus. In genotype 3, strains were related to a virus from Pakistan. Our analysis indicates that, to stop transmission of HAV in Amsterdam, the entire MSM population and travelers to countries where HAV is endemic, especially children, should be vaccinated. Prevention strategies need not include the vaccination of all children living in Amsterdam.
study of 33 acute index cases in 1997 and 1998, we isolated and sequenced HAV RNA from stool samples and found 2 distinct subtypes: genotype 1B, which was introduced by children from Morocco, and genotype 1A, which was endemic among homosexual men [9]. On the basis of all incident reported cases during a 2-year period, we present here a complete picture of the molecular epidemiology of HAV in Amsterdam. Our findings are important for policy decisions regarding the most effective HAV prevention strategy.

PARTICIPANTS AND METHODS

Participants
From 1 August 2000 to 30 August 2002, 156 index patients with community-acquired acute symptomatic HAV infection were reported to the Municipal Health Service (MHS), Amsterdam. An additional 26 individuals with serologically confirmed acute infection were traced as contacts. Reporting criteria are clinical signs and symptoms with laboratory confirmation of acute infection, as measured by the presence of IgM antibodies to HAV. Reported patients are typically approached with active surveillance, including source and contact tracing, passive immunization of susceptible contacts at risk, and hygienic advice. Information was obtained with regard to possible risk factors during the 2–6 weeks before the onset of disease. For our study, individuals were classified with a hierarchical algorithm as to the probable mode of transmission. The first group included individuals returning from countries where HAV is endemic 2–6 weeks before the onset of disease (designated “IMP”). The second group included household or family members of patients with confirmed HAV infection (designated “FAM”). The third group included children who had school contact with a confirmed case patient (designated “SCH”). The fourth group included men who had sex with men (designated “MSM”) who visit “dark rooms” or other venues for anonymous sex. The fifth group included drug users, for whom the drug type, frequency, and route of administration were registered. The sixth group included patients for whom none of these risks were identified and transmission was classified as unknown (designated “UNK”). In addition to transmission risk factors, we recorded demographic data for each case patient, including age, sex, country of origin, food history (especially regarding fresh berries, shellfish, and any possibly contaminated water sources), and the identified source person, if any. After informed consent was obtained from reported patients and 26 contacts, we tried to retrieve serum and/or plasma samples from the diagnosing laboratory. Subjects were asked to send stool samples to our laboratory. Permission for the study was granted by the Medical Ethical Commission of the Academic Medical Center Amsterdam on the basis of the study design and the informed consent of participants.

Isolation, Amplification, and Sequencing of HAV RNA

HAV RNA isolation. Serum and plasma samples were aliquoted in 1.7-mL vials, and stool samples were aliquoted as a 10%–20% suspension in PBS. All samples were stored at −80°C until the isolation of RNA was performed by use of TriPure Isolation Reagent (Roche), according to the manufacturer’s protocol. Isolated RNA was resuspended in 50–μL Tris HCl (10 mmol/L [pH 8.0]) and stored at −80°C. A control for the RNA isolation and the subsequent nested reverse-transcriptase (RT) polymerase chain reaction (PCR), 1 sample was spiked with 5 μL of an HAV-positive culture supernatant (HAV cyt HB1.1) [10] and processed in parallel with the studied serum, plasma, and stool samples.

Oligonucleotide primers. Random hexamer primers were used for RT. PCR primers targeting the VP1-P2a region and the more variable VP3-VP1 region of the HAV genome were derived and modified from published sequences (table 1) [9, 11–13]. The second-round PCR was performed with primers located internal to the first-round PCR primers. All primers were synthesized by Life Technologies (Gibco BRL).

HAV RNA amplification. Nested RT-PCR was performed in a PTC-200 DNA Engine Thermal Cycler (MJ Research, through BiozymTC), as described elsewhere [9]. Sequencing was performed directly on the second-round PCR products by use of second-round primers and Big Dye Terminator chemistry (versions 2.0 and 3.0; PE Biosystems).

Sequencing and phylogenetic analysis. Sequencing products were analyzed on an ABI 310 automated sequencer (PE Biosystems) and aligned with BioEdit Sequence alignment Editor software [15]. Aligned sequences were then used to generate a phylogenetic tree by use of Molecular Evolutionary Genetics Analysis software (version 2.1) [16]. The tree was constructed by use of neighbor-joining and Kimura-2 parameter models, and reproducibility was tested by performing 1000 bootstraps.

Nucleotide-sequence accession numbers. The nucleotide-sequence data reported in the present article have been deposited in the GenBank sequence database under accession numbers AY343685–AY343785, AY101267–AY101271, AY343-786–AY343888, and AY101276–AY101280. The reference types used were obtained from GenBank: genotype 1A (accession numbers X75214 and AB020564), genotype 1B (accession numbers M59808 and M20273), and genotype 3A (accession number M66695).

Statistical Analyses
Where appropriate, the χ² test was used to compare characteristics between groups. To calculate risk factors for the non-availability of HAV RNA sequences, SPSS logistic regression was used to obtain univariate and multivariate odds ratios and 95% confidence intervals. In multivariate modeling, all relevant factors were included.
Table 1. Primer sequences and locations in the hepatitis A virus (HAV) genomic regions VP1-P2a and VP3-VP1.

<table>
<thead>
<tr>
<th>Region, primer</th>
<th>Sequence, 5′–3′</th>
<th>Nucleotide no. [14]</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VP1-P2a</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First-round PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR-5 [13] sense</td>
<td>TTG TCT GTC ACW GAA CAR Tcw G&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2950–2972</td>
<td></td>
</tr>
<tr>
<td>BR-9 [13] antisense</td>
<td>AGT CAC WCC TCT CCA RGA AAA YTT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3310–3286</td>
<td></td>
</tr>
<tr>
<td>Second-round PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RJ-3 [11] sense</td>
<td>TCC YAG AGC WCC WTT RAA&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2984–3002</td>
<td></td>
</tr>
<tr>
<td>BR-7 antisense</td>
<td>ACT TCA TTT GAC AAC TCT TCC TGA</td>
<td>3179–3202</td>
<td></td>
</tr>
<tr>
<td><strong>VP3-VP1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First-round PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–4 [9] sense</td>
<td>YGT TGC TCC YCA TGT YAG AGT&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2115–2135</td>
<td></td>
</tr>
<tr>
<td>VP1–6 antisense</td>
<td>CAT ATG ATC TGA TGT ATG TCT</td>
<td>2436–2456</td>
<td></td>
</tr>
<tr>
<td>Second-round PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–2 [12] sense</td>
<td>GTT TTG CTC CTC TTT ATC ATG CTA TG</td>
<td>2168–2194</td>
<td></td>
</tr>
<tr>
<td>VP1–1 [12] antisense</td>
<td>GGA AAT GTC TCA GGT ACT TTC TTT G</td>
<td>2390–2415</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Coded primer sequences were derived and modified from published articles as indicated in the list of references. The nested polymerase chain reaction (PCR) primers were used also for sequencing analysis.

<sup>a</sup> R = A + G; W = A + T; Y = C + T.

**RESULTS**

**Participants.** Of 156 reported hepatitis A index cases, 120 (77%) were male, and 73 (47%) were of non-Dutch origin. Index cases were a mean age of 24.6 years (range, 1–69 years). Of these, 61 were MSM, and their mean age was 38.0 years (range, 20–63 years). No drug use was reported. Five individuals were hospitalized for 1–10 days. The percentage of jaundice was lower in children aged <10 years (37/44 [84%]) and higher in adults aged >40 years (27/28 [96%]). At least 1 sample type (serum, plasma, feces, or a combination of samples) was obtained from 110 of 156 index patients and from 14 of 26 contact cases. HAV RNA was sequenced from 14 contact samples, but these were not included in the analysis. In 10 contact cases, HAV RNA was sequenced both in index and contact cases. Nine sequence pairs showed homology, but, in the tenth, we identified a minor difference in the VP1-P2a region. For 4 contacts, the index case did not participate. On the basis of the homology in 9 of 10 index/contact pairs, we assumed that the HAV RNA of these 4 contacts without an index-case sample was identical to that of the nonparticipating index patient and added the samples into the index group. We thus analyzed 114 (73%) of 156 samples. Index case patients with available sequence data represented in the phylogenetic tree in figure 1A were 78% male and 47% of non-Dutch origin. Table 2 lists the age specifications per risk group of these participants.

**Isolation and sequencing analysis.** In 104 (91%) of 114 samples, HAV RNA could be isolated and sequenced for the more conserved VP1-P2a region. For individuals with 2 sample types, this rate was 94% (62/66), and it was 87% (42/48) for individuals with 1 sample type. For the more variable VP3-VP1 region, sequences could not be determined for 8 individuals (positivity rate, 93%). Table 2 shows the probable transmission source for 156 reported cases related to the availability of a sequence in the VP1-P2a region. Availability was not biased toward sex (P > .4), country of origin (P > .8), source of transmission (P > .25), or study period (P > .5). A sequence was available in a lower percentage of adults aged 20–29 years (4/14 [29%]), but this was not statistically significant (P = .05).

In a logistic-regression model that included all of the variables listed above, the availability of sequences was statistically not related to any of these variables. For both regions, HAV RNA sequencing results were identical when they were derived from different samples (feces or blood) from the same individual.

**Molecular epidemiology.** HAV sequences from the VP1-P2a region are displayed in a phylogenetic tree constructed by the neighbor-joining method (figure 1A) that includes reference sequences for the distinct genotypes. Overall, strains clustered into 3 genotypes: 1A, 1B, and 3. The phylogenetic tree based on sequences of the VP3-VP1 region is shown in figure 1B. The sequences from the less-conserved VP3-VP1 region showed a pattern of clustering similar to that of the VP1-P2a region but, as expected, with more variability. Figure 2A, 2B, and 2C, respectively, shows subsets of sequences in genotypes 1A, 1B, and 3 of the VP1-P2a tree. Each sequence is designated by calendar year and week of onset of disease, followed by source of transmission (IMP, FAM, SCH, MSM, or UNK).
Figure 1.  

A, Neighbor-joining phylogenetic tree of the nucleotide sequences in the VP1-P2a region of isolates of 104 cases of acute hepatitis A from individuals in Amsterdam. The tree includes reference viruses GBM and AH-1 in genotype 1A, HM175 in genotype 1B, and GA76 in genotype 3A. Sample nos. show successive entry no. and origin. The 2-digit nos. in the figure represent the bootstrap values obtained by generating 1000 trees. Only bootstrap values $\geq 70$ are shown. The samples are numbered in order of arrival in the laboratory.

B, Neighbor-joining phylogenetic tree of the nucleotide sequences in the VP3-VP1 region of the same isolates as in panel A, including the same reference viruses, with a similar representation of bootstrap values. MSM1, a cluster of 10 isolates with 100% similarity; MSM2, a cluster of 33 isolates with 100% similarity; SCH1, a cluster from a primary school of 6 isolates with 100% similarity; SCH2, a cluster from a primary school of 4 identical strains; SCH3, a cluster from a primary school of 5 identical strains.
Figure 1. (Continued.)
Table 2. Availability of sequences of reported cases with acute hepatitis A infection in Amsterdam, according to source of transmission, with respective average age and age distribution of the separate transmission groups.

<table>
<thead>
<tr>
<th>Probable source of infection</th>
<th>No. of index cases</th>
<th>Available sequences in VP1-P2a region, no. (% available)</th>
<th>Age, average (range) [SD], years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Import</td>
<td>48</td>
<td>29 (60)</td>
<td>12 (1–38) [11.7]</td>
</tr>
<tr>
<td>Family</td>
<td>9</td>
<td>8 (89)</td>
<td>24 (7–42) [17.3]</td>
</tr>
<tr>
<td>School</td>
<td>9</td>
<td>8 (89)</td>
<td>8 (6–10) [1.4]</td>
</tr>
<tr>
<td>Male homosexual activity</td>
<td>61</td>
<td>42 (69)</td>
<td>40 (27–63) [8.4]</td>
</tr>
<tr>
<td>Unknown</td>
<td>29</td>
<td>17 (59)</td>
<td>22 (4–36) [12.8]</td>
</tr>
<tr>
<td>Total</td>
<td>156</td>
<td>104 (67)</td>
<td>25 (1–63) [17]</td>
</tr>
</tbody>
</table>

...continued...
Figure 2. Subset of genotypes 1A (A), 1B (B), and 3 (C) from the neighbor-joining phylogenetic tree of the nucleotide sequences in the VP1-P2a region of figure 1. The sample numbers indicate the year and week of disease onset and probable source of transmission. In clusters of 100% similarity, isolates are represented in chronological order. ~, isolates from men who have sex with men (MSM) not engaging in high-risk behavior; #, isolates from non-MSM, with strains in MSM clusters; *, isolates of contact cases with strains that did not have 100% similarity; +, isolates with possible epidemiological relationship, confirmed by molecular virology of isolates; IMP, returning from areas of high/medium hepatitis A virus (HAV) endemicity during the 6 weeks preceding disease onset; FAM, family or household contact of hepatitis A case; SCH, school-related (i.e., acute hepatitis A in children from the same classroom or using the same toilet facilities in school); UNK, unknown source of transmission.
Genotype 1B, VP1-P2a region

Figure 2. (Continued.)
tions occur without symptoms [17], especially among children. In addition, symptomatic infections might go undiagnosed, and confirmed cases might go unreported. The Amsterdam Sentinel Project estimated the rate of the underreporting of hepatitis A in 1979 at 42% [18]. The national sentinel project found, during the period 1994–1997, a rate of underreporting >55% during the first 2 years. However, during the second 2 years, the incidence of notifications was higher than sentinel estimates [19]. In Amsterdam, the Public Health Laboratory, which also works with general physicians in the area, is incorporated within the MHS and directly reports positive laboratory results to the Division of Public Health (DPH). Along with other Amsterdam laboratories, administrative arrangements are made to report positive findings directly to the DPH. The incidence of reported cases of hepatitis A in Amsterdam exceeds the incidence of reported cases of hepatitis A in the rest of the country annually by 2–5 times. This might partly reflect a true difference but is also attributed to better reporting.

**MSM.** During the study period, 4 distinct HAV strains entered the population of MSM. After cocirculating endemically among MSM, the virus seems to disappear after several months or a year, which suggests a reproductive rate just <1. Through the European research network for rapid the detection of foodborne outbreaks (QLK1-1999-00594), we found that the MSM2 sequence was identical to strain HAV/SA/10/2000/DE in both the VP1-P2a and the VP3-VP1 regions (GenBank accession numbers AY028976 and AY027537). It had been isolated 4 months earlier in Berlin from tourists returning from Ibiza, Spain, without specific information on homosexual behavior. This throws new light on the interpretation of isolate 2001WK36_UNK-Spain speculated~. Its homology with MSM strains suggests unreported homosexual activities in The Netherlands. Travel to Spain, including Barcelona, where this strain has also been identified, could equally have been the source of the infection, with or without homosexual risk behavior.

From MSM not reporting high-risk sexual behavior, we isolated strains identical to the MSM strains. Our molecular data confirm epidemiological findings from Columbus, Ohio, where, during an epidemic of hepatitis A among MSM, high-risk behavior was not an additional risk factor for infection [20]. Our findings also confirm an epidemiological suggestion from Rotterdam, where, during an epidemic of hepatitis A among MSM, travel history was subordinate to local risk behavior [21]. From the MSM travelers to Australia and the Republic of South Africa, we isolated strains that were circulating in the MSM population in Amsterdam before and after their trips. Two transmissions were speculated to have originated in the workplace, where in both instances a homosexual colleague with hepatitis A was speculated as the possible source. Molecular data confirmed an MSM connection.
Travelling children. Our results show that, over a 2-year period, multiple distinct HAV strains are introduced through children returning from visits to their country of parental origin. Such children transmit the virus to siblings, to Dutch schoolmates, and to their susceptible Dutch parents. There were 24 new viral introductions by children and 3 school clusters but no ongoing transmission for any of these strains. The control policy (active source and contact tracing with immunization of possibly exposed contacts) seems to keep the HAV reproductive rate close to 1. In genotype 1A, a child with HAV infection from an unknown source (2001WK50_UNK), was the index case for a cluster in a primary school (SCH1, figure 2A). A highly similar strain had been isolated the previous year. This strain might have been reintroduced from the same origin in Morocco, given that we saw in a cluster in genotype 1B. Genotyping strains from children returning from Morocco in other areas of The Netherlands showed homology with several of our isolated strains (data not shown). We conclude that regional strains circulate in Morocco and are exported regularly to various parts of our country and, presumably, to other countries in Europe.

The first import case with infection by genotype 3 (2000WK40_IMP) had returned from a family holiday in Spain 6 weeks before illness onset. The isolated strain suggests a possible link to Pakistan. The children attended a secondary school that was also attended by Pakistani children, but no symptomatic cases were reported in this school.

Drug users. Contrary to other areas of the world [22–24], we did not find hepatitis A infection among drug users. In Amsterdam, hepatitis A was reported in drug users annually up to 1995, but, since that year, hepatitis A has not been confirmed in drug users in Amsterdam, although drug users with jaundice are still referred to our laboratory or to the DPH. In recent years, hepatitis B, C, and, occasionally, E related to travel and Epstein-Barr virus infections are confirmed.

Vaccination policy. We present strong arguments for 2 distinct active immunization policies—for MSM and for children who import strains. Regarding the first group, the HAV disease burden increases with age [1, 2]. The average age of reported cases was 24.6 years, but it was 38 years for MSM. Universal vaccination is economically justifiable in areas of intermediate endemicity [25], but The Netherlands overall has low endemicity. Nationwide vaccination is thus infeasible on economic grounds [26], even if it is justifiable on medical grounds. Without such vaccination as primary prevention, active source and contact tracing is the cornerstone of HAV control. This is adequate intervention for the general population but not for MSM, because, in this setting, sexual transmission occurs mostly in anonymous settings such as dark rooms. Our results show continued transmission of a single HAV strain among MSM, including MSM who do not engage in high-risk behavior, but with virtually no spread outside this group. We conclude that all MSM in Amsterdam should be vaccinated against hepatitis A. Because the nationwide vaccination program for groups at high risk for hepatitis B virus (HBV) includes MSM [27], we advise the use of a combined HBV and HAV vaccine [28]. Data from our study show an HAV reproductive rate just <1 and suggest that elimination of HAV in this group would be easy to achieve with only a modest increase in the protection rate.

To stop HAV transmission by children who import strains, we advise vaccination before departure for all children going to areas in which HAV is endemic. Our study shows that current prevention strategies, which are based on the voluntary vaccination of children, with coverage <50%, do not stop the introduction of HAV in Amsterdam. The existing secondary prevention strategy, which consists of the active tracing and immunizing of contacts, prevents further spread of the virus, but occasional clusters, without symptomatic index cases, continue to occur. In The Netherlands, starting in 2003, all children with 1 or both parents born in countries of medium or high HBV endemicity will receive HBV vaccination during their first year of life. This policy includes children originating from Morocco and Pakistan, the countries that were a major source of HAV in Amsterdam. Adding hepatitis A to this program, as a combined hepatitis B and A vaccine [29], would have prevented 17 (59%) of 29 primary HAV import cases during our study period. The percentage of secondary cases that might have been prevented can only be speculated. Our data suggest that 2 of 3 school clusters were directly related to an import virus from Morocco, as well as several secondary transmissions to household contacts (data not shown). In 5 Amsterdam cases of unknown transmission, molecular data suggested an association with HAV import cases. The proposed targeted childhood vaccination program will also reduce cases now classified as unknown. On this basis, a vaccination program for all children living in Amsterdam is not required.

Virological typing for public health. The VP3-VP1 and VP1-P2a regions show similar patterns of clustering (figure 1A and 1B), but VP3-VP1 shows more variability. The latter can be explained by changes over time caused by 1 or 2 nucleotide differences (cluster MSM2). The similarity shows that both regions can be used for public health purposes. The more variable region, however, might invoke uncertainties in confirming transmission routes, because natural changes over time can obscure existing true relations. Our results further show that, for clarification of transmission patterns of a direct feco-orally transmitted pathogen, molecular typing can supplement epidemiological information, as it can in sexually transmitted HBV [22] and airborne transmitted tuberculosis [30]. With the present data set, molecular typing could not reveal the transmission route in all cases. A structural collection of strains might further
reveal relationships that now remain obscure. The European research network for the rapid detection of foodborne outbreaks is effectively contributing to such data collection. Molecular data have added value in outbreak investigation, either in confirming suspected relationships [13, 31–37] or in ascertaining, in retrospect, that several different strains were involved in epidemiologically uniform outbreaks [38–41].

The major achievement of the present study lies in its decisive arguments for targeted vaccination policy. Sound epidemiological studies have assisted in developing differentiated vaccination policies [42, 43]. Universal childhood vaccination is warranted, especially in areas of intermediate HAV endemicity. A targeted approach reduces costs but introduces uncertainties in overall protective efficacy. Because the molecular data failed to associate isolates for only 7 cases of unknown transmission, we conclude that in The Netherlands, an area of low endemicity, the addition of the 2 proposed targeted vaccination strategies could optimally prevent >90% of new cases of hepatitis A.

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