and the competitor concentration was set at 2×10^{12} copies/μl.

Serial tenfold dilutions of the competitor RNA were divided into aliquots with Tris-HCl (10 mM, pH 8.0) containing 0.5 U/μl Rnasin. The quantitative RT-PCR was performed according to the HAV RT-PCR protocols described previously [van Steenbergen et al., 2004; Tjon et al., 2005] in an end volume of 25 μl with an input of 2 μl competitor RNA and 2 μl RNA from patient material. Amplification of the competitor resulted in an amplicon of 267 bp, while the amplicon size of patient-derived HAV cDNA was 247 bp. The qualitative RT-PCR had a higher sensitivity than the quantitative RT-PCR concerning detection of patient-derived HAV RNA because no competitor RNA was added. Hence, HAV RNA detected with the qualitative RT-PCR was not always quantifiable. The number of HAV copies in a stool sample were calculated per millilitre of a 10%–20% suspension of fresh stool in phosphate buffered saline; hereafter referred to as "faeces suspension". Quantitation was possible from virus load levels of 2×10^3 copies/ml faecal suspension and from 1×10^3 copies/ml blood.

Statistical Analysis

Statistical analyses were performed with SAS [SAS statistical package, 2005b], R [R statistical package, 2005a] and SPSS 12.0 [SPSS 12.0, 2005c] software for Windows. The distribution of the time until virus loads in faeces and blood were below a certain level was estimated, for example undetectable by RT-PCR, using the nonparametric maximum likelihood estimator for interval censored data [Groeneboom, 1996].

Relations between the three markers (ALT and virus levels in blood and in faeces) were analysed in a linear regression model after transformation to the log-scale. For each marker combination, two models were fitted; each marker was used as dependent variable to predict the other.

The Mann–Whitney test was used to determine if HAV genotype influenced duration of HAV excretion in faeces and/or jaundice. The test was also used to determine if liver damage influenced the duration of HAV excretion, comparing patients with ALT levels above and below the median of 1,000 IU/ml blood.

RESULTS

General Characteristics of Participants

Follow-up study. Of the anti-HAV IgM positive cases reported from August 2003 to 2004 at the participating health services, 27 people participated in the follow-up study. As shown in Table III, these included 18 men and 9 women (median age 33 years; interquartile range (IQR) 23–42 years), of whom 17 persons were infected with subtype 1a strains (63%), 7 with subtype 1b strains (26%) and 3 with subtype 3a strains (11%).

All but two participants reported jaundice. Of these, 84% (21/25) also reported the duration of jaundice, which lasted for a median period of 17 days (IQR: 5–74 days). Comparing patients infected with genotype 1a versus genotype 1b, we found no significant difference in duration of jaundice (Mann–Whitney test: $P = 0.89$) or duration of HAV excretion in faeces ($P = 0.13$).

TABLE III. Characteristics of the Participants in the Follow-Up and the HAV Typing Study

	Follow-up study	HAV typing study
No. of patients	27	55
Median age (years)	33 (IQR 23-42)	32 (IQR 12-38)
Gender		
Male	18 (67%)	43 (78%)
Female	9 (33%)	12 (22%)
Genotype		
1a	17 (63%)	39 (71%)
1b	7 (26%)	14 (25.5%)
3a	3 (11%)	2 (3.6%)
Jaundice	25/27 (93%)	49/55 (89%)
Median duration of jaundice (days)	17	NA
Median duration of HAV excretion (days)	81	NA

NA, not applicable.

HAV-Typing Study Participants

These 55 patients consisted of 43 men and 12 women (median age: 32 years; IQR 12–38 years) (Table III). There were 39 persons infected with subtype 1a strains (71%), 14 with subtype 1b (25.5%) and 2 with subtype 3a (3.6%). Of the total, 49 persons (89%) reported jaundice.

Time Course of Virus Load Levels and ALT Among Patients

In concordance with the comparisons mentioned above, there were no observable differences per genotype concerning the course of HAV excretion. In The Netherlands, there are two main risk groups among whom different HAV genotypes circulate: homosexual men are infected with genotype 1a strains, while travelers to HAV endemic countries are infected mainly with either genotype 1b strains (Morocco, Turkey, etc.) or genotype 3a strains (Pakistan, India) [van Steenbergen et al., 2004]. The patterns of patients infected with genotype 1a, 1b or 3a are shown in Figure 1a–c.

Figure 1a represents a 26-year-old homosexual man infected with a genotype 1a strain. He reported anonymous sex in saunas as a risk factor. He was jaundiced for 16 days and a HAV faecal excretion was detected up to 98 days after disease onset.

Figure 1b depicts a 31-year-old patient who was infected in Morocco with a genotype 1b strain. He was jaundiced for 21 days, and virus excretion was detected until 70 days after onset of illness.

Figure 1c represents an 11-year-old patient who became infected in Pakistan with a genotype 3a strain. The patient was jaundiced for 18 days and HAV excretion was detected for 76 days.

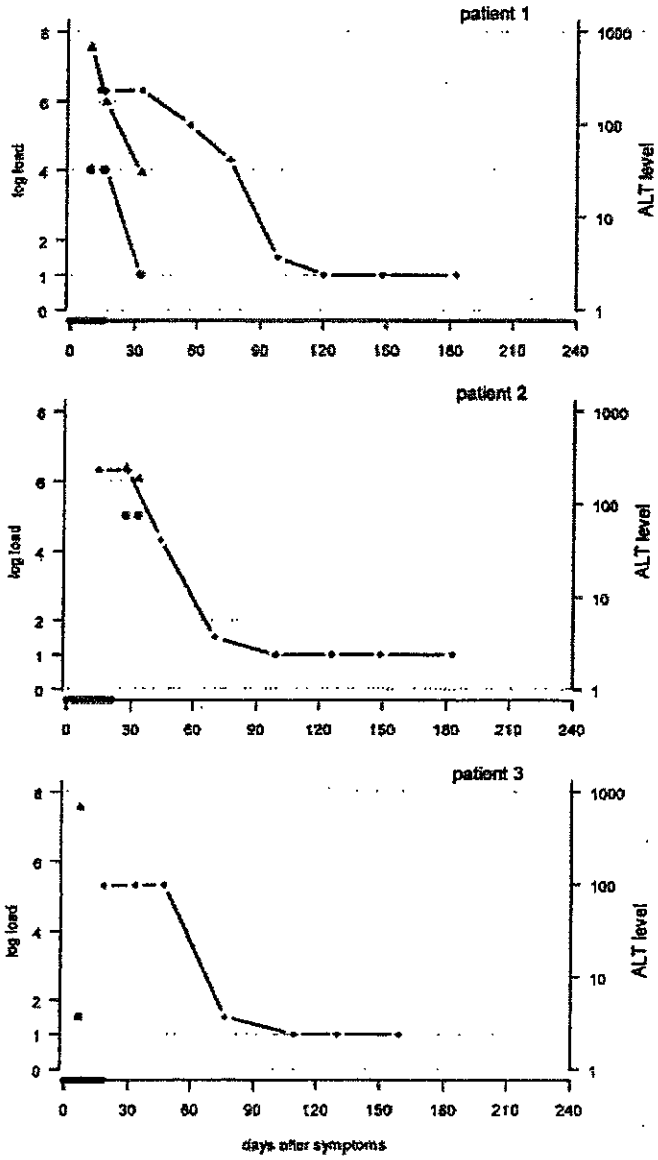

Fig. 1. a–c: Time course of virus load and ALT level in three patients. The left Y-axis shows the logarithm of virus load in linear scale per ml faeces suspension or per ml blood. Logload = 1: detection limit qualitative RT-PCR. Logload = 1.5: detection limit quantitative RT-PCR. Logload = 2: Quantification limit. The right Y-axis shows the ALT levels in log scale in IU/ml blood. The X-axis shows the number of days after onset of symptoms. Duration of jaundice is depicted by the grey bar on the X-axis. a: Patient 1 is a 26-year-old homosexual man who got infected with a type 1a virus by anonymous sex in public venues. He was jaundiced for 16 days. b: Patient 2 is a 31-year-old man who got infected with a genotype 1b strain in Morocco. He was jaundiced for 21 days. c: Patient 3 is an 11-year-old boy who got infected with a genotype 3a strain in Pakistan. He was jaundiced for 18 days.

HAV Detection and Quantitation in Faeces

Hepatitis A virus (HAV) faecal excretion was detected for an estimated median period of 81 days after onset of symptoms in samples of the 27 follow-up study participants (Fig. 2). Faecal excretion was never shorter than 57 days, with a maximum of 127 days. Fifty percent of the participants still excreted at least 2×10^6–2×10^8 copies/ml faeces suspension at 36 days after onset of

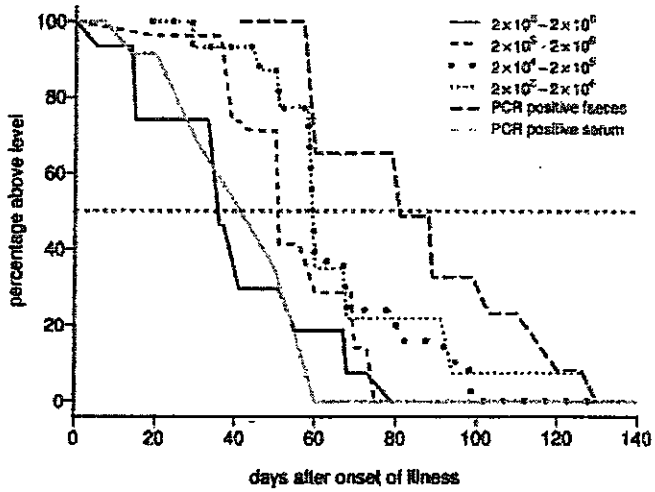

Fig. 2. Curves for virus excretion levels at different time points. The black curves show the estimated percentage of patients over time that have a virus load in faeces above a certain limit. The grey curve shows the last time points at which sera were HAV-positive in the qualitative RT-PCR. The horizontal line is the median value.

symptoms. After 65 days this had declined to 10% of patients. The lowest quantifiable level, 2×10^3 copies/ml faeces suspension, was detected for a median period of 60 days.

HAV Detection and Quantitation in Blood

In the follow-up study (n = 27), serum samples were provided from 1 day before onset of illness to 74 days after the onset of symptoms. The estimated median period in which HAV was detected in blood was 42 days after disease onset (Fig. 2). At 60 days after the onset, all blood samples were estimated to be HAV-negative.

The PCR results of the sera provided by participants of both the follow-up and HAV typing study are shown in Figure 3. In total, 119 samples taken from 82 participants were tested, since 22 follow-up patients donated more than one blood sample. Again, we found that the proportion of PCR-positive sera declined over time. It

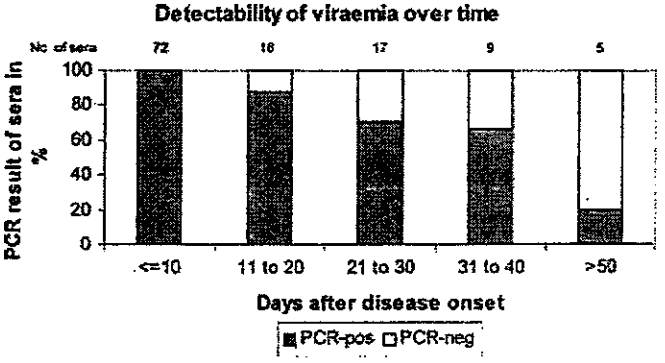

Fig. 3. Detectability of HAV viraemia according to days after onset of illness. The Y-axis shows the percentage of PCR-positive (grey) blood samples donated by participants of the follow-up and the HAV-typing study. All 72 sera (100%) donated within the first 10 days of illness were PCR-positive, whereas 80% of the sera donated more than 40 days after onset were PCR-negative.

was 100% in the period up to 10 days after onset of illness and declined to 20% after 40 days (Fig. 3).

Taking into account one serum sample per patient obtained at different time points after the onset of disease, it was found that the median virus load levels varied at different time points. In the first 10 days of illness, the median virus load level was 1×10^6 copies/ml blood (95% confidence interval (CI): 5.2×10^5–1.9×10^6 copies/ml blood; n = 69). After 10 days, the median virus load of the donated sera was below quantitation level ($<10^3$ copies/ml).

Relation Between ALT Levels and Virus Levels in Blood and in Faeces

ALT levels and virus load in blood. To determine if the degree of liver damage influenced virus levels in blood in the first 10 days of illness and vice versa, data were analysed from follow-up and HAV typing-study participants (n = 64). Spearman analysis revealed a significant association between liver damage and the HAV level in serum ($\rho = 0.354$; $P = 0.004$). This was corroborated by the results from linear regression analysis, which showed that if ALT levels decreased with $1\log_{10}$ IU/L blood, viraemia levels decreased with $0.81\log_{10}$ copies/ml blood ($P = 0.017$), and vice versa, if viraemia levels decreased with $1\log_{10}$ copies/ml blood, ALT levels decreased with $0.11\log_{10}$ IU/L blood ($P = 0.017$). To minimize the confounding effect of time, these analyses were repeated with data of the first 5 days of illness, which yielded similar results.

ALT levels and virus load in faeces. Nine participants provided blood and faeces in the same period (5 days) within the first 10 days of illness. Using Spearman analysis, a strong positive correlation was found between the virus level in blood and in faeces ($\rho = 0.942$; $P < 0.01$). No significant correlation was found between ALT levels and virus load in faeces during this period ($\rho = 0.248$; $P = 0.520$).

ALT levels and duration of HAV excretion in faeces. The possible relation between liver damage and the duration of HAV excretion in faeces was of interest. Upon analysis, no difference was found in duration of HAV excretion between persons with ALT levels above the median (1,000 IU/ml blood) versus ALT levels below the median in the first 10 days of illness (Mann–Whitney test: $P = 0.5$; n = 16).

DISCUSSION

Due to the acute and self-limiting nature of HAV infection, sequential collection of samples combined with clinical data from patients is uncommon. Information on the duration and level of HAV excretion in blood and in faeces is therefore limited despite its importance for disease control.

To elucidate these matters, clinical data and serial blood and faeces samples were collected from 27 acute hepatitis A patients for half a year. The biochemical and clinical data were combined with quantitative RT-PCR results to study possible relations between symptomatic disease and the duration and level of HAV excretion.

In other studies, the real-time PCR method was used to quantify HAV loads in sera. These real-time PCRs target the 5′ noncoding region and virus loads were quantified with an external standard [Costa-Mattioli et al., 2002b; Rezende et al., 2003; Normann et al., 2004].

At the onset of this study, a real-time PCR was compared with a competitive nested RT-PCR, targeting the VP3-VP1 region (data not shown). An internal standard was used for both virus quantitation, and as an inhibition control for PCR of faeces-derived RNA samples. Tests using the real-time PCR showed a significant increase in the fluorescent signal representing amplification of the internal standard, whereas almost no increase in fluorescent signal was detected for amplification of the wild-type HAV cDNA. Nevertheless, both the internal standard and the wild-type HAV cDNA were amplified during the reaction, since amplicons of the expected sizes were visible on acryl-amide gels. Unfortunately, we were not able to solve this problem and therefore chose to use the competitive nested RT-PCR that is described in the present study, as this yielded more reproducible and robust results.

The results show that HAV is detectable in faeces for a median period of 81 days after onset of symptoms, with a maximum of 127 days. Although the literature on this subject is sparse, studies with fewer patients describe similar findings of HAV excretion periods in faeces from 52 days post-onset up to 89 days in healthy individuals [Yotsuyanagi, 1996; Polish et al., 1999; Flehmig et al., 2000; Chitambar et al., 2001; Costa-Mattioli et al., 2002a]. HIV-positive persons may excrete the virus for even longer periods, since one study found that an HIV-1 infected person still excreted 5×10^6 copies/g faeces 106 days after the onset of illness [Costa-Mattioli et al., 2002a].

Of our patients, 50% were still shedding HAV in faeces at 36 days, at the same high levels as at disease onset (Fig. 2). One study reported successful infection of two tamarins by inoculation with a suspension of faeces donated 27 days after the ALT peak of the human patient [Polish et al., 1999]. The animals seroconverted and excreted HAV in blood and in faeces after 15 and 21 days, respectively. Thus, patients still excreting high levels of HAV in faeces after 1 month may still be infectious.

Sera collected during the present study were HAV RNA-positive for a median period of 42 days after illness. Other studies report viraemic periods varying from 22 up to 490 days [Bower et al., 2000; Kwon et al., 2000; Ida et al., 2002; Costa-Mattioli et al., 2002b; Sagnelli et al., 2003; Normann et al., 2004].

Although HAV transmission by blood products is rare, there have been several reports on HAV outbreaks due to contaminated blood products [Mannucci et al., 1994; Robertson et al., 1998; Soucie et al., 1998; Chudy et al., 1999; Gowland et al., 2004; Heitmann et al., 2005]. To prevent contamination of blood products in The Netherlands, HAV patients are excluded from donating

blood for 4 weeks after clinical symptoms are resolved, or 6 weeks in the case of contact with a HAV patient. Since the patients had jaundice for a median period of 17 days, and elevated ALT levels for a median period of 16 days, the present study suggests that most HAV patients are probably HAV RNA-negative by the end of the 4–6 weeks of exclusion. However, blood donors without any HAV symptoms and those with prolonged viraemia must also be taken into account, especially since donated blood is not tested for the presence of HAV in The Netherlands. An even greater risk to blood supplies is present during the period before HAV antibodies, increased liver enzyme levels and HAV symptoms are detected [Gowland et al., 2004; Heitmann et al., 2005]. Weimer et al. [2002] reported a window period of 2 weeks in which HAV-infected patients had high HAV levels in blood with normal ALT levels before onset of illness. PCR techniques can reduce this window period and increase the safety of blood products, especially since HAV is 10,000 times more infectious by the intravenous route than orally, and contaminated blood products can increase morbidity and mortality in already ill patients treated with these products [Purcell et al., 2002].

It was found that in the first 10 days after onset of illness, ALT levels were correlated positively with viraemic levels in blood. However, no indications were found that the degree of liver damage during this period influenced duration of HAV excretion in faeces. Other studies have found that ALT and HAV levels in blood are correlated positively [Fujiwara et al., 1997; Normann et al., 2004; Sainokami et al., 2005]. The results of the present study support the findings of Bower et al. [2000] who detected HAV levels of 10^4–10^5 plaque forming units (pfu)/ml up to 20 days prior to the ALT peak. These levels declined to 1–10 pfu/ml 30 days after the ALT peak.

The factors that cause liver damage during HAV infection are still poorly understood. One hypothesis is that hepatic disease is caused by the patient's immune system as it attempts to clear the virus [Vallbracht et al., 1986; Brack et al., 2002; Rezende et al., 2003]. Age and underlying disease, as well as medication, can influence disease outcome. Rezende et al. [2003] reported that patients with HAV-related fulminant hepatitis had low HAV levels in blood in spite of hepatocyte necrosis. This finding is in agreement with Sainokami et al. [2005] who found that ALT levels and virus load were significantly correlated in patients with mild HAV-related symptoms, but not in those with severe symptoms. In contrast to the position in vivo, wild-type HAV can persistently infect human cell lines without causing observable cytopathogenicity [Vallbracht et al., 1984]. Cytotoxic peripheral blood lymphocytes taken from HAV patients have been shown to induce cytolysis in HAV-infected cell cultures, whereas lymphocytes from noninfected persons did not have this effect [Vallbracht et al., 1986; Maier et al., 1988]. Accordingly, one would expect immunocompromised persons to have persistent HAV infection. Reportedly, immunocompromised HIV-infected persons are HAV-viraemic for a longer period than expected [Ida et al., 2002; Costa-Mattioli et al., 2002a] and one study found that they show less elevated ALT levels and are longer viraemic than HIV-negative patients [Ida et al., 2002]. Another study found that neonates still excreted HAV 4–5 months after infection [Rosenblum et al., 1991]. Hence, our finding that the virus load in blood is associated positively with liver damage supports the notion that HAV clearance in hepatocytes is immunomodulated.

On the other hand, increased disease severity may be associated with viral factors, such as genotype and nucleotide changes in, for example the 5′-non-translated-region of the virus [Fujiwara et al., 2002]. No indication was found in this study that genotype influenced duration of jaundice or HAV excretion, nor did other studies looking for an association between HAV genotype and disease severity [Fujiwara et al., 2003; Rezende et al., 2003; Normann et al., 2004]. The findings show that HAV faecal excretion is high in the first month of illness, suggesting that patients may be infectious for a longer period than currently assumed. Furthermore, HAV patients can be viraemic for up to 2 months, which has to be taken into account by blood banks. Finally, a positive association was found between ALT and viraemia levels during the first 10 days of illness.

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No. 37

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研究報告の概要

○分子疫学的試験によりヨーロッパにおける活動的男性同性愛者のA型肝炎エンデミックが示されている
1997年～2005年に、デンマーク、ドイツ、オランダ、ノルウェー、スペイン、スウェーデン、英国において、男性同性愛者にA型肝炎の大規模アウトブレイクが発生した。このA型肝炎アウトブレイクに関与するウイルス株に遺伝子学的な関連性があるかどうかを確かめるため、これら国々の間で共同試験が実施された。当該株のVP1およびVP1/P2A領域のN末端の配列を解析し比較した。ヨーロッパのさまざまな国の男性同性愛者に認められた株のほとんどは、MSM1と名づけられたgenotype IA に属する近縁のクラスターを形成していた。同じ時期に、これらの国々の別のリスク集団には異なるHAV株が流行しており、このことは男性同性愛者だけに特定のHAV株が流行したことを示している。1997年～2005年の期間に男性同性愛者に同一の株が認められたことは、これらHAV株が男性同性愛者間に長く流行していることを示している。各国内の同性愛者コミュニティーはその集団内にHAVを長期間維持するにはおそらく小さすぎる。しかし、ヨーロッパ全土の同性愛者コミュニティーは相同性の高いHAV 株の流行が何年も持続するのに十分な大きさであるため、男性同性愛者間での流行が引き起こされたのであろう。

使用上の注意記載状況・その他参考事項等

解凍赤血球濃厚液「日赤」
照射解凍赤血球濃厚液「日赤」
解凍赤血球-LR「日赤」
照射解凍赤血球-LR「日赤」

血液を介するウイルス、細菌、原虫等の感染
vCJD等の伝播のリスク

報告企業の意見	今後の対応
1997年～2005年に、ヨーロッパにおいて男性同性愛者間で特定のHAV株によるA型肝炎の大規模アウトブレイクが発生したとの報告である。	日本赤十字社は、輸血感染症対策として、問診で肝炎の既往があった場合、A型肝炎については治癒後6ヶ月間、家族に発症した人がいる場合は1ヶ月間献血不可としている。また、男性と性的接触を持った男性は1年間献血不可としている。今後も引き続き情報の収集に努める。

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Molecular Epidemiological Studies Show That Hepatitis A Virus Is Endemic Among Active Homosexual Men in Europe

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Large outbreaks of hepatitis A have occurred in Denmark, Germany, the Netherlands, Norway, Spain, Sweden, and the United Kingdom during the period 1997–2005 affecting homosexual men. A collaborative study was undertaken between these countries to determine if the strains involved in these hepatitis A outbreaks were related genetically. The N-terminal region of VP1 and the VP1/P2A region of the strains were sequenced and compared. The majority of the strains found among homosexual men from the different European countries formed a closely related cluster, named MSM1, belonging to genotype IA. Different HAV strains circulated among other risk groups in these countries during the same period, indicating that specific strains were circulating among homosexual men exclusively. Similar strains found among homosexual men from 1997 to 2005 indicate that these HAV strains have been circulating among homosexual men for a long time. The homosexual communities are probably too small within the individual countries to maintain HAV in their population over time, whereas the homosexual communities across Europe are probably sufficiently large to sustain continued circulation of homologous HAV strains for years resulting in an endemic situation among homosexual men.
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KEY WORDS: molecular epidemiology; HAV; outbreaks; homosexual men

INTRODUCTION

Hepatitis A is transmitted primarily by the fecal-oral route from person to person. The seroprevalence in Europe varies from intermediate levels in Southern and Eastern Europe to low levels in Western Europe, and very low levels in Scandinavia [Jacobsen and Koopman, 2004]. In low endemicity countries, hepatitis A mainly affects groups at high risk of infection such as homosexual men, intravenous drug users, and travellers to high endemicity areas. In recent years, several outbreaks occurred among homosexual men in several countries in Europe: United Kingdom in 1997 [Bell et al., 2001], Norway in 1997–98 [Stene-Johansen et al., 2002], Germany in 1997 [Fengler, 1997], and 2002–2003 (unpublished), France in 2000 [Delarocque-Astagneau, 2004], and Spain in 2002 and 2003–2004 (unpublished), and in addition, the Netherlands reported sporadic cases in 1997–1998 [Bruisten et al., 2001] and 2000–2004 [De Jager and Heijne, 2004; Reintjes et al., 1999; van Steenbergen et al., 2004]. In 2004, outbreaks of hepatitis A among homosexual men were reported in Denmark [Mazick et al., 2005], Norway [Blystad et al., 2004], Sweden (unpublished), the United Kingdom [O'Sullivan, 2004], and Spain (unpublished).

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