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一般的名称	（製造承認書に記載なし）	研究報告の公表状況	Zayc-Schmidt EM, Pichl L, Laue T, Heitmann A, Schottstedt V. Transfusion. 2005 Jun;45(6):1037-8.	公表国 ドイツ
販売名（企業名）	合成血「日赤」（日本赤十字社） 照射合成血「日赤」（日本赤十字社）			

研究報告の概要

○A型肝炎ウイルスRNAの供血者スクリーニングにより検出された旅行関連A型肝炎
異なる地域の2名の供血者（A：2004年7月22日、B：同8月12日）でHAV-NAT陽性の結果が示された。いずれも無症状で、A型肝炎の予防接種は受けていなかった。2名ともエジプトのフルガダへの旅行から帰国したばかりで、隣接したホテルに滞在していた。フルガダは感染のリスクがある地域とされておらず、供血延期にならなかった。
血漿のNATスクリーニングは、96検体ミニプールを用いてリアルタイムPCRにより行われ、NATで陽性となったミニプールは、chessboardプーリングにより追跡し、個別NATにより確認が行われる。この2名では、さらにワンチューブRT-PCRを行い、配列データ解析をCLUSTAL W法により行った。供血者の新鮮凍結血漿のHAV定量化により、力価2.58 EE2 IU/mL（A）、5.3 EE3 IU/mL（B）であることがわかった。GenBankの配列で最も一致したのは、1984年フランスの孤発例のHAV株2F84（97%）であった。両者の遺伝子配列は100%の相同性を示し、同一の感染源と確認された。配列アラインメントは遺伝子型1Bと一致した。
2名には検査結果を通知し、追跡調査のための検体を依頼したが、供血者Aの検体は得られなかった。Aは8月5日に黄疸などを発症して入院し、抗体検査からA型肝炎と診断され、2週間後に回復した。8月19日に入手した供血者Bの検体は、抗体検査とNATの双方で陽性であった。9日後に発熱などの症状を発症し、2日間持続した。感染源は特定されていないが、宿泊したホテルの一つがヨーロッパ人旅行者のHAV感染300例以上の感染源であることが後に判明した。
A型肝炎のリスクが認識されていない地域においても、予防接種を受けていない旅行者には感染のリスクがある。HAVのハイリスク地域滞在直後に供血する場合は、輸血による感染を予防するため、NATによるHAV検査または供血延期のいずれかが適切である。

報告企業の意見	今後の対応
エジプトへの旅行中にHAVに感染し、供血時のNAT検査で検出されたとの報告である。	日本赤十字社は、輸血感染症対策として献血時に海外渡航歴の有無を確認し、帰国後4週間は献血不適としている。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。今後も引き続き情報の収集に努める。

使用上の注意記載状況・その他参考事項等

合成血「日赤」
照射合成血「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

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Travel-related hepatitis A detected by hepatitis A virus RNA donor screening

We read with interest the report by Gowland and coworkers[1] describing molecular and serologic tracing of transfusion-transmitted hepatitis A virus (HAV) between a blood donor and recipient. We describe the application of reverse transcription–polymerase chain reaction (RT-PCR) and phylogenetic analysis to tracing acute hepatitis A infections in two blood donors to a common source of HAV infection during their vacations in Egypt.

Within a 3-week period, two blood donations from different regions of Germany (Donor A, July 22, 2004; and Donor B, August 12, 2004) tested positive by HAV PCR during routine processing at the Red Cross Blood Transfusion Service West. Both donors were asymptomatic and neither had been vaccinated against hepatitis A. Both had recently returned from holiday trips to Hurghada, Egypt, where they stayed in different, but neighboring, Red Sea beach hotels. They were not deferred as blood donors, because Hurghada is not a known risk area.

Routinely, all blood donated at the Red Cross Blood Transfusion Service West is tested by PCR for HCV, human immunodeficiency virus-1 (mandatory by German Guidelines), hepatitis B virus, parvovirus B19 DNA, and HAV RNA in accordance with requirements of the plasma fractionation industry.[2] PCR screening of plasma is conducted using 96-sample minipools at the Laboratory of the Red Cross Blood Transfusion Service Baden-Wuerttemberg-Hessen by real-time PCR. PCR-positive minipools are traced back by chessboard-pooling and confirmed by single-donation PCR.

Additional testing for these two donors included quantitative PCR performed by one-tube RT-PCR (RealArt HAV LC RT-PCR kits, artus GmbH, Hamburg, Germany). Full-length RNA was reverse-transcribed and subsequently amplified and sequenced (DNA Cloning Service, Hamburg, Germany). Sequence data analysis was performed by CLUSTAL W algorithm. Quantification of HAV with the donors' fresh frozen plasma revealed titers of 2.5 EE2 IU per mL (Donor A) and 5.3 EE3 IU per mL (Donor B). The closest sequence match to GenBank was HAV strain 2F84 (97%) from a sporadic case in France in 1984.[3] Sequence analysis of both strains isolated from the plasma units revealed 100 percent homology, confirming a common source of their infections. Sequence alignment to known strains of HAV matched genotype 1B for both donations (Fig. 1). Typically, genotype 1A predominates in Germany.

The donors were informed about their test results, asked for follow-up blood samples, and interviewed by telephone to investigate the possible sources for their HAV infections. No follow-up sample was available from Donor A. His family doctor reported that he was hospitalized on August 5, 2004, because of jaundice, abdominal pain, fatigue, and loss of appetite. Hepatitis A was diagnosed and confirmed by the presence of anti-HAV immunoglobulin M (IgM) and immunoglobulin G (IgG). His apartase aminotransferase level was 11,120 U per L and his alanine aminotransferase level was 5,664 U per mL. He recovered in 2 weeks. A follow-up sample from Donor B from August 19, 2004, was positive for both anti-HAV IgM and IgG and HAV PCR. He was asymptomatic, but 9 days later, he developed fever, pain in the upper abdomen, arthralgies, and one episode of dark urine. This illness lasted 2 days. The specific source of their infections has

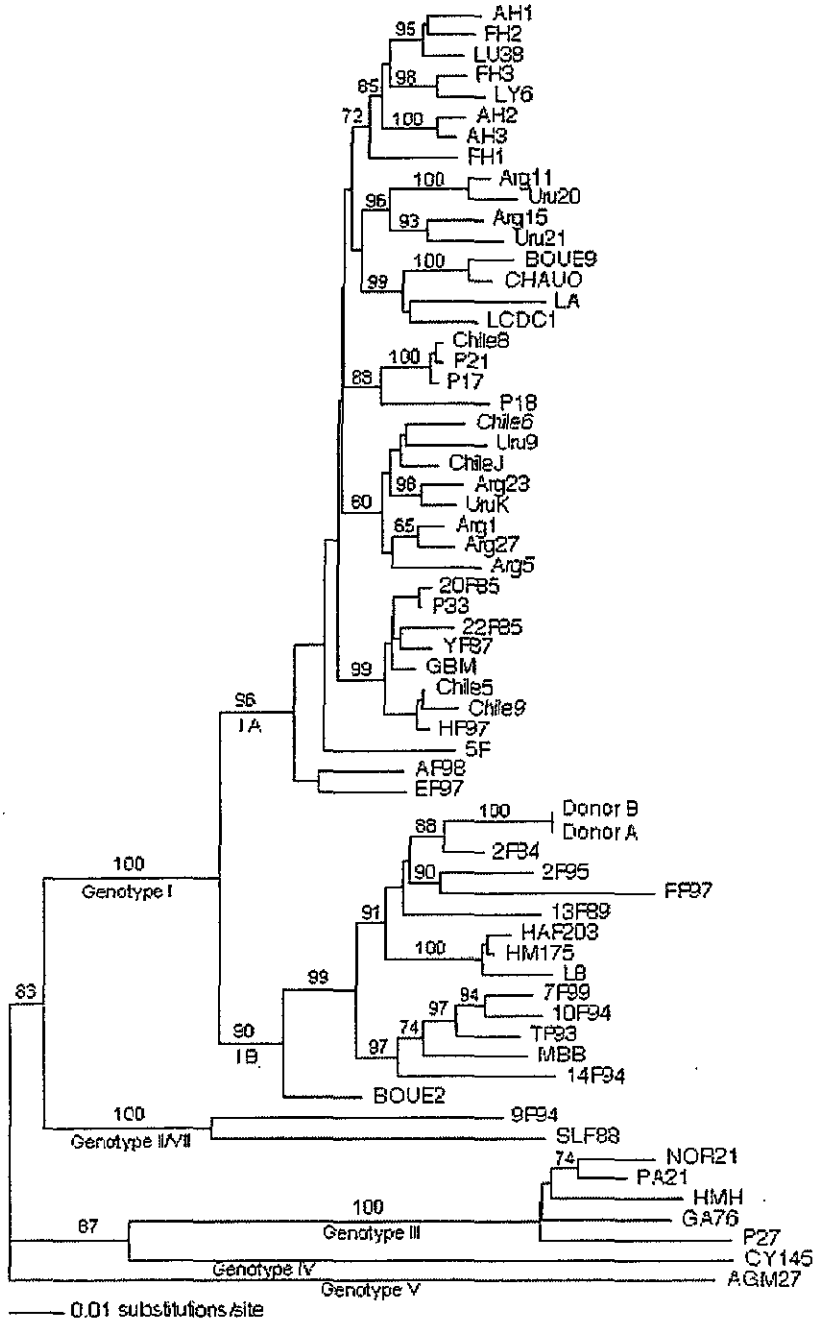

Fig. 1. Phylogenetic analysis of the two HAV strains isolated of the plasma unit (Donors A and B). Numbers at the branches indicate bootstrap percentage after 1000 replications of bootstrap sampling. The bar indicates genetic distance.

not been identified.[4] Four weeks after the donors departed, however, one of the hotels was identified as the source of more than 300 cases of hepatitis A among European tourists.[5]

This experience alerts us that there is a substantial risk for hepatitis A among nonvaccinated tourists not only in known regions of known high risk for hepatitis A, but also in certain areas where the risk has not been recognized. When blood is donated shortly after a stay in an HAV high-risk region, either testing donations by HAV NAT or a temporary deferral of donors (when common source outbreaks are recognized) is appropriate to prevent transfusion-transmitted infections.

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一般的名称	①②ポリエチレングリコール処理人免疫グロブリン ③人免疫グロブリン	研究報告の公表状況	Transfusion, 45(7), 1097-1105, 2005	公表国 ドイツ
販売名 （企業名）	①献血ヴェノグロブリン-IH ヨシトミ（ベネシス） ②ヴェノグロブリン-IH（ベネシス） ③グロブリン-Wf（ベネシス）			

研究報告の概要

ドイツで 2003 年に HAV 感染のウインドピリオド期に得られた血液は、市販の RT-PCR (reverse transcription-polymerase chain reaction)の検査では HAV 陰性であった。その後、この感染ウイルスはほぼ 100%に近い遺伝子配列の特徴から HAV ⅢA 型と確認された。この HAV ⅢA 型はドイツで初めて検出され、HMH 株と命名された。市販の診断検査システムではこの HAV 型を検出できないため、すべての HAV 型を定量的及び定性的に確認できる real-time RT-PCR キットが開発された。HAV ⅢA 型に利用できるほぼ 100%近い全核酸配列が得られた。血液及び血液製剤の安全性を保証するためには、HAV の遺伝子には多様性があるので、遺伝子の絶え間ない監視及び核酸測定法を新たな遺伝子型に適応させることが必要である。

使用上の注意記載状況・その他参考事項等

代表として献血ヴェノグロブリン-IH ヨシトミの記載を示す。

2. 重要な基本的注意

(1)本剤の原材料となる献血者の血液については、HBs 抗原、抗 HCV 抗体、抗 HIV-1 抗体、抗 HIV-2 抗体、抗 HTLV-I 抗体陰性で、かつ ALT（GPT）値でスクリーニングを実施している。更に、プールした試験血漿については、HIV-1、HBV 及び HCV について核酸増幅検査(NAT)を実施し、適合した血漿を本剤の製造に使用しているが、当該 NAT の検出限界以下のウイルスが混入している可能性が常に存在する。本剤は、以上の検査に適合した血漿を原料として、Cohn の低温エタノール分画で得た画分からポリエチレングリコール 4000 処理、DEAE セファデックス処理等により人免疫グロブリンを濃縮・精製した製剤であり、ウイルス不活化・除去を目的として、製造工程において 60℃、10 時間の液状加熱処理及び濾過膜処理（ナノフィルトレーション）を施しているが、投与に際しては、次の点に十分注意すること。

報告企業の意見	今後の対応
市販のRT-PCRの検査ではHAV陰性であった血液ドナーからHAV ⅢA型が見つかった。HAV ⅢA型は市販の診断システムでは検出できないために、すべてのHAV型を定量的及び定性的に確認できるreal-time RT-PCRキットが開発されたとの報告である。 万一、原料血漿にHAVが混入したとしても、HAVと類似した特徴を有しているEMCのウイルスバリデーション試験成績から、本剤の製造工程において十分に不活化・除去されると考えている。	弊社では、最終製剤の試験としてHAV NATを実施しているために、この報告の検査法の調査を行っている。

Occurrence of hepatitis A virus genotype III in Germany requires the adaptation of commercially available diagnostic test systems

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BACKGROUND: A blood donation, obtained in 2003 in Germany during the preseroconversion diagnostic window period of a hepatitis A virus (HAV) infection, tested HAV-negative by commercially available HAV reverse transcription–polymerase chain reaction (RT-PCR) detection assays.

STUDY DESIGN AND METHODS: The virus responsible for this infection was identified as HAV genotype IIIA by characterization of the nearly complete genome sequence.

RESULTS: Thereby, this HAV variant, which was named strain HMH, was detected in Germany for the first time. Because the commercially available HAV RNA detection systems failed to detect this genotype, a real-time RT-PCR kit was developed that allows quantification and detection of all HAV genotypes. The first nearly full-length nucleotide sequence so far available for HAV genotype IIIA is also provided.

CONCLUSION: This case demonstrates that owing to the genetic variability of HAV, constant monitoring and adaptation of the diagnostic nucleic acid assays are required to guarantee the safety of blood and blood products.

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In recent years the safety of blood and blood products has been improved significantly by the application of the nucleic acid amplification technology (NAT). In comparison with serologic blood screening procedures, this method is more sensitive and able to directly detect pathogens before the occurrence of detectable pathogen antibodies, a period known as diagnostic window period.[1]

Particularly after infection with hepatitis A virus (HAV), a hepatotropic picornavirus that causes acute viral hepatitis in humans and has an extraordinary particle stability, the diagnostic window period for serologic screening tests, which are designed to detect liver transaminases or anti-HAV, is remarkably long.[2] During this period of 3 weeks after the initial infection, infectious virus is already present in significant amounts in blood and represents a serious risk for HAV transmission by blood and blood products prepared from donations collected during this phase.[2,3] Although in general the risk of HAV transmission via blood or blood products is considered to be low, there are several reports of this transmission. The recent study of Gowland and coworkers[4] proves that HAV can be transmitted by labile blood components, and there are several reports proving transmission of HAV by plasma-derived coagulation factor (F)VIII and F IX concentrates to hemophilia patients.[5-7] Therefore, to shorten the HAV window period and to reduce the risk of transmission, polymerase chain reaction (PCR) technology was applied. Also cost- and time-saving commercially available reverse transcription (RT)–PCR detection assays were developed, which are routinely used especially for the detection of HAV in plasma for fractionation. In Western Europe and in the United States, plasma fractionation companies have established HAV NAT as a quality assurance tool. In Germany, all Red Cross Blood Transfusion Services that distribute plasma to fractionators have meanwhile implemented HAV NAT screening methods.

In this context it is important to know that although all HAV strains known belong to only one serologic group, phylogenetic analysis of the VP1-coding sequence from worldwide HAV isolates revealed that five distinct HAV gen-

otypes, which include several subgenotypes, can be distinguished by the degree of their genetic heterogeneity.[8]

These genotypes correlate with the geographic origin of the virus isolates.[9,10] For example, all HAV isolates from South America belong to genotype IA. For Europe, a more heterogenous pattern is observed, with genotypes IA and IB and recently genotype III in the Scandinavian and Mediterranean regions as well as in France.[11] This genetic variability may influence the sensitivity or even the detectability of HAV in blood.

To confirm a positive screening result for HAV obtained by the German Red Cross Blood Transfusion Service of Baden-Württemberg-Hessen, PCR-Laboratory, Frankfurt/M, by an in-house RT-PCR, we received in 2003 a plasma sample from a blood donation collected by the blood donation service of the German Red Cross in Hagen, Germany, in which neither transaminases nor anti-HAV were detectable. With a commercially available HAV quantification kit (LightCycler, Roche Diagnostics), which is not certified for diagnostic purposes and is therefore applicable for research only, we were not able to detect HAV RNA. Four other independent laboratories could also not detect HAV RNA. Two weeks after blood donation, however, the donor developed clinical hepatitis A, which shows that the plasma sample was collected during the diagnostic window period of an HAV infection.

Because of the false nucleic acid testing by commercially available RT-PCR kits, we characterized the virus present in the plasma sample by sequencing and found a genotype that was not shown in Germany until then. Based on these results, we developed a HAV RT-PCR screening assay that is able to detect all known HAV genotypes.

MATERIALS AND METHODS

RNA extraction

With a viral RNA mini kit (QIAamp, Qiagen, Hilden, Germany), viral RNA was extracted from 140 µL of plasma (sample derived from a donation collected by the German Red Cross) and was eluted in 60 µL AVE buffer (Qiagen). Five microliters of the viral RNA was quantified by the LightCycler HAV quantification kit (Roche Diagnostics) and by a HAV LC RT-PCR kit (RealArt, artus, Hamburg, Germany).

LightCycler HAV quantification kit

Real-time RT-PCR was performed according to the manufacturer's instructions.

cDNA synthesis

Five microliters of viral RNA was used to accomplish full-length cDNA synthesis with the a first-strand synthesis system for RT-PCR (Superscript, Invitrogen, Karlsruhe, Germany). We used a primer-mix composed of oligo(dT)$_{12-18}$ primer and the random hexamer at a ratio of 3:1.

Long-range PCR

PCR was performed in 200 µL thin-wall PCR tubes in a total volume of 50 µL. The long-range PCR contained 1.5 µL of 5 mmol per L dNTP-mix (Roche Diagnostics), 1.5 µL $MgSO_4$ (50 mmol/L; Sigma-Aldrich, Taufkirchen, Germany), 10 µL 5× PCR buffer (artus), 1 µL of 10 µmol per L forward primer HAV$_{181-204}$, and 1 µL of 10 µmol per L reverse primer HAV$_{7261-7240}$ and as template 2 µL of cDNA. Three different DNA polymerases were used. Every PCR procedure contained 1.25 U TaKaRa *Taq* Hot Start Version (Takara, Gennevilliers, France), 0.5 U TaKaRa *Taq* (Takara), and 0.1 U of proofreading Isis DNA polymerase (Qbiogene, Heidelberg, Germany). Amplification occurred in a master cycler (Eppendorf, Hamburg, Germany) with the following thermal conditions: denaturation at 95°C for 2 minutes and 40 cycles of 1 minute of denaturation at 95°C, 1 minute of annealing at 55°C, and 10 minutes of extension at 72°C. Additionally, a final extension step of 10 minutes at 72°C was performed. To synthesize the 5'-end and the 3'-end of the genome we used primer pair HAV$_{1-18/399-380}$ and primer pair HAV$_{7096-7119/7431-7403}$, respectively. For this we modified the extension step of the thermal conditions in the cycles and at the end to 2 minutes at 72°C. After cycling, the PCR products were checked in a 1 percent agarose gel electrophoreses. Primer sequences are shown in Table 1.

TABLE 1. Primer sequences

Amplicon	Forward primer	Reverse primer
7-kb fragment	HAV$_{181-204}$ 5'-ACACTCATTTCACGCTTTCTGTCT-3'	HAV$_{7261-7240}$ 5'-CATGCATAAAGGCAAACCACTG-3'
5'-end fragment	HAV$_{1-18}$ 5'-TTCAAGAGGGGTCTCCGG-3'	HAV$_{399-380}$ 5'-AGCCTACCCCTTGTGGAAGA-3'
3'-end fragment	HAV$_{7096-7119}$ 5'-GGTTGAAGATAGAATTAGGCCTGC-3'	HAV$_{7431-7403}$ 5'-ATTAGTGACGAAAGAAATAAACAAACCT-3'

Sequencing

Sequencing was done by the DNA Cloning Service (Hamburg, Germany). The 7-kb fragment was sequenced in both directions with the primer-walking approach. The 5′-end fragment was sequenced in antisense direction, whereas the 3′-end fragment was sequenced in sense direction. Sequence length is 7166 bp; it lacks the 3′-UTR and a small part of the three-dimensional region of the HAV genome. The sequence was submitted under Accession Number AY644337.

Sequence data analysis

The complete VP1 nucleotide sequences were aligned with the CLUSTAL W program.[12] The sequences used are listed in Table 2. The phylogenetic tree was generated with the neighbor-joining method. Distances were calculated by the Juke-Cantor formula. The reliability was assessed by bootstrap resampling (1000 pseudo-replicates).

RealArt HAV LC RT-PCR kit

HAV RNA was quantified by one-tube RT-PCR with the LightCycler instrument (Roche Diagnostics). The real-time RT-PCR was performed according to the instruction manual. PCR characteristics in terms of sensitivity, specificity, and performance were determined as follows.

Sensitivity. The detection limit (sensitivity limit) of the RealArt HAV LC RT PCR kit was determined with the international HAV standard (WHO International Standards for HAV RNA NAT assays, NIBSC Code 00/560). The international standard was spiked in HAV-negative plasma, and a half-logarithmic dilution series has been set up from 571.5 to 0.181 IU per mL (eight dilutions). Twenty-four replicates of each dilution were extracted with the QIAamp viral RNA mini kit (extraction volume, 140 µL; elution volume, 80 µL) and were analyzed with the RealArt HAV LC RT PCR reagents. The internal control was added to the 24 samples of each dilution according to the instruction

TABLE 2. Origins of HAV strains used in the phylogenetic analysis

Strain	Accession number	Date of isolation	Geographical location	Genotype
P33-99	AJ437227	1999	France	IA
20F85	AJ437241	1985	France	IA
5F	AJ437190	Not known	France	IA
EF97	AJ437218	1997	France	IA
22F85	AJ437242	1985	France	IA
YF87	AJ437243	1987	France	IA
AF98	AJ438162	1998	France	IA
HF97	AJ438160	1997	France	IA
BOUE9	AJ437234	2000	France	IA
3F	AJ437189	Not known	France	IA
9F	AJ437185	Not known	France	IA
P21-99	AJ438166	1999	France	IA
P18-99	AJ438164	1999	France	IA
P17-99	AJ438165	1999	France	IA
GBM	X75214	1976	Germany	IA
Chile9	AJ437163	1999	Chile	IA
Chile5	AJ437160	1999	Chile	IA
ChileJ	AJ437164	1999	Chile	IA
Chile8	AJ437162	1999	Chile	IA
Arg5	AJ437152	1999	Argentina	IA
Arg1	AJ437151	1999	Argentina	IA
Arg23	AJ437157	1999	Argentina	IA
Arg11	AJ437155	1999	Argentina	IA
Arg15	AJ437156	2000	Argentina	IA
Arg8	AJ437154	2000	Argentina	IA
UruK	AJ437178	1999	Uruguay	IA
Uru20	AJ437176	2000	Uruguay	IA
Uru17	AJ437175	1999	Uruguay	IA
Uru21	AJ437177	2000	Uruguay	IA
Chauo	AJ437179	Not known	Mexico	IA
LCDC1	X14666	Not known	China	IA
LU38	AF357222	Not known	China	IA
LY6	AF485328	Not known	China	IA
LA	K02990	1975	United States	IA
FH3	AB020569	1994	Japan	IA
FH2	AB020568	1993	Japan	IA
FH1	AB020566	1992	Japan	IA
AH1	AB020564	1992	Japan	IA
AH2	AB020565	1991	Japan	IA
AH3	AB020566	1993	Japan	IA
Val15	AF396405	1999	Spain	IA
Val17	AF396407	1999	Spain	IA
Val11	AF396401	1999	Spain	IA
FF-97	AJ437249	1997	France	IB
2F95	AJ437256	1995	France	IB
13F89	AJ437245	1989	France	IB
2F84	AJ437244	1984	France	IB
7F99	AJ437250	1999	France	IB
10F94	AJ437255	1994	France	IB
TF93	AJ437247	1993	France	IB
14F94	AJ437247	1994	France	IB
BOUE2	AJ437315	2000	France	IB
HAF203	AF268396	1992	Brazil	IB
HM175	M14707	1976	Australia	IB
MBB	M20273	1978	Northern Africa	IB
9F94	AJ437317	1994	France	II
HMH	AY644337	2003	Germany	IIIA
P27-99	AJ437316	1999	France	IIIA
GA76	L07668	1976	United States	IIIA
NOR21	AJ299464	not known	Norway	IIIA
PA21	M34084	1980	Panama	IIIA
CY145	M59286	1988	Philippines	IV
AGM27	D00926	1985	Kenya	V
SLF88	AY032861	1988	Sierra Leone	VII

TABLE 3. Virus material for cross-reactivity test

Virus	Concentration	Origin
Poliovirus 2	10^4 copies/mL	Cell culture material
Coxsackievirus A9	10^7 copies/mL	Cell culture material
Coxsackievirus A16	Not quantified	Cell culture material
Coxsackievirus B3	8×10^7 copies/mL	Cell culture material
Coxsackievirus B5	2×10^5 copies/mL	Cell culture material
Echovirus 6	Not quantified	Cell culture material
Echovirus 9	10^8 copies/mL	Cell culture material
Echovirus 11	2×10^5 copies/mL	Cell culture material
Echovirus 22	Not quantified	Cell culture material
Echovirus 30	10^8 copies/mL	Cell culture material
Rhinovirus	Not quantified	Cell culture material
Enterovirus 71	2×10^3 copies/mL	Cell culture material
HBV	10^6 IU/mL	Positive plasma
HCV	10^3 IU/mL	Standard ACCURUN 305 (BBI Diagnostics, Boston, MA)

manual and passed the purification process. The sensitivity limit was determined by a probit analysis. The experiment was carried out on 3 different days with eight replications per dilution. The analysis was performed by the program PriProbit (Version 1.63).

Quantitation standards were generated by RT-PCR from HAV-positive cell culture supernatant (strain HM-175). The PCR product was sequenced and in vitro transcribed with T7 RNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). Transcripts were photometrically quantified, and the respective RNA copy number was calculated. To adapt the transcripts to *IU* we used a quantitative RT-PCR approach, whereas the purified international standard served as a calibrator.

Specificity. The specificity of the HAV LC RT-PCR system is ensured by the selection of primers and probes, as well as the selection of stringent reaction conditions. The primers and probes are selected with computer software (Primer Express, Version 1.5, Applied Biosystems, Weiterstadt, Germany) and are checked for possible homologies to other known sequences by sequence comparison analysis with the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). To determine the specificity of the RealArt HAV LC RT-PCR kit, HAV genotypes IA (strain HAV GBM), IB (strain HM-175), and III (strain HAV HMH) were tested. Because from genotypes II and VII no original virus was available, we synthesized DNA fragments to imitate nucleic acid of genotypes II and VII. Based on sequence information of the database (genotype II strain 9F94, Accession No. AJ437317; and genotype VII strain SLF88, Accession No. AY032861), the target region (95 bp) was divided in two oligonucleotides that overlap in 25 bp. To complete the free ends of the oligonucleotide construct, the oligonucleotides (10 pmol each), 5 mmol per L $MgSO_4$, 0.2 mmol per L dNTP-mix, and 0.25 U *Taq* polymerase were incubated for 10 minutes at 73°C. These DNA fragments were in vitro transcribed and tested with the RealArt HAV LC RT-PCR kit. Potential cross-reactivity was tested with some enteroviruses (poliovirus, coxsackievirus, echovirus, rhinovirus, and enterovirus 71), which are nearly related to HAV, as well as with hepatitis B virus (HBV) and hepatitis C virus (HCV), which cause similar disease patterns. The virus material (listed in Table 3) was extracted with the QIAamp DSP virus kit (extraction volume, 500 µL; elution volume, 60 µL) and tested with the RealArt HAV LC RT-PCR kit.

Precision. The total variance consists of the intraassay variability, the interassay variability, and the interlot variability. To determine the intraassay variability, eight replicates with 571.5 IU per mL were extracted with the QIAamp viral RNA mini kit (extraction volume, 140 µL; elution volume, 80 µL) and analyzed in one PCR procedure with the same lot of the HAV LC RT-PCR kit. Data for interassay variability were generated through three different PCR procedures with eight replicates (571.5 IU/mL) each. Every procedure was handled by a different operator on three different LightCycler instruments, but with the same lot of the RealArt HAV LC RT-PCR kit. For determination of the interlot variability, three different lots of HAV LC RT-PCR kit were tested with eight replicates each of 571.5 IU per mL spiked samples. From the generated data, the standard variation, the variance, and the coefficient of variation (CV) were determined.

Robustness. Thirty HAV-negative plasma samples were spiked with 47 IU per mL HAV RNA (threefold concentration of the analytical sensitivity limit) and extracted with the QIAamp DSP virus kit (extraction volume, 500 µL; elution volume, 60 µL). Internal control was added according to the instruction manual and passed the purification process. These samples were analyzed with the RealArt HAV LC RT-PCR kit.

RESULTS

Clinical information

The donation in question of a male donor, born in 1944, was positive for the presence of HAV RNA. Initial pool-

ing and PCR were performed as described elsewhere.[13] The pool was created by ethylenediaminetetraacetate-plasma aliquots from 96 donations. The positive result from the pool was traced back to the viremic single donation by subpooling via x-/y-intersection approach ("chessboard pooling"), testing, and subsequent confirmation testing of a different aliquot of the identified single donation.

TABLE 4. Comparison of nucleotide identities of VP1 and full-length genomes between strain HMH and the mean of different reference isolates of each genotype

	Mean identity			
Genotype:	IA	IB	VII/II*	III
VP1	80.6	80.4	80.0	95.3
Full-length	82.5	82.8 (only genotype VII)	82.9	Not available

* Genotypes II and VII were combined because recently it was speculated that these two genotypes might be one or two subgenotypes of the same genotype (see also Fig. 1).[8]

Serologically, neither immunoglobulin M (IgM) nor immunoglobulin G antibodies could be detected with the ETI-HA-IGMK PLUS and ETI-AB-HAVK PLUS assays (DiaSorin, Saluggia (VC), Italy). The determination of the alanine aminotransferase (ALT) level resulted in a value of 135 U per L. A blood sample that was taken 1 week after the donation showed a positive result for HAV IgM antibodies. Two later blood samples confirmed these findings. Two weeks after the first blood donation the person developed jaundice with an ALT value of greater than 4000 U per L. Hospitalization was not necessary. The results of these molecular biologic and serologic tests show that this donation was taken during the diagnostic window period. Thus, it is the first HAV RNA–positive diagnostic window period donation detected in Germany.

To confirm this result, we received a sample that we analyzed with the LightCycler HAV quantification kit (Roche Diagnostics). In contrast to the in-house PCR performed at the PCR laboratory of the German Red Cross Blood Transfusion Service, Baden-Württemberg–Hessen, the Roche HAV kit failed to detect the HAV RNA. The standards for quantification and the international HAV standard (NIBSC Code 00/560) were detected, and the internal control showed that the samples were not inhibited for the PCR procedure (results not shown). The RealArt HAV LC RT-PCR kit quantified 130 IU per µL HAV RNA in this sample, which is equivalent to 6500 copies per reaction. Because the sensitivity of the LightCycler HAV quantification kit was stated as at least 50 copies of HAV RNA per reaction (95% cutoff value), the sensitivity limit is not the cause for failed detection.

Characterization of the virus

To obtain more information about the characteristics of the virus present in the blood donation, RNA was extracted from the original sample, and HAV cDNA was synthesized and sequenced. For classification, the VP1-coding sequence was compared with all HAV genotypes already known. As shown in Table 4, the virus was identified as HAV genotype III (95.3% nucleotide identity), a HAV lineage not detected in Germany until now. This strain was named HAV strain HMH. Alignment analysis of the complete VP1-coding region with 60 other isolates, which are listed in Table 2, revealed that HAV HMH shows its closest relationship with strain NOR-21 and P27-99 (Fig. 1). Taking into consideration that no VP1 nucleotide sequence of HAV subgenotype IIIB is available in the database and that HAV HMH clustered directly in sequences of subgenotype IIIA, this strain was classified as HAV subgenotype IIIA.

The nearly complete nucleotide sequence and the corresponding amino acid sequence were also compared with those of reported isolates. In this case, the highest nucleotide identity of 82.9 percent was found with HAV genotype VII strain SLF-88 and the lowest identity (81.9%) with genotype IA strain AH3. Table 4 shows the mean nucleotide identity of HAV HMH in comparison with different strains of each genotype. The comparison with full-length HAV genotype III was not possible, because no full-length sequence of this genotype is available.

Development of the RealArt HAV LC RT-PCR kit

Because commercially available PCR assays were not able to detect HAV genotype III and the epidemiologic situation in Europe is obviously heterogenic, it was necessary to have a PCR detection assay that is able to detect all described HAV genotypes. Therefore, we searched for conserved genomic regions of HAV that would allow constructing primers and probes for a PCR assay, which would be able to detect all HAV genotypes.

The mean nucleotide identities of the 5'-NTR are higher than the mean nucleotide identities of the full-length nucleotide sequences. The highest nucleotide identity was found with a mean of 96 percent in the VP4-coding region, followed by the VP3 region with 84 percent identity. Therefore, these regions are more conserved than the regions encoding VP1 (80% identity) and VP2 (82% identity). Among the nucleic acid sequences coding for the nonstructure proteins, the highest similarity was observed for the 3C region (84% identity).

Based on this information, HAV primers and probes for detection of HAV RNA as well as for an internal control amplification system were synthesized and a real-time PCR on the LightCycler instrument was developed. The internal control (second amplification system) identifies a possible PCR inhibition and moni-