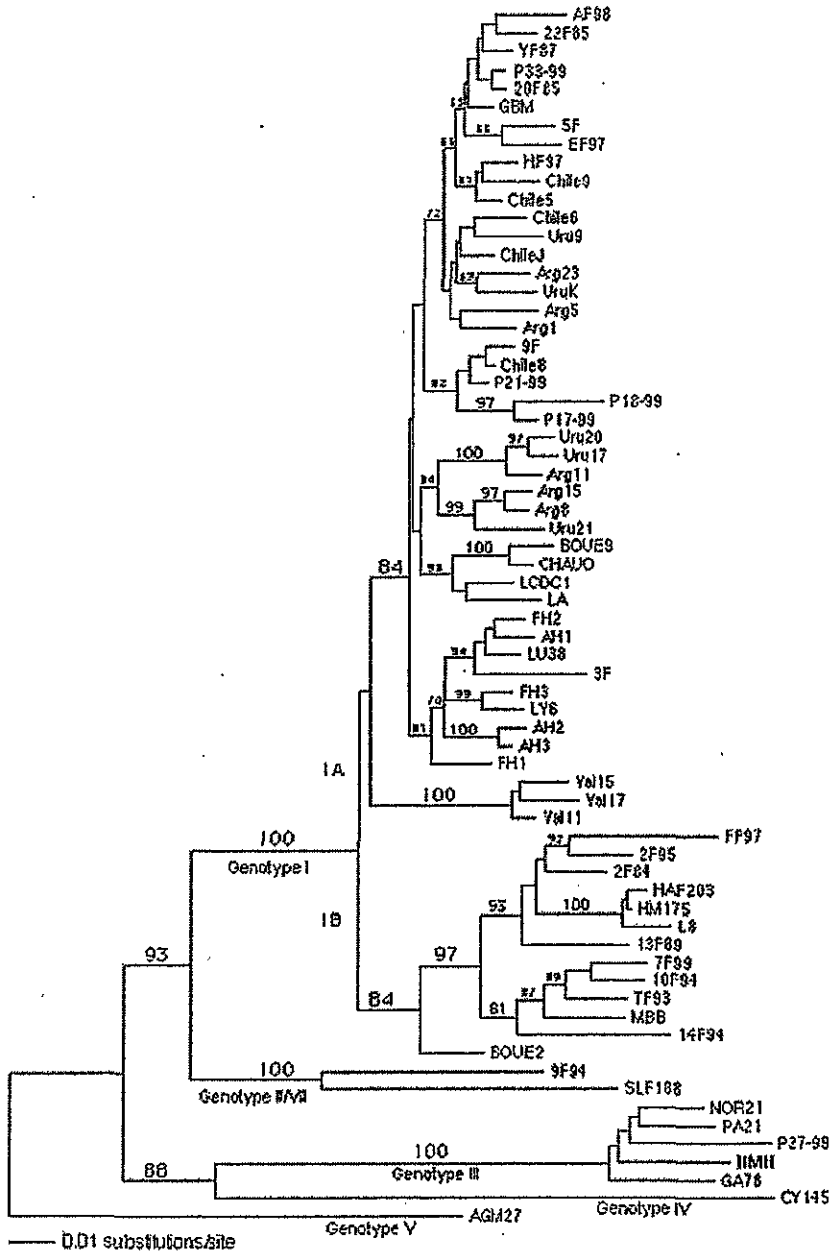

Fig. 1. Phylogenetic analysis of the complete VP1 region. Numbers at the branches indicate bootstrap percentage after 1000 replications of bootstrap sampling. The bar indicates genetic distance.

tors the RNA isolation procedure. For generation of quantitation standards, a HAV PCR product was in vitro transcribed, photometrically quantified, and converted into the copy quantity. Comparison with the international HAV standard (WHO International Standards for HAV RNA NAT assay, NIBSC Code 00/560) showed that 10 copies of the in vitro transcript correspond to 1 IU. A quantitation standard series (QS 1-4) was established (1000-1 IU/μL).

Sensitivity. With 5 μL of sample material extracted with the QIAamp viral RNA mini kit (extraction volume, 140 μL; elution volume, 80 μL), the HAV LC RT-PCR kit showed a detection limit of 183.02 IU per mL of the international HAV standard per reaction (95% probability) (Fig. 2).

Specificity. The available HAV genotypes (genotype IA strain HAV GBM, genotype IB strain HAV HM175, and genotype III strain HAV HMH) have been tested with the HAV LC RT-PCR kit and could be detected with similar sensitivity. Genotypes II and VII were not available but the detectability is ensured by detection of the synthesized genotype II (strain 9F94) and genotype VII (strain SLF88) in vitro transcripts. For possible cross-reactivity, some enteroviruses (poliovirus, coxsackievirus, echovirus, rhinovirus, and enterovirus 71), HBV, and HCV material was tested. The used virus material is listed in Table 3. Only the closest related enterovirus, enterovirus 71, showed a weak cross-reactivity with the HAV LC RT-PCR kit.

Precision. The precision data allowed the determination of the total variance of the assay. The data for HAV were generated with 571.5 IU per mL plasma extracted with the QIAamp viral RNA mini kit (extraction volume, 140 μL; elution volume, 80 μL). According to the results (Table 5), the intraassay variability was 28.23%, the interassay variability was 30.91%, and the maximum variation of two samples of the same concentration was 31.40%.

Quantitation of the HAV strain HMH

The HAV RNA of the window period blood donation was quantified with the HAV LC RT-PCR kit. The viral load of this blood donation was 5.6×10^4 IU per mL (Fig. 3).

DISCUSSION

This first HAV RNA–positive, antibody-negative blood donation in Germany found by the German Red Cross clearly shows the importance of screening blood donations

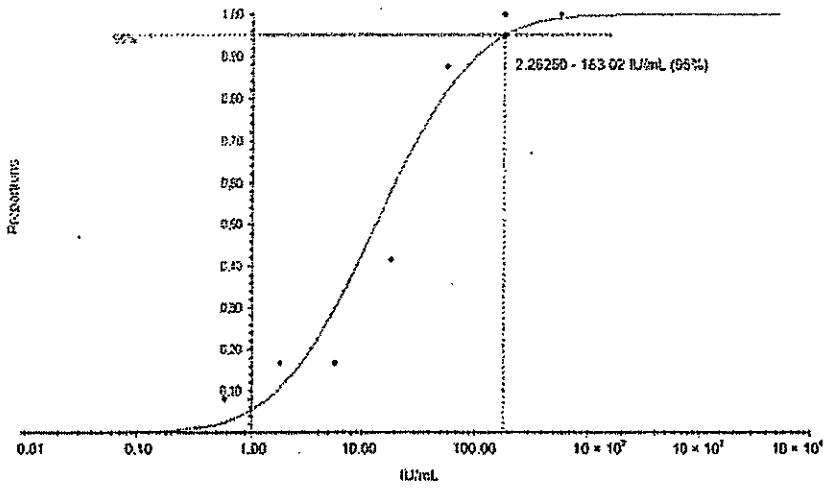

Fig. 2. Diagram of the statistical analysis: From the international HAV standard, a dilution series from 571.5 to 0.181 IU per mL was extracted (QIAamp viral RNA mini kit, Qiagen; extraction volume, 140 µL; elution volume, 80 µL) and analyzed with the RealArt HAV LC RT-PCR system. The experiment was carried out on 3 different days with eight replicates. Analysis was performed by the program PriProbit (Version 1.63).

TABLE 5. Precision data generated with 571.5 IU per mL for quantitation results (IU/mL)

HAV	SD	Variance	CV (%)
Intraassay variability	167.71	2.8×10^4	28.23
Interassay variability	184.62	3.4×10^4	30.91
Interlot variability	183.80	3.4×10^4	30.69
Total variance	188.20	3.5×10^4	31.40

for HAV by PCR. Until now there are several reports of HAV transmission via blood and especially due to the extraordinary particle stability via blood products.[4-6] The clinical consequences for recipients of HAV contaminated blood or blood products are serious, and the fact that there always exists a period where serologic tests failed requires the introduction of HAV NAT for testing of blood donations. Screening of plasma and/or blood by PCR can significantly reduce the diagnostic window period. Schreiber and associates calculated a reduction of 72 percent of the residual risk for HCV transmission via blood or blood products by screening via NAT.[14] The established HAV RT-PCR screening method for blood donations of the German Red Cross Blood Transfusion Service, Baden-Württemberg–Hessen, showed feasibility of such nucleic acid–based assays for routine screening purposes in a large blood bank setting. A prerequisite for the increase of the safety of blood and blood products by PCR screening is the constant monitoring and adaptation of the tests to the genetic variability of the pathogens, as well as the observation of the geographic distribution and relevance of different genotypes. The fact that only four out of eight independent laboratories detected the HAV infection shows that most of the PCR assays used are not able to detect all genotypes of HAV. Comparative studies of the nucleotide sequences of different HAV strains have suggested that sequence relatedness can be correlated with the geographic origin of the virus.[15,16] Genotype I was mainly found in North America, China, Japan, and the former USSR and Thailand.[15,17] Most of the HAV isolates found in western Europe belong to genotypes IA and IB but genotype III was also found. Genotype IIIA occurred during the 1980s in Sweden[17] and at the end of the 1990s also in Norway.[18] So far, genotype III was found predominantly in India, Sri Lanka, Nepal, Malaysia, and the United States.[15,17,19] Based on the work of Costa-Mattioli and coworkers,[8] which suggests a new classification of the HAV genotypes, we selected the complete VP1 region for the phylogenetic analysis of strain HMH. Our analysis shows that the HMH strain belongs to genotype III, subgenotype IIIA. Thus this isolate was the first documented HAV genotype III in Germany. Phylogenetic analysis of an outbreak in 1999 in the north of Brittany revealed the presence of subgenotype IIIA for the first time in western Europe.[20] Tallo and colleagues[21] reported that, although genotype I was the predominant one in Estonia during the past years, the last HAV outbreak was associated with a genotype IIIA strain. These cases suggest that genotype IIIA is becoming more prevalent among the HAV-infected population than formerly assumed. This obvious shift of genotype dissemination makes it indispensable to check and adapt the available PCR assays for detection of all HAV genotypes.[14]

We show that in contrast to other commercial real-time HAV PCR assays, the newly developed RealArt HAV RT-PCR kit is able to detect all genotypes. An implemented second amplification system, the internal control, identifies a possible PCR inhibition and controls the RNA isolation procedure. Internal controls minimize the risk of false-negative results and make additional labor-intensive and costly external control reactions unneccessary.[22] The sensitivity of this assay has been determined by probit regression analysis. This calculation has been proved adequate for the description of sensitivity limits of PCR assays.[23] The detection limit in consideration of the purification with the QIAamp viral RNA mini kit (extraction volume, 140 µL; elution volume, 80 µL) is consistently 183.03 IU per mL ($p = 0.05$). The assay is highly reproducible with intra- and interassay variability of 28.23 and 30.91 percent and interlot variability of 30.69 percent, respectively.

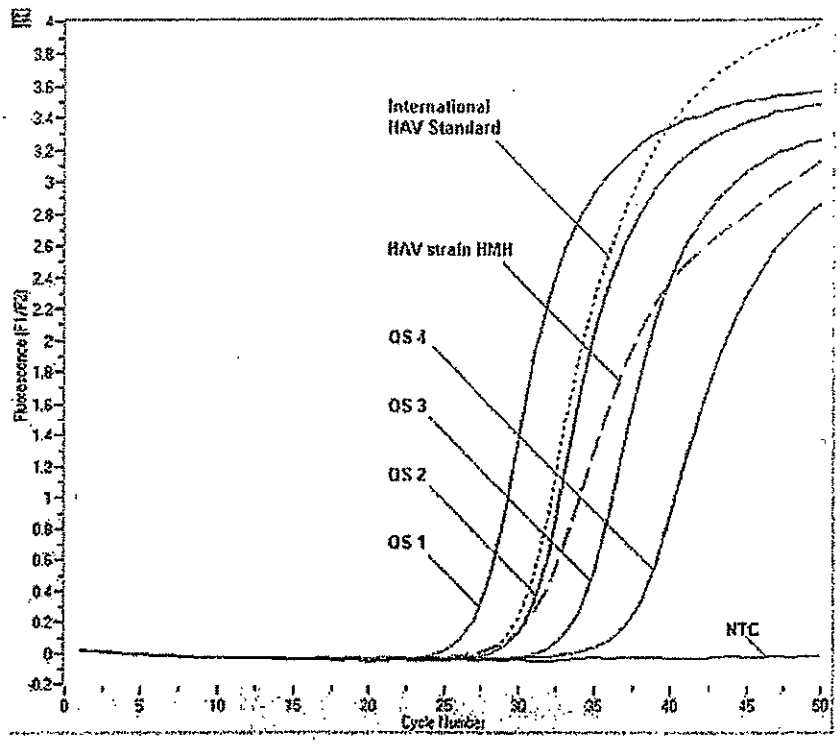

Fig. 3. LightCycler analysis of HAV strain HMH with the RealArt HAV LC RT-PCR kit (artus). The quantitation standards (QS) 1-4 contain in vitro transcript HAV RNA in following concentrations: QS 1, 1000 IU per μL; QS 2, 100 IU per μL; QS 3, 10 IU per μL; and QS 4, 1 IU per μL. The dotted line shows the international HAV standard, and the interrupted line shows quantitation of HAV strain HMH. Strain HMH was quantified with 130 IU per μL eluate, which is equivalent to 5.6×10^4 IU per mL plasma. The employed international standard shows 145 IU per μL eluate, which is equivalent to 6.2×10^4 IU per mL. NTC = nontemplate control.

With the serum sample described, we were able to show that blood screening by the HAV LC RT-PCR kit reduces the window phase during which HAV infection fails to be diagnosed by serologic assays. Real-time PCR assays are able to detect an infection during this window phase already and thus definitely earlier than serologic assays. With regard to the genetic variability, this study shows that amplification assays for the detection of HAV must be more refined than previously thought.

ACKNOWLEDGMENTS

We thank the German Red Cross Blood Transfusion Service of Baden-Württemberg–Hessen, PCR-Laboratory, Frankfurt/M, for identification and providing of the HAV-positive blood donation.

REFERENCES

1. Chamberland ME, Alter HJ, Busch MP, et al. Emerging infectious disease issues in blood safety. Emerging Infect Dis 2001;7:552-3.
2. Bower WA, Nainan OV, Han X, Margolis HS. Duration of viremia in hepatitis A virus infection. J Infect Dis 2000;182:12-7.
3. Krugman S, Ward R, Giles JP. The natural history of infectious hepatits. Am J Med 1962;32:717-28.
4. Gowland P, Fontana S, Niederhauser C, Manouri Taleghani B. Molecular and serologic tracing of a transfusion-transmitted hepatitis A virus. Transfusion 2004;44:1555-61.
5. Chudy M, Budek I, Keller-Stanislawski B, et al. A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated plasma pool. J Med Virol 1999;57:91-9.
6. Soucie JM, Roberston BH, Bell BP, et al. Hepatitis A virus infections associated with clotting factor concentrate in the United States. Transfusion 1998;38:573-9.
7. Normann A, Graff J, Gerritzen A, Brackmann HH, Flehmig B. Detection of hepatitis A virus RNA in commercially available factor VIII preparation. Lancet 1992;340:1232-3.
8. Costa-Mattioli M, Cristina J, Romero H, et al. Molecular evolution of hepatitis A virus: a new classification based on the complete VP1 protein. J Virol 2002;76:9516-25.
9. Lemon SM, Jansen RW, Brown EA. Genetic, antigenic and biological differences between strains of hepatitis A virus. Vaccine 1992;10(Suppl 1):S40-4.
10. Apaire-Marchais V, Robertson BH, Aubineau-Ferre V, et al. Direct sequencing of hepatitis A virus strains isolated during an epidemic in France. Appl Environ Microbiol 1995;61: 3977-80.
11. Costa-Mattioli M, Monpoeho S, Schvoerer C, et al. Genetic analysis of hepatitis A virus outbreak in France confirms the co-circulation of subgenotypes Ia, Ib and reveals a new genetic lineage. J Med Virol 2001;65:233-40.
12. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673-80.
13. Roth WK, Buhr S, Drosten C, Seifried E. NAT and viral safety in blood transfusion. Vox Sang 2000;78(Suppl 2): 257-9.
14. Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ. The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. N Engl J Med 1996;334:1685-90.

15. Jansen RW, Siegl G, Lemon SM. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proc Natl Acad Sci U S A 1990;87:2867-71.
16. Robertson BH, Khanna B, Nainan OV, Margolis HS. Epidemiologic patterns of wild-type hepatitis A virus determined by genetic variation. J Infect Dis 1991;163:286-92.
17. Robertson BH, Jansen RW, Khanna B, et al. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J Gen Virol 1992;73: 1365-77.
18. Stene-Johansen K, Skaug K, Blystad H, Grinde B. A unique hepatitis A virus strain caused an epidemic in Norway associated with intravenous drug abuse. The Hepatitis A Study Group. Scand J Infect Dis 1998;30:35-8.
19. Khanna B, Spelbring JE, Innis BL, Robertson BH. Characterization of a genetic variant of human hepatitis A virus. J Med Virol 1992;36:118-24.
20. Costa-Mattioli M, Ferre V, Monpoeho S, et al. Genetic variability of hepatitis A virus in South America reveals heterogeneity and co-circulation during epidemic outbreaks. J Gen Virol 2001;82:2647-52.
21. Tallo T, Norder H, Tefanova V, et al. Sequential changes in hepatitis A virus genotype distribution in Estonia during 1994-2001. J Med Virol 2003;70:187-93.
22. Cone RW, Hobson AC, Huang ML. Coamplified positive control detects inhibition of polymerase chain reactions. J Clin Microbiol 1994;32:2633.
23. Drosten C, Weber M, Seifried E, Roth WK. Evaluation of a new PCR assay with competitive internal control sequence for blood donor screening. Transfusion 2000;40:718-24.