High-throughput HBV DNA and HCV RNA detection system using a nucleic acid purification robot and real-time detection PCR: its application to analysis of posttransfusion hepatitis

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BACKGROUND: A high-throughput detection system was developed for HBV DNA and HCV RNA.
METHODS: A combination of real-time detection PCR using an automated system (PRISM 7700, PE Biosystems, Foster City, CA) and automatic viral nucleic acid extraction (BioRobot 9604, Qiagen, Hilden, Germany) was used as the high-throughput detection system. An internal control for HBV DNA detection was also developed.
RESULTS: Testing of 96 samples for HBV and HCV was completed within 5 hours. The sensitivity of this system almost equals that of the manual method using nested PCR. The addition of an internal control for HBV detection did not affect the sensitivity of the method and confirmed the accuracy of results. It was possible to quantify HBV in HBV+ samples that contain more than 500 genome equivalents per mL. We started using this system from June 1999 for testing stored donor and patient samples to analyze cases of posttransfusion hepatitis and identified three HBV+ donations that were implicated in posttransfusion hepatitis B.
CONCLUSION: The high-throughput detection system is a useful tool for HBV DNA and HCV RNA detection because it enables rapid and reliable testing of a large number of samples.

HBV and HCV are transfusion-transmitted viruses. Serologic screening tests have been performed to eliminate the blood component contaminated by these viruses. However, some contaminated blood donations are not detected by conventional serologic screening tests, resulting in posttransfusion viral transmission. In order to prevent such incidences of posttransfusion viral transmission, considerable effort has been directed as follows. One way is to improve or develop serologic methods by increasing their sensitivity using a chemiluminescence detection system[1,2] (PRISM, Abbott, Abbott Park, IL) and detection methods for the HCV core antigen.[3,4] The other way is the adoption of NAT.

Recently, the NAT has been introduced in blood screening in many countries to detect window-period infection.[5–7] However, the NAT cannot detect all window-period infections because there had been cases in which blood components that tested negative by PCR caused posttransfusion hepatitis.[8–10] Moreover, pooled plasma of 8 to 96 or more samples is generally used for NAT. Therefore, even though the introduction of NAT to blood

ABBREVIATIONS: AR = automated purification plus real-time PCR; bDNA = branched DNA; FAM = 6-carboxy-fluorescein; geq = genome equivalent; MN = manual nucleic acid purification plus nested PCR; MR = manual nucleic acid purification plus real-time PCR; PHA = passive hemagglutination assay; RPHA = reversed passive hemagglutination assay; TAMRA = 6-carboxy-tetramethyl-rhodamine.

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screening will significantly reduce the incidence of posttransfusion hepatitis or viral transmission, it is very difficult to eliminate completely the possibility of posttransfusion viral transmission. In order to grasp the present state of posttransfusion viral transmission and evaluate and/or improve the present screening system, particularly in terms of the pooling size of NAT, it is important to identify samples that escaped blood screening, including NAT, by investigating all of the samples implicated in posttransfusion viral transmission and to characterize the viruses in these samples.

Japanese Red Cross Blood Centers started storing a sample (6 mL of whole blood) of each donation from September 1996. When hospitals inform us of a hepatitis case suspected to be associated with blood transfusion, we perform NAT on the implicated stored samples as well as serologic tests for viral markers. The nested PCR method is one of the most sensitive methods for virus detection. However, it is very laborious and time consuming and requires skill to test a large number of samples. Moreover, the probability of contamination is relatively high because it requires opening of PCR reaction tubes containing the first PCR products. Recently, a real-time detection PCR system[11–13] that can perform amplification and detection automatically without opening the PCR reaction tubes and an automatic nucleic acid purification system[14] were developed. Then we developed a high-throughput blood-borne virus detection system by combining the automatic nucleic acid purification system and the amplification and detection system. This system enables rapid and reliable testing of a large number of samples.

MATERIALS AND METHODS

Sample materials

An HBV+ plasma obtained from donor screening was used as a positive control, which is determined to be of genotype C based on a phylogenetic tree of S-region sequences constructed using the neighbor-joining method. The HBV quantity in the positive control was determined by a branched DNA (bDNA) assay (Chiron Diagnostics, Emeryville, CA). An HCV+ control was also obtained from donor screening and was determined to be of genotype 1b by the SSP method[15] and based on the constructed phylogenetic tree of the core region sequences. The HCV quantity of the HCV+ control was determined (HCV AMPLICOR Monitor, Roche Molecular Systems, Branchburg, NJ). One of the two HIV+ samples used in this study was determined to belong to clade B by sequencing of the *gag* region and was based on the phylogenetic tree. The clade of the other HIV+ sample was not determined. The HIV quantity was determined (HIV AMPLICOR Monitor, Roche Molecular Systems). Samples of each donation were stored at −30°C until use. Reference panels and a run control were purchased (PELICHECK and PELISPY, respectively, from the Central Laboratory of the Blood Transfusion Service, Netherlands).

Primers and probes

Primers and probes for HBV and HCV detection were designed to correspond with the core region and the 5′ noncoding region, respectively, based on the sequences stored in GenBank databases to detect all genotypes (subtypes) of each virus. Probes for detecting amplicons of viral nucleic acid were labeled with 6-carboxyfluorescein (FAM) as a reporter dye at the 5′ end and with TAMRA (6-carboxy-tetramethyl-rhodamine) as a quencher dye at the 3′ end. Probes for detecting amplicons of internal control were labeled with VIC at the 5′ end and with TAMRA at the 3′ end. The oligonucleotide sequences of the primers and probes are shown in Tables 1 (for HBV DNA detection) and 2 (for HCV RNA detection). A mixture of two forward primers, two reverse primers, and five probes was used for HBV detection. Similarly, a mixture of one forward primer, one reverse primer, and two probes was used for HCV detection.

Internal control

An internal control for nucleic acid purification and HBV DNA amplification was constructed by amplifying a plasmid (pCR 2.1-TOPO, Invitrogen, San Diego, CA). The oligonucleotide sequences of primers for constructing the internal control are shown in Table 3. By using this primer set, a PCR product with sequences the same as

TABLE 1. Oligonucleotides for HBV detection

Oligonucleotide	Function	Sequence
HB-FP1	Forward primer	5′-CCTATCTTATCAACACTTCCGG-3′
HB-FP2	Forward primer	5′-CCTATCCTATCAACGCTTCCGG-3′
HB-RP1	Reverse primer	5′-TTGAGATCTTCTGCGACG-3′
HB-RP2	Reverse primer	5′-TTGAGATCTTCTGCGACCG-3′
BP-1	Probe	5′-(FAM)-TACTGTTGTTAGACGACGAGGCAGGTT-(TAMRA)-3′
Bp1-2	Probe	5′-(FAM)-TTGTGTTGTTAGACAACGAGGCAGGT-(TAMRA)-3′
Bp1-3	Probe	5′-(FAM)-TACTGTTGTTAGAGGAAGGGGAAGGG-(TAMRA)-3′
Bp-3	Probe	5′-(FAM)-TGTTGTTAGACGACGGGACCGAGG-(TAMRA)-3′
Bp3-2	Probe	5′-(FAM)-TGTTGTTAGATGACGAGACCGAGG-(TAMRA)-3′

TABLE 2. Oligonucleotides for HCV detection

Oligonucleotide	Function	Sequence
C53P	Forward primer	5′-A(T/C)CACTCCCCTGTGAGGAACT-3′
C33P3	Reverse primer	5′-GG(T/G)CCTGGAGG(C/T)TG(C/T)ACG-3′
CTPR5	Probe	5′-(FAM)-TGTCTTCACGCAGAAAGCGTCTAGCCAT-(TAMRA)-3′
CTPR52	Probe	5′-(FAM)-TGTCTTCACGCGGAAAGCGCCTAGCCAT-(TAMRA)-3′

TABLE 3. Oligonucleotides for internal control preparation and detection

Oligonucleotide	Function	Sequence
HB-FP1TA1	Forward primer	5′-CCTATCTTATCAACACTTCCGGAGGGCGAATTCTGCAGATAT-3′
HB-FP2TA1	Forward primer	5′-CCTATCCTATCAACGCTTCCGGAGGGCGAATTCTGCAGATAT-3′
HB-RP1TA3	Reverse primer	5′-TTGAGATCTTCTGCGACGGGCCTCTTCGCTATTACGCC-3′
HB-RP2TA3	Reverse primer	5′-TTGAGATCTTCTGCGACCGGGCCTCTTCGCTATTACGCC-3′
TAP-1	Probe	5′-(VIC)-CATCACACTGGCGGCCGCTC-(TAMRA)-3′

those of the HBV primers at the 5′ and 3′ ends but not at the inner part was obtained. The amplicon of the internal control was detected with the probe TAP-1 (Table 3).

Nucleic acid purification

HBV DNA was purified manually (EX-R&D, Sumitomo Metal Industries, Tokyo, Japan) with 100 μL of plasma. HCV RNA was purified by the acid guanidinium thiocyanate-phenol-chloroform purification method[16] using 100 μL of plasma. Purified HBV DNA or HCV RNA was suspended in 20 μL of distilled water, and the extracts were used for PCR amplification and detection. Nucleic acid was purified (BioRobot 9604, Qiagen, Hilden, Germany) based on the manufacturer's protocols using 250 μL of plasma. The purified nucleic acid was suspended in 60 μL of distilled water, and two 25-μL aliquots were used for real-time detection PCR in duplicate testing. When automation was used for nucleic acid purification, an internal control for HBV detection was added to the lysis buffer to obtain 50 genome equivalents (geq) per PCR.

Nested (seminested) PCR

The seminested PCR method for HBV detection was described elsewhere.[17] The reverse transcription-nested PCR method for HCV detection was carried out with the previously reported method[18] combined with the acid guanidinium thiocyanate-phenol-chloroform method[16] for nucleic acid purification.

Real-time detection PCR

Master mix (25 μL, *Taq*Man 2 × PCR, PE Biosystems, Foster City, CA) and 3.5 μL of the mixture of primers and probes was dispensed automatically to each well of a 96-well reaction plate (MicroAmp, PE Biosystems) using the automated handler. Then 25 μL of purified viral nucleic acid solution or control plasmid solution for HBV quantification was also added to each well with the automated handler. The final concentration of the total primers or probes is 600 n*M* for forward and reverse primers and 200 n*M* for the detection probes. In the case of HCV testing, a different core reagent (*Taq*Man EZ RT-PCR, PE Biosystems) was used instead of the master mix. The PCR conditions for HBV testing were as follows: 50°C for 2 minutes, 95°C for 10 minutes, and 50 cycles of 95°C for 15 seconds, 55°C for 1 minute, and 72°C for 15 seconds followed by 72°C for 7 minutes. In HCV detection, the RT-PCR conditions were as follows: 50°C for 2 minutes, 60°C for 30 minutes, 95°C for 5 minutes, and 50 cycles of 95°C for 15 seconds, 55°C for 1 minute, and 72°C for 15 seconds followed by 72°C for 7 minutes.

In order to quantify HBV, a cloned HBV DNA in which the geq was determined based on the absorbance at 260 nm, and molecular weight was used to obtain a calibration curve by plotting the geq against the threshold cycle of real-time detection PCR. The geq of the unknown sample was determined based on the threshold cycle of the unknown sample and the calibration curve.

Serologic tests

Serologic screening tests have been performed in Japanese Red Cross Blood Centers using reversed passive hemagglutination assay (RPHA) for HBsAg, passive hemagglutination assay (PHA) for anti-HBs, hemagglutination inhibition assay for anti-HBc, and PHA (Dainabot, Tokyo, Japan) or particle agglutination assay (Ortho-Clinical Diagnostics, Tokyo, Japan) for anti-HCV. All reagents for the HBV screening tests were prepared in house. Patient and donor samples implicated in posttransfusion HBV or HCV infection cases were tested for the presence of HBsAg, anti-HBc, anti-HBs, and anti-HCV using EIAs (Abbott, Abbott Park, IL).

Statistical analyses

The two-tailed Pearson chi-square tests were performed to calculate the p value using software (SPSS software, SPSS, Chicago, IL).

RESULTS

Sensitivity

An HBV+ sample and an HCV+ sample, which have been used as positive controls in our laboratory, were tested with three methods to compare the sensitivity of each method (Tables 4 and 5): manual nucleic acid purification followed by (seminested) nested PCR (MN), manual nucleic acid purification followed by real-time detection PCR (MR), and automatic nucleic acid purification followed by real-time detection PCR (AR). The sensitivity of the MN is estimated to be 30 geq per mL for HBV detection using an HBV-positive sample quantified by the bDNA assay as well as for HCV detection using the run control and an HCV+ sample quantified (HCV AMPLICOR Monitor, Roche Molecular Systems and a bDNA method (data not shown).

When an in-house-prepared HBV+ control containing 30 geq per mL was tested, the p values obtained using two-tailed Pearson chi-square tests between the two methods were as follows: AR versus MR, 0.315; MN versus AR, 0.004; and AR versus MR, 0.082. Thus, AR showed a higher sensitivity than MN in the testing of samples containing 30 geq per mL of HBV. There was no significant difference in results when an in-house-prepared HBV+ control containing 100 geq per mL was tested.

Similarly, when an HCV+ control containing 30 geq per mL was tested, the p values between the two methods were as follows: MN versus MR, 0.165; MN versus AR, 0.115; and AR versus MR, 0.002. There was no significant difference between AR and MN, although the sensitivity of AR was less than that of MR, which is different from the result of HBV detection sensitivity.

The sensitivity of AR was also examined by testing the reference panels. There was no notable difference between AR and MN (Tables 4 and 5). Comparing the results of the three methods and those of testing panels, it was suggested that the sensitivity of the combination of AR is comparable to that of MN, although the HCV detection sensitivity is less than that of the combination of MR.

Specificity and crosscontamination

Two HBV+ samples (genotype C, 1000 geq/mL; genotype A, 90 geq/mL), two HCV+ samples (genotype 1b, 1000 geq/mL; genotype 3, 100 geq/mL), two HIV+ samples (clade B, 200 geq/mL; undetermined clade, 1000 geq/mL), and 20 other samples were HBV–, HCV–, and HIV– by the conventional screening tests used for confirming the specificity and the absence of crosscontamination during AR. All experiments for confirming the specificity and the absence of crosscontamination were performed not using blind tests. Twenty samples dispensed from one HBV+ sample, 20 samples dispensed from one HCV+ or HIV+ sample, and 20 negative controls were arranged in a checkerboard pattern where HBV– samples were placed before and after HBV+ samples. Then nucleic acid extraction and amplification and detection were performed simultaneously using AR. The HCV detection method was also tested in the same manner. All of the HCV+ samples, HIV+ samples, and the screening test-negative samples were negative in HBV detection PCR. Also, all of the HBV+ samples, HIV+ samples, and screening test-negative samples were negative in HCV detection PCR.

We also carried out the simultaneous extraction and amplification and detection using 48 HBV– samples derived from one HBV screening-negative sample and 48 HBV+ samples derived from one HBV screening-positive sample that was estimated to contain 50,000 geq per mL of HBV. These were arranged to a checkerboard pattern and were used for an experiment. This experiment was carried out twice; namely, 96 extractions and 192 amplifications and detections were performed for each positive and negative sample. One well for amplification and detection of HBV– sample was judged to be positive (one crosscontamination). Considering these results, there is no crossreaction observed thus far, and there is a very low crosscontamination rate when using this method.

TABLE 4. Comparison of HBV detection sensitivity among the three methods

Method		Sample (positive/total)				
		In-house-prepared positive control			PELICHECK panel	
DNA purification	Amplification/detection	100 geq/mL	30 geq/mL	Normal human plasma	100 geq/mL	30 geq/mL
Manual (MN)	Nested PCR/PAGE	10/10 (100%)	5/11 (45%)	0/10 (0%)		
Manual (MR)	PRISM 7700	12/12 (100%)	11/17 (65%)	0/22 (0%)	2/2 (100%)	1/2 (50%)
BioRobot (AR)	PRISM 7700	45/52 (87%)	65/78 (83%)	0/42 (0%)	40/40 (100%)	30/44 (68%)

TABLE 5. Comparison of HCV detection sensitivity among the three methods

Method		Sample (positive/total)				
		In-house-prepared positive control			PELICHECK panel	
DNA purification	Amplification/detection	100 geq/mL	30 geq/mL	Normal human plasma	100 geq/mL	30 geq/mL
Manual (MN)	Nested PCR/PAGE	20/20 (100%)	20/36 (56%)	0/40 (0%)		
Manual (MR)	PRISM 7700	33/35 (94%)	25/35 (71%)	0/35 (0%)	1/2 (50%)	2/2 (100%)
BioRobot (AR)	PRISM 7700	64/80 (80%)	31/78 (40%)	0/76 (0%)	44/44 (100%)	28/40 (70%)

Addition of internal control and its effect on sensitivity

The internal control for HBV detection was purified by extraction of the electrophoresed PCR products from the agarose gel. When the internal control was added to the lysis buffer to a final quantity of 50 geq per PCR, the internal control was detected in all the PCR reaction wells (42 positive/42 PCR). Therefore, we used an internal control of 50 geq per PCR for HBV detection.

In order to examine whether the internal control affects the sensitivity of the method, we tested the positivity rate with or without an internal control (Table 6). It was confirmed that the addition of an internal control of 50 geq per PCR to the lysis buffer for nucleic acid purification in HBV detection does not affect the sensitivity of the method.

Analyses of HBV and HCV infection cases that occurred after transfusion

We started using the system described here in June 1999 for testing all donor and patient samples, which were implicated in posttransfusion HBV or HCV infection. The maximum number of samples in one experiment was 96 because both pieces of automated equipment used are 96 well-formatted systems. Testing of 96 samples was completed within 5 hours. In conventional experiments, however, we performed each test using less than 48 samples, including five positive controls and one negative control per nine samples because the real-time detection PCR was performed in duplicate by dividing the purified nucleic acid solution into two aliquots. Seventeen and 39 cases reported to us to be implicated in HBV infection and HCV infection, respectively, were tested with this system. The total numbers of donors were 357 for HBV (range, 1-185; average, 21) and 551 for HCV (range, 1-130; average, 14). HBV DNA was detected in the stored donor sample in three cases suspected to be posttransfusion HBV infection. HBsAg was not detected in two cases but was detected in one case using an overnight EIA method, and anti-HBs and anti-HBc were not detected in all three cases by the EIA method. The sequences of the pre-S region determined by direct sequencing of the PCR products were completely identical between the HBV DNA samples obtained from donors and patients. We have not detected HCV RNA in all cases suspected to be HCV infection after transfusion until now.

Related data on three cases associated with HBV DNA-positive donors were identified as follows. Case A was associated with Donor A, who was anti-HBs positive (RPHA and EIA), anti-HBc positive (hemagglutination inhibition assay), and anti-HBe positive (EIA) but HBsAg negative (EIA) and HBeAg negative (EIA) 9 months after the implicated donation, which was his first donation. Two patients transfused with blood components from Donor A died within 1 month from the transfusion due to a primary disease. Case B was associated with Donor B who was anti-HBs positive (EIA), anti-HBc positive (EIA), and anti-Hbe positive (EIA) but HBsAg negative (EIA) and HBeAg negative (EIA) 10 months after the implicated donation. We could not look back to any of her previous donations. No patients were transfused with blood components from Donor B. Case C was associated with Donor C. We could not follow up testing of Donor C, and we did not perform look back study because he donated approximately 1 year previous to the implicated donation. Two patients were transfused with blood components from the same donation of Donor C. One patient died because of a primary disease. The other patient who was transplanted with a liver and transfused with 160 mL of FFP from Donor C was HBsAg negative, anti-HBs negative, anti-HBc negative, and HBV DNA negative 9 months after the transfusion.

Quantitation of HBV DNA

We used 5, 50, 500, 5000, and 50,000 geq of cloned HBV DNA per PCR to obtain the calibration curve. Although 50 to 50,000 geq of HBV DNA were always detected, 5 geq HBV DNA per PCR were occasionally not detected. That is, more than 50 geq of HBV per PCR can be quantified definitely. Because the final volume of the purified viral nucleic acid from 250 μL of plasma is approximately 60 μL, and 25 μL is used for one PCR, the purified nucleic acid in 100 μL of plasma is used per PCR. Considering these factors, more than 500 geq of HBV DNA per mL of plasma can be quantified accurately. We identified three HBV-positive donations associated with posttransfusion

TABLE 6. Effect of internal control addition on the sensitivity of the combined method of automatic nucleic acid purification and real-time detection PCR (AR)

Internal control	HBV positive rate (positive/total)*
Present	31/40 (78%)
Absent	27/40 (68%)

* HBV-positive control containing 30 geq/mL HBV DNA was used.

HBV infection, as described. The quantities of HBV DNA in these three cases were 1,400 geq per mL (case A), 130,000 geq per mL (case B), and 9,900 geq per mL (case C).

DISCUSSION

The NAT is a prominent method for detecting viral nucleic acid, and PCR has been used widely for this purpose. Amplifying the first PCR product by nested PCR, the sensitivity of the test increases, and virions at very low levels can be detected. In the case of nested PCR, however, the probability of contamination (false positive) is relatively high because it entails opening the reaction tube containing the first PCR product. Real-time detection PCR is an excellent method in this context because the PCR product can be detected without opening the reaction tube. On the other hand, viral nucleic acid extraction and purification is also a very important step in the NAT because the recovery rate of nucleic acid from virions directly affects the sensitivity of the test. The purification of nucleic acid from a large number of samples is still laborious and requires skill even if a commercially available nucleic acid purification kit is used. Therefore, an automatic device with a high recovery rate of nucleic acid is needed for nucleic acid purification using a large number of samples. Thus, the combination of real-time detection PCR and automatic viral nucleic acid extraction and purification described here is suitable for use as a high-throughput detection system.

Although the combination of AR showed a higher sensitivity for HBV detection than MN ($p = 0.004$), AR showed a sensitivity comparable to MN ($p = 0.115$) but a lesser sensitivity than MR ($p = 0.002$) for HCV detection. These results suggest that the viral RNA recovery rate using an automated handler is slightly lower than that using manual extraction.

There are many reports on viral nucleic acid detection using the *Taq*Man system for HCV detection,[12,19] HBV detection,[20,21] and both HBV and HCV detection.[22] Our results on the sensitivity described here agreed well with those of these previous reports. The detection limits of *Taq*Man PCR for HCV detection are 13 geq per sample quantified using bDNA,[12] 152 to 8000 RNA molecules per mL using spectrophotometery or HCV Monitor assay,[19] and less than 50 molecules per mL[22] using commercially available HCV panel controls and Amplicor HCV Monitor. The detection limits of *Taq*Man PCR for HBV detection are 1 molecule (54.2% of the reactions were positive),[21] less than 50 molecules per mL,[22] and a linear response in the assay from 10^1 to 10^9 copies per reaction.[20]

The Japanese Red Cross Blood Centers started the NAT for HBV, HCV, and HIV on all donations from October 1999 using pooled plasma (n = 500) and reduced the pooling size from 500 to 50 in February 2000. We identified three HBV+ donations that were implicated in posttransfusion hepatitis B, as described previously here. The HBV DNA quantities in these donations were 1400 geq per mL, 130,000 geq per mL, and 9900 geq per mL. The former two donations occurred before the start of the NAT using pooled plasma (n = 500), and the last one occurred before reducing the pooling size to 50. Considering the HBV DNA quantity, all three donations would not have escaped the present NAT using pooled plasma from 50 samples because the sensitivity is sufficiently high to detect HBV DNA in all three cases.

With the cooperation of Japanese Red Cross Blood Centers, we carried out a study on the impact of sample handling and storage conditions on the stability of HCV RNA. There were no significant changes in HCV RNA concentration among the whole-blood specimens that were stored at 4, −20, and −80°C for 4 weeks, as quantified using an HCV AMPLICOR Monitor (Iizuka H et al., unpublished data). These data agree with the report of Gessoni et al.[23] that HCV RNA was stable in whole-blood samples stored at 4°C for 168 hours. On the other hand, Halfon et al.[24] reported that a significant decrease in the quantity of HCV RNA by 15.6 or 23 percent was observed when sera were stored at −20 °C for 5 days or 6 months, respectively. Although we have not observed a drastic decrease in HBV DNA or HCV RNA quantity in sera stored at –30°C and −80°C for several years, further studies on the long-term stability of HBV DNA and HCV RNA are required to evaluate the present storage conditions of donated samples.

We started using this system in June 1999 for analyzing the cases suspected to be infected with HBV or HCV after transfusion; we did not experience any distinct contamination caused by the use of either automated system. The high-throughput detection system described here is a useful tool for HBV and HCV genome detection because it enables rapid and reliable testing of a large number of samples.

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