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Reverse Transcription-PCR Detection of Hepatitis G Virus

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Hepatitis G virus (HGV) was recently identified as a new member of the *Flaviviridae*, but its clinical significance is still unclear. Since no immunoassay for the diagnosis of HGV is available, we developed a sensitive reverse transcription-PCR (RT-PCR) assay to facilitate the detection of the viral genome by mass screening in the clinical laboratory. Sequences within the 5′-noncoding region and within the putative NS5a region are independently amplified in the presence of digoxigenin-11-dUTP and are detected by hybridization with biotinylated capture probes binding to a streptavidin-coated matrix. Semiquantitative Enzymun-Test DNA detection via chemiluminescence can be performed either in a microtiter plate format or on fully automated ES 300 machines. We were able to detect at least 8×10^2 genome equivalents per ml of serum using both primer pairs. HGV was shown to be present in 43 of 130 (33%) serum samples from intravenous drug abusers with a high risk of parenteral exposure. However, only two of the patients were positive when the NS5a primers only were used, and only one patient was positive when only the 5′-noncoding region primers were used, demonstrating the increased sensitivity of HGV detection with two sets of primers. Among these patients, there was no obvious correlation with other viral infections like hepatitis B virus, hepatitis C virus, or human immunodeficiency virus. Within a blood donor panel, 3 of 92 (3%) samples were found to be HGV positive, suggesting that donated blood may need to be screened for HGV.

Viral hepatitis remains an important public health problem throughout the world. The so far well-characterized hepatitis viruses can be divided into two categories, those associated with acute, self-limited illness only (hepatitis A virus and hepatitis E virus) and those associated with acute and chronic disease (hepatitis B virus [HBV], hepatitis C virus [HBC], and hepatitis D virus) (for a recent review, see reference 8). Persistent HBV and HBC infection may cause chronic liver failure and are contributing factors in the development of hepatocellular carcinoma (4). HDV is an incomplete virus and can only replicate in the presence of HBV (11).

Despite the availability of sensitive immunoassays and nucleic acid assays for the detection of infection with these known agents of viral hepatitis, the etiology of 10 to 20% of posttransfusion non-A-E hepatitis and community-acquired non-A-E hepatitis cases has remained unaccounted for, suggesting the existence of one or more additional infectious agents associated with hepatitis (2).

Using expression cloning and immunoscreening, Linnen et al. (7) identified a new positive-sense RNA virus from the plasma of a patient (designated PNF2161) with posttransfusion chronic non-A, non-B hepatitis, which became the source of the prototype virus. Since this patient was coinfected with HCV, a second cloning source (designated R10291) was identified from blood donations deferred because of abnormal alanine aminotransferase levels and negative for markers for HBV and HCV but with a 4-year history of abnormal liver functions (7). Sequence analysis showed that the genomes of both viruses share 90.5% amino acid identity. The viral genome of PNF2161 encodes a single, continuous open reading frame of 2,873 amino acids with a number of characteristic motifs, i.e., a helicase motif, two chymotrypsin-like protease motifs, and an RNA-dependent RNA polymerase motif. Overall, sequence homologies with flaviviruses like GB virus type A (GBV-A) (43.8%), GBV-B (28.4%), and HCV type 1 (HCV-1) (26.8%) suggested the discovery of a new member of the *Flaviviridae* family (3) which was provisionally designated hepatitis G virus (HGV). In contrast to HCV and HGV, GBV-A and GBV-B were isolated from tamarins infected with serum from a surgeon with acute icteric hepatitis (9, 12, 14). Whether both are common human hepatotrophic viruses is still under discussion. In using degenerate primers, Leary and colleagues (6, 13) were able to identify a third isolate, designated GBV-C, from a human source, and nucleotide sequence identities of approximately 85% suggest that GBV-C and HGV are independent isolates of the same virus (2, 15).

The disease association of HGV, the course of HGV infection, and whether there is detectable immune response that can be used in a serological screening system are still under investigation (2). So far, the only tool for the diagnosis of HGV infection is based on the detection of the viral genome, a tedious and time-consuming method hardly desirable for use for routine diagnosis. In the study described in this report, however, we combined the reverse transcription-PCR (RT-PCR) assay with the Enzymun-Test DNA detection system (Boehringer Mannheim GmbH, Mannheim, Germany) and made this technique applicable for mass screening in the clinical laboratory. For the greatest sensitivity, we amplified by PCR two independent regions of the viral genome, i.e., the 5′-noncoding region (5′-NCR) and the NS5a region. Subsequently, both amplicons are hybridized with internal biotinylated capture probes and are detected by chemiluminescence, which can be performed either in a 96-well microtiter plate format or on fully automated ES 300 machines.

MATERIALS AND METHODS

Human material and biochemicals. Sera were collected from high-risk intravenous drug abusers (IVDAs) in Berlin, Germany, and the blood donor bank in Salzburg, Austria. All reagents used were from Boehringer Mannheim GmbH

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TABLE 1. PCR primers and capture probes used for amplification and detection of HGV-RNA[a]

Primer or probe	Sequence	Position[a]
5′-NCR forward primer	5′-CGGCCAAAAGGTGGTGGATG-3′	100
5′-NCR reverse primer	5′-CGACGAGCCTGACGTCGGG-3′	285
5′-NCR capture probe	5′-biotin-GGTAGCCACTATAGGTGGG-3′	161
NS5a forward primer	5′-CTCTTTGTGGTAGTAGCCGAGAGAT-3′	6904
NS5a reverse primer	5′-CGAATGAGTCAGAGGACGGGGTAT-3′	7059
NS5a capture probe	5′-biotin-GTTACTGAGAGCAGCTCAGAT-3′	6980

[a] Nucleotide positions refer to the 5′ positions in the sequence that is GenBank accession number U44402 (7).

with the exception of the QIAamp HCV kit and the QIAquick gel extraction kit (Qiagen Inc., Chatsworth, Calif.). Oligonucleotide primers for PCR amplification and biotinylated capture probes for detection were synthesized and purified by high-pressure liquid chromatography at the DNA synthesis group of Boehringer Mannheim.

Oligonucleotides. The 5′-NCR primer set (Table 1) was designed on the basis of an alignment of 36 nucleotide sequences of the 5′-NCR of HGV which were kindly provided by Genelabs Technologies, Redwood City, Calif. The NS5a primer-probe set (Table 1) is the original primer set used by Linnen et al. (7); it was derived from clone 470-20-1. The primer sets delineate PCR amplification products of 186 bp within the 5′-NCR and 156 bp within the NS5a region. The biotinylated capture probes are internal sequences within both amplicons. Biotin was introduced to the 5′ end of the DNA via an aminolinker by D-biotin-*N*-hydroxysuccinimide. The six primers and capture probes described above were combined in the Hepatitis G Virus-Probe set (catalog no. 1782 720; Boehringer Mannheim GmbH). Primers c4f and c4r for the detection of GBV-B were synthesized as described previously (12).

RNA extraction. For the preparation of total RNA from human sera, we used the QIAamp HCV kit according to the manufacturer's instructions. In brief, 140 μl of serum was incubated with 560 μl of lysis buffer containing chaotropic salts and carrier RNA for 10 min at room temperature. After the addition of 560 μl of ethanol, the precipitated RNA was applied onto a silica-based spin column for purification and was finally eluted with 50 μl of diethyl pyrocarbonate-treated water.

RT-PCR. For first-strand cDNA synthesis, 10 μl of the RNA preparation described above was supplemented in a total reaction volume of 20 μl with 1× RT buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 1 mM dithioerythritol [pH 8.5]), 100 μM PCR nucleotide mixture, 200 nM random hexanucleotide mixture, 1 U of RNase inhibitor, and 20 U of Moloney murine leukemia virus reverse transcriptase (final concentrations). The mixture was incubated for 10 min at room temperature; this was followed by incubation for 30 min at 42°C and finally for 5 min at 94°C for denaturation of the products.

For performing a hot PCR start, the RT mixture was supplemented at 80°C with 80 μl of preheated PCR mixture. The composition of the PCR mixture was 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ [pH 8.3]), 1× PCR digoxigenin labeling mix (200 μM [each] dATP, dCTP, and dGTP, 190 μM dTTP, and 10 μM digoxigenin-11-dUTP), 200 nM (each) primers, and 2.5 U of the Expand High Fidelity PCR system. Either 35 cycles of amplification under semiquantitative conditions or 45 cycles of amplification under qualitative conditions were run, with stepwise denaturation for 15 s at 94°C, annealing for 30 s at 55°C, and elongation for 30 s at 72°C.

All of the concentrations presented above are final values. Reaction conditions were adapted to the GeneAmp PCR System 9600 thermal cycler (Perkin-Elmer, Foster City, Calif.).

Detection of amplification products. The amplification products were analyzed by agarose gel electrophoresis and were visualized under UV illumination after staining with ethidium bromide. Subsequently, the gels were blotted onto positively charged nylon membranes, immobilized by UV cross-linking, and visualized with a nonradioactive digoxigenin DNA detection kit (Boehringer Mannheim GmbH). In brief, the blots were blocked for 1 h (0.1 M maleic acid, 0.15 M NaCl, 1% blocking reagent [pH 7.5]) and were incubated for another 1 h with anti-digoxigenin–alkaline phosphatase conjugate, Fab fragments, diluted 1:20,000 in blocking buffer. After extensive washing, CDP-*Star*, diluted 1:2,000, was used as a fast and sensitive substrate for alkaline phosphatase. Usually, 15 min was sufficient for exposure to X-ray-sensitive films.

For greater convenience and mass screening, detection can be performed by the Enzymun-Test DNA detection system (Boehringer Mannheim GmbH) either in a microtiter plate format or on a fully automated ES 300 machine. The amplification products are diluted 1:10 with denaturation solution (50 mM NaOH). A total of 100 μl of this sample was mixed with 400 μl of hybridization solution (phosphate-buffered saline [pH 6.5]) which contained the biotinylated capture probe at a concentration of 75 ng/ml. The subsequent hybridization for 120 min at 37°C was performed either in a streptavidin-coated microtiter plate or in a streptavidin-coated sample cup of an ES 300 instrument. After washing, the detection of HGV-specific DNA was detected by binding the biotin-labelled nucleic acids to the streptavidin solid phase and adding anti-digoxigenin–peroxidase conjugate in Tris-HCl buffer (pH 7.5). The mixture was incubated for 30 min at 37°C, and after a final washing, the enzyme substrate [1.9 mM 2,2′-azino-di(3-ethylbenzthiazoline sulfonate), diammonium salt; ABTS] in 100 mM phosphate citrate buffer (pH 4.4)–3.2 mM H_2O_2 (as sodium perborate) was added. The color was allowed to develop for 30 min, and the A_{405} was measured. Positive samples were determined by using a cutoff value of three times the absorbance of an HGV-negative serum sample.

Nucleotide sequencing. The amplicons were purified by agarose gel electrophoresis and were excised with a QIAquick gel extraction kit. Sequencing was carried out on an automated sequencer (Applied Biosystems Inc., Foster City, Calif.).

Statistical analysis. The prevalence of HGV infection between different subgroups of IVDAs were compared by chi-square tests. A P value of <0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Test principle. The objective of the present study was the development of a convenient, specific, and sensitive method for the diagnosis of HGV RNA in human specimens such as blood samples. Viral RNA was isolated by standard tech-

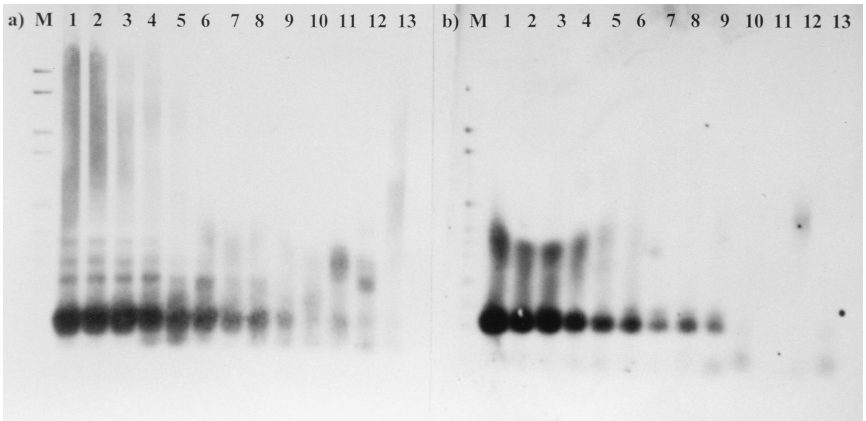

FIG. 1. Specificity and sensitivity of the RT-PCR assay for the detection of HGV. Amplicons of PCRs performed with 5′-NCR (a) and NS5a (b) primers were separated by agarose gel electrophoresis, blotted onto nitrocellulose, and detected by a nonisotopic assay with digoxigenin. The fragments had the expected lengths of 156 bp (a) and 186 bp (b). The consecutive dilution series was as follows: lane 1, undiluted sample (10 μl of the PCR result); lane 2, 2^{-1} dilution; lane 3, 2^{-2} dilution; and so forth. Lane 13, negative control; lane M, molecular size marker. The autoradiograph presented reveals a clear signal up to the dilution of 2^{-8} (lane 9) with both sets of primers.

TABLE 2. Alignment of the prototype 5′-NCR nucleotide sequence with five amplicon samples[a]

Sample	Sequence
a	GGTGATGACA GGGTTGGTAG GTCGTAAATC CCGGTCACCT **TGGTAGCCAC TATAGGTGGG** TCTTAAGAGA
b	T·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T··
c	T···T··G·C ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T··
d	·····T·· ·····T·· ·····T·· ·····T·C ·····T·· ·····T·· ·····T··
e	·····GT· ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T··
f	·GT· ·····T·· ·····T·· ·····T·· :·····T··· ·····T·· ·····G··
a	AGGTTAAGAT TCCTCTTGTG CCTGCGGCGA GACCGCGCAC GGTCCACAGG TGTTGGCCCT ACCGGTGGGA
b	·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ··C·····T· ·····T··
c	····C····· ·····C··· ·····T·· ·····T·· ·····T·· ·····T·· ·····T··
d	·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T··
e	·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T··
f	·····T·· ·····T·· ····T····· ···A····· ·····T·· ·····T·· ·····T··

[a] Samples: a, prototype (7); b and c, IVDAs; d to f, blood donors. Sequences start adjacent to the end of the forward primer. Dots represent sequence identity. The internal capture probe is indicated in boldface type.

niques, e.g., by using the QIAamp HCV kit. Subsequent RT with Moloney murine leukemia virus reverse transcriptase and PCR were performed under standard conditions.

We chose parallel PCR amplification with two pairs of HGV-specific primers (Table 1). This strategy of amplification of two independent parts of the viral genome offers at least two advantages: (i) the ability to overcome the unknown variability of the virus and therefore to increase the overall sensitivity of the assay and (ii) a guarantee of the highest degree of specificity by double-checking each sample rather than verifying of the result by a second test with the same primer set. The 5′-NCR primer set was derived from an alignment of 36 HGV sequences of the 5′-NCR which so far is genetically the best characterized region. While the forward primer and the capture probe match all 36 sequences, the reverse primer matches 35 of the 36 sequences. The second set is basically identical to the original Genelabs primer set, which was derived from clone 470-20-1, coding for the putative NS5 protein (7). For mass screening, both RT and PCR were performed in the 96-well format of the GeneAmp PCR System 9600.

During the amplification reaction, a label (digoxigenin-11-dUTP) is incorporated into the amplicon for nonisotopic detection. The amplified products are denatured and hybridized with an internal biotinylated capture probe. Specific hybrids are bound to a streptavidin-coated solid phase which can be either a 96-well microtiter plate or the reaction tube of an ES 300 machine (both available from Boehringer Mannheim GmbH). Subsequently, anti-digoxigenin–horseradish peroxidase-labelled conjugate and the ABTS substrate as the indicator are used for photometric measurement.

Specificity. The presence of the expected amplification products obtained by RT-PCR by using 5′-NCR- and NS5a-specific primer pairs was confirmed by agarose gel electrophoresis. After blotting onto nitrocellulose, the incorporated digoxigenin-11-dUTP was detected by chemiluminescence, resulting in unique bands of the expected size (Fig. 1). As discussed above, increased specificity was ensured by the use of biotinylated hybridization probes, which were selected from appropriate internal sequences.

Cross-reactivity, i.e., amplification of other viral agents like HCV, was not observed since the presence of the correct HGV fragments was further verified by nucleotide sequencing. Tables 2 and 3 present the sequences of five amplicons derived from clinical specimen (see below) with both 5′-NCR and NS5a primers. Although single point mutations were found, the sequences of these amplicons proved to be mostly identical to the sequence of the prototype virus (7). Mismatches of one nucleotide within the sequence of the internal capture probe still lead to successful hybridizations that may be due to the relatively low hybridization temperature of 37°C. Whether single point mutations are due to the different geographical origins of the samples or whether they represent different subtypes of HGV remains to be established.

Sensitivity. To evaluate the sensitivity of the RT-PCR assay in combination with the Enzymun-Test DNA detection system, the lower detection limit of the template RNA was determined by endpoint dilution of a synthetic RNA standard (Table 4). The endpoint concentration was reached when three of five assays gave signals above the background signal. The cutoff was defined as three times the absorption of an HGV-negative serum sample, which is approximately 150 mE. In using purified in vitro transcripts as the template for amplification with the 5′-NCR primer pair in a 45-cycle PCR, the assay had a sensitivity of at least 20 RNA copies per reaction (Table 4). Assuming RNA recovery of 90% (the manufacturer of the QIAamp HCV kit indicates 90 to 95% RNA recovery) and an

TABLE 3. Alignment of the prototype NS5a nucleotide sequence with five amplicon samples[a]

Sample	Sequence
a	GACATCCCCC GTACTCCATC GCCAGCACTT ATCTCG**GTTA CTGAGAGCAG CTCAGAT**GAG AAGACCCCGT CGGTGTCCTC CTCGCAGGAG G
b	C·····T··· ·····T·· ·····T·· ·G····· ·····T·· ·····T·· ·····A·· ·····T·· ·
c	·T····· ·C····· ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·····T·· ·
d	···CG ·C····· ·····T·· ·····T·· ·G····· ·····T·· ·····T·· ·····T·· ····A····· ·
e	···G····· ·····T·· ·····T·· ·····T·· ·A····· ·····T·· ····T····
f	·····T·· ·C····· ·····T·· ·····T·· ·C····· ·····T·· ·····T·· ·····T·· ·A····· ·

[a] Samples: a, prototype (7); b and c, IVDAs; d to f, blood donors. Sequences start adjacent to the end of the forward primer. The internal capture probe is indicated in boldface type.

TABLE 4. Determination of detection limit of RT-PCR assay[a]

Amount of RNA	No. of copies/ assay	Detectionlimit (mE)				
		Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
1 fg	2,000	3,278	3,180	3,253	3,313	4,641
100 ag	200	2,988	3,069	2,991	3,138	4,471
10 ag	20	2,858	3,544	3,225	734	3,685
1 ag	2	2,761	83	2,930	66	23
0.1 ag	0.2	1,613	49	38	69	14
0.01 ag	0.02	15	35	14	9	17
0.001 ag	0.002	11	16	13	10	29
H_2O	0	15	14	12	11	14
1 fg wild-type RT	2,000	11	8	11	13	13

[a] Serial dilutions of a synthetic RNA standard were amplified and detected on an ES 300 machine by using the 5′-NCR primer-probe set in five independent experiments. The cutoff was determined to be 150 mE, which is three times the absorbance of an HGV-negative serum sample.

RT-PCR efficiency of 5%, we would be able to detect 8×10^2 genome equivalents per ml in a 45-cycle PCR. Elevation of the primer concentration from the 200 nM used in the standard protocol to 1 μM did not enhance the sensitivity (data not shown).

On the basis of this calculation, we established calibration curves for both primer pairs using a dilution series of a high-titer serum sample (data not shown). The experiment demonstrated that despite the exponential amplification during PCR, our detection system allows quantification by our RT-PCR assay within the range of about 3 log steps when 35 cycles are used. The results were reproducible, and semiquantitative determination of HGV RNA in patient samples can thus be performed. For qualitative screening of clinical samples, 45 cycles of PCR were performed; this resulted in a clear discrimination between positive and negative samples.

The assumption that the amplification of two different regions would increase the overall sensitivity of HGV detection was indeed evident for some clinical samples (Table 5). Repeatedly positive results for the same sample with either primer pair should be considered HGV positive. Serum samples 1 and 2 taken from the panel of IVDAs (see below) were detected with the NS5a primers only, and serum sample 3 was positive by using the 5′-NCR primers only (Table 5).

Prevalence of HGV. Samples of sera from panels of IVDAs recruited at multiple sites and healthy blood donors in Central Europe were selected and tested for HGV. To exclude false-positive signals, all samples were tested with both pairs of primers, and subsequently, all HGV-positive samples were prepared and assayed a second time. HGV RNA was detected in 43 of 130 (33%) IVDAs. Twenty-three of 77 (30%) IVDAs were anti-HBV core antigen positive and 20 of 53 (38%) were anti-HBV core antigen negative ($P = 0.8$); 32 of 107 (30%) IVDAs were anti-HCV positive and 11 of 23 (48%) were anti-HCV negative ($P = 0.1$); and 7 of 23 (30%) IVDAs were anti-HIV positive and 36 of 107 (34%) were anti-HIV negative ($P = 0.9$). Comparisons between groups were performed by chi-square tests. Although HGV was present in 30% of the HBV-, HCV- and human immunodeficiency virus-positive subjects, there was no statistically significant correlation between the occurrence of those viruses and coinfection with HGV. Among the blood donors, 3 of 92 (3%) proved to be HGV positive with both primer sets. These observed prevalences of HGV are in accordance with the ones found by Linnen et al. (7) as well as those found in similar studies performed with primer pairs derived from the NS3 helicase domain of GBV-C (1, 5, 10, 13).

TABLE 5. Increased sensitivity of HGV RNA detection by using two primer sets[a]

Sample no.	Sensitivity of detection (mE)				Interpretation
	First PCR		Second PCR		
	5′-NCR primer	NS5a primer	5′-NCR primer	NS5a primer	
1	56	2,879	200	1,328	NS5a positive
2	26	743	62	540	NS5a positive
3	2,197	46	3,227	40	5′-NCR positive
4	43	37	51	38	Both negative
5	5,010	4,411	5,909	5,806	Both positive

[a] Extinctions of five selected RT-PCR samples as measured on an ES 300 machine. Discrepant results were tested twice (first and second PCRs).

The IVDA panel was also screened for the presence of GBV-B by using published primers (12) and the same RT-PCR conditions used for the detection of HGV. We were not able to detect GBV-B in human specimens, a result in agreement with those of Nuebling and Loewer (10). Control RNA, which had been prepared from a GBV-B-infected tamarin, gave a clear positive signal.

Conclusions. HGV is a new *Flavivirus*-like virus that is commonly distributed among individuals with a high level of parenteral exposure to blood or blood products, such as drug addicts, hemophiliacs, and patients receiving multiple transfusions (1, 2, 7, 15). Numerous questions remain unanswered (e.g., the site of replication of HGV and its disease association). HGV detection extends the differential diagnosis of viral hepatitis, and therefore, donated blood or blood products may need to be screened for HGV. So far, direct detection of the viral genome is possible only by RT-PCR. The combination of simple RNA preparation, RT-PCR with digoxigenin labelling, liquid hybridization, and chemiluminescence detection allows for the routine diagnosis of HGV RNA in human specimens. HGV can be detected on fully automated ES 300 machines, and the results are sufficiently reproducible for semiquantitative determination of the analyte. On the basis of amplification of two independent viral regions, our test principle has at least two advantages: increased overall sensitivity and the ability to verify HGV-positive (or HGV-negative) specimens by a second assay.

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