

Reliability of Methods for Hepatitis B Virus DNA Detection

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A quality assurance program has been established by the European Group for Rapid Viral Diagnosis and the European Expert Group on Viral Hepatitis for monitoring nucleic acid detection methods for hepatitis B virus (HBV) DNA in serum samples. Thirty-nine laboratories participated in this quality program and generated 43 data sets. Of the participating laboratories, all but one used the PCR technique to detect HBV DNA. A coded panel was tested that was composed of seven undiluted HBV DNA-positive serum samples and five HBV DNA-negative donor serum samples. Furthermore, two dilution series, one from a positive patient and one from a full-length recombinant DNA, were included. Twenty-six data sets (60.5%) had faultless results with both dilution series. Twelve data sets (27.9%) recognized the undiluted serum samples, and 19 data sets (44.2%) had false-negative and/or false-positive results. Ten data sets (23.3%) performed well with the entire panel of samples. From these results, it can be concluded that in a large group of laboratories HBV detection by PCR shows specificity and sensitivity problems; therefore, PCR test interpretation should be done with great care.

Advances in molecular biology and biotechnology are creating exciting possibilities for DNA diagnosis. A number of instruments to facilitate routine DNA diagnostic procedures are available (6). Conventional hybridization procedures have now been used widely for the detection of hepatitis B virus (HBV) DNA in serum, tissues, and mononuclear blood cells. HBV DNA is the most direct marker for viral multiplication, and several commercial semiquantitative tests based on liquid hybridization assays have been developed.

Amplification techniques such as the PCR are extremely sensitive methods for detecting nucleic acid sequences of HBV DNA in serum. The PCR can certainly modify the approach used for the diagnosis of viral hepatitis. In chronic hepatitis B, PCR is particularly useful for the identification of individuals with active HBV replication who are hepatitis B surface antigen positive and anti-hepatitis B e-antigen positive. For clinical use, quantitative values will be necessary. Follow-up of HBV infections in liver transplantation programs will benefit from the use of the PCR technique (2). Also, the efficacy of antiviral treatment can be monitored with PCR beyond the level of 10 pg of HBV DNA per ml. In addition, PCR in connection with direct sequencing can be used for the identification of genetic variability of HBV. While PCR provides a direct and highly sensitive identification of viral genomes, limitations such as the risk of false-positive results due to contamination and difficulties in developing quantitative tests should be mentioned also. Additionally, false-negative results due to suboptimal reaction conditions, incomplete denaturation of DNA, or errors in sample collection and/or processing may occur.

The aim of the combined efforts of the European Expert Group on Viral Hepatitis (Eurohep) and the European Group for Rapid Viral Diagnosis was a comparison of the specificity and sensitivity of “in-house”-developed nucleic acid detection methods for HBV DNA. This report describes the results from

the 39 laboratories that participated in this quality control program. A test panel consisted of 25 samples with and without HBV DNA and included 12 undiluted samples; two dilutions series of 5 samples each were constructed. Three of the 25 samples had to be returned to the organizers. Two of these samples were strongly positive, containing more than 3,000 particles per ml; the third one contained less than 3 particles per ml. Analysis of these samples by the reference laboratory ensured that no loss of sensitivity was due to postal and handling procedures. The 12 undiluted samples were from five HBV DNA-negative donors and seven HBV DNA-positive patients. The HBV DNA content of the positive serum samples ranged from 3.5 to 222 pg/ml as determined by a liquid hybridization assay (Genostics; Abbott) using the internal standard of the manufacturer. The HBV DNA-negative serum samples were PCR and hybridization assay (Genostics) negative as determined by two reference laboratories. Table 1 shows the serological characterization of the 12 undiluted samples. One HBV DNA-negative serum sample was anti-hepatitis B surface antigen positive and was obtained from an HBV-vaccinated person. In order to determine the detection limit of the assays used, 10 samples consisting of two 100-fold dilution series from the Eurohep HBV standard (4) and a recombinant plasmid were prepared. Theoretically, each dilution series consisted of 3×10^6 particles down to 0.03 particle per ml. The dilution series were made in HBV DNA-negative donor serum.

Forty-nine laboratories requested material; from these, 39 laboratories participated, with 4 laboratories submitting two data sets each. Each laboratory detected HBV DNA according to their in-house-developed nucleic acid detection method. Of the 39 participating laboratories, all but 1 laboratory used the PCR technique to amplify HBV DNA. One laboratory (code 8; see Fig. 1) used a hybrid capture assay (Digene Diagnostics Inc.). Twenty-five laboratories used a single-round PCR, and seven used a nested PCR technique, while three laboratories used both single-round and nested PCRs to obtain their results. Three laboratories did not give adequate information about the system used. Approximately 50% of the laboratories extracted the DNA by using proteinase K digestion followed by

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TABLE 1. Characterization of 12 undiluted samples^a

Sample	Positive or negative for:						HBV DNA determined by:	
	HBsAg	Anti-HBc	Anti-HBs	HBeAg	Anti-HBe	IgM anti-HBc	Reference PCR	Radioimmunoassay (pg/ml)
1	+	+	-	-	+	-	+	3.5
2	-	-	+	-	-	-	-	-
3	+	+	+	+	-	± (grey area)	+	26
4	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-
6	+	+	-	+	-	± (grey area)	+	39
7	+	+	-	+	-	-	+	92
8	-	-	-	-	-	-	-	-
9	+	+	-	+	+	+	+	3.9
10	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	32
12	+	+	-	+	-	-	+	222

^a HBsAg, hepatitis B surface antigen; HBeAg, hepatitis Be antigen; anti-HBc, antibody to hepatitis B core antigen; anti-HBs, antibody to HBsAg; anti-HBe, antibody to HBeAg; IgM, immunoglobulin M. The presence of IgM anti-HBc was determined with the microparticle enzyme-linked immunosorbent assay (Abbott Laboratories, North Chicago, Ill.). PCR was done in two reference laboratories. The radioimmunoassay was Genostics. +, positive; -, negative.

standard organic extractions. Four laboratories used the guanidinium isothiocyanate denaturation method followed by adsorption to silica particles (1). Four laboratories used heat denaturation, four used treatment with NaOH followed by neutralization, two laboratories used only organic extractions, one used the microwave, one used proteinase K combined with deoxycholic acid treatment, and one used affinity capture. All laboratories analyzed their PCR products by gel electrophoresis.

Eighteen of the laboratories confirmed their amplified material by Southern blot analysis, and 10 of these used a radioactive probe labeling system. PCR was done at three different locations in all laboratories, to prevent contamination. None of the laboratories used the uracil *N*-glycosylase system (3, 8, 10).

To assure confidentiality, all laboratories sent their results to a neutral office (S. W. Schalm, University Hospital Rotterdam Dijkzigt, Rotterdam, The Netherlands), which assigned a code to each participant. Anonymous results were analyzed at the coordinating laboratory (Diagnostic Center SSDZ, Delft, The Netherlands). Two laboratories (W. Gerlich, Giessen, Germany, and H. Niesters, Rotterdam, The Netherlands) were assigned to function as reference laboratories for the coordinating laboratory.

A "quality score" was assigned to each participant. The quality score (x, y) is a combination of the performance score " x " and the detection score " y ." Performance score points were awarded as follows: one point if the five strong-positive undiluted serum samples and the six negative samples are correctly determined; one point if, in addition to the above, both weak-positive samples (3.5 and 3.9 pg/ml) were found

positive; and one point for each correct dilution series. A dilution series was considered to be tested correctly if one or more of the least diluted samples in the series were found to be positive and all higher dilutions from this series were found to be negative. The maximum theoretical performance score is therefore a total of 4 points. Detection score points were awarded for each dilution tested correctly in the two dilution series. The maximum detection score for the two dilution series is 8 points, 4 points for each series, although it has to be mentioned that a dilution containing three particles per ml is not likely to be detected.

Analysis of the 12 undiluted samples, the 7 HBV DNA-positive samples and the 5 HBV DNA-negative samples, revealed that only 12 of 43 data sets (27.9%) showed all 12 samples correctly. Twelve data sets (27.9%) showed sensitivity problems by not recognizing one of the two low-range positive samples. Furthermore, 15 data sets (34.9%) showed false-positive results. Four (9.3%) data sets had one or more high-range positive samples incorrect. Performance with the two dilution series showed that in 60.5% of the data sets a correct sequence of the dilution in both series could be obtained. In 16.3%, one of the two dilution series was correct.

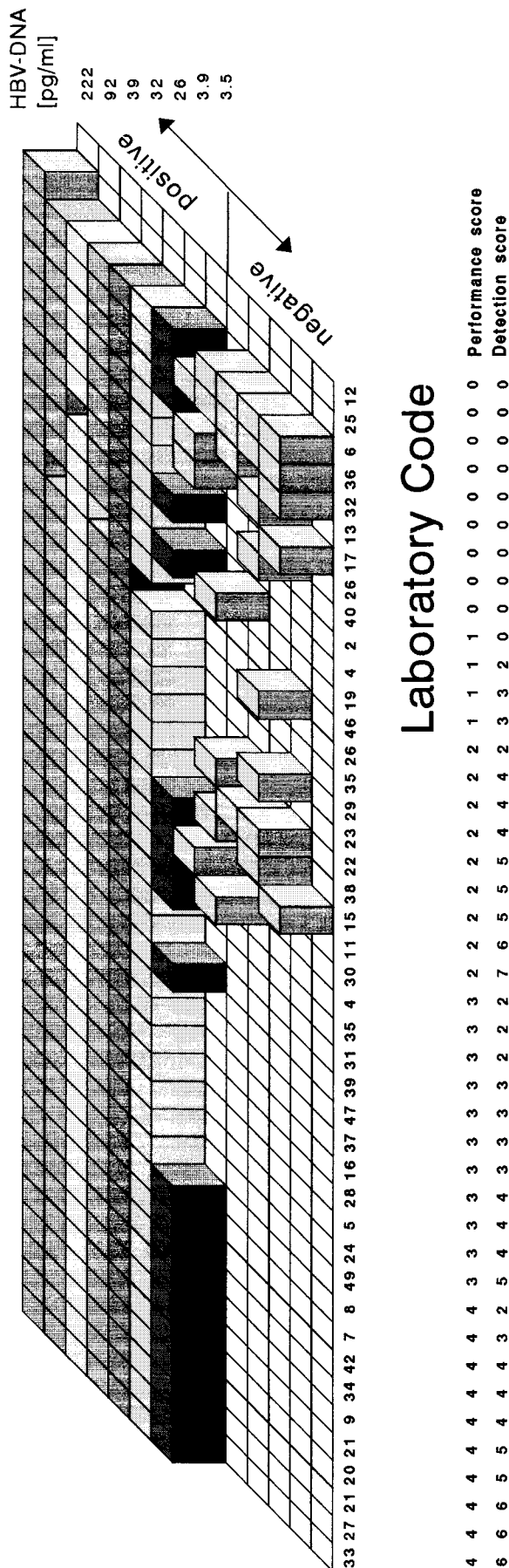
Taking the panel of samples as a whole, 10 laboratories (23.3%; Table 2) reported a good performance, 9 (20.9%) failed to detect one of the two weak-positive undiluted serum samples, and the remaining 24 (55.8%) produced false-positive and false-negative results for dilution series and/or undiluted samples. Figure 1 shows the test results from all laboratories on the 12 undiluted samples sorted by quality score. The quality scores ranged from 4.6 to 0.0.

From the information given by the participants, it could be

TABLE 2. Performance of 43 data sets for HBV DNA detection with a panel of two dilution series and 12 undiluted samples

Results with dilution series	Results with undiluted samples (no.)			Total no.
	All 12 correct	Only weak-positive incorrect	Errors with positive and/or negative samples	
Both series correct	10 (23.3) ^a	9 (20.9)	7 (16.3)	26 (60.5)
One series correct	2 (4.7)	2 (4.7)	3 (7.0)	7 (16.3)
Errors in both series	0	1 (2.3)	9 (20.9)	10 (23.3)
Total	12 (27.9)	12 (27.9)	19 (44.2)	43

^a Percentages are given in parentheses.



concluded that a good quality score was not associated with a particular DNA extraction procedure, the primers used in the PCR, use of nested PCR, or detection by Southern blot analysis with radioactive or nonradioactive probes. Also, the laboratories using a nested PCR were not more prone to generate false-positive results. However, some remarks should be made about the interpretation of the results. First, the panel of 12 undiluted samples was included to get information about the specificity and sensitivity of the test. The seven positive samples, which contained 222 to 3.5 pg of HBV DNA per ml, were selected on the basis of the Genostics liquid hybridization test (Abbott). In the case of this Genostics (Abbott) test, however, the internal standard of the assay gives an underestimation of the actual number of HBV DNA molecules by a factor of 10 is given (5). Within the pathobiology group of Eurohep, reference samples for HBV DNA were generated and analyzed by several reference laboratories. This will introduce reference samples which can be used as real gold standards for the detection of HBV DNA.

Since no HBV DNA liquid hybridization-negative and PCR-positive samples were included in this proficiency panel, in principle, DNA tests based on conventional HBV DNA detection would be able to detect in this panel the low- and high-positive undiluted samples. The technical sensitivity of the in-house DNA test was determined on two 100-fold dilution series on an HBV standard serum sample and HBV genomic recombinant plasmid DNA. Analysis of the three samples which had to return to the coordinator revealed that in all cases false-negative results observed in several data sets were not due to shipment problems.

Conclusions. Our results clearly show that the main performance problem for HBV DNA detection seems to be false positivity, as was also found in a recent HCV quality control study (11) with RNA as a target for PCR. Besides false positivity, sensitivity also is a problem in many laboratories. Similar problems of specificity and sensitivity have been reported in methods for the detection of *Mycobacterium tuberculosis* (9). All of these studies show clearly that results obtained with an in-house PCR test should be interpreted with great care. Quality control for all PCR assays, and especially those used for diagnostic purposes, begins with the in-house laboratory practices. In-house aspects for quality control of PCR assays include well-trained personnel, laboratory design, stock solution preparations, operating procedures, and control measures (6). These procedures not only reduce potential contamination but also help to ensure the reproducibility of PCR assays. The results of this quality control study show the desperate need for an effective quality assessment scheme before PCR can be used reliably in the diagnosis of infectious diseases. A useful step in the case of HBV may be the selection of HBV DNA-positive and -negative reference sera or panels of sera which can be used to standardize HBV DNA detection.

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FIG. 1. Results from the 39 participating laboratories; 4 laboratories each submitted two data sets (codes 21, 35, 4, and 26). Of the 39 laboratories, one laboratory (code 8) used a hybrid capture assay; the others performed PCR. Results on 12 undiluted samples are shown. Positive samples containing 3.5 to 222 pg of HBV DNA per ml by liquid hybridization assay (Genostics) are shown on the right. The laboratory codes are shown at the bottom of the grid. Performance and detection scores are also given.

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