HBV load in treated and untreated individuals

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A new marker of active HBV infection: quantitative detection of HBV RNA in serum by real-time NASBA.

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In preparation
Abstract

In a previous report we proposed that quantification of HBV RNA in serum or plasma might have diagnostic value (Penning et al, submitted for publication). Here we describe a quantification method for this HBV RNA. The method is based on NASBA technology and quantification is possible through real-time beacon detection as described by Leone et al. This real-time NASBA (Retina™) has a detection range of $10^2$ to $10^8$ HBV RNA copies/reaction, which is equivalent to $10^4$ to $10^{10}$ HBV RNA copies per ml of plasma or serum, using the equivalent of 10 µl sample, with good reproducibility and precision. With this Retina™, we longitudinally studied several patients infected with HBV that showed different courses in the infection and associated disease.

We show that our assay can be used to easily monitor HBV replication in patients by quantifying the HBV RNA load in their serum.
Introduction

Infection with the hepatitis B virus (HBV) can lead to different diseases, varying from fulminant or chronic hepatitis, to liver fibrosis, cirrhosis or hepatocellular carcinoma\(^2,3\). Nowadays, it is estimated that worldwide more than 350 million people are infected with HBV and 1 to 2 million people die each year as a consequence of infection with HBV\(^4,6\). In western countries, people at risk for infection are often vaccinated. Apart from that there still is no medicine for HBV that gives satisfying results, either because too few patients respond to therapy\(^2\), or because patients develop resistance\(^7,9\). To give patients the best possible treatment, it is important to follow the progression of the disease in infected patients as good as possible.

HBV is a DNA virus that replicates through an RNA intermediate. This mRNA is reverse transcribed to form cDNA in a core particle inside the cell. At the same time the RNA template is degraded. Only when formation of the second DNA strand has started, the core particle is enveloped and secreted from the cell\(^2,6,10,12\). Therefore it was rather surprising that some studies reported the finding of HBV RNA in serum \(^13\text{-}15\) and M. Penning et al., submitted for publication). We showed that the RNA is particle associated and suggested that it might be used as a diagnostic marker (M. Penning, et al., submitted for publication).

To be able to study the quantity of the HBV RNA in the serum of patients infected with HBV, we describe in this study the development of a sensitive and reproducible method for the quantification of HBV RNA. This assay is based on the nucleic acid sequence based amplification (NASBA). One of the advantages of our assay is the one-tube, isothermal nature of the method that allows high-throughput applications for nucleic acid detection. The homogeneous real-time detection allows a closed-tube format of the assay, avoiding any post-amplification handling of amplified material and therefore minimizing the risk of contamination of subsequent reactions.
Chapter 6

Materials and methods

Extraction methods

Nucleic acids were extracted from 100 µl of serum, plasma, or other liquid medium by applying the well-known silica-guanidinium thiocyanate protocol Y\textsuperscript{16}, using L6 (5.25 M GuSCN, 50 mM Tris HCl [pH 6.4], 20 mM EDTA, 1.3% [wt/vol] Triton X-100) as lysis buffer. Alternatively, samples were extracted using one of three modifications of this method, or using TRizol (Invitrogen life technologies, Breda, The Netherlands). The first modified method is referred to as Y\textsuperscript{17}, and involves involving addition of 1 ml lysis buffer L7A and 30 µl of size-fractionated silica particles before following the classic Y protocol. L7A is prepared from buffer L6 by the addition of α-casein to a final concentration of 1 mg/ml.

In the second modified method, we applied protocol H\textsuperscript{18}, which involves pre-digestion of the sample with 50 µl freshly prepared buffer L8 (445 µl H\textsubscript{2}O, 50 µl of 5 M NaCl, 50 µl of 20% [wt/vol] sodium dodecyl sulfate, 50 µl of 1 M Tris HCl-0.1 M EDTA [pH 8.0], 5 µl of denatured salmon sperm DNA [2mg/ml] and 400 µl of proteinase K) at 56°C for 30 min, followed by the classic protocol Y. The last modified method, protocol H\textsuperscript{+} is a modification of protocol H in which lysis buffer L6 is replaced by L7A. Each sample was always eluted in 50 µl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]).

The TRizol method for the isolation of RNA from serum was performed according to the manufacturer’s instructions.

DNase and RNase treatment

For DNase treatment, 3 µl DNase I (10 U/µl) and 2 µl of 0.5 M MgCl\textsubscript{2}, was added to 100 µl of nucleic acid solution. This mix was incubated at 37°C for one hour and consequently at 70°C for 10 minutes to stop the DNase I.

For RNase treatment we added 10 µl of 50 mg/ml RNase A to 100 µl of nucleic acid solution, followed by incubation for 1 hour at 37°C. Subsequently, we added 1 µl proteinase K (20 mg/ml), and incubated 15 more minutes at 37°C.

Primers and probes

Nucleic acid extracted from sera or plasma of HBV-infected individuals was amplified with real-time NASBA (Retina™, Primagen, Amsterdam, The Netherlands) using a primer set for HBV RNA amplification, spanning the region upstream of the HBV polyadenylation
signal. As 5'-primer we used HBV PolA P1.4 (5'-AAT TCT AAT ACG ACT CAC TAT AGG GAG AAG GTT TTT TTT TTT TTT AGC T-3). The stretch of 17 T's makes this primer specific for the detection of poly(A) RNA molecules. The 4 HBV specific nucleotides at its 3'-end increase the specificity for HBV RNA significantly (results not shown). As 3'-primer we used the HBV specific primer HBV PolA P2.1 (5'-GTA CTA GGA GGC TGT AGG CA-3'). With this primer set we amplify a product of 199 nucleotides. For detection of the amplified products, we used beacon HBV RNA WT1 (5'-CGA CTG CGC CGA TGG TGG CGT CG-3'), which is labeled with 6-fluorescein (6-FAM) as a fluorescent label at its 5'-end and with [4-(dimethylamino)phenyl]azo]benzoic acid (DABCYL) as a quencher at its 3'-end.

**HBV RNA Retina™**

The amplification in the NASBA reaction involves the action of three enzymes: AMV RT, T7 RNA polymerase, and RNAsé H. Two specific oligonucleotide primers, one of which contains a bacteriophage T7 RNA polymerase promoter site, are added to amplify nucleic acids more than 1012-fold in 90 – 120 minutes. The primer with the T7 tail binds to the single stranded RNA and is being extended by the AMV RT. After degradation of the template by RNase H, the second primer can bind to the cDNA, and will be extended to form a double stranded product with a double stranded T7 promoter sequence. From this double stranded product, single stranded RNA can be transcribed which can then enter a new cycle of amplification. Real-time detection is possible because the RNA amplicons that are generated during the NASBA process are detected by sequence-specific 6-FAM-labeled molecular beacon probes.\(^1\)

Retina™ reactions were started by mixing 5 μl extracted nucleic acid and 10 μl Retina™ amplification mix in microtubes. The final concentrations were: 40 mM Tris-HCl (pH 8.5), 12 mM MgCl₂, 70 mM KCl, 5 mM DTT, 1 mM dNTP's (each), 2 mM rATP, 2 mM rUTP, 2 mM rCTP, 1.5 mM rGTP, 0.5 mM ITP, 0.75 mM EDTA, 15% (v/v) DMSO, 0.2 μM primer P1, 0.2 μM primer P2, 0.2 μM molecular beacon probe and 0.375 M sorbitol.

Subsequently, the reactions were incubated at 65°C for 5 minutes for denaturation, followed by incubation at 41°C for 5 minutes. Then the enzyme mix (2.1 mg BSA, 0.01 units RNase H, 37 units T7 RNA polymerase, 7.5 units AMV-RT) was pipetted into the lids of the microtubes. The tubes were centrifuged briefly (10 sec) at 1000 rpm in an Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany) to collect the enzyme mix. After gentle mixing by tapping, the tubes were incubated at 41°C in a fluorimeter (Cytofluor 4000, Perkin Elmer, Wellesley MA, USA) for 120 minutes, with measurement of
the fluorescent signal every minute. The 20 µl reactions were excited at 485 nm, and fluorescence was measured at 530 nm. Readings were normalized to the background of a reaction containing water instead of template.

**Calculation**

The amount of HBV RNA present in samples was calculated using a calibration curve generated from in vitro RNA, transcribed from a plasmid containing a fragment of the HBV genome of 772 nucleotides around the polyadenylation signal. The standard curve indicated the relation between time-to-positivity (TTP) and input amount. The in vitro RNA was used in 10-fold serial dilutions ranging from $10^2$-$10^8$ copies per reaction. The concentration of HBV RNA in the samples was expressed in log copies/reaction. We used 100 µl of input material (serum or plasma) in the extraction (factor 10), and then eluted in 50 µl of TE buffer of which we used 5 µl in a Retina™ reaction (additional factor 10). Therefore, to calculate the concentration per ml of serum or plasma, we multiplied the copies/reaction by a factor 100.

**Test evaluation**

Precision, linearity and reproducibility of the HBV RNA Retina™ assay were evaluated as described below. The serial dilutions of the in vitro RNA (see above) were prepared freshly for each run from an aliquotted stock solution. The in vitro RNA concentration of this solution had previously been determined by OD$_{260}$ measurement.

(a) Precision: Various replicates (see results) of HBV in vitro RNA serial dilutions representing seven different RNA concentrations in the range from $10^2$ – $10^8$ copies per reaction were analyzed. Mean response values as well as between-run variation were estimated.

(b) Linearity and reproducibility: Standard response curves were generated from HBV in vitro RNA serial dilutions with known concentrations in the range from $10^2$ – $10^8$ copies per reaction. Data from 19 runs were analyzed in a linear mixed model, with input concentration modeled as a fixed-effects term and between-run variation modeled as a random-effects term. Results from the Retina™ assay were compared with the RNA concentrations expected from the HBV in vitro RNA serial dilutions by Bland-and-Altman analysis, which performs a regression of the difference between outcome and expected concentration onto their average value.

Data were analyzed using the SAS System version 6.12 (SAS Institute, Inc., Cary, NC) and SPLUS 2000 professional release 2 (MathSoft, Inc., Cambridge, MA).
Patients

Participants from the Amsterdam cohort studies on HIV-1 infection and AIDS among homosexual men and intravenous drug users (IVD) were tested for markers of past or present HBV infection. By the end of 1998 a total of 1,275 participants had entered the Amsterdam cohorts. Of 1,253 participants, 804 from the cohort of homosexual men and 449 from the cohort of drug users, HBV status was determined. Patients were selected for this study when stored samples were available during a period of at least 3 years on 6 monthly base. The last available sample of each individual was tested for antibodies against HBC. If tested positive, the first available sample was tested. Subsequently, in patients with a first sample tested negative and a last sample tested positive, the moment of anti-HBC seroconversion was determined by testing all available samples in between. Patients with missing samples in a time interval period of longer than 1 year where seroconversion took place were excluded from further analysis. In addition, HIV-1 infected individuals were also excluded if antiretroviral therapy was started before or during HBV seroconversion. Patients whose latest sample tested negative or first sample tested positive for antibodies against HBC were also excluded from the study.

Sero logical and biochemical markers of HBV infection

Serum HBsAg, HBeAg, and anti-HBc were measured using commercially available enzyme immunoassays (EIA; Hepanostika HBsAg/HBeAg/anti-HBc, Organon Teknika bv., Boxtel, the Netherlands) as indicated by the manufacturer. Hepatotoxicity was studied by monitoring alanine aminotransferase (ALT) and aspartate aminotransferase (AST) blood levels around anti-HBC seroconversion. ALT and AST values were assessed in all sera according to standard procedures.


**Results**

**Specificity**

To show the specificity of the HBV RNA Retina™ we used a serum quantified by branched DNA (bDNA) to a load of \(3 \times 10^9\) copies HBV DNA per ml. The load of HBV RNA in this serum was not determined before with other methods. Nucleic acids from six fractions of 200 µl were isolated by protocol Y\(^{16}\). The eluates were pooled and split in 3 fractions of 100 µl. The first fraction was treated with DNase I, the second fraction with RNase A, and the third fraction was mock incubated at 37°C for 1 hour. After these treatments, the fractions were extracted with protocol Y once more to remove enzymes and eluted in 50 µl. 5 µl of these eluates and a 10-fold dilution of them were used in a Retina™ reaction. Real-time monitoring of NASBA reactions yields fluorescence plots, with the typical initial exponential rates followed by a plateau phase. The exponential rate is consistent with the amplification phase of the NASBA reaction in which the products of amplification function as templates.

Figure 1 shows that the sample treated with DNase I gives the same result as the untreated sample. The sample treated with RNase A however, is completely negative. Thus the HBV RNA Retina™ we developed specifically detects HBV RNA. This is not surprising, since the 5'-primer was developed to amplify only poly(A) containing RNA molecules.

**Comparison of isolation protocols**

We compared different isolation protocols to determine which was the most suitable in
our experiments. First we tested the standard protocol Y, and investigated the beneficial effect of adding α-casein in the lysis buffer. This beneficial effect could lead to a lower detection limit. The addition of α-casein to the lysis buffer is referred to as protocol Y* and H*, respectively, when used in protocol Y or H (see Materials and Methods section for details). We compared these extraction protocols to a commercial assay, known to specifically isolate RNA. This method was the TRIzol isolation, of which we followed the protocol for RNA isolation from serum. For these comparisons, we used the same serum that we used to show the HBV RNA specificity of the Retina™. We tested 10-fold serial dilutions of this serum, prepared in HBV-negative serum, followed by extraction with protocol Y, Y*, H, H*, or TRIzol.

Figure 2. Comparison of isolation protocols
Protocol Y, standard silica-guanidiniumthiocyanate extraction method; Protocol Y*, addition of α-casein to lysis buffer; Protocol H, proteinase K digestion prior to extraction; and protocol H*, both proteinase K digestion prior to extraction and addition of α-casein to lysis buffer; figure 2E: TRIzol protocol.

Figure 2 shows the results of HBV RNA Retina™ after extraction of samples in the serum dilution series. We found no enhanced detection of HBV RNA with an extraction method incorporating a proteinase K digestion step, as in protocol H and H* (see protocol Y vs. H and Y* vs. H*). However, the addition of α-casein to the lysis buffer improved detection by HBV RNA Retina™ 10-fold (see protocol Y vs. Y* and H vs. H*). Because we found little or no difference between results after protocol Y* and TRIzol, further experiments were
performed using the more convenient and rapid Y+ protocol. With each extraction, an HBV RNA-negative serum sample and an HBV RNA-positive sample (10^6 copies/ml) were included as controls to ensure extraction quality.

**Quantitative performance of HBV RNA Retina™**

In order to start all reactions simultaneously, enzyme mix was pipetted into the lids of the microtubes and subsequently spun down. HBV RNA copy numbers were calculated relative to an HBV in vitro RNA 10-fold dilution series, which was included in every run. The input of this in vitro RNA ranged from 10^2 till 10^8 copies per reaction, and H2O was used as the no template-control.

![HBV RNA standard curve](image1)

**Figure 3A:** RNA standard curve generated from HBV in vitro RNA 10-fold dilution series. Curves are normalized for background (H2O). The inverse relationship between time-to-positivity (TTP) and copy number is demonstrated.

![Calibration Curve](image2)

**Figure 3B:** Calibration curve, generated from the standard curves in figure 3A. The known copy numbers are plotted against the measured TTP. Using this calibration curve, the load in a given sample can be extrapolated from the linear TTP curve of the standards used in that experiment.

Data were processed in an Excel 97/98 spreadsheet to correct for background as measured in the no-template control sample and to determine the intercept of the slope with the x-axis. Calibration curves were generated and fitted with at least 5 points of the dilution series of in vitro RNA using the same spreadsheet (Figure 3). Only if the calculated R^2 was greater than or equal to 0.94, we considered it a valid experiment. The
HBV RNA loads of the samples were then interpolated using these calibration curves. The cut-off of each individual assay was set to be equal to the value of the last positive sample in the dilution series.

**Precision**

Figure 4 shows the levels of HBV RNA obtained by analysis of high-titer HBV in vitro RNA after dilution in water to concentrations in the range from $10^2$ – $10^8$ copies per reaction.

![Figure 4](image)

**Figure 4.** Accuracy of HBV RNA Retina™ assay at input levels in the range from 1x$10^2$ to 1x$10^8$ copies/reaction. Samples were analyzed in numbers of replicates according to Table 1. Mean values and standard deviations are shown together with the line of equality.

Table 1 shows the mean HBV RNA load as measured by the Retina™ as well as the range, standard deviation, and coefficient of variation (CV) at seven standardized HBV input levels for varying numbers of replicates. The mean values measured in the range of $10^2$ – $10^8$ copies/reaction, fit very well to the line of equality. In the $10^4$ – $10^7$ copies/reaction, standard deviations are very small. At lower and higher concentrations, standard deviations increase to almost 1 log at an input of $10^3$ and $10^8$ copies/reaction.

<table>
<thead>
<tr>
<th>Input HBV RNA</th>
<th>Mean response</th>
<th>Range</th>
<th>Std. dev.</th>
<th>C.V.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (n = 11)</td>
<td>1.818</td>
<td>0.502 – 2.583</td>
<td>0.544</td>
<td>29.9%</td>
</tr>
<tr>
<td>3 (n = 19)</td>
<td>2.807</td>
<td>-0.110 – 3.932</td>
<td>0.898</td>
<td>32.0%</td>
</tr>
<tr>
<td>4 (n = 19)</td>
<td>3.993</td>
<td>3.278 – 4.772</td>
<td>0.336</td>
<td>8.42%</td>
</tr>
<tr>
<td>5 (n = 19)</td>
<td>5.147</td>
<td>4.525 – 5.745</td>
<td>0.303</td>
<td>5.88%</td>
</tr>
<tr>
<td>6 (n = 19)</td>
<td>6.079</td>
<td>5.709 – 6.368</td>
<td>0.175</td>
<td>2.87%</td>
</tr>
<tr>
<td>7 (n = 19)</td>
<td>7.065</td>
<td>6.846 – 7.339</td>
<td>0.120</td>
<td>1.69%</td>
</tr>
<tr>
<td>8 (n = 19)</td>
<td>7.570</td>
<td>4.921 – 8.081</td>
<td>0.841</td>
<td>11.1%</td>
</tr>
</tbody>
</table>

**Table 1.** Precision of HBV RNA Retina™ assay. All values are Log10 transformed copies/reaction, except for n, the number of replicates used for the analysis, and C.V., the inter-assay coefficient of variation. Std. dev.: standard deviation; C.V.: coefficient of variation.
Linearity and reproducibility

Figure 5 shows the mean response curve as calculated from 19 different runs, with assay variability modeled as a random term in a linear mixed model.

![Figure 5](image)

From the estimates in Table 2, it can be inferred that the intercept of the mean response curve is not significantly different from 0 and that its slope is not significantly different from 1. The standard deviation of the intercept due to random assay variability is 0.765 (95% cfi: 0.348 - 1.677) and the standard deviation of the slope due to random assay variability is 0.136 (95% cfi: 0.062 - 0.297). The correlation between intercept and slope is -0.975, so if the intercept of a standard response curve is larger than average, its slope will tend to be smaller. In the Bland-and-Altman analysis, both intercept and slope are not significantly different from zero, which shows that there is no general tendency of overestimation or underestimation.

<table>
<thead>
<tr>
<th></th>
<th>Intercept (95% cfi)</th>
<th>Slope (95% cfi)</th>
<th>Regr. line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear</td>
<td>0.005 (-0.432 - 0.442)</td>
<td>0.986 (0.908 - 1.064)</td>
<td>y = x</td>
</tr>
<tr>
<td>Bland-and-Altman</td>
<td>-0.167 (-0.570 - 0.236)</td>
<td>0.020 (-0.049 - 0.089)</td>
<td>y = 0</td>
</tr>
</tbody>
</table>

Table 2. Linearity and reproducibility of HBV RNA Retina™ assay as confirmed by linear regression and Bland-and-Altman comparisons of 19 standard response curves. Bland-and-Altman analysis performs a regression of Log10(Retina™/Input) onto 0.5×Log10(Retina×Input). cfi: confidence interval. Regr. line: regression line.

Patient studies

To show that our Retina™ can be used to monitor HBV RNA loads in patients, we
quantified HBV RNA and HBV DNA in longitudinally serum samples in a number of patients. HBV DNA was quantified with another Retina™ assay, specific for HBV DNA19. When we found a sample that had a load below the quantification limit for either RNA or DNA, and the result for the other Retina™ was positive, both DNA and RNA loads were tested again. We further determined HBsAg, HBeAg and ALT and AST levels in all samples. In figure 6, we show two patients, from who the moment of anti-HBc seroconversion is known. In figure 6A an example of a patient with a typical acute infection is shown, and in figure 6B a patient with a chronic infection. In the patient with the acute infection, it can be seen that all markers peak around anti-HBc seroconversion. HBeAg peaks shortly before, and HBsAg at the moment of anti-HBc seroconversion. HBV DNA and HBV RNA levels peak several months after anti-HBc seroconversion. All these markers disappear in a relatively short time. ALT and AST levels do not increase significantly in the samples tested. There may be a peak that we do not detect due to the sampling intervals.

In the chronically infected patient (Figure 6B), it is observed that HBsAg and HBeAg become positive at the moment of anti-HBc seroconversion. Three months later, a peak in ALT and AST levels can be seen, followed by an increase in the load of HBV DNA and HBV RNA, which both remain present at a high level for a period of at least 17 months.
Figure 6. Patient examples
In the upper panel load profiles of HBV DNA and RNA are shown. In the lower panels other markers for HBV infection are depicted.

Fig. 6A. Patient with acute HBV infection, Fig. 6B. Patient with chronic HBV infection.
Discussion

Following reports in literature in which HBV RNA in serum was found (13-15 and M. Penning, et al., submitted for publication), we would like to perform studies on this RNA to determine if HBV RNA in serum can be used as a diagnostic marker. Therefore, we developed a real-time assay for the quantification of HBV RNA, based on NASBA technology. This Retina™ is a sensitive, reproducible, and specific assay, that is easy to perform. Quantification is possible by the use of molecular beacon probes. These are used in the NASBA reactions to generate a fluorescent signal for direct amplicon detection during the amplification process. Due to the continuous measurement of fluorescence in the HBV DNA real-time NASBA, the time point at which a reaction reaches the threshold of detection, and thus becomes positive, is easily determined. There is an inverse correlation between the time needed for a reaction to become positive, the so-called time-to-positivity (TTP), and the initial copy number of HBV RNA used as input in the reaction. The higher the input, the less time the reaction needs to amplify to a detectable level. Results indicate that the HBV RNA Retina™ has a dynamic range from $10^2$ to $10^8$ HBV RNA copies/reaction, but that the assay is at its best in the range from $10^4$ to $10^7$ copies/reaction, and has in this range a very good reproducibility and precision. It is known that the HBV DNA present in virions, has a protein covalently attached to it. Furthermore, the HBV RNA in virions is probably attached to the HBV-encoded polymerase (Penning et al, submitted for publication). Such proteins may interfere with silica-guanidinium thiocyanate extraction methods, if they become irreversibly attached to the silica and are therefore not eluted during the final elution step. That is why we compared our standard protocol (protocol Y) with a method in which extraction was preceded by proteinase K digestion (protocol H). This step would digest protein present in the sample and eliminate its interfering influences on extraction. It has been reported that for extraction of HBV DNA addition of α-casein to the lysis buffer increases the yield of the extraction. We tested this protocol Y* also for the extraction of HBV RNA. These modifications of the standard silica-guanidinium thiocyanate extraction method were performed to see if any of them would improve the overall performance of the HBV RNA Retina™. The addition of α-casein to the lysis buffer improved detection by HBV RNA Retina™ 10-fold (compare protocol Y with Y* and H with H*). This is in agreement with previous results for isolation efficiency of HBV DNA17,19.

In a 96 well format run (80 specimens plus standards), the complete assay, including extraction of HBV RNA from plasma or serum samples, can be completed in five hours (or
three hours without extractions). The HBV RNA Retina™ has been tested in patient sera and has proven to be easy and relatively quick to perform and turned out to be an easy and accurate method to monitor the HBV RNA load in patient samples. Based on the results presented in this study and previous results (Penning et al, submitted for publication), HBV DNA and RNA seem to follow more or less the same dynamics in acutely and chronic infected patients. To draw more definite conclusions, more extensive studies in a larger group of patients are being performed right now. Then we hope to be able to more precisely link the presence or dynamics of HBV RNA in serum to the status or development of HBV infection.

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References


