COMPARISON OF AN IN-HOUSE QUALITATIVE PCR ASSAY FOR DETECTION OF HEPATITIS B VIRUS DNA WITH REAL TIME PCR USING SYBR® GREEN I

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ABSTRACT
The HBV infection is a very serious health problem and there is need of sensitive and precise methods for diagnostics. The measurement of HBV DNA in serum with various molecular techniques has become an important part of the diagnostics procedure of this infection. In this study an in-house PCR method was compared with the Real Time PCR technique for detection of HBV DNA. It was found in this case that they are both accurate but the Real Time technique has some important advantages.

Introduction
Hepatitis B virus (HBV) infection is a major health problem, with about 5% of the world’s population being chronically infected. Of these, about 1 million die each year due to progression to cirrhosis or hepatocellular carcinoma (1). HBV is the smallest DNA virus known, and its genome shows a highly compact organization. A unique aspect in the HBV replication cycle is that pregenomic mRNA serves as a template for synthesis of the first viral DNA strand by the reverse transcriptase (RT) polymerase of HBV (2). The RNase H activity of the HBV DNA polymerase removes the mRNA during this process, and synthesis of the complementary second DNA strand is then started, generating a partially double-stranded DNA molecule for packaging in virions. When the virus enters the host, this molecule is extended into a fully double-stranded DNA molecule, thus starting a new replication cycle (3, 4, 5). HBV DNA can be detected in the blood in more than 90% of infected hosts who are positive for hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg).

The use of polymerase chain reaction (PCR) in molecular diagnostics has increased to the point where it is now accepted as the golden standard for detecting nucleic acids from a number of origins and it has become an essential tool in the research laboratory. The method utilizes a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a double-stranded DNA (dsDNA) target, with the pair spanning a region that will be exponentially reproduced. The hybridized primer acts as a substrate for a DNA polymerase (most commonly derived from the thermophilic bacterium Thermus aquaticus and called Taq), which creates a complementary strand via sequential addition of deoxynucleotides. The process can be summarized in three steps: (i) dsDNA separation at temperatures > 90°C, (ii) primer annealing at 50-75°C, and (iii) optimal extension at 72-78°C (6). Traditional detection of amplified DNA relies upon electrophoresis of the nucleic acids in the presence of ethidium bromide and visual or densitometric analysis of the resulting bands after irradiation by ultraviolet light (7). On the other hand, the Real Time PCR
relies upon detection of fluorescence trig-
gerated by different mechanisms (8, 9). In
this experiment SYBR® Green 1 was used.
It is fluorophore which fluoresce when as-
associated with dsDNA which is exposed to a
suitable wavelength of light (6).

Materials and Methods

Patients and clinical samples

Serum was obtained from eight patients
with chronic HBV infection and cirrhosis,
which were positive for both HBsAg and
HBeAg. Serological determination of HBV
markers was performed with commercially
available ELISA kits (HBsAg Monolisa +
3.0; HBeAg Monolisa 3.0/BIO-RAD).

Extraction of HBV DNA

Extraction of the virus DNA from the sera
was made by the phenol-chloroform
method(10). A 300 µl portion of serum was
incubated at 65°C for 2 hours in the pre-
sence of proteinase K, 0.2M EDTA, 1.5 M
NaAc, 10% sodium dodecyl sulfate (SDS),
tRNA and salmon DNA. To each sample
was added equal quantity of Phen-
ol/Isoamyl alcohol/Chloroform
(25/24/1)/AppliChem/. The probes were
centrifuged for 10min at 12 000 rpm (4 ºC)
and the upper phase was transferred into
new tubes. This procedure was repeated 3
times in order to achieve clear upper phase.
Chloroform/Isoamyl alcohol (24/1) was
added afterwards and the samples were
centrifuged for 1min and only the clear
upper layer was extracted as previously.
Then, 3M NaAc and 100% ethanol was
added to the samples and they were cen-
trifuged for 20 min. The liquid phase was
removed from the tubes and 70% ethanol
was added. After 5 min centrifugation the
ethanol was removed and the samples were
dried for about 40min. To the dried sam-
dles 20 µl of sterile water was added and
the samples were left overnight at 4°C.

Polimerase Chain Reaction (PCR)

In-house PCR

The serum DNA sample (2 µl) was ampli-
fied in a 50 µl reaction volume containing
primers Hbv-1 and Hbv-2 - 2 µl each, Re-
action Buffer - 5 µl, MgCl - 3 µl, dNTP’s -
4 µl, Taq-P – 0.25 µl, and sterile water –
31,75 µl. The reaction was performed in a
programmable DNA MiniCycler™ (MJ
Research, USA) by the following protocol:
5 min. at 94°C; 1min. at 94°C; 1min. at
45°C; 2 min. at 72°C. The last three steps
were repeated 35 times and were followed
by the last step – 10 min. at 72°C. 10 µl of
each amplified sample was fractionated by
SYBR® Green I (Invitrogen™) and 13
µl sterile water. The reaction volume was
50 µl. The reaction was performed follow-
ing the protocol: 10 min. at 95°C; 15 sec. at
95°C; 30 sec. at 55°C; 30 sec. at 72°C. The
last three steps were repeated for 45 times
and at the end melting curve analysis was
made between 55°C-95°C. The last step
was 10 min. at 72°C. The Real Time PCR
was performed using DNA Engine Opti-
con™ Version 1.0 (MJ Research, USA).

In both experiments along with the pa-
tient sera, an HBV DNA standard 10³ IU/
µl was used for comparison with the re-

Results and Discussion

In the first experiment (in-house PCR) was
found that all of the tested patients were
positive for HBV DNA. The results were
shown by agarose gel electrophoresis. The
results from the second experiment con-
formed the data from the first experiment-
all the sera were positive for HBV DNA.
The Melting curve analysis was performed
and it was shown that the curves of the sera
were almost identical with the curve of the
positive HBV 10³ IU/µl standard (Fig. 1
and Fig. 2). For clearer image the graphic
of the Melting curve analysis was divided

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into two separate tables – Fig. 1 shows the first five sera, and Fig. 2 shows sera № 6, 7, 8, 10 and the standard as № 9.

After comparison of the two PCR techniques it was shown in this case that both of them are accurate and reliable. It has been suggested that in-house PCR assays often suffer from problems with standardization, false positive results, or contamination (11). Sample preparation is still the most difficult step to perform and to automate for both commercial and in-house assays and complete abolishment of post-amplification handling is an additional...
factor in limiting contamination. The Real Time PCR has several advantages, which makes it more useful in the practice:

The time needed to complete the amplification protocol by in-house method for detection of HBV DNA is longer (3 h) compared with the time that Real Time PCR technology consumes (1.45 h) but the last method is more sensitive, being able to detect less than 100 HBV DNA copies/ml.

The Real Time technique enables to be distinguished the wild type HBV and mutations in the viral genome by the changes in DNA thermal stability, which results in a different melting curve. It is important to monitor this process because of the emergence of drug resistant HBV strains carrying mutations associated with the treatment (12,13).

The technique reduces the risk of contamination and false positive results and there is not direct exposure to any toxic substances. The amount of PCR product amplified is monitored in each cycle and the method provides rapid analyses of absolute template amounts without post-PCR step.

In conclusion, the real-time PCR with SYBR® Green I for detecting HBV DNA is accurate, rapid and highly reproducible assay.

REFERENCES