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VERSANT Hepatitis B Virus DNA 3.0 검사와 Digene Hybrid Capture II Hepatitis B Virus DNA 검사의 비교 및 B형 간염 임상상과의 관련성

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Comparison of VERSANT Hepatitis B Virus DNA 3.0 Assay with Digene Hybrid Capture II Hepatitis B Virus DNA Test in Relation to Clinical Status of Hepatitis B Virus

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Background : Some differences exist among various Hepatitis B virus (HBV) DNA quantification assays due to lack of standardization and besides clinical usefulness has not been firmly elucidated in Korean HBV patients.

Methods : We compared Bayer VERSANT HBV DNA 3.0 Assay (VERSANT 3.0) with Digene Hybrid Capture II HBV DNA Test (HC-II) according to HBeAg status and ALT levels in 232 HBV-infected Korean patients. One hundred and seventeen sera with undetectable DNA levels by HC-II were further analyzed by Real-Q HBV quantification assay (BioSewoom).

Results : Although VERSANT 3.0 and HC-II showed an excellent correlation (r=0.9739), the results (copies/mL) by VERSANT 3.0 were 0.45 $\log_{10}$ higher than those by HC-II. HBV DNA levels were higher in HBeAg-positive group than in HBeAg-negative group (P=0.002), and in abnormal ALT group than in normal ALT group (P<0.0001). The detection rate of HBV DNA by VERSANT 3.0 was lower in HBeAg-negative and normal ALT group (n=68) than in HBeAg-positive or abnormal ALT group (n=164) (35.3% vs 89.6%, P<0.0001). Fifty two sera out of 61 sera with undetectable DNA by VERSANT 3.0 were measurable by Real-Q with mean value of 3.26 $\log_{10}$ copies/mL.

Conclusions : VERSANT 3.0 and HC-II showed an excellent correlation, but a little difference (0.45 $\log_{10}$) existed. VERSANT 3.0 effectively measured clinically relevant HBV DNA levels in most HBV-infected patients in Korea. However, more sensitive assays are needed for patients with negative HBeAg and normal ALT to see the low copies of HBV DNA levels. *(Korean J Lab Med 2007;27:451-7)*

Key Words : *Hepatitis B virus, DNA, Quantification, HBe antigen, Alanine aminotransferase*

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INTRODUCTION

Quantitative measurement of hepatitis B virus (HBV) is valuable in predicting disease progression, monitoring HBV replication activity and infectivity, and assessing the response to antiviral treatment[1]. A number of commercial assays are currently available for the quantification of HBV DNA including hybridization-, signal-, and target-amplification-based technologies. However, their results are expressed with different units (e.g. mEq/mL, copies/mL, WHO IU/mL) and some differences exist among them due to lack of standardization[2, 3], which causes some confusions to clinicians.

Hybrid Capture II test (HC-II, Digene Corp., Beltsville, MD, USA) had better clinical sensitivity than the initial version of branched DNA (bDNA) assay (VERSANT 1.0, Bayer HealthCare LLC, Tarrytown, NY, USA)[4]. A new version of the bDNA assay, VERSANT HBV DNA 3.0 Assay (VERSANT 3.0), was recently developed with better sensitivity than the previous version. Although these assays are widely used in Korea, the difference between them has been studied in only one study[5]. The main purpose of our study was to compare the results of VERSANT 3.0 and HC-II to know the conversion factor between these two assays in Korean HBV-infected patients.

In addition, with the availability of new and potent antiviral drugs, it becomes more important to measure low levels of HBV DNA in order to predict the emergence of drug-resistant HBV strains[6, 7]. VERSANT 3.0 was reported to have a wide dynamic range (2×10^3-10^8 copies/mL) enough to cover clinically relevant HBV levels[5]. To see the practical usefulness of VERSANT 3.0 in Korean HBV-infected patients and to investigate how much the results of HBV DNA assays are affected by the clinical status of patients, the results of VERSANT 3.0 were also analyzed according to HBeAg status and alanine aminotransferase (ALT) levels, and those samples with undetectable HBV DNA levels by HC-II were further analyzed with another HBV DNA assay based on real-time PCR, Real-Q HBV Quantification Assay (Real-Q, BioSewoom Inc., Seoul, Korea).

MATERIALS AND METHODS

1. Patients

A total of 232 patients with chronic HBV infection were consecutively recruited from the hepatitis clinic of Hallym University Medical Center in January 2006. They were 143 males and 89 females, and their median age was 40 yr (range, 17-86 yr). They were all HBV surface antigen (HBsAg) positive, and 133 patients (57.3%) were HBV e antigen (HBeAg) positive and 99 patients (42.7%) were HBeAg negative. HBV antigen tests were performed at the time of recruitment using AxSYM analyzer (Abbott Laboratories, Abbott Park, IL, USA). Serum ALT levels (normal range, <40 IU/L) were measured in all patients using Neo-200FR autoanalyzer (Toshiba Medical Systems Co., LTD., Tokyo, Japan).

One serum sample taken from each patient was used for HBV DNA quantification by both VERSANT 3.0 and HC-II. Samples with undetectable DNA levels by HC-II were further analyzed by Real-Q (BioSewoom Inc.). Informed consent was obtained from each patient, and the institutional review board approved the use of clinical samples in this study.

2. HBV DNA quantification assays

1) VERSANT 3.0

VERSANT 3.0 was performed according to the manufacturer's instructions. The assay procedure consists of two major activities, hybridization of the probes and measurement of the light output, which occurs within a semi-automated instrument, Bayer System 340 branched DNA (bDNA) Analyzer. After denaturation, HBV genome was hybridized to capture probes and target probes, which were also hybridized to pre-amplifier probes. Amplifier probes were subsequently hybridized to preamplifier probes, forming a bDNA complex. This immobilized complex was then hybridized to alkaline phosphatase (AP)-labeled probes, and light emission was detected by incubating the AP-bound complex with a chemiluminescent substrate. The concentration of HBV DNA was determined by a standard curve, which was generated by six standards with known concentrations of recombinant DNA. The results were expressed in both copies/mL and the World Health Organization (WHO) International Units (IU/mL). The dynamic range of results was from 2.0×10^3 to 1.0×10^8 HBV DNA copies/mL. The relationship between both values was 5.6 copies/mL=1 IU/mL for WHO reference material NIBSC code 97/746 (5×10^5 IU/vial).

2) HC-II

HC-II is a nucleic acid hybridization antibody capture microplate test with signal amplification utilizing chemiluminescent detection, and was performed according to the manufacturer's instructions. After denaturation, HBV DNA was hybridized to RNA probe mix (specific for HBV *ad* and *ay* strains). The resultant RNA-DNA hybrid was captured onto the surface of microwell coated with antibodies specific for RNA-DNA hybrid, and this immobilized hybrid was then reacted with AP-labeled antibodies specific for it. The emitted light was measured on a luminometer after an incubation of AP-bound hybrid with a chemiluminescent substrate. The results were expressed in pg/mL according to the plot of standards, and were converted to copies/mL (1 pg/mL $=2.83\times10^5$ copies/mL). The dynamic range of the results was from 0.5 to 6,000 pg/mL (from 1.42×10^5 to 1.70×10^9 copies/mL).

3) Real-Q

Real-Q is a real-time PCR method using TaqMan technology, which amplifies and quantifies a 112-bp region of the HBV genome with a sensitivity of 50 copies/mL. When Real-Q was evaluated by Clinical and Laboratory Standards Institute guidelines[8, 9], the precision of total coefficient of variation (CV) ranged from 1.29% to 1.88% (for 6 log and 3 log levels, respectively), and the linearity from 1×10^2 to 1×10^{10} copies/mL. Briefly, 2 μL of internal control (IC) was added to 200 μL of serum samples, and viral DNA was extracted using QIAamp MinElute Virus Spin kit (QIAGEN Inc., Valencia, CA, USA). Real-time PCR was undertaken with a final volume of 25 μL (5 μL of extracted DNA, 12.5 μL of PCR mixture, 3 μL of HBV probe and primer mixture, 3 μL of IC probe and primer mixture and 1.5 μL of sterile water) using ABI PRISM 7000 system (Applied Biosystems, Foster City, CA, USA). After initial denaturation at 95°C for 10 min, amplification was achieved by 45 cycles consisting of 95°C for 20 sec, 58°C for 30 sec and 72°C for 30 sec. Five HBV standards calibrated against a commercial HBV DNA control (ACCURUN 325 HBV DNA control: Boston Biomedica, Inc., West Bridgewater, MA, USA) were used to generate a standard curve, by which quantification data were calculated. Throughout the whole PCR procedure, competitive homologous IC and uracil-N-glycosylase were used to identify possible PCR inhibition and to prevent possible carryover contamination, respectively.

3. Statistical analysis

Correlation and regression data were analyzed to define the relationship between continuous variables. Bland and Altman plot was used for the analysis of data agreement [10]. The difference in $\log_{10}$ values for each matched pair of results was plotted against the average of the $\log_{10}$ values for each pair. Horizontal lines were drawn at the average difference, and at the average difference plus and minus 1.96 times the standard deviation (SD) of the differences. The differences of DNA levels between two groups were analyzed by student's t test. For the statistical analysis, MedCalc software (version 9.20, MedCalc software, Mariakerke, Belgium) was used. $P\leq0.05$ was considered statistically significant.

RESULTS

1. Comparison of VERSANT 3.0 with HC-II

Distribution of HBV DNA levels measured by VERSANT 3.0 and HC-II is summarized in Table 1. When HBV DNA levels higher than or within the dynamic range were combined together, they were detectable in 171 patients (73.7%) by VERSANT 3.0, and in 115 patients (49.6%) by HC-II. In all of these 115 patients, HBV DNA was detected by VERSANT 3.0. The overall concordance rate between these two assays was 75.9% (176/232). All of the discordant results (24.1%, 56/232) were positive by VERSANT 3.0 while negative by HC-II.

Correlation and consistency of the difference between VERSANT 3.0 and HC-II were analyzed in 54 patients with HBV DNA levels within the dynamic ranges of both assays (Fig. 1A). The scatter plot of the $\log_{10}$ quantitative values by VERSANT 3.0 versus those by HC-II showed a good linear relationship and a close data agree-

Table 1. Distribution of HBV DNA levels measured by VERSANT 3.0 and HC-II

	VERSANT 3.0 (copies/mL), N (%)			Total
	HD ($>10^8$)	WD (2×10^3-10^8)	LD ($<2\times10^3$)	
HC-II (pg/mL)				
HD (>6,000)	0 (0)	0 (0)	0 (0)	0 (0)
WD (0.5-6,000)	61 (26.3)	54 (23.3)	0 (0)	115 (49.6)
LD (<0.5)	0 (0)	56 (24.1)	61 (26.3)	117 (50.4)
Total	61 (26.3)	110 (47.4)	61 (26.3)	232 (100.0)

Abbreviations: HD, higher than detection limit; WD, within detection limit; LD, lower than detection limit.

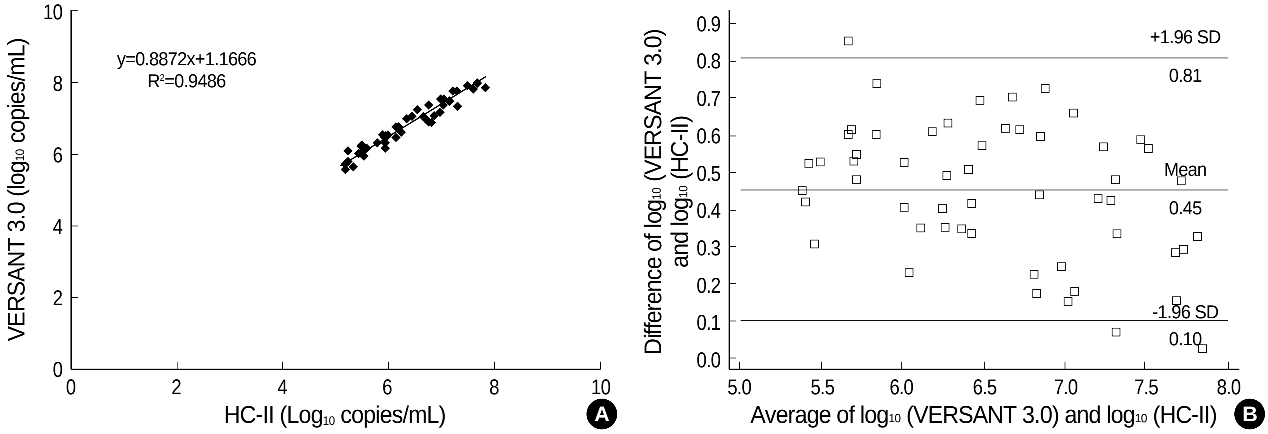

Fig. 1. (A) Correlation of $\log_{10}$ quantitative HBV DNA levels between VERSANT 3.0 and HC-II. (B) Differences in $\log_{10}$ quantitative HBV DNA levels between VERSANT 3.0 and HC-II in relation to average $\log_{10}$ quantification (n=54).

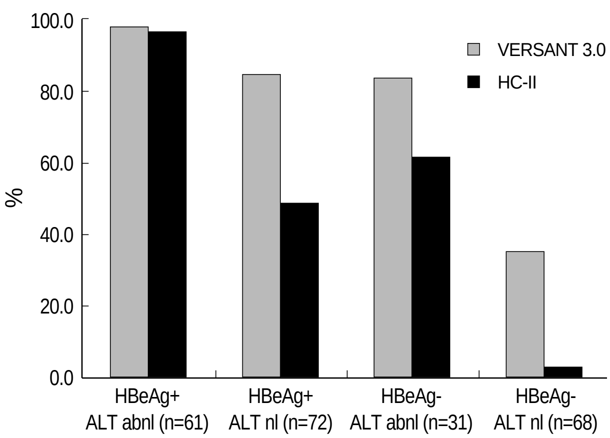

Fig. 2. Detection rates of HBV DNA by VERSANT 3.0 and HC-II in relation to clinical status of HBV.
Abbreviations: HBeAg, hepatitis B virus e antigen; abnl, abnormal; nl, normal.

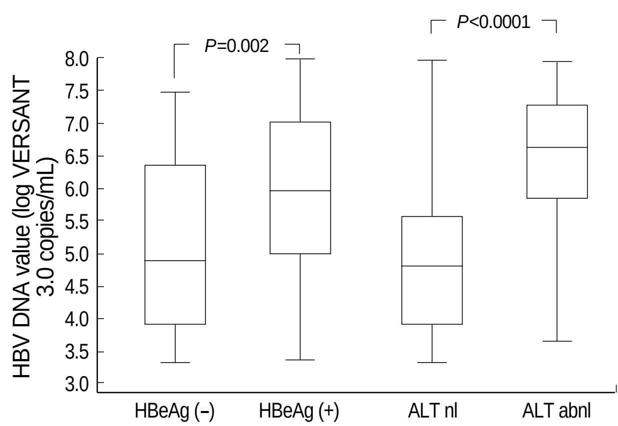

Fig. 3. HBV DNA levels by VERSANT 3.0 in relation to clinical status of HBV. The plots display the median values (lines inside boxes), 25th and 75th percentiles (lower and upper limits of boxes), and the minimum and 99th percentiles.
Abbreviations: nl, normal; abnl, abnormal.

ment ($r=0.9739$, $r^2=0.9486$, slope$=0.8872$). The formula for interassay conversion between them was: HBV DNA by VERSANT 3.0 ($\log_{10}$ copies/mL)$=0.8872\times$HBV DNA by HC-II ($\log_{10}$ copies/mL)$+1.1666$. In Bland-Altman plot (Fig. 1B), the average difference between data pairs from the two assays was 0.45 $\log_{10}$ (about 2.82 times higher) with 95% confidence interval (CI) from 0.10 $\log_{10}$ to 0.81 $\log_{10}$, and it did not differ in both HBeAg-positive and HBeAg-negative patients (0.48 $\log_{10}$ and 0.39 $\log_{10}$, respectively).

2. Performance of VERSANT 3.0 and HC-II according to HBeAg status and ALT levels

The detection rates of HBV DNA by VERSANT 3.0 and HC-II were further analyzed in four groups according to the HBeAg status and ALT levels (Fig. 2). The detection rate of VERSANT 3.0 in patients with negative HBeAg and normal ALT level (n=68) was significantly lower than in patients with positive HBeAg or abnormal ALT level (n=164) (35.3% vs 89.6%, $P<0.0001$). Such a significant difference was mainly due to the differences of HBV DNA levels in each group (Fig. 3). In 110 patients whose HBV DNA levels were within the dynamic range of VERSANT 3.0, HBV DNA levels were significantly higher in HBeAg-positive group (n=66) than in HBeAg-negative group (n=44) (5.92 $\log_{10}$ vs 5.11 $\log_{10}$, $P=0.002$) and in abnormal ALT group (n=48) than in normal ALT group (n=62) (6.47 $\log_{10}$ vs 4.93 $\log_{10}$, $P<0.0001$). There was also a moderate correlation between HBV DNA levels and serum ALT levels in both HBeAg-positive (r=0.4651, P=0.001) and HBeAg-negative groups (r=0.4264, P=0.004).

3. Comparison of VERSANT 3.0 with Real-Q

In 117 patients with undetectable HBV DNA levels by HC-II, Real-Q was further applied and their results were compared with those by VERSANT 3.0. In 55 sera with detectable HBV DNA levels by VERSANT 3.0, HBV DNA were detected by Real-Q with the mean value±2SD of 4.69 $\log_{10}$±0.68 $\log_{10}$ (Fig. 4). Out of 61 sera with undetectable HBV DNA levels by VERSANT 3.0, 52 sera showed detectable HBV DNA by Real-Q with mean±2SD of 3.26 $\log_{10}$±0.51 $\log_{10}$, which was significantly lower than in former 55 sera with detectable DNA levels by VERSANT 3.0 (P<0.0001) (Fig. 4). The correlation between VERSANT 3.0 and Real-Q in 55 sera was also satisfactory (r=0.8329, r^2=0.6937, P<0.0001) (Fig. 5A). Check for between-method outliers for two points which showed differences higher than mean+1.96 SD in Fig. 5 revealed that they were within the test limit (four times average of absolute differences between two methods), therefore could be used for comparison analysis. In Bland-Altman plot, the average difference between data pairs from these two assays was -0.23 $\log_{10}$ (95% CI=-1.04-0.57 $\log_{10}$) (Fig. 5B).

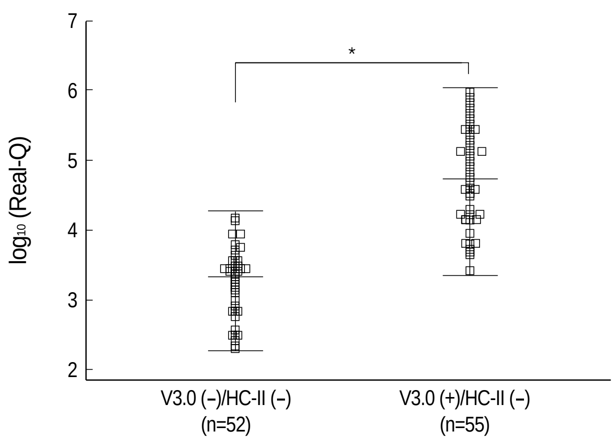

Fig. 4. Scatter plot shows HBV DNA levels measured by Real-Q ($\log_{10}$ copies/mL) in sera with undetectable DNA by both of VERSANT 3.0 (V3.0) and HC-II (n=52) (left) and in sera with detectable DNA by VERSANT 3.0 (V3.0) but undetectable by HC-II (n=55) (right). Horizontal bars represent the means and±2SD. The difference between two groups was tested by Student's t test. *P< 0.0001.

DISCUSSION

A new version of bDNA assay, VERSANT 3.0, has been recently introduced and showed a high specificity (99.3%), an excellent reproducibility (between-run CV= 1.6-9.4%; within-run CV=6.5-20.7%), and a broad linear range of quantification (2.0×10^3-1.0×10^8 HBV DNA copies/mL)[5]. However, there is a lack of standardization among different HBV DNA quantification assays to date. The previous version of VERSANT HBV DNA assay usually showed definitely higher estimates than Hybrid Capture assays or PCR-based assays[11-14]. The new VERSANT 3.0 has been also reported to show slightly higher values than Hybrid Capture assays[5]. In our study, the comparison between VERSANT 3.0 and HC-II showed a good correlation coefficient (r^2=0.9486). How-

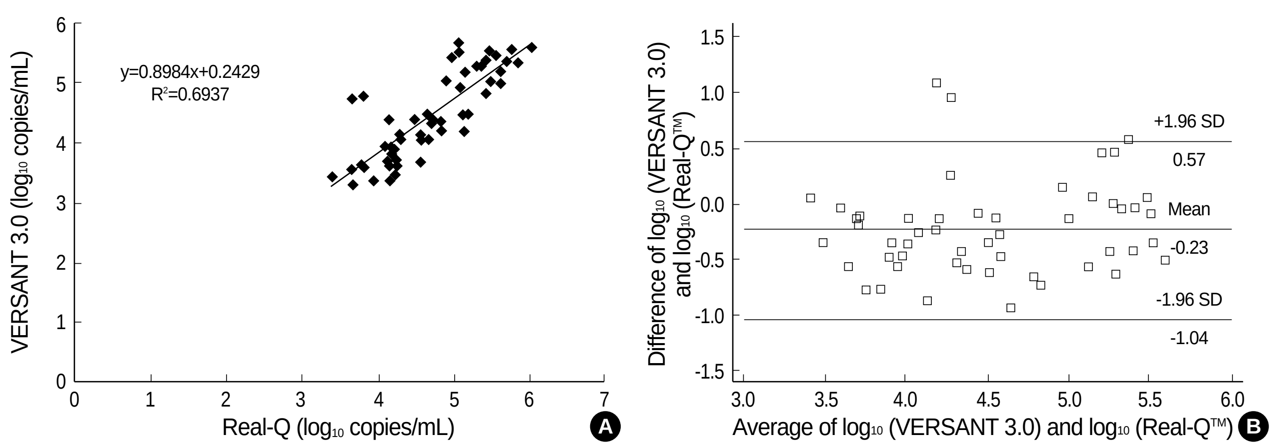

Fig. 5. (A) Correlation of $\log_{10}$ quantitative HBV DNA levels between VERSANT 3.0 and Real-Q (n=55). (B) Differences in $\log_{10}$ quantitative HBV DNA levels between VERSANT 3.0 and Real-Q in relation to average $\log_{10}$ quantification (n=55).

ever, the average $\log_{10}$ difference between these two assays was slightly higher with the value of 0.45 $\log_{10}$ (95% CI, 0.10-0.81) than the previous report (0.103 $\log_{10}$)[5].

The substantial difference may be due to different detection principles and a lack of standardization. VERSANT 3.0 has standard materials calibrated against WHO reference material 97/746, and the results are converted to copies/mL (1 IU=5.6 copies/mL). HC-II has standard materials (pg/mL), which are converted to copies/mL using a conversion factor (1 pg/mL=2.83×10^5 copies/mL), but not standardized with WHO standard HBV DNA[3]. VERSANT 3.0 has been also reported to show a relatively small average difference of 0.13 $\log_{10}$ compared with Cobas Taqman HBV test (Roche Diagnostics, Meylan, France), which also uses internal quantification standard (QS), and is standardized with WHO standard HBV DNA[15]. Further studies and efforts for standardization are still needed in this field.

In our study, 52 out of 61 sera with undetectable HBV DNA levels by VERSANT 3.0 were measurable by Real-Q with a mean value of 3.26 $\log_{10}$ (about 1819.7) copies/mL, most of them were below the lower detection limit of VERSANT 3.0 (2×10^3 copies/mL). Therefore, from a practical point of view, VERSANT 3.0 is satisfactory because it has a lower detection limit (2×10^3 copies/mL) enough to cover the clinically relevant HBV DNA levels, considering that viral breakthrough is defined as an increase of HBV DNA levels above 5 $\log_{10}$ copies/mL[16, 17] and more sensitive real-time PCR still could have relatively higher CVs at very low HBV DNA levels according to extraction methods or manufacturers[2, 18].

We tried to find an appropriate strategy regarding the suitable HBV DNA quantification assay in routine clinical settings in Korea, and analyzed the clinical sensitivity of VERSANT 3.0 according to HBeAg status and ALT levels. Our data shows that VERSANT 3.0 can be primarily used for the patients with positive HBeAg or abnormal ALT levels (70.7%, 164/232 consecutive samples). However, the low detection rate (35.3%) by VERSANT 3.0 in patients with negative HBeAg and normal ALT levels (n=31) implies that a more sensitive assay is required to see the very low HBV DNA levels (below 2×10^3 copies/mL) in these patients.

In summary, the correlation between VERSANT 3.0 and HC-II was good across their overlapping dynamic ranges, and the results (copies/mL) by VERSANT 3.0 were 2.82 times higher than those by HC-II. In routine clinical settings, VERSANT 3.0 seems to be a satisfactory choice for HBV DNA quantification in most of patients with positive HBeAg or abnormal ALT levels. However, to see low HBV DNA levels in patients with negative HBeAg and normal ALT levels, more sensitive assays are needed.

요 약

배경 : B형 간염 바이러스(hepatitis B virus, HBV) DNA 정량검사는 임상검사실에서 널리 이용되고 있으나, 서로 다른 검사방법 간에 표준화가 되어 있지 않으며, 한국인 B형 간염 환자에서 임상적 유용성에 대해 명확히 규명되지 않았다.

방법 : 232명의 한국인 B형 간염 환자를 대상으로 Bayer VERSANT HBV DNA 3.0 Assay (VERSANT 3.0)를 Digene Hybrid Capture II HBV DNA 검사(HC-II)와 비교하고 HBeAg과 혈청 ALT 농도에 따라 평가하였다. HC-II로 검출한계 이하인 117예를 Real-Q 정량 PCR (BioSewoom, Korea)로 추가 분석하였다.

결과 : VERSANT 3.0과 HC-II는 우수한 상관관계를 보였으나(r=0.9739), VERSANT 3.0의 결과(copies/mL)는 HC-II의 결과(copies/mL)에 비해 0.45 $\log_{10}$ 높았다. HBV DNA 농도는 HBeAg 양성군에서 HBeAg 음성군에 비해 유의하게 높았으며 (P=0.002), 비정상 ALT군에서 정상 ALT군에 비해 유의하게 높았다(P<0.0001). VERSANT 3.0의 HBV DNA 검출률은 HBeAg 음성이고 정상 ALT군(n=68)에서 HBeAg 양성이고 비정상 ALT군(n=164)에 비해 유의하게 낮았다(35.3% vs 89.6%, P<0.0001). VERSANT 3.0으로 HBV가 검출되지 않은 61예 중 52예는 Real-Q에서 검출 가능하였으며 평균 3.26 $\log_{10}$ copies/mL의 값을 보였다.

결론 : VERSANT 3.0과 HC-II는 우수한 상관관계를 보였으나, 결과 간에 약간의 차이(0.45 $\log_{10}$)가 존재하였다. VERSANT 3.0은 대부분의 한국인 B형 간염환자에서 임상적으로 유용하게 HBV DNA 정량이 가능하였다. 그러나, HBeAg 음성이고 정상 ALT를 보이는 환자들에서의 낮은 HBV DNA 농도를 정량하는 목적으로는 좀 더 예민한 검사가 필요할 것으로 생각된다.

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