Letter to the Editor

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Inconsistent virus DNA quantification results in clinical hepatitis B and the verification by genotyping and sequencing: a case report

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To the Editor,

Hepatitis B virus (HBV) infection is the leading cause of hepatocellular carcinoma (HCC). Approximately 700,000 people die of HCC each year worldwide and HCC is thus the third leading cause of cancer related deaths [1]. Despite the HBV vaccine program established in the early 1990s, an epidemic of HBV infection in China still causes a rigorous public health problem [2]. Given the high risk of developing liver cirrhosis and HCC after chronic HBV infections, there is a critical need for screening, diagnosis and monitoring of HBV [3]. In addition to tests for HBV serological markers, real-time PCR based HBV DNA quantification has been widely used in clinical practice to predict disease activity and monitor virus treatment response [4]. We herein present a case with opposite results of HBV DNA quantification between the Sansure method and the Roche method using the same patient serum.

A 52-year old male with a history of HBV infection and cirrhosis for seven years was admitted to Qianfoshan hospital on February 9th, 2019. The patient was given a diagnosis of HCC. His serum HBV DNA quantification results (Sansure HBV DNA quantification kit, China; ABI 7300 Plus instrument, USA) revealed that the virus concentration was $1.28\text{E} + 05$ IU/mL. Three days later, a second serum of this patient was drawn and examined by Roche (COBAS® AmpliPrep®/COBAS® TaqMan® 48, HBV Test v2.0, USA) HBV DNA quantification test due to a temporary shortage of Sansure kits and the result was $<2.00\text{E} + 01$ IU/mL. The inter-laboratory comparison between Sansure method and Roche method was performed every three months, and there were no inconsistent results observed ever. On the other hand, it was uncommon to see a dramatic decline of HBV copies in clinic. The sample was further confirmed to have neither hemolytic nor lipid interference, and the patient was overnight fasting before blood collection. The instruments used for quantification were under quality inspection and maintained daily. The internal controls in experiments and the amplification curves were normal (Figure 1). To eliminate the possibility of operating error, each determination method was repeated twice more. Results with the Sansure method were consistent with the first test ($1.08\text{E} + 05$ IU/mL, $1.13\text{E} + 05$ IU/mL), and results with the Roche method were consistent with the prior Roche results (undetermined, undetermined).

To help analyze the results, the patient's related clinical and laboratory parameters were reviewed. The patient has a seven year history of HBV infection and has been taking anti-HBV drugs. The HBsAg level regularly monitored two months ago was 127 IU/mL, but HBV DNA was negative (Sansure method). As shown in Table 1, all of HBsAg, HBeAb and HBV-PerS1 were positive at admission and the patient's liver function was affected. However, regarding HBV, the results between immunological tests and nucleic acid detection are not always positively correlated, which makes clear interpretation difficult. High expression of HBsAg does not necessarily predict active

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Figure 1: The amplification curves of hepatitis B virus DNA quantification.

The amplification curve of HBV is indicated in red and IC is indicated in blue. (A) HBV DNA quantification with the Sansure HBV quantification kit determined in ABI 7300 Plus on February 9th. (B) The hypersensitive DNA quantification of HBV was measured using the Roche CAP/CTM system on February 12th.

HBV DNA replication [5], and HBsAg level could be negative while HBV DNA is positive [6].

In pursuit of an explanation for the HBV DNA quantification inconsistency between testing methods, we carefully verified the patient's identification information, then drew a third blood sample on February 13th, 2019. DNA quantification by the Sansure method showed the virus

load was $8.97\text{E} + 04$ IU/mL. Interestingly, the result from the Roche method was still lower than the limit of detection. These data suggested that a false positive or false negative result had been generated.

HBV is classified into 10 genotypes, designated A to J based on phylogenetic analysis. Genotypes B and C are the most common in Asia-Pacific region. In chronic hepatitis B patients, genotype C and D have a higher frequency of basal core promoter mutations than genotype A and B, and a higher risk of cirrhosis and HCC, leading to a poorer clinical outcome [7]. We then performed the HBV genotyping and drug resistant mutations assay (Yaneng, China) to confirm the HBV status in this patient. The significance of the research was fully explained to the patient and informed consent was obtained. The further studies were conducted in accordance with the World Medical Association Declaration of Helsinki principles. As shown in Figure 2A, the HBV DNA was confirmed as genotype C. Remarkably, the minimum detection limit of this assay was $1.00\text{E} + 03$ IU/mL, which indicated that (i) this patient is HBV positive; (ii) the virus load must be at least $1.00\text{E} + 03$ IU/mL; (iii) an amplification failure occurred in the Roche method.

Both the Sansure method and the Roche method use the real-time TaqMan PCR technique for HBV DNA quantification. The primers used for Sansure are located in the HBV S gene, and for Roche they are located in the Pre-core gene. The detailed sequences are unknown. Further, we designed two individual primers in the S gene that would cover the Sansure amplification products and performed PCR. We examined these PCR products using Sanger sequencing. After analyzing on NCBI Blast, the data showed 100% similarity to HBV genotype C (Figure 2B).

The HBV genome is replicated by reverse transcription of the encapsidated pregenomic RNA with viral encoded polymerase and lacks proofreading activity, which makes the HBV mutation rate higher than in other DNA viruses [8].

Table 1: Related laboratory testing parameters on February 9th, 2019.

Parameter, unit	Test result	Local reference
Clinical chemistry testing		
ALT, U/L	27	9–50
AST, U/L	317	15–40
GGT, U/L	132	10–60
ALP, U/L	178	45–125
TBIL, $\mu\text{mol/L}$	146	5–24
DBIL, $\mu\text{mol/L}$	120.2	0.24–7.10
UBIL, $\mu\text{mol/L}$	25.8	2.8–23.8
TP, g/L	48.2	65–85
ALB, g/L	22.6	40–55
GLB, g/L	25.6	20–40
Tumor markers		
CEA, ng/mL	1.7	0–5.0
AFP, ng/mL	>2,000	0–8.8
CA199, U/mL	>1,200	0–37.0
HBV serological markers		
HBsAg, IU/mL	>250.0	<0.05
HBsAb, mIU/mL	<0.9	<10.0
HBeAg, S/CO	0.327	<1.0
HBeAb, S/CO	1.74	>1.0
HBcAb, S/CO	6.72	<1.0
HBV-PreS1Ag, S/CO	253.1	<1.0

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ -glutamyltransferase; ALP, alkaline phosphatase; TBIL, total bilirubin; DBIL, direct bilirubin; UBIL, unconjugated bilirubin; TP, total protein; ALB, albumin; GLB, globulin; CEA, carcinoembryonic antigen; AFP, α -fetal protein; CA, cancer antigen; HBV, Hepatitis B virus.

Figure 2: Verification experiments for the conflicting HBV DNA quantification results. (A) The serum on February 13th was examined by HBV genotyping and drug resistant mutations assay. The specific HBV sequences were amplified by PCR, followed by hybridization with probes fixed on nylon membrane and then produced a color reaction with tetramethylbenzidine. (B) The serum on February 13th was verified by Sanger sequencing. The sequencing primers were designed in the S region and the products were 429 bp long. Sequencing data was analyzed on NCBI Blast with graphic display (left panel) and hit list (right panel).

Amplification failure of the Roche method might be due to variation in the HBV Pre-core gene. In Asia Pacific, around 50% of patients with chronic HBV infection and HBeAg negative serology have HBV Pre-core gene variation, which is higher than the rate in the United States and Northern Europe (24%) [9]. Since the Roche primer sequences are unknown, we must speculate, but we believe that the undetermined result from the Roche CAP/CTM system is due to a mismatch between primers and HBV gene variants, which in turn led to a failure of annealing or low efficiency amplification during PCR.

This case reminds that a false negative result can be generated in HBV DNA quantification. If contradictory results occur, they need to be promptly confirmed with clinical parameters and verification experiments so that the diagnosis and treatment will not be misinformed. An HBV DNA quantification kit would more

effectively have primers designed in the most conserved regions of virus among local population. Dual primers or probes may work well in detecting viruses with high mutation rates like HBV [10]. Their application in clinical diagnosis is promising and could provide increased reliability.

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