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## Stool vs. Serum Hepatitis B Virus DNA in Patients with Chronic Hepatitis B

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**Background:** Serum hepatitis B virus (HBV) DNA and hepatitis B e antigen (HBeAg) liver function in patients with chronic hepatitis B (CHB) are significantly associated. A comparison of clinical significance of fecal HBV DNA and serum HBV DNA has not yet been reported.

**Material/Methods:** Stool and serum samples were collected from 66 patients with CHB. Fecal HBV DNA, serum HBV DNA, and intestinal microbiota DNA were detected by real-time quantitative fluorescence polymerase chain reaction (PCR). Liver function and HBeAg were analyzed.

**Results:** The stool and serum HBV DNA were positively correlated ( $r=0.57$ ,  $P=0.001$ ). Fecal HBV DNA was higher in the HBeAg-positive group than in the HBeAg-negative group ( $P=0.02$ ). Fecal HBV DNA was negatively correlated with alkaline phosphatase (ALP) ( $r=-0.41$ ,  $P=0.001$ ) and TBIL ( $r=-0.29$ ,  $P=0.02$ ), and was positively correlated with *Enterococcus* ( $r=0.38$ ,  $P=0.002$ ). Serum HBV DNA was negatively correlated with alanine aminotransferase (ALT) ( $r=-0.30$ ,  $P=0.02$ ), aminotransferase (AST) ( $r=-0.26$ ,  $P=0.049$ ), and *Lactobacillus* ( $r=-0.31$ ,  $P=0.01$ ).

**Conclusions:** These observations suggest that fecal HBV DNA and serum HBV DNA in patients with CHB have different effects. Fecal HBV DNA might be associated with changes in *Enterococcus* concentrations, but serum HBV DNA is not.

**MeSH Keywords:** **Hepatitis, Chronic • Liver Function Tests • Nucleic Acid Amplification Techniques**

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## Background

HBV remains the primary pathogen causing chronic hepatitis B. More than 350 million people are infected with HBV worldwide. Patients with HBV infection have an increasing risk of liver cirrhosis and hepatocellular carcinoma, especially in China and other Asian countries, where over 1 million people die of end-stage liver disease or liver cancer every year.

Distribution of HBV in the body is systemic and HBV occurs in blood, milk, sweat, gastric juice, saliva, urine, and other body fluids. There are different concentrations of HBV particles and different infections in different body fluids. The concentration of HBV particles in the blood is the highest and infection in the blood is the most serious [1,2].

HBV exists in intestinal tract because of the presence of bile, gastric juice, and intestinal juice.

Serum HBV DNA is useful for understanding HBV infection and replication, and predicts the prognosis. Serum HBV DNA and HBeAg are closely related in patients with chronic hepatitis B. There are many reports of serum HBV DNA and liver function, and several reports showed that serum HBV DNA loads and liver function parameters are significantly associated [3,4].

The relationship among stool HBV DNA and serum HBV DNA, HBeAg, and liver function in patients with CHB has not yet been reported. It remains to be determined whether fecal HBV DNA is associated with the degree of liver damage. The liver and gut share a common embryological origin; therefore, their structures and functions are intrinsically associated with each other, forming a "gut-liver axis". Some gut bacteria and the metabolites of gut flora might reach the liver on more than just rare occasions, activate TLR/NLR in the liver, and result in liver inflammation [5]. It is possible that HBV and its metabolites in the intestinal tract might reach the liver, resulting in liver inflammation, and might be associated with the degree of liver damage.

The intestinal tract contains a vast pool of bacteria and is a major site for induction of regulatory T cells, which secrete immunosuppressive cytokines. The intestinal microbiota plays a significant role in human nutrition, immunology, and pathological processes, and the intestinal flora help fight infection and carcinogenesis [6–8]. Intestinal dysfunction in liver disease is well known and translocation of bacterial peptidoglycan, microbial products, and metabolic byproducts has been suggested to aggravate the clinical course of patients with chronic liver disease [9,10]. It is reported that levels of *Bifidobacterium* and *Lactobacillus* are decreased, while *Enterobacteriaceae*, *Enterococcus*, and *Candida albicans* are obviously increased in chronic HBV infection [5,11]. There is a report that serum HBV

is associated with both altered gut permeability and alterations in the gut microbiota composition [5], but it is unclear whether HBV in the gut can change the intestinal microbiota.

The present study aimed to determine whether fecal HBV DNA had different significance from serum HBV DNA. These were investigated by comparing the various correlations of fecal HBV DNA and serum HBV DNA with *Bifidobacterium*, *Lactobacillus*, *Enterobacteriaceae*, *Enterococcus*, *Candida albicans*, and liver function.

## Material and Methods

### Sample source

Patients with CHB were from the Third Affiliated Hospital of Anhui Medical University. Clinical diagnosis was based on the diagnostic standards of viral hepatitis, as jointly revised by the Society of Infectious Diseases and Parasitic Diseases and the Chinese Society of Hepatology, CMA in 2000.

The inclusion criteria for CHB patients were: (1) having positive hepatitis B surface antigen for over half a year, and glutamic-pyruvic transaminase twice the normal upper limit; (2) no gastrointestinal hemorrhages in the past week and negative stool occult blood test; (3) imaging examination did not indicate cirrhosis; (4) had not taken liver-damaging drugs in the past month; (5) laboratory tests excluded hepatitis A virus, hepatitis C virus, hepatitis E virus, and cytomegalovirus, as well as Coxsackie and Epstein-Barr virus infections. Exclusion criteria were: (1) previously diagnosed and treated cases; (2) received antibiotics, antiviral drugs, or interferon in the past month; (3) had a history of other liver diseases, inflammation, autoimmune diseases, lung, heart, and other diseases. Finally, 66 CHB patients were included in this study – 46 males and 20 females – with an average age of 42.5±20.7 years.

All CHB patients lived in Hefei, China and maintained their habitual diet, typical of the Hefei region, in the month before the study. The characteristics and clinical information of the participants are summarized in Table 1.

All patients were informed that urine would contaminate the stool sample and were required to collect the stool sample after urination. The interval between the blood and stool sample collection did not exceed 2 days.

This study was conducted according to the Declaration of Helsinki. Approval was obtained from the hospital ethics committee (No. 2009[2]). Informed consent was obtained from all patients.

**Table 1.** The clinical data of the patients with CHB ( $\bar{x} \pm s$ ).

Items	Chronic hepatitis B
No. of subjects (n)	66
Gender (male/female)	46/20
Age (year)	42.5±20.70
Stool wet weight (mg)	200±5.61
TBIL (μmol/L)	44.20±41.66
ALT (U/L)	695.61±658.86
AST (U/L)	518.60±640.18
ALB (g/L)	39.7±4.42
GGT(U/L)	130.88±77.45
ALP (IU/L)	113.14±59.81
Log of stool HBV DNA	5.88±1.63
Log of serum HBV DNA	6.05±1.07
<i>Enterobacteriaceae</i>	8.38±1.04
<i>Enterococcus</i>	6.94±1.00
<i>Bifidobacterium</i>	7.74±1.15
<i>Lactobacillus</i>	7.22±1.65
<i>Candida albicans</i>	6.34±0.35

**Sample collection**

Patients were diagnosed with CHB, and blood and stool samples of the patients were collected before treatment. The stool samples were collected in the morning. After the sample was stirred evenly, 400 mg (as weighed by electronic balance) were divided into 2 EP (Eppendorf) tubes (200 mg each) and stored

at -80°C. In addition, fasting venous blood (8 ml) was collected, and the serum was separated and preserved at -20°C.

**DNA extraction**

DNA was extracted from the stool and the serum using the Column Stool DNA Out kit (Beijing Tiandz Inc., China) and HBV DNA determination kit (Shanghai ZJ Bio-Tech Co., Ltd, China), respectively, and the steps were performed in accordance with the kits' instructions. The stool and serum DNA were stored at -20°C. The integrity of DNA was confirmed by agarose gel electrophoresis and UV-light photography with ethidium bromide staining.

**Detection of HBV DNA**

Fecal HBV DNA and serum HBV DNA were detected by quantitative real-time PCR method and the HBV DNA determination kit (as mentioned above) was used. The detection steps were performed in strict accordance with the HBV DNA determination kit instructions. The Mx3000p quantitative fluorescence PCR (Stratagene Division, Agilent technologies, Inc., USA) and the matching Mxpro software of the Mx3000p quantitative fluorescence PCR were used for quantitative detection of HBV DNA.

According to the kit instructions, the detection range was  $0.5 \times 10^3 \sim 5 \times 10^8$  IU/ml for serum. A result over  $0.5 \times 10^3$  IU/ml was defined as a positive result, and the copy numbers of HBV DNA were recorded at the same time. The serum HBV DNA unit was IU/ml and the stool HBV DNA unit was IU/g wet weight. The stool HBV DNA (IU/g)=measurements (IU/ml)×(70÷1000)÷(200÷1000). "70÷1000" shows a total volume of stool DNA extract, unit: ml; 200÷1000 shows stool mass, unit: g.

**Table 2.** Primers and conditions used for qualitative and quantitative PCR.

Target group	Sequence(5-3')	Amplicon size (bp)	Annealing temp (°C)	Reference
<i>Enterobacteriaceae</i>	F: CATTGACGTTACCCGCGAGAAGAAGC	195	55	[12]
	R: CTCTACGAGCTCAAGCTTGC			
<i>Enterococcus</i>	F: CCCTTATTGTTAGTTGCCATCATT	144	54	[13]
	R: ACTCGTTGTACTIONCCATTGT			
<i>Bifidobacterium</i>	F: CTCCTGGAACGGGTGG	550	52	[14]
	R: GGTGTTCTCCCGATATCTAC			
<i>Lactobacillus</i>	F: AGCAGTAGGGAATCTTCCA	341	52	[15]
	R: CACCGCTACACATGGAG			
<i>Candida albicans</i>	F: TTTATCAACTGTACACCAGA	273	50	[16]
	R: ATCCCGCCTTACCACTACCG			

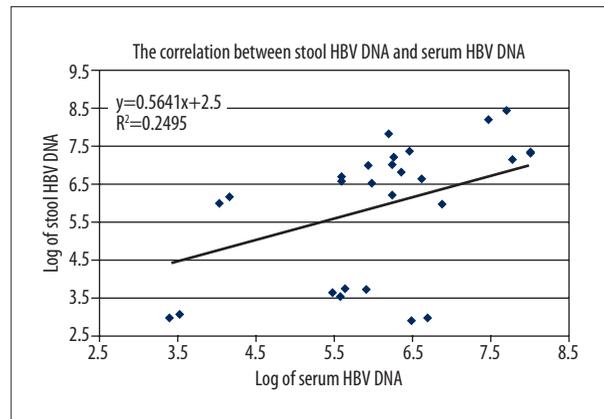
## Quantitative PCR to detect stool microbiota populations

Oligonucleotide primers were based on the references [12–16] and are listed in Table 2. These species-specific primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Qualitative PCR was used to detect the existence of various bacteria and to prepare qPCR standards. PCR was carried out in a TProfessional Thermocycler (Biometra, Göttingen, Germany) and performed in a 25- $\mu$ l mixture (2.5  $\mu$ l 10 $\times$ PCR buffer, 2  $\mu$ l dNTP mixture [2.5 mM], 1  $\mu$ l forward primer, and 1  $\mu$ l reverse primer [0.1  $\mu$ M], 0.2  $\mu$ l TaKaRa TaqTM [5 U/ $\mu$ l] and 1  $\mu$ l of template DNA). All enzymes and chemical materials were provided by TaKaRa (Dalian, China). The amplification program used was as follows: preheating at 94°C for 8 min; 32 cycles of denaturation at 94°C for 1 min, annealing for 45 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min. The annealing temperature for each pair of primers is shown in Table 2. The size of amplified fragments was checked through electrophoresis on 1% agarose gel containing ethidium bromide.

PCR products of different bacterial primer sets were purified by using the TaKaRa MiniBEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China) and cloned into the pMD™18-T Vector (TaKaRa, Dalian, China). The ligation mixtures were transfected into competent JM109 cells (TaKaRa, Dalian, China) and the correct plasmid insertions were screened according to the manufacturer's instructions. Plasmid DNA was extracted and purified with a plasmid extraction kit (TaKaRa, Dalian, China). Finally, plasmid DNA strands were sent for commercial sequencing (TaKaRa, Dalian, China). The sequences received from the company were compared directly with those in GenBank using BLAST [<http://www.ncbi.nlm.nih.gov/blast/>].

The concentration of plasmid DNA was detected by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Shanghai, China). Plasmid DNA was serially diluted 10-fold in EASY Dilution (TaKaRa, Dalian, China) from 10<sup>2</sup> to 10<sup>7</sup> copies/ $\mu$ l for use as plasmid standards in real-time PCR.

Intestinal microbiota was analyzed by using Rotor-Gene3000 (Qiagen, Hilden, Germany) associated with Rotor-Gene software (version 6.1). A 20- $\mu$ l amplification reaction was completed with 10  $\mu$ l SYBR Premix Ex TaqII (TaKaRa, Dalian, China), 0.8  $\mu$ l of each primer (10 $\mu$ M), 2  $\mu$ l of the respective crude template DNA or water (negative control), and 0.4  $\mu$ l ROX reference dye. Amplifications were performed with the following reaction procedures: 1 cycle at 95°C for 30 s, 40 cycles of denaturation at 95°C for 8 s, annealing for 20 s (Table 2) and 72°C for 10 s (Bifidobacterium, 72°C for 30 s). To avoid the interference of primer-dimers, spurious priming, or a secondary structure, fluorescence was measured after the extension phase of each cycle at an appropriate temperature for 10 s. Following amplification, melting temperature analysis of the



**Figure 1.** The correlation between fecal HBV DNA and serum HBV DNA. Notes: The Log of fecal HBV DNA was  $5.88 \pm 1.63$ . The Log of serum HBV DNA was  $6.05 \pm 1.07$ . The copy numbers of HBV DNA from the two types of samples were positively correlated ( $r=0.57$ ,  $P=0.001$ ), correlation is significant at the 0.01 level (2-tailed).

PCR products was performed to confirm the specificity of the PCR. The melting curves were obtained by slow heating at 0.2°C/s increments from 58°C to 95°C with continuous fluorescence collection.

The copy number of target bacterial DNA in each  $\mu$ l of crude DNA template was determined by comparison with serially diluted standards running on the same plate. The correlation coefficient values of the standard curves were limited from 0.99 to 1.0. Plasmid standards and samples were assayed simultaneously in duplicate and  $\Delta C(t) < 0.5$  is requisite. The intestinal microbiota DNA unit was copies/ $\mu$ l.

## Detection of liver function

Liver function, including ALT, aspartate aminotransferase (AST), glutamyl transferase (GGT), ALP, TBIL, and ALB, was analyzed using an automatic biochemistry analyzer (Hitachi 7600-020, Japan), and operated in strict accordance with the instructions of the manufacturer.

## Statistical analysis

The data were processed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). The quantitative data of HBV DNA and bacterial DNA were non-normally distributed, were logarithmically transformed to fit normal distribution, and are expressed as mean  $\pm$  standard deviation (SD). Other data are shown as mean  $\pm$ SD. The fecal HBVDNA in the HBeAg-positive group and the HBeAg-negative group was compared by independent sample *t*-test. The correlation was analyzed with Pearson's correlation. Statistical significance was defined at  $P < 0.05$ .

**Table 3.** The difference of HBV DNA between the HBeAg-positive group and the HBeAg-negative group.

Items	HBeAg-positive group	HBeAg-negative group
Log of fecal HBV DNA	6.27±1.70*	5.45±1.44

In Independent samples test, \* P=0.02<0.05.

**Table 4.** The correlation between HBV DNA and liver function (n=66).

Items	Log of fecal HBV DNA		Log of serum HBV DNA	
	r	p	r	p
ALT	-0.07	0.58	-0.30	0.02*
AST	-0.05	0.68	-0.26	0.049*
ALB	0.03	0.80	0.09	0.46
GGT	-0.20	0.11	-0.11	0.39
ALP	-0.41	0.001**	-0.02	0.89
TBIL	-0.29	0.02*	-0.19	0.13

In Pearson's correlation analyses, \* P<0.05; \*\* P<0.01.

**Table 5.** The correlation between HBV DNA and intestinal microbiota (n=66).

Items	Fecal HBV DNA		Serum HBV DNA	
	r	p	r	p
<i>Enterobacteriaceae</i>	0.08	0.50	-0.13	0.29
<i>Enterococcus</i>	0.38	0.002**	-0.01	0.93
<i>Bifidobacterium</i>	0.16	0.21	-0.09	0.46
<i>Lactobacillus</i>	0.19	0.13	-0.31	0.01*
<i>Candida albicans</i>	0.20	0.11	0.08	0.53

In Pearson's correlation analyses, \* P<0.05, \*\* P<0.01.

## Results

**Correlation analysis of stool and serum HBV DNA.** Logarithm (Log) of stool HBV DNA was 5.88±1.63 and Log of serum HBV DNA was 6.05±1.07. Pearson's correlation analyses indicated that the HBV DNA copies from the 2 sources were positively correlated (r=0.57, P<0.01) (Figure 1).

**Difference in HBV DNA between the HBeAg-positive group and the HBeAg-negative group.** Log of the stool HBV DNA in the HBeAg-positive group was higher than in the HBeAg-negative group, and the difference was statistically significant (P=0.02) (Table 3).

**Correlation analysis of HBV DNA and liver function.** Pearson's correlation analysis indicated that fecal HBV DNA was negatively correlated with ALP (r=-0.41) and TBIL (r=-0.29), and was

not correlated with ALT, AST, ALB, or GGT (P>0.05). Correlation analyses indicated that serum HBV DNA was negatively correlated with ALT (r=-0.30) and AST (r=-0.26) and was not correlated with TBIL, ALB, GGT, or ALP (P>0.05) (Table 4).

**Correlation analysis of HBV DNA and intestinal microbiota.** Pearson's correlation analysis indicated that fecal HBV DNA was positively correlated with *Enterococcus* (r=0.38) and was not correlated with *Enterobacteriaceae*, *Bifidobacterium*, *Lactobacillus*, *Clostridium*, or *Candida albicans* (P>0.05). Correlation analysis indicated that serum HBV DNA was negatively correlated with *Lactobacillus* (r=-0.31) and was not correlated with *Enterococcus*, *Enterobacteriaceae*, *Bifidobacterium*, or *Candida albicans* (P>0.05) (Table 5).

## Discussion

Quantitative PCR with high operability, sensitivity, and repeatability to detect HBV DNA can reflect the replication of HBV in patients. With the development of stool DNA extraction technology, the quantitative detection of stool HBV DNA has been reported [17]. HBV exists in saliva, gastric juices, and other body fluids [1,2]. It is possible that HBV particles exist and replicate in the intestinal tract. The positive result of fecal HBV DNA might indicate the replication of HBV in the intestinal tract. Thus, HBV DNA can be detected in the stool of patients with CHB.

Positive HBeAg in serum indicates that HBV is actively replicating and highly infectious [18,19]. We found that fecal HBV DNA in patients with positive HBeAg was higher than in patients with negative HBeAg, and stool HBV DNA and serum HBV DNA were positively correlated. This might indicate that HBV in the intestinal tract is also actively replicating when HBV in serum is actively replicating. Previous studies confirmed that serum HBV DNA can be detected in HBeAg-negative patients [20–22] because mutations in the precore (G1896A) and basic core promoter (A1762T and G1764A) regions affect the production of HBeAg in HBeAg-negative patients. However, HBV replication is not affected [23,24]. We found that HBV DNA can be detected in both serum and stool in HBeAg-negative patients. This indicates that the replication of HBV might take place in both serum and intestinal tract in HBeAg-negative patients. Our observation suggests that serum HBV DNA and fecal HBV DNA might have the same ability to show the replication of HBV.

The outcome of HBV infection and the pathogenesis of liver disease are determined by dynamic interactions between the virus and the host immune system [22,25]. There is no consistent conclusion about the relationship between serum HBV DNA and the degree of liver injury [3,4,26]. Some reports showed that serum HBV DNA loads are associated with liver function parameters [3,4]. We found that serum loads of HBV

DNA were negatively correlated with ALT and AST, indicating that serum HBV DNA might be associated with the degree of liver cell damage, but loads of fecal HBV DNA were not correlated with ALT and AST and were negatively correlated with ALP and TBIL. Our study findings suggest that fecal HBV DNA might be associated with the degree of biliary damage. Our research indicates that stool and serum HBV DNA might differ in ability to indicate the degree of liver damage.

Changes in intestinal microbial ecology can occur at various stages of HBV infection, including asymptomatic carriage of hepatitis B virus, chronic hepatitis B, and decompensated HBV cirrhosis [5,11]. Decrease in beneficial bacteria and increase in harmful bacteria in the intestine have been confirmed in liver disease with HBV infection. The cause of this and the influence of HBV on intestinal microbial ecology are unclear. Our study indicated that fecal HBV DNA was positively correlated with *Enterococcus* and that serum HBV DNA was negatively correlated with *Lactobacillus*. This suggests that the replication of HBV in serum might be associated with the degree of the disorders of *Lactobacillus*, and the replication of HBV in the intestinal tract might be associated with the degree of the disorders of *Enterococcus*. The present study indicates that serum HBV DNA and stool HBV DNA might have different abilities to indicate the degree of disorders of gut flora.

## Conclusions

Serum HBV DNA and stool HBV DNA might have the same significance in understanding HBV replication, but they might have different abilities to reflect the degree of liver damage and disorders of gut flora. Our study is just a preliminary investigation of the clinically relevant differences between stool HBV DNA and serum HBV DNA. Assessment of the role of detection of stool HBV DNA in HBV-related liver disease requires further studies with larger sample sizes and hierarchical dynamic observations.

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