Journal of Medical Diagnostic Methods
ISSN: 2168-9784

Yu., J Med Diagn Meth 2015, 4:2
DOI: 10.4172/2168-9784.1000.168

Research Article Open Access

# PARV4 Co-Infection is Associated with Disease Progression in HBV Patients in Shanghai.

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**Received Date:** Feb 09, 2015, **Accepted Date:** Mar 05, 2015, **Published Date:** Mar 12, 2015



## Abstract

Human parvovirus 4 (PARV4) has been detected in blood and tissue samples from HIV/AIDS patients who are injecting drug users and we have reported PARV4 co-infection of hepatitis B or hepatitis C virus (HBV, HCV) infected individuals. In this study, we have investigated the consequences of co-infection with PARV4 for HBV infected individuals.

The aim of this study was to investigate the consequences of co-infection with PARV4 for HBV infected individuals.

Serum samples from healthy controls and HBV infected subjects were retrieved from Shanghai Center for Disease Control and Prevention Sample Bank. HBV genotypes were determined and sequences compared with reference sequences to derive phylogeny trees. All samples were tested for PARV4, parvovirus B19 and HCV; serum alanine transaminase (ALT) in chronic HBV patients was used as an indicator of disease severity. Relevant cytokines were measured in sera from healthy controls and HBV carriers.

HBV infected subjects had higher prevalence of PARV4 compared to healthy controls. PARV4 prevalence was not associated with HBV genotype, negatively correlated with serum HBV DNA, but positively correlated with serum ALT level. Additionally, serum IL-8 was up-regulated in HBV carriers co-infected with PARV4.

Co-infection with PARV4 may increase the risk of liver disease in HBV patients through up-regulation of inflammatory cytokines, but the underlying mechanism remains to be elucidated.

**Keywords:** Parvovirus 4; Hepatitis B virus; HBV-parvovirus co-infection

## Introduction

Parvoviruses are non-enveloped viruses with linear, single-stranded DNA genomes and among the smallest of the animal DNA viruses [1]. Human Parvovirus 4 (PARV4) was identified in 2005 [2]; prior to that, parvovirus B19 was the only known human pathogenic parvovirus. The pathogenic mechanisms of PARV4 remain un-defined.

PARV4 DNA has been detected in blood samples from cadavers of hepatitis C virus (HCV) RNA positive injecting drug users [3] and liver tissues [4] of patients with chronic HCV infection. We have previously observed an association between PARV4 infection and HCV or hepatitis B virus (HBV) infection [5]. Parvovirus B19 is associated with acute hepatitis and may cause a wide spectrum of clinical complications in immunocompetent patients and in pregnant women with primary infection [6]. Sharing 30% nucleotide identity with B19, PARV4 might also be able to cause clinical complications in infected subjects.

High PARV4 prevalence has been detected in HIV, HCV and HBV infected individuals. Little is known about clinical features that accompany co-infection of PARV4 [5, 7, 8] in at-risk population. Recently PARV4 was linked to encephalitis [9] and symptoms such as pharyngitis, vomiting, and arthralgias were observed in the individuals with PARV4 [2]. Similar to Cytomegalovirus (CMV) and parvovirus B19 (B19), PARV4 antigen persists in the host organ[10] and PARV4 shows greater resistance to pasteurization than B19 [11]. As a common agent co-infection with other pathogens, PARV4 may have an more important impact on clinical disease than B19. Simmons and his colleagues observed no correlations between PARV4 serostatus and HCV outcomes, but a significant association between PARV4 serostatus and early HIV-related symptom [12]. Our previous study found a significant higher PARV4 seroprevalence in HBV and HCV infected persons in Shanghai. But due to limitations of the previous study we did not investigate the clinical disease of the PARV4 infected HBV and HCV infections.

In this study we confirmed the prevalence of PARV4 DNA in the healthy and HBV infected subjects in Shanghai, as well as the association between PARV4 and HBV infection. To analyze the consequences of PARV4 co-infection.in HBV infected individuals in Shanghai, we investigated the association between PARV4 prevalence

and serum ALT concentration, as well as the association between PARV4 prevalence and HBV DNA concentration. We also determined and analyzed the serum levels of inflammatory cytokines of the study subjects.

## Results

### HBV genotype C dominates in Shanghai and is associated with higher ALT levels.

HBV genotype was determined in 194 serum samples with the commercial genotype kit. Overall, the genotyping results showed that 36.1% (70/194, 95%CI:29.3-43.3%) of HBV isolates belonged to B genotype, 57.2% (111/194,95%CI:49.9-64.3%) belonged to C genotype, 1.5% (3/194,95%CI:0.3-4.5%) were mixed B and C genotype; genotypes of the remaining 5.2% (10/194, 95%CI:2.5-9.3%) of isolates could not be determined.

Sequence analysis of a subset of isolates (19 B genotype, 19 C genotype, 3 B/C mixed type and 4 undefined isolates) showed that the commercial kit correctly identified the 19 B genotype and 19 C genotype isolates, while the three isolates classed as B/C mixed genotype belonged to C genotype and the four isolates with undefined genotype belonged to B genotype. Our data suggest that although the commercial kit can be used for genotyping of HBV, those isolates identified as B/C mixed or undefined type by the commercial kit should be further analysed by sequencing.

46.0% (52/113, 95%CI:36.6-55.6%) of HBV carriers were infected with HBV genotype B and 51.3% (58/113, 95%CI:41.7-60.8%) were infected with HBV genotype C, p=0.432. Of the chronic HBV patients, 65.4% (53/81, 95%CI:54.0-75.7%) were infected with HBV genotype C and 22.2% (18/81, 95%CI:13.7-32.8%) with HBV genotype B. The data suggest that HBV C genotype is dominant in Shanghai and is associated with disease progression in HBV infected subjects.

The complete genome sequences of 19 B and 17 C genotype isolates were generated and submitted to Genbank (accession number JX 661485 to JX 661474) and with reference sequences downloaded from Genbank, were used to build a phylogenetic tree （see supplement figure 1）. All virus isolates belonged to B or C genotypes, no other genotypes were identified.

The serum HBV DNA load of the chronic HBV patients infected with genotype B (1.08x108; 95%CI, 2.72x105-4.44x10$^9$) or C viruses (9.75x108; 95%CI, 3.05x106-3.70x1010) were similar (t test, p=0.402). The serum ALT levels of the chronic HBV patients infected with genotype C viruses (258.8 U/l,95%CI:101.6-420.8 U/l) were significantly higher than those infected with genotype B viruses (43.3 U/l, 95%CI:29.3-59.7 U/l, t test, p=0.012, see Figure 1A). Serum ALT levels did not correlate with serum HBV DNA load in chronic HBV patients (r(coefficient of correlation)=0.011, p=0.387, Figure 1B). Our data suggest that HBV genotype C may cause severe liver damage in chronic patients but this may not be through direct damage by the virus.


Figure1. Phylogenetic tree of HBV sequences from HBV infected subjects in Shanghai.

**Supplement Figure 1:** Phylogenetic tree of HBV sequences from HBV infected subjects in Shanghai. The whole genome sequences of the 45 isolates were determined by Biosune using 4 pairs of previously reported primers [34]. Sequences were edited by Seqman and ContigExpress, resulting in whole genome sequences of 36 isolates. The 36 sequences were used to construct a phylogenetic tree with MEGA 5. To facilitate phylogenetic analysis, reference sequences for the eight HBV genotypes (A, B, C, D, E, F, G, H) were downloaded from GenBank (Genbank accession numbers AB076678, AB126580, AB010291, AB033554, EF134945, EF134946, EU939562, AB112063, EU939647, GQ377563, AB049609, AB033559, AB126581, AB091255, AB106564, AB166850, AY090459, AB056513, AB064310, AB205010 and AY090460). The nucleotides sequence of the entire genome of HBV were amplified, sequenced and analyzed using. Bootstrap value are given at the node of each branch (1000 replicates). Viruses from HBV carriers were defined as SHCA with respective numbers, such as SHCA1, SHCA2, SHCA4, SHCA6, SHCA8, SHCA13, SHCA17, SHCA18, SHCA23, SHCA24, SHCA29, SHCA31, SHCA32, SHCA37, SHCA46, SHCA49, SHCA53, SHCA56, SHCA59, SHCA62, SHCA66, SHCA69, SHCA75, SHCA84, SHCA91, SHCA92 and SHCA99. Those sequences from chronic HBV patients were named as SHCR, such as SHCP3, SHCP9, SHCP11, SHCP22, SHCP23, SHCP26, SHCP31, SHCP34, SHCP38 and SHCP39. Sequences of 8 different HBV genotype viruses (with Genbank code) were downloaded from Genbank and analyzed as the reference viruses. The dashed circles in black represent sequences of HBV references genotype of A、B、C、D、E、F、G and H. The open circles represented viruses from Shanghai.

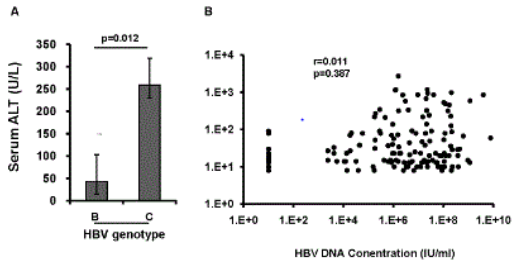


**Figure 1:** Serum ALT levels, HBV genotype and HBV DNA concentration. Alanine transaminase (ALT) was assayed in sera from chronic hepatitis B virus (HBV) patients using commercial kits as described in Methods. Serum specimens of HBV antigen (HBsAg) positive study subjects were tested for presence of HBV DNA with Real Time PCR Quantitation kit (ZJ Bio-Tech, Shanghai) using the Roche Light Cycler 480. HBV genotype was determined with a commercial HBV genotype (B & C) detection Real Time PCR kit (KL Bio-Tech, Shanghai) using the Roche Light Cycler 480. Average ALT levels in the serum samples collected from HBV B genotype infected subjects and HBV patients infected with C genotype are shown. Significant difference between B genotype and C genotype HBV infected patients is shown ($\chi 2$ test, $p \leq 0.05$). B. Association between ALT levels and HBV DNA concentration in the serum samples collected from chronic HBV patients were analyzed by Pearson correlation r (coefficient of correlation) and p value is indicated.

## PARV4 Positive detection rate of HBV infected subjects is higher than healthy controls.

To explore the PARV4 prevalence in HBV infected study subjects, we tested for the presence of PARV4 DNA in sera from 50 healthy controls, 92 HBV carriers and 70 chronic HBV patients. 16.0% (8/50, 95%CI:7.2-29.1%) of healthy controls and 31.5% (51/162, 95%CI: 24.1-40.0%) of HBV infected subjects were PARV4 DNA positive (MH $\chi 2$=4.53, p=0.033, Figure 2A). The PARV4 DNA positive detection rate was similar in HBV carriers (28.3%, 26/92, 95%CI:17.9-39.6%) and chronic HBV patients (35.7%, 25/70, 95%CI:24.6-48.1%, MH $\chi 2$=1.02, p=0.311, Figure 2C). There was no difference (MH $\chi 2$=1.75, p=0.186, see Figure 2B) in serum PARV4 DNA prevalence between subjects infected with HBV genotype B (23.2%, 13/56, 95%CI: 13.0-36.4%) or genotype C (33.8% 27/80, 95%CI:23.6-45.2%).

## Abnormal liver function is related to higher PARV4 prevalence in chronic HBV patients.

To investigate the association between PARV4 prevalence and serum ALT concentration, we categorized chronic HBV patients into 2 groups based on their serum ALT concentration. Patients with serum ALT concentration lower than 40U/L were termed the Liver Function Normal group; patients with serum ALT concentration higher than or equal to 40U/L were termed the Liver Function Abnormal group. Compared with the Liver Function Normal group (25%, 10/40, 95%CI: 12.7-41.2%), the Liver Function Abnormal group (50%, 15/30, 95%CI: 31.3-68.7%) had higher PARV4 prevalence (MH $\chi 2$=4.60, p=0.032, Figure 2D). Our data show that co-infection with PARV4 is associated with liver abnormality in chronic HBV patients.

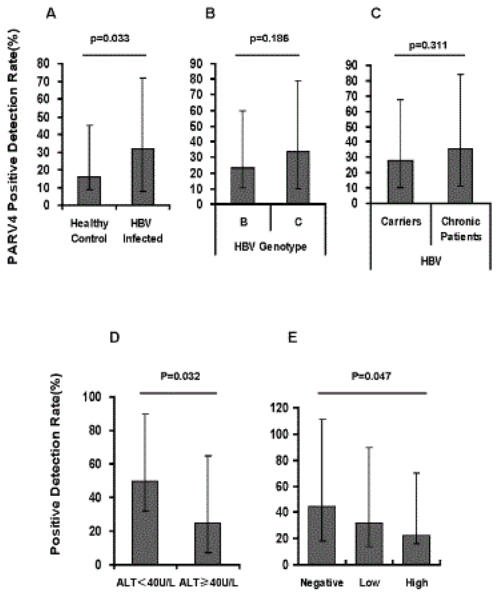


**Figure 2:** HBV infected subjects have higher PARV4 prevalence than healthy controls but PARV4 positive detection rate decreases with increasing HBV DNA concentration. Parvovirus (PARV) nested PCR was performed in all sera using the outer primers PV4ORF1F and PV4ORF1R and the inner primers PV4NS1Fn2 and PV4NS1Rn2 as described in Methods. The PARV4 DNA positive detection rate in the serum samples collected from HBV infected subjects and healthy controls (A) or subjects infected with HBV B or C genotype (B) or chronic HBV patients and HBV carriers (C) are shown. Significant differences between study groups ($\chi 2$ test, $p \leq 0.05$) are indicated. Data shown are percentage ±95% CI. (D) ALT was assayed in sera from chronic HBV patients using the alanine aminotransferase Kit (UV-LDH Method) from Kehua Cooperation, Shanghai). The PARV4 DNA positive detection rate in serum samples collected from patients with serum ALT concentration lower than 40U/l (the Liver Function Normal group) and patients with serum ALT concentration higher than or equal to 40U/l (the Liver Function Abnormal group) are shown. Significant differences ($\chi 2$ test, $p \leq 0.05$) between 2 study groups are indicated. (E) HBV DNA was determined in sera from HBsAg positive study subjects. The PARV4 DNA positive detection rate in serum samples negative for HBV DNA (serum HBV DNA concentration ≤5×103 IU/ml); with Low HBV DNA (HBV DNA concentration between 5×103 IU/ml and 5×108 IU/ml) and with High HBV DNA (HBV DNA concentration ≥5×108 IU/ml) are shown. The $\chi 2$ test for linear trend was used to analyze statistical differences among the 3 groups and is indicated on the graph. Data shown are mean±95% CI.

## PARV4 positive detection rate decreased with increasing HBV DNA concentration.

HBV infected study subjects were categorized into 3 groups based on their serum HBV DNA concentration. PARV4 prevalence in the HBV DNA Negative group, Low HBV DNA group and High HBV DNA group was 46.2% (12/26, 95%CI: 26.6-66.6%), 29.8% (37/124, 95%CI: 22.0-38.7%) and 16.7% (2/12, 95%CI: 2.1-48.4%), respectively. When the High HBV DNA group was set as reference, the odds ratio (OR) of the HBV DNA negative group and Low HBV DNA group were respectively 2.13 and 4.29; suggesting that with the decrease of the serum HBV DNA concentration, the PARV4 infection rate increased, ($\chi 2$ for linear trend=3.92, p=0.047, see Figure 2E). Our data suggest that patients with lower HBV virus load are more likely to be co-infected with PARV4. HBV infection may, directly or indirectly, inhibit the replication of co-infecting viruses like PARV4.

## Serum IL-8 is up-regulated in HBV carriers when compared to healthy controls.

Serum levels of IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α were 3.08 pg/ml (95%CI: 1.82-4.33 pg/ml), 3.82 pg/ml (95%CI: 3.15-4.49 pg/ml), 41.48 pg/ml (95%CI: 26.52-64.85 pg/ml), 1.93 pg/ml (95%CI:1.39-2.46 pg/ml), 2.47 pg/ml (95%CI:1.59-3.34 pg/ml) and 1.64 pg/ml (95%CI: 1.05-2.22 pg/ml), respectively in healthy controls and 2.40 pg/ml (95%CI:1.94-2.85 pg/ml), 3.17 pg/ml (95%CI: 2.66-3.68 pg/ml), 102.71 pg/ml (95%CI:60.39-174.66 pg/ml), 1.49 pg/ml (95%CI:1.13-1.85 pg/ml), 2.21 pg/ml (95%CI:1.35-3.07 pg/ml) and 1.35 pg/ml (95%CI:0.45-2.24 pg/ml), respectively in HBV carriers (Figure 3A). Compared with healthy controls, serum IL-1 (p=0.236), IL-6 (p=0.161), IL-10 (p=0.181), IL-12 (p=0.724) and TNF-α (p=0.688) were unchanged, but IL-8 (p=0.041) was markedly up-regulated in HBV carriers (Figure 3B).

There was no difference in serum levels of IL-1, IL-6, IL-8, IL-10, IL-12 and TNF- α, (IL-1 (p=0.106), IL-6 (p=0.811), IL-8 (p=0.309), IL-10 (p=0.705), IL-12 (p=0.379) and TNF-α (p=0.111), between HBV carriers infected with B or C genotype virus (Figure 3CD).

## Serum IL-8 is up-regulated in HBV carriers co-infected with PARV4

Serum levels of IL-1, IL-6, IL-8, IL-10, IL-12, TNF-α were 2.41 pg/ml (95%CI:1.87-2.94 pg/ml), 2.91pg/ml (95%CI:2.47-3.37 pg/ml), 82.41 pg/ml (95%CI:69.09-108.35 pg/ml), 1.40 pg/ml (95%CI:1.03-1.78 pg/ml), 2.17 pg/ml (95%CI:1.23-3.11 pg/ml) and 1.72 pg/ml (95%CI: 0.59-2.84 pg/ml), respectively in HBV carriers not infected with PARV4 and 2.62 pg/ml (95%CI:2.29-2.94 pg/ml), 3.74 pg/ml (95%CI: 3.08-4.39 pg/ml), 182.77 pg/ml (95%CI:153.98-221.23 pg/ml), 1.50 pg/ml (95%CI: 1.13-1.86 pg/ml), 2.58 pg/ml (95%CI:1.78 -3.39pg/ml) and 0.90 pg/ml (95%CI: 0.56-1.25 pg/ml), respectively in HBV carriers infected with PARV4.

There was no difference in the serum levels of IL-1, IL-6, IL-8, IL-10, IL-12 and TNF- α IL-1 (p=0.636), IL-6 (p=0.083), IL-10 (p=0.775), IL-12 (p=0.618) and TNF- α (p=0.360), Figure 3E) between HBV carriers with and without co-infection with PARV4. However, HBV carriers co-infected with PARV4 displayed up-regulated serum IL-8 (p=0.025) compared to HBV carriers without PARV4 infection (Figure 3F).

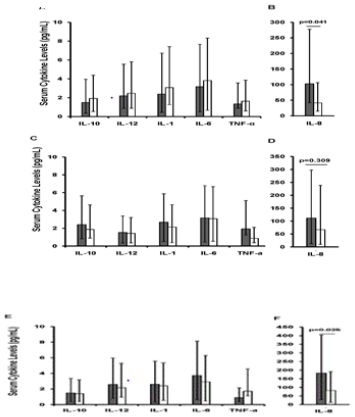

**Figure 3**: Serum IL-8 is up-regulated in HBV carriers compared to healthy controls and in HBV carriers co-infected with PARV4. Serum levels of interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12 and tumor necrosis factor (TNF)-α were assayed using a Human Inflammatory Cytokine Cytometric Bead Array（CBA）kit as described in Methods. Kruskal-Wallis H test was used to determine the statistical differences between study groups, significant differences ($p<0.05$) are indicated. Data presented are mean±95% CI. Serum levels of IL-1, IL-6, IL-10, IL-12 and TNF-α in healthy controls (white bars) and HBV carriers (grey bars). Serum levels of IL-8 in healthy controls (white bars) and HBV carriers (grey bars). Serum levels of IL-1, IL-6, IL-10, IL-12 and TNF-α in HBV genotype C infected carriers (white bars) and HBV genotype B infected carriers (grey bars). Serum levels of IL-8 in HBV genotype C infected carriers (white bars) and HBV genotype B infected carriers (grey bars). Serum levels of IL-1, IL-6, IL-10, IL-12 and TNF-α in HBV carriers not co-infected with PARV4 (white bars) and those co-infected with PARV4 (grey bars). Serum levels of IL-8 in HBV carriers not co-infected with PARV4 (white bars) and those co-infected with PARV4 (grey bars).

## The serum nucleic acid concentration of HCV and B19 in co-infected patients is low

Only 2 study subjects, both male, were positive for B19 DNA (Table 1). Both of them were diagnosed as chronic HBV patients; HBV DNA load could be estimated in only one patient (1.8×105 IU/mL). The serum B19 DNA concentration of the two HBV/B19 co-infected patients was 6.7×104 and 8.0×104 IU/mL.

Similarly, only 2 study subjects were positive for HCV RNA (Table 1). One was male HBV carriers and the other was chronic HBV patient with HBV DNA concentration of 3.4×105 and 1.8×105 IU/mL and HCV RNA concentration of 5.0×104 and 5.3×104 IU/mL respectively. None of the study subjects were positive for HBoV DNA.

| Patient ID. | CP10 | CP21 | CA78 | CP37 |
|---|---|---|---|---|
| Gender | Male | Male | Male | Male |
| Age | 49 | 50 | 21 | 48 |
| HBsAg | Pos | Pos | Pos | Pos |

| | | | | |
|---|---|---|---|---|
| **HBeAg** | Pos | Neg | Neg | Neg |
| **HBV DNA (IU/mL)** | 1.8x105 | Neg | 3.4x105 | 1.8x105 |
| **HBV Genotype** | C | / | B | C |
| **HCV IgG** | Neg | Neg | Neg | Neg |
| **HCV RNA (IU/mL)** | Neg | Neg | 5.0x104 | 5.3x104 |
| **PARV4 DNA** | Pos | Neg | Pos | Neg |
| **B19 DNA (IU/mL)** | 6.7x104 | 8.0x104 | Neg | Neg |
| **ALT (U/L)** | 52 | 12 | 10 | 31 |
| **Clinical Diagnosis** | Chronic HBV | Chronic HBV | Carrier | Chronic HBV |

**Table 1:** The characteristics of HBV infected study subjects co-infected with B19 or HCV

## Discussion

We have used serum samples from HBV carriers and chronic HBV patients to analyze the consequences of PARV4 co-infection in HBV infected individuals in Shanghai. Using phylogenetic analysis combined with serological assays, we have shown that HBV genotype C predominates in Shanghai and may cause more severe disease than genotype B and that co-infection with PARV4 is associated with increased liver damage in chronic patients. Previously, we have reported that co-infection with PARV4 may not be associated with increased disease severity in HBV patients [5]; this discrepancy with our current findings is probably due to the different criteria used to define HBV carriers and chronic patients in the two studies. Previously, we defined chronic HBV patients as those positive for both HBsAg and HBeAg, however, chronic HBV patients can be HBeAg negative or positive [13]. In the current study, we have used strict clinical criteria including liver biopsy, resulting in a more robust differentiation of chronic HBV patients from HBV carriers. Similar to our previous report, we found that HBV infected subjects had higher prevalence of PARV4 infection compared to healthy subjects. However, no difference in prevalence of PARV4 co-infection was found between HBV carriers and chronic patients and between those infected with HBV genotype B or genotype C.

Previous studies have suggested a significant correlation between HBV and parvovirus B19 co-infection [14, 15] while PARV4 was originally detected in a HBV infected patient, raising the possibility of an association with HBV infection [2,16]. The frequency of PARV4 DNA detection was significantly higher among HBV infected subjects than matched healthy controls, which is in agreement with our previous study [5] and similar to results from a cohort of hemodialysis and lung transplant patients [17]. There was no difference in PARV4 DNA positive rate between HBV carriers and chronic HBV patients as we have reported previously [5]. There was also no association of HBV genotype with PARV4 co-infection, with patients infected with genotype B or C being equally affected. However, within the chronic HBV patients group, the Liver Function Abnormal group had markedly higher PARV4 prevalence than the Liver Function Normal group, suggesting that co-infection with PARV4 may promote liver damage in chronic HBV patients.

The current study also showed that with decreasing serum HBV DNA levels, PARV4 infection rate increased gradually, and displayed a dose-dependent trend ($\chi 2$ for linear trend of 3.99, p=0.046). Our data suggest a reciprocal inhibition of infection and/or replication of HBV and PARV4; this is consistent with a previous study showing that the translation machinery of hepatocytes may be down-regulated after infection with HCV, through the activity of the core protein [18]. Elucidation of the underlying mechanisms would require analyses of liver biopsies from infected individuals, which was beyond the scope of this study. In our study, the prevalence of HCV and B19 in HBV infected subjects was similar (0.5%, 2/383). The serum nucleic acid concentrations of HBV, HCV and B19 in co-infected patients were all lower than 5×105 IU/ml, suggesting that the replication not only of HBV but of HCV and B19 was suppressed to some extent in HBV co-infected study subjects.

A recent study found that PARV4 infection elicits strong T cell responses [19], while we have observed markedly higher serum IL-8 levels in HBV carriers co-infected with PARV4, a feature typically associated with persistent, contained infections similar to cytomegalovirus and human immunodeficiency virus -1 co-infection [20]. Together, the findings suggest that persistence of PARV4 in HBV/HCV infected individuals and the associated activated antiviral T cell response, as well as the elevated serum IL-8 production may contribute to disease pathogenesis.

Interestingly, we found a PARV4 seropositive detection rate of 16.0% (8/50) in healthy subjects in Shanghai. Previous findings have suggested parenteral acquisition of PARV4 infections [8,21-23]. However, our current study, taken with similar studies showing 26.15% (51/195) of lots of source plasma pools from healthy donors in Beijing were positive for PARV4 [24] and 16.7% to 17.6% PARV4 IgM seropositivity in healthy adults in Taiwan [25] suggest that PARV4 may be transmitted through non-parenteral routes. Similar PARV4 prevalence has also been reported from Africa [26,27]; importantly, there was no parenteral risk in either of the African studies, further supporting this idea. Given the high prevalence of PARV4 DNA in sera from healthy population, other nonparenteral transmission routes may exist and need to be investigated.

HBV genotypes have distinct geographic distributions and maybe associated with disease severity [28]. We found that more study subjects were infected with HBV C genotype than B genotype. We also found that genotypes B and C almost equally accounted for the HBV carriers; but in chronic HBV patients genotype C accounted for 3 times more infections than the genotype B, suggesting that genotype C, but not B, is associated with a more aggressive disease, paralleling other studies within [29] and outside China [30]. HBV genotype C has been reported to be more prevalent in southern China [30], more frequent in patients with chronic HBV infection and hepatocellular carcinoma (HCC) [29, 30], and associated with more severe liver damage in HBeAg-negative patients [31]. In this context, HBV genotype C may more readily cause liver damage and disease progression in HBV infected patients.

HBeAg-negative patients with detectable HBV DNA have been shown to have more severe liver damage [31] and elevated serum HBV DNA level (≥10,000 copies/ml) is a strong risk predictor for HCC [32]. However, in our study serum HBV DNA load between genotype B and C HBV patients was similar, consistent with another study which suggested that viral genotypes may not have a direct influence on HBV DNA levels [30]. There was no correlation of serum ALT levels with HBV DNA load in chronic HBV patients suggesting that HBV may not cause liver damage directly in chronic HBV patients.

In conclusion, there was no association of HBV genotype with PARV4 prevalence in co-infected subjects. PARV4 infection was negatively correlated with serum HBV DNA concentration, but positively related to serum ALT production in co-infected subjects. Importantly, we found an association between serum IL-8 level and PARV4 infection in HBV carriers. Co-infection with PARV4 in HBV infected subjects may induce immune responses through up-regulation of inflammatory cytokine production and contribute to disease pathogenesis, but the underlying mechanisms still need to be clarified.

Our study was a cross-sectional, retrospective assessment of a limited number of HBV infected subjects and our ability to define the interactions of PARV4 and HBV, as well as the subsequent clinical outcomes was limited. A longitudinal study of PARV4 and HBV co-infection would provide a better understanding of the effect of co-infection on disease progression.

## Methods

### Study Groups

Serum samples of healthy control and HBV infected subjects were retrieved from Shanghai Center for Disease Control and Prevention (SCDC) sample bank. Two hundreds and fourteen serum samples of patients who were clinically diagnosed as HBV carriers (113) or chronic HBV patients (101) according to the case definition from the Viral Hepatitis B Diagnostic Criteria from Ministry of Health of China (WS299 2008) were retrieved. Chronic HBV patients were defined as those who were positive for HBsAg ≥6 months, positive for serum HBeAg or HBV DNA, with persistent or intermittent elevation in ALT/AST levels; or those who were positive for HBsAg ≥6 months, positive for serum HBeAg or HBV DNA, with liver biopsy showing chronic hepatitis with moderate or severe necro-inflammation. HBV carriers were defined as those positive for HBsAg ≥6 months, positive or negative for serum HBeAg, serum HBV DNA ≤2,000 IU/ml, persistently normal ALT/AST levels with liver biopsy showing absence of significant hepatitis. At the same time we chose 50 serum samples collected from healthy subjects in 2010 that were negative for both anti-HCV IgM and HBsAg as the healthy controls.

### Serological ELISA

Anti-HCV IgG and HBsAg were assayed in all serum samples and HBeAg was assayed in all sera positive for HBsAg using commercial ELISA kits (Diagnostic Kit for Anti-HCV IgG, HBsAg and HBeAg from Kehua Cooperation, Shanghai) as described previously [5].

### PARV PCR

The total DNA of each serum sample (200μl) was isolated and 60μl of eluted nucleic acid used as template for PARV nested PCR using published PCR primers and reaction conditions [33] as described previously [5].

### B19 and Bocavirus Real-time PCR

Human Parvovirus (B19) Real Time PCR kit and human bocavirus (HBoV) Real Time PCR kit (Both from Shanghai ZJ Bio-Tech Co., Ltd., People's Republic of China) were used to perform B19 and HBoV real-time PCR respectively, in all sera.

### HBV DNA /HCV RNA Test and ALT Assay

Serum specimens of HBsAg positive subjects were tested for presence of HBV DNA and HCV RNA with Real Time PCR Quantitation kit (ZJ Bio-Tech, Shanghai) using the Roche Light Cycler 480 (Roche, Switzerland). As per kit protocols, specimens with HBV DNA ≤ 5×103 IU/ml are accepted as negative for HBV DNA; specimens with results between 5×103 IU/ml and 5×108 IU/ml are accepted as positive for HBV DNA; those with HBV DNA > 5×108 IU/ml need to be eluted and tested again. Therefore, we grouped study subjects into 3 groups based on their HBV DNA concentration; subjects with HBV DNA ≤ 5×103 IU/ml were defined as the HBV DNA Negative group those with serum HBV DNA concentration > 5×103 IU/ml but ≤ 5×108 IU/ml were termed Low HBV DNA group; those with serum HBV DNA concentration > 5×108 IU/ml were termed High HBV DNA group.

ALT was assayed in sera from chronic HBV patients using a commercial kit (UV-LDH Method, Kehua Cooperation, Shanghai). Experiments were performed as per instructions; positive and negative controls provided by the company were included in each test. According to kit protocol, serological positivity was accepted at titers ≥40 U/l.

### HBV Genotyping

HBV genotype was determined with a commercial HBV genotype (B & C) detection Real Time PCR kit (KL Bio-Tech, Shanghai). Experiments were performed as per instructions; strong positive control for B/C, positive control for B, positive control for C and negative controls provided by the company were included in each test. The PCR was run on Roche Light Cycler 480. The results were analyzed and interpreted according to kit protocols.

### Sequencing and Phylogenetic Analysis

The presence of PARV in sera was confirmed by DNA sequencing of amplification products. PCR amplicons from the position 1564 to 1724 of ORF1 were sequenced by Biosune using internal primers (BioSune, Shanghai). Sequences were blasted in Genbank.

To confirm results from the commercial HBV genotype (B & C) kit, we selected 19 sera from each of the groups identified as B or C, all 3 sera identified as mixed B/C, and 4 of the 10 sera where the genotype was undefined (6 samples were insufficient for further genotyping). The whole genome sequences of the 45 isolates were determined by Biosune using 4 pairs of previously reported primers [34]. Sequences were edited by Seqman and Contig Express, resulting in whole genome sequences of 36 isolates. The 36 sequences were used to construct a phylogenetic tree with MEGA 5. To facilitate phylogenetic analysis, reference sequences for the eight HBV genotypes (A, B, C, D, E, F, G, H) were downloaded from GenBank (Genbank accession numbers AB076678, AB126580, AB010291, AB033554, EF134945, EF134946, EU939562, AB112063, EU939647, GQ377563, AB049609, AB033559, AB126581, AB091255, AB106564, AB166850, AY090459, AB056513, AB064310, AB205010 and AY090460).

### CBA Assays

Serum IL-1, IL-6, IL-8, IL-10, IL-12 and TNF-α levels were assayed in sera from healthy controls and HBV carriers using a Human Inflammatory Cytokine Cytometric Bead Array (CBA) kit (BD

Biosciences, San Jose, CA, USA) as described previously [35]. The means±95% confidence interval (CI) are shown.

## Data Analysis

Data were processed by EpiInfo version 3.5. Differences between nominal or ordinal variables were tested by the Mantel-Haenszel (MH) $\chi 2$ test. Continuous numeric data were compared by t test or nonparametric Kruskal-Wallis $\chi 2$ test. The $\chi 2$-test for linear trend was employed to evaluate the relationship between serum HBV DNA load and the PARV4 positive detection rate of the HBV infected study subjects. Significance was accepted at $p \leq 0.05$.

This study was approved by the Ethical Review Committee of Shanghai Municipal Center for Disease Control and Prevention (SCDC).

## Competing Interests

The authors do not have any financial, professional or personal competing interests.

## Acknowledgments

This work was supported by the Shanghai Committee of Science and Technology (grant number 09ZR1426800), and Shanghai Municipal Health Bureau (grant number GWDTR201201).

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