Turkish Journal of Medical Sciences

Volume 47 | Number 3 Article 23

1-1-2017

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Recommended Citation

AMIRAHMADI, FERESHTE; SARVARI, JAMAL; HOSSEINI, SEYED YOUNES; PIRBONYEH, NEDA; and GORZIN, ALI AKBAR (2017) "Frequency of human parvovirus 4 (PARV4) viremia among HBV-infected patients and healthy donors in Shiraz, Iran," *Turkish Journal of Medical Sciences*: Vol. 47: No. 3, Article 23. https://doi.org/10.3906/sag-1603-83
Available at: https://journals.tubitak.gov.tr/medical/vol47/iss3/23

TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2017) 47: 868-873
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doi:10.3906/sag-1603-83

Frequency of human parvovirus 4 (PARV4) viremia among HBV-infected patients and healthy donors in Shiraz, Iran

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Received: 12.03.2016 • **Accepted/Published Online:** 06.12.2016 • **Final Version:** 12.06.2017

Background/aim: PARV4, a small DNA virus belonging to the family *Parvoviridae*, was first isolated in an HBV injecting drug user. Several studies have investigated PARV4 co-infection with HBV and HCV and its effect on the progression of liver disease. The aim of this study was to determine the frequency of PARV4 among HBV-infected patients and healthy individuals.

Materials and methods: A group of 90 HBV patients and a group of 90 healthy subjects were included in this study. Samples were selected after screening tests such as HBsAg ELISA, anti-HCV ELISA, and anti-HIV ELISA. Nested-PCRs were conducted to detect the PARV4 genome. Positive samples were then subjected to DNA sequencing.

Results: PARV4 DNA was detected in 4.4% of HBV patients in comparison with 1.1% of healthy individuals (P-value: 0.36). DNA sequencing results revealed that PARV4 in all five positive samples was genotype I.

Conclusions: Although this pilot study showed no significant difference between the frequency of PARV4 among HBV patients and healthy donors, further studies with a larger sample size are suggested to determine the association of PARV4 with HBV co-infection and the impact of this virus on the progression of liver disease in patients with hepatitis B.

Key words: *Parvoviridae*, PARV4, nested-PCR, genotyping

1. Introduction

Human parvovirus 4 (PARV4) genotype I was first identified in 2005 in plasma from a homeless, intravenous drug user simultaneously infected with hepatitis B virus (HBV) and showing symptoms of an acute viral infection that included fatigue, nausea, diarrhea, sore throat, and joint pain (1). Genotypes II and III were identified in 2006 and 2008, respectively (2,3). The nucleotide difference between genotypes I and II is about 8% in sequenced regions (3,4). Two large nonoverlapping open reading frames, ORF1 and ORF2, organized the genome of PARV4 and encode respectively a nonstructural and a capsid protein (5). Initially it was suggested that this virus is transmitted only through parenteral routes because it is more common in people who have been exposed to blood. It had been detected in hemophiliacs that received nonvirally inactivated clotting factors (5–8). However, a study in Africa detected PARV4 DNA in nasal and fecal samples; thus respiratory and fecal–oral transmission of the virus was suggested (9). Nonparenteral transmission has also been reported in South Africa and India (10,11). In addition, researchers that conducted a study in Taiwan suggested placental transmission (12). So far, the virus has been isolated from many tissues including bone marrow of HIV-positive individuals (13) and the liver of HBV- and HCV-infected patients (14,15). Moreover, the virus has been diagnosed in plasma, cerebrospinal fluid (CSF), skin, myocardium, and blood products around the world (1,3,7,8,11,15–17). Despite the detection of viral nucleic acid in many organs, PARV4 has scarcely been linked to specific symptoms, except for a recent study that was conducted in Africa linking PARV4 to encephalitis (11) and another study in which the virus was diagnosed in people with symptoms including pharyngitis, nausea, and joint pain (1). However, it is difficult to determine a pathological association because PARV4 was often found in individuals with HCV, HBV, or HIV infection. Nevertheless, as a common co-infection with these pathogens, PARV4 may have an important role in clinical disease because of recent experiments that are indicative of an immune response to the virus (18,19). In spite of the short duration of viremia, the virus is detectable for a long time in the tissue, causing a latent infection with PARV4 (13,14,20). In the present study, considering the results

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of previous studies that indicate a significant association between B19 and hepatitis B and C viruses (21,22), and also according to the possibility of PARV4 related with these viruses, we compared the prevalence of PARV4 viremia in healthy subjects and HBV-infected individuals and determined its genotype in positive cases.

2. Materials and methods

2.1. Sample collection

We altogether analyzed 180 samples in two groups. The first group consisted of 90 cases in patients with hepatitis B who were not co-infected with HCV or HIV. These samples were consecutively collected during a 5-month period (October 2012–January 2013) from patients referred to Motahari Clinic in Namazi Hospital, Shiraz. Sampling was carried out by professionals with written informed consent of patients. The second group (control) consisted of 90 samples from healthy individuals that were collected from staff and students of Shiraz University of Medical Sciences and they tested negative for HBs Ag.

In the HBV-infected group women formed 25.5% of patients (23/90), with a mean age of 42 years, and men consisted 74.5% (67/90) of patients, with an average age of 47 years. Other information regarding the participants in this group is as follows: 10 healthy carriers of hepatitis requiring no treatment, 2 patients with liver cirrhosis caused by hepatitis B virus who were on the waiting list for a liver transplant, 20 patients who received a liver transplant due to liver cirrhosis caused by HBV, and 54 subjects with chronic hepatitis B receiving treatment with Tenofovir. Figure 1 shows the status of hepatitis B in members of this group.

In the healthy group 26.6% (24/90) were women, with a mean age of 40 years, and 73.4% (66/90) were men, with a mean age of 43 years.

2.2. Screening tests

Anti-HCV Ab and anti-HIV Ab were the first tests done on all samples using ELISA kits (Pishtaztb, Tehran, Iran). Positive samples were excluded from our study and negative samples were examined by HBsAg test using an ELISA kit (Pishtazteb, Tehran, Iran). Based on the positive or negative results, subjects were divided into two distinct groups. After that, nucleic acid was extracted from all 180 samples (using Cinnapure Viral kit); then HBV-PCR was performed on samples that tested positive for HBsAg that gave approval for hepatitis B (using Cinnagen kit, Tehran, Iran).

2.3. PARV4 DNA detection

The DH5α strain of *E.coli* was transformed by a plasmid containing the genome of PARV4 genotype I (GenBank: AY622943.1) by electroporation. The transformed bacteria were cultured in LB medium containing ampicillin (50 µg/mL). Afterwards, nucleic acid was extracted and used as a positive control to set up PARV4 nested-PCR. PCR was performed using specific primers (16) targeting the first open reading frame (ORF1) (Table 1). Time and temperature for both the PCR steps were similar and as follows: 96 °C for 9 min, followed by 45 cycles of 96 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final extension of 7 min at 72 °C (16). In the first round 5 µL of extracted nucleic acid was used in a final volume of 50 µL and subsequently 5 µL of the first round PCR product was used as a template for the second round amplification. The final PCR product was analyzed on 1% agarose gel electrophoresis. The PCR products of the positive samples were purified from the gel using a DNA extraction kit (Bioneer Co., South Korea) and were then sequenced using internal primers.

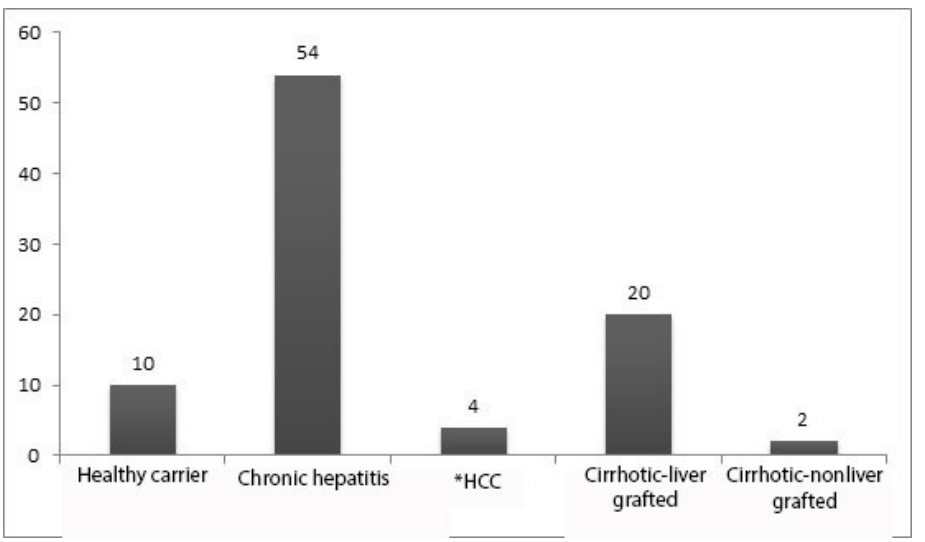

Figure 1. HBV patients according to disease status. *HCC: Hepatocellular carcinoma.

Table 1. Primers used in this study to detect PARV4.

Primer name	Primer sequence
PV4NS1F	5′-AAGACTACATACCTACCTGTG-3′
PV4NS1R	5′-TGCCTTTCATATTCAGTTCC-3′
PV4NS1Fn2	5′-GTTGATGGYCCTGTGGTTAG-3′
PV4NS1Rn2	5′-CCTTTCATATTTCAGTTCCTGTTCAC-3′

3. Results

To specify groups, initially all samples were tested for HBsAg by ELISA, the results of which were negative. Another test performed on all samples was anti-HCV Ab ELISA, the results of which were also negative.

The lack of correlation between PARV4 and HBV co-infection: One sample of 90 subjects in the control group (1.1%) that belonged to a 40-year-old man and 4 samples out of 90 (4.4%) in HBV patients were positive for PARV4 DNA. Statistical analysis (using Fisher's exact test) showed no significant difference between the two groups (P-value = 0.36). Figure 2 shows the PARV4 nested-PCR results (a 168 bp band) for five positive samples along with negative and positive controls.

Positive samples of HBV-infected subjects included a healthy carrier female (10% of carriers) and 3 males, 2 patients with chronic hepatitis B (3.7% of patients with chronic hepatitis B) and 1 who received liver transplant due to liver cirrhosis caused by HBV (4.5% of cirrhotic patients). Table 2 show details of the 5 PARV-infected persons in this study. Sequencing results revealed that the virus in the 5 positive cases was PARV4 genotype I (Figure 3). The sequence was submitted to GenBank and is accessible under KP216530.

4. Discussion

Previous studies have shown an important association between HBV and B19; and also PARV4 was first identified in an HBV-infected person. In the present study to determine the possible association between PARV4 and HBV co-infection we examined the PARV4 viremia in healthy subjects and HBV-infected individuals. In addition, we specified the genotype of PARV4 in our positive samples.

A similar study was conducted in 2013 in north China and reported the same molecular abundance of PARV4 in both healthy subjects and patients with hepatitis B. Altogether, 1626 blood samples from healthy individuals and HBV patients were collected and DNA from three human parvoviruses, B19, Bocavirus (HBoV) and PARV4, was detected. Of all blood samples tested, 138 (8.49%) were found to exhibit HPAV (human parvoviruses) viremia, including 3.51% with B19, 3.75% with HBoV, and 2.52% with PARV4 although no significant correlation between HBV and HPAV including PARV4 was found (23). These results correspond to those observed in our study. However, other studies have reported different results in comparison to our study. A study in 2012 characterized the association of PARV4 with HBV and HCV infection in Shanghai (24). Sera from 51 out of 153 (33.3%) of HCV-infected subjects compared to only 16.7% (16/96) matched controls were positive for PARV4 DNA (P = 0.004). Similarly, PARV4 DNA detection was significantly higher in HBV-infected individuals (41.5%, 78/188) compared with the control group (P < 0.0001). However, in that study, the measurement of ALT (alanine aminotransferase) level showed that PARV4 does not have any effect on the severity of HBV disease. In our study, ALT level in the 2 patients with chronic hepatitis B, co-infected with PARV4, was higher than the HBV chronic

Figure 2. PARV-PCR results. From left to right are marker, negative control, positive control, and five positive samples.

Table 2. Demographic information of PARV4-infected subjects.

No.	Hepatitis B status	Sex	Age	ALT (U/L)
1	healthy	male	40	12
2	healthy carrier	female	31	10
3	chronic hepatitis	male	24	62
4	chronic hepatitis	male	34	46
5	liver cirrhosis	male	86	13

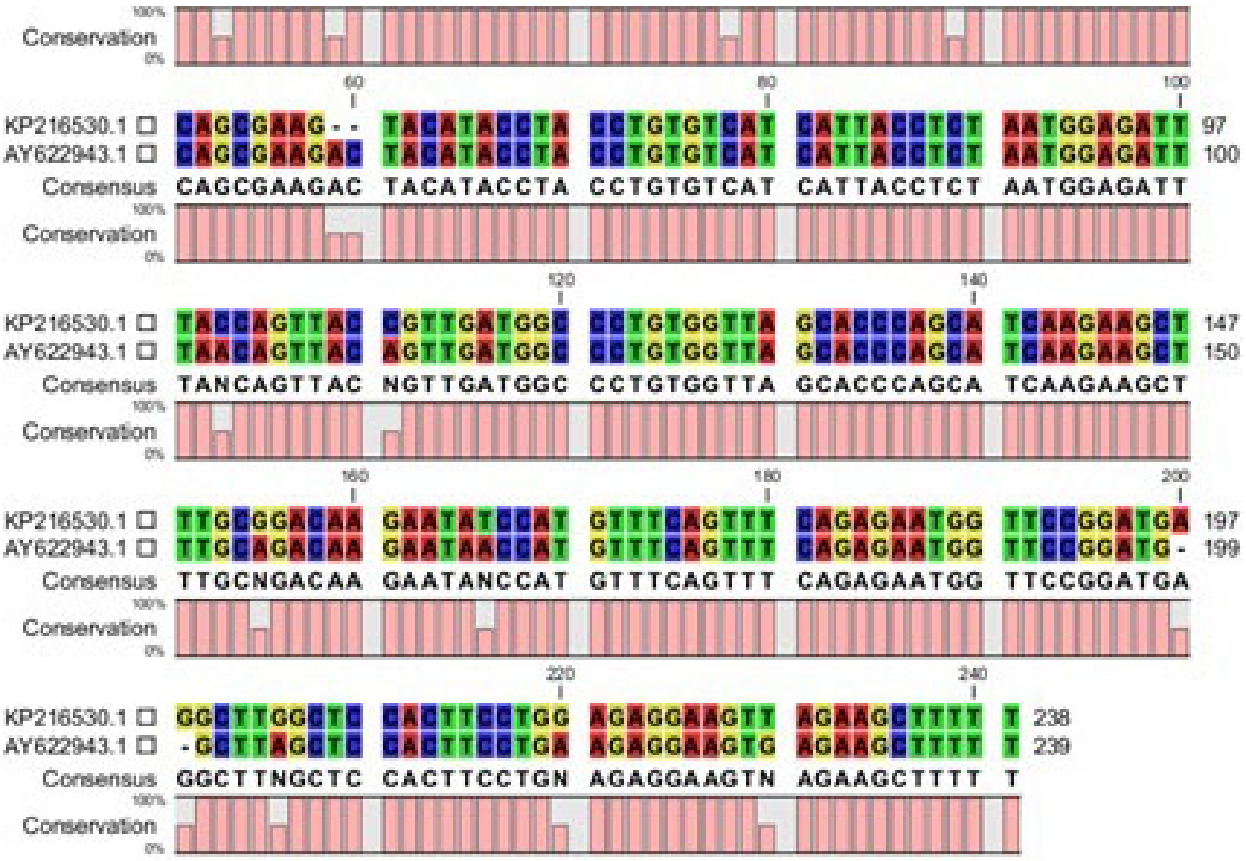

Figure 3. A representative of sequence alignment. One of our positive samples (GenBank: KP216530.1) is aligned against the positive control (GenBank: AY622943.1).

infected patients who were not positive for PARV4 DNA. However, statistical analysis on the liver enzymes required larger groups. In another study in 2007 in London, PARV4 DNA frequency was reported in 30% of the blood samples (3 out of 10) from cadavers of injecting drug users who were also infected with HCV (25). A study in the United Kingdom in 2007 detected PARV4 DNA from 70% (17/24) of bone marrow, lymphoid tissues, and brains of HIV-infected persons, but plasma samples of these patients were negative for PARV4. Presence of PARV4 DNA in tissues in the absence of viremia suggests that persistent infection with the virus in tissue occurs more than acute infection. PARV4 DNA sequencing showed a significant difference in HIV-infected individuals in age between those that carried PARV4 genotype I and those infected with genotype II. As all people infected with genotype I were born in 1968 or later, they were 38 years old or less. This result is similar to the results obtained in our study. Samples infected with genotype II in the mentioned study belong to the natives in 1956 and later (13). In 2011, Touinssi et al. detected PARV4 DNA in 27.3% (6 out of 22) of HCV-infected individuals and 38.7% (29 out of 75) of HBV patients (26). It is thought that the high prevalence of PARV4 viremia in HIV-, HCV-, and HBV-infected subjects may actually reveal a commonly higher exposure to infectious agents in these groups (24). Regarding the immune response to the virus and since viral antigens express on lymphoid tissue and liver cells, it suggests that PARV4 may affect HBV, HCV, and HIV pathogenesis (13,14).

Prior to 2007, PARV4 was not isolated from tissues of individuals without HIV infection but in this year a study was done on the livers of 87 non-HIV-infected persons and samples from 13 of them (15%) were positive for the presence of PARV4 DNA (14). The results of that study showed that PARV4 can cause a persistent infection in the tissue like B19 does. All specimens examined, except one, were derived from those who were not infected with HIV. In addition none, except one, had a history of intravenous drug use. The samples from both individuals tested negative for PARV4 DNA. These findings imply that infection with this new member of the family *Parvoviridae* is not restricted to HIV-infected subjects. PARV4 detection of blood donor samples also confirms this theory (16). Briefly this study demonstrates the presence of PARV4

in the liver of a wide range of people, although further studies are needed to clarify the importance of the stability of the virus and its impact on liver disease. Note that the sequencing results of this study showed that all samples infected with genotype I belonged to those aged between 56 and 64 years and all samples infected with genotype II were from those aged between 64 and 85 years. The results of our study showed infection with PARV4 genotype I in those aged 25 to 40 years except for one sample from an 86-year-old male. The difference in distribution of PARV4 in relation to the region can be justified by the following parameters including the epidemiology of PARV4 and the study population.

Our study has some shortcomings including small sample size and not separating hepatitis B clinical presentation such as inactive carrier, chronic active, and HBe Ag positive.

In conclusion, this is the first study on PARV4 in Iran and our data showed infection with this virus in this country. Future studies are recommended to investigate the abundance of PARV4 in different regions in Iran; frequency of different genotypes; routes of transmission; impact of the virus in co-infection with viruses such as HBV, HCV, and HIV; pathogenesis; etc. It is also suggested to examine the presence of PARV4 DNA in the liver of people who have liver manifestation of unknown etiology.

Acknowledgments

The authors would like to thank Dr Klaus Hedman, the Haartman Institute, University of Helsinki, Finland, for the valuable help to provide the plasmid containing PARV4 genome. We would also like to thank the staff of Motahari Clinic in Namazi Hospital, Shiraz, for their help in collection of samples from HBV patients. This article is extracted from the student's dissertation (F. Amirahmadi. 9012097001, Grant Number: 91-6446), supported by Shiraz University of Medical Sciences.

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