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PREVALENCE OF HEPATITIS B VIRUS AMONG APPARENTLY HEALTHY BLOOD DONORS AND AMONG PATIENTS OF MOLECULAR BIOLOGY LABORATORY OF KIRAN HOSPITAL, KARACHI

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ABSTRACT

Hepatitis B virus affects millions of people worldwide and can lead to serious diseases as liver cirrhoses and hepatocellular carcinoma (HCC).The local prevalence of HBV infection among healthy population has not been well studied. The aim of the present study was to determine the seroprevalence of HBV and HBV DNA by PCR among apparently healthy blood donors and among patients of a molecular diagnostic laboratory in Karachi.

Serological test for Hbs-Ag were carried out by ICT (immunochromatography) rapid screening test and further by ONE STEP HBV COMBO 5 TEST CARD (Chemtrue, USA) as per supplier's instructions and molecular tests for HBV DNA were performed by nested PCR.

The prevalence of HBV infection determined by HBs-Ag reactivity and HBV DNA tests by nested PCR in apparently healthy blood donors of Karachi was found to be 2.39%. Among 124 samples of KIRAN PCR and molecular biology laboratory 45 (36.3%) were found HBV positive in which forty samples were HBs-Ag positive and 5 were negative. All 45 samples were positive by HBV DNA test by nested PCR.

Key Words: Hepatitis B, Hbs-Ag, HBV DNA, HBV PCR.

INTRODUCTION

Hepatitis B Virus (HBV) is endemic in South and South-East Asia with carrier state ranging from 1 to 15 % of the general populations. HBV infection is associated with significant morbidity and mortality related to chronic liver disease and hepatocellular carcinoma (HCC).

It is estimated that about 500 million persons have chronic HBV infection (Hannoun *et al.,* 2000) and about one million persons die of HBV related viral diseases annually (Francis, 1999). The virus causes acute hepatitis of varying severity (Heerman *et al.,* 1999) and persists in 2-10% of adult patients (Bowyer *et al.,* 2000) leading to chronic liver disease, cirrhoses and hepatocellular carcinoma (Abe *et al*., 1999).

In Pakistan, HBV infection rate is increasing day by day. The reason may be the lack of proper health facilities or poor economical status and less public awareness about the transmission of major communicable diseases like Hepatitis B virus, Hepatitis C virus and Human Immunodeficiency Virus (Alam *et al.,* 2007). Studies indicated that the hepatitis B is a crucial public health problem in Pakistan with increased morbidity and mortality. Exposure rate of Hepatitis B virus in Pakistan is not known clearly but limited data shows 35-38% prevalence with 4% being carriers and 32% having anti-Hepatitis B virus surface antibodies through natural conversion. (Hakim *et al*., 2007).

According to a review from PMRC (Anon., 2002), HBV affects 28% adults at any stage of life and about 6% children. Nearly a quarter of Pakistanis are immune to hepatitis B and 3-5% are carrier (Zuberi, 1998).

Many hepatitis B virus markers are used for diagnosing and monitoring hepatitis B patients. Immunoassays are generally used for HBV diagnosis. HBs-Ag by EIA is highly sensitive assay that indicate the presence of HBV. However the hepatitis B virus concentration shows little or no correlation with HBV DNA (Guillou *et al*, 2000) therefore HBs-Ag is not a good marker of HBV detection. Some time HBs-Ag may fail to detect the presence of HBV infection. The reason of lack of HBs-Ag is not known and some time it may give false positives (Weber *et al*., 2001).

Serological markers of HBV infection may vary depending on whether the infection is acute or chronic. A diagnosis of acute HBV infection can be made on the basis of the detection of IgM class antibody to hepatitis B core antigen (IgM anti HBc) in serum; IgM anti-HBc is generally detectable at the time of clinical onset and declines to sub detectable levels within 6 months. IgG anti-HBc persists indefinitely as a marker of past infection. Anti-HBs become detectable in patients who do not progress to chronic infection. The presence of anti-HBs after acute infection generally indicates recovery and immunity from reinfection. In patients with chronic HBV infection, both HBs-Ag and IgG anti-HBc remain persistently detectable, generally for life. In addition, a negative test for IgM anti-HBc together with a positive test for HBs-Ag in a serum specimen usually indicates that an individual has chronic HBV infection. HBe-Ag negative mutants are an important subset of patients. In the natural course of chronic HBV

infection, the loss of HBe-Ag expression and the appearance of antibodies directed against it (Anti-HBe) are usually accompanied by cessation of viral replication. However such a serology profile may also be seen in individuals who harbor precore (PC) and basal core promoter (BCP) mutants where replicative infection continues. The frequent genomic mutation that leads to HBe-Ag negativity is the mutation of the nucleotide, this mutation thus prevents HBe-Ag from being expressed (Lok *et al*., 1994). PC variants are more common among patients with genotype D (65 to 75 percent) which is most prevalent genotype in Pakistan, than genotype A (9 to 18 percent) (Rodriguez-Frias *et al*., 1995; Grandjacques *et al*., 2000; Choudhary *et al*., 2009).

Occurrence of these mutations results in increase in viral load (Hunt *et al*., 2000; Buckwold *et al*., 1997; Buckwold *et al*., 1996; Moriyama *et al*., 1996). These changes were initially thought to be related to a "HBe-Ag-negative phenotype" but recent studies showed that they may also be found in some HBe-Ag-positive patients, especially those with chronic hepatitis (Okamoto *et al*., 1994; Kidd-Ljunggren. *et al*., 1997).

Detection of HBV-DNA from blood is useful in the diagnosis when response to treatment is assessed. This is also important in the diagnosis of the occasional cases where HBs-Ag disappears after the acute HBV infection with failure to develop anti-HBs protective antibodies. This is generally thought to be due to the presence of very low level infection. HBV-DNA can be detected by amplification of HBs gene in nested PCR.

In the present study we determine the seroprevalence of HBV among apparently healthy blood donors and among patients of a diagnostic lab in Karachi; we also developed a PCR based assay for HBV DNA detection and assessed the relation of HBV DNA with clinical and serological features of the subjects.

MATERIALS AND METHODS

Collection of blood samples:

A total of 14537 blood samples were collected from volunteer blood donors in four months period from three blood banks of Karachi in which 9393 from JPMC blood bank 3784 from LNH blood bank and 1360 from Burhani blood bank and screening tests were carried out.

One hundred and twenty four more patients' samples were collected from KIRAN PCR and molecular biology laboratory.

Serology/Biochemistry tests:

Serological test for Hbs-Ag were carried out by ICT (immunochromatography) rapid screening test and further by ONE STEP HBV COMBO 5 TEST CARD (Chemtrue, USA) as per supplier's instructions.

Serum alanine aminotransferase (ALT/SGPT) was tested with routine automated Biochemistry analyzer (Selectra-E, Nethrland). These tests were performed at KIRAN PCR and molecular biology lab, Karachi.

HBV DNA tests by PCR:

We use the polymerase chain reaction for detections of hepatitis B virus DNA in the serum/plasma of the subjects. Part of the surface antigen gene was amplified using two sets of primes (one for regular and other for nested PCR) (Table 3).

Extraction of DNA:

HBV DNA was extracted form 100 µl of the serum/plasma samples by methods (DNA purification system, PUREGENE, Gentra USA) as per supplier's instructions and used for PCR amplification.

PCR Amplification:

Two round PCR was performed for HBV DNA detection using two set of primers given in table 3. PCR master mixture was prepared allowing 40 micro liter mixture per reaction with concentrations1X PCR Buffer, $MgCl_2$ 2.5mM, dNTPs 0.15mM, forward and reverse primers 0.5pM each per reaction, and Taq DNA polymerase 1 unit per reaction and template. 10 µl of the template DNA extracted from 100µl plasma sample was added in the PCR reaction mixtures for regular PCR and 2µl of the regular PCR product was added in nested PCR.

Amplification was performed on MBS 0.2 thermal cycler from thermo hybaid with following temperature profile; denaturation at 95ºC for 5 min one cycle followed by 35 cycles of denaturation at 95ºC for one min, annealing at 55ºC for one minute and extension at 72ºC for 1minute. Then final elongation at 72ºC for 5 minutes one cycle and then hold at 4ºC. Same profile was used both for regular and nested PCR amplifications.

After completion of the PCR 10µl of the reaction mixture was loaded on 1.5% agarose gel, and DNA bands were separated by electrophoresis. The bands were stained with ethidium bromide and visualized on UV Tran illumination.

RESULTS

A total of 14537 blood samples were investigated from volunteer blood donors during four months period in three blood banks of Karachi in which 243 out of 9393 from JPMC blood bank 82 out of 3784 from LNH blood bank and 23 out of 1360 from Burhani blood bank were found positive. Overall prevalence of HBV PCR in apparently healthy blood donors of Karachi was found to be 2.39%. All HBs-Ag positive samples were also found positive by HBV DNA test by nested PCR. Similarly 98% samples were HBe-Ag and Anti HBs negative while 95% samples were anti HBe and anti HBc positive (Table 1). The demographic data shows that all the donors were males; age range was 18-45 years with median age of 27 years (Table 1). It was also found that HBV positive donors were higher in some areas of Karachi. The alanine amino transferase (ALT/SGPT) level of 98% HBV positive donors was within normal range (10-40U/L) with mean level of 22 U/L (Table 1).

Among 124 samples of KIRAN PCR and molecular biology lab 45 (36.3%) were found HBV positive in which forty samples were HBs-Ag positive and 5 were negative. All 45 samples were positive by HBV DNA test by nested PCR (Table 2).

The demographic data of KIRAN PCR and molecular biology lab shows 72.5% were males and 27.5% were females; age range was 14-70 years with median age of 36.4 years (Table 2). The alanine amino transferase (ALT) level of 98% HBV positive samples was higher than normal range (10-40 U/L) with mean level of 68 U/L (Table 2).

Serological picture of KIRAN samples is slightly different. Here 88.8% samples are HBs-Ag positive and 11.1% are negative whereas only 55.5% samples are negative for HBe-Ag and 57.7% for Anti HBs. All 45 samples were positive for anti HBc and anti HBe (Table 2).

Table 1. **Demographic, biochemical, serological and HBV DNA PCR data among volunteer blood donors in three blood banks of Karachi.**

	JPMC Blood bank	**LNH Blood bank**	**BURHANI Blood bank**	**TOTAL (%)**
Total	9393	3784	1360	14537
Median age (years)				27 (range 18-45)
Median ALT U/L				22 (10-69)
S Antigen positive	243	82	23	348
S Antigen Negative	0	0	0	0 (0)
Anti HBs Negative	240	80	20	340 (98%)
E Antigen Negative	239	79	21	339 (98%)
Anti HBe positive	235	77	18	330 (95%)
Anti HBc positive	240	70	22	332 (95%)
HBV DNA positive	243	82	23	348 (100%)

Table 2. Demographic, biochemical, serological and HBV DNA PCR data of samples of KIRAN PCR and molecular biology laboratory.

	KIRAN LABORATORY	**Total (%)**
Total	124	124
Male	90	72.5%
Female	34	27.5%
Median age (years)		27 (range 14-70)
Median ALT U/L		68(10-40 Normal range)
S Antigen positive	40/45	88.8%
S Antigen Negative	5/45	11.1%
Anti HBs Negative	26/45	57.7%
E Antigen Negative	25/45	55.5%
Anti HBe positive	45/45	100%
Anti HBc positive	45/45	100%
HBV DNA positive	45/124	36.3%

Table 3. Primers used for amplification of target sequences of HBV surface antigen.

Primers	Round	Sequence (5'-----3')	Position	Band
Outer Sense primer (HBV 409)	1st	5′ CAT CCT GCT GCT ATG CCT CAT CT 3′	5′ 283 to 305 3′	296bp
Outer Antisense primer (HBV 703)	1st	5′CGA ACC ACT GAA CAA ATG GCA CT 3′	5′ 556 to 578 3′	
Inner Sense primer (HBV 485)	2nd	5′ GGT ATG TTG CCC GTT TGT CCT CT 3′	5′ 332 to 354 3′	230bp
Inner Antisense primer (HBV 686)	2nd	5′ GGC ACT AGT AAA CTG AGC CA 3′	5′ 542 to 561 3′	

DISCUSSIONS

Hepatitis B virus affects millions of people worldwide, and can lead to serious diseases as liver cirrhoses and hepatocellular carcinoma (HCC). Hepatitis B infection is found worldwide but the prevalence varies enormously between different countries. It is estimated that one-half of the world population has experienced infection and there are 350 million chronically infected individuals. Hepatitis B is responsible for 1.5 million deaths per year. Around 40% of chronically infected individuals will die as a result of their infection. Blood and blood products are the main routes through which the virus is transmitted. Any technique that allows the transfer of blood or serum from one individual to another is potentially likely to transmit HBV. HBV infection is especially common amongst IV drug abusers. Unfortunately, very limited research work on HBV has been done in Pakistan. HBV infection rate in Pakistan is increasing day by day; the reason may be the lack of proper health facilities or poor economical status and less public awareness about the transmission of major communicable diseases like Hepatitis B virus. Screening of healthy blood donors of 14537 individuals resulted in 348 infected persons with hepatitis B virus representing 2.39 % of the total subjects whereas 124 samples of KIRAN PCR and molecular biology lab resulted 36.3% HBV positives. This represents quite a higher number of infected individuals. Different studies have been conducted in the country to assess the disease burden in the indigenous population. All such studies present a varying rate of infection based on the study design, population selected, diagnostic assays and demographical and epidemiological variation. According to various study groups, the HBV prevalence rate has been reported as 2–10% among healthy blood donors; 5–9% among health care personnel; 3.6–18.66% among the general population; 3.16% among the pregnant women; 10–20% in patients with provisional diagnosis of hepatitis and 3.16–10.4% among professional blood donors (JCPSP 2002).

According to World Health Organization report Pakistan falls under the endemic region with 3% HBV infected country population, this figure is sensible as far as healthy blood donors data of our study on the other hand 36.3 % infected population of the study subjects of KIRAN PCR and molecular biology lab are controversial because almost all of the individuals included in the this study were clinically infected patients who were referred to KIRAN hospital for their HBV DNA test. This figure, however, does not represent the overall picture of the total country's population prevalence rate infected with hepatitis B virus.

It is also worth mentioning that all blood donors were males; the prevalence of HBV infection among subjects of KIRAN PCR and molecular biology lab was found to be higher in males (73%) than females (27%). Leung (2002) also reported that men aged 40 years or old were most affected HBV patients as compared to females. The reason may be that the men are more exposed to the risk factors. Males are more educated and employed working outsides their homes or in agricultural lands while women are mostly involved in house activities based on the cultural and religious preferences and influences. Males are more commonly involved in the trends and practices leading to the transmission of infections like blood transfusions and barber visiting so becoming more prone to get the infection at a much frequent rate as compared to females.

The early aged patients who were positive for HBV DNA they show comparatively low level of the alanine amino transferase (ALT). In early life, immune tolerance to HBV is common with normal serum ALT levels, despite HBV DNA levels (Leung; 2002). In our study the ALT level of healthy blood donors was almost within normal and among patients of KIRAN lab was very high (median range 68 U/L).

Similarly, horizontal transmission is a major risk factor for the infection acquisition as vertical transmission of HBV infection in Pakistan has been reported to be very low. It has already been documented that the HBs-Ag prevalence rate in pregnant women was 2.5% in Pakistan, out of which 17% and 61% were HBe-Ag and anti-HBe-

Ag positive thus indicating the vertical transmission a less important cause of HBV transmission (Abbas *et al*., 2004).

The incidence of HBe-Ag negative chronic hepatitis B has increased in many countries (Bowyer and Sim; 2000).In our study HBe-Ag negative patients predominated (98% in blood donors and 55.5% in KIRAN lab patients). This may possibly not represent the true prevalence of the HBe-Ag negative disease as the samples were collected from apparently healthy donors.

Detection of hepatitis B virus DNA in the serum or plasma provides a mean of measuring the viral infection in the blood and monitoring of the treatment of hepatitis B virus (HBV) infection in the patients before during and after antiviral therapy studies, and management of HBV (Nowak *et al*., 1996; Heerman *et al.,* 1999; Paraskevis *et al*., 2002). A highly sensitive means of detection of HBV DNA is useful for the early diagnosis of hepatitis B virus infection and for detection of HBV in therapeutic plasma protein preparation (Heerman *et al.,* 1999) Highly sensitive and reliable PCR assay could not only be used for monitoring hepatitis B patients also for the early diagnosis of the HBV infection. Since HBe-Ag negative patients predominated in our study, DNA testing by PCR was the only alternate and confirmatory test as well. This is also important in the diagnosis of the occasional cases where HBs-Ag disappears after the acute HBV infection with failure to develop anti-HBs protective antibodies. This is generally thought to be due to the presence of very low level infection. In our study of 45 positive samples, five were HBs-Ag negative and were detected by PCR.

We found that samples which were positive for HBs-Ag and HBV DNA by PCR, almost all of them were HBe-Ag negative moreover five samples, which were negative for HBs-Ag, were positive for HBV DNA by PCR, similar results were also found by Pfeffl *et al.,* (1997). HBs-Ag detection by ELISA is a highly sensitive immunoassay that indicates the presence of HBV. However HBs-Ag concentration shows a little or no correlation with HBV DNA. HBs-Ag may give false negatives, the reason for lack of HBs-Ag is not clear (Weber *et al.,* 2001), and several explanations have been suggested. HBs-Ag may be hidden in circulating immune complexes (Ackerman *et al.,* 1994; Joller-Jemelka *et al.,* 1994). Variation in pre S region or mutation in the surface antigen itself or especially in the a determinant, which is recognized by anti HBs may render HBs-Ag undetectable by conventional assays (Melegari *et al.,* 1994; Carman *et al.,* 1997; Seddigh-Tonekaboni *et al.,* 2000; Zuckerman *et al.,* 2000 and Weber *et al*., 2001).

As we have reported data of healthy blood donors for HBV which help not only to estimate burden of disease in a population and helpful for epidemiological studies, therefore, it is of worth important consideration for clinicians to adopt better strategies for appropriate prevention and cure of infection. Moreover, data of diagnostic lab embody true picture of the prevalence of HBV. Furthermore, molecular techniques of diagnosis such as PCR are important for precise epidemiological as well as developing effective therapeutic management against such infections.

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