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# Occult Hepatitis B virus Infection (OBI) predominated by genotype D among chronic liver disease patients: The first report from Odisha, Eastern India

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**Abstract.** Detection of Occult hepatitis B infection (OBI) has higher significance for treatment and management. There is no information available about the OBI and its genotype associated with chronic hepatitis patients in Odisha, India. We aimed to determine the association of OBI and its genotype among the chronic hepatitis patients in Odisha. In a hospital based study, 175 serum samples of chronic hepatitis patients were screened for Hepatitis surface antigen (HBsAg). All the HBsAg - negative samples were tested for hepatitis B core antibodies (HBcAb). HBcAb-positive samples were tested further for Hepatitis surface antibodies (HBsAb), hepatitis B virus HBV-DNA and HBV genotyping. Of the 89 HBsAg negative samples, 79 (88.8%) were found positive for HBcAb and these patients were presumed to have OBI. Among the total HBcAb positive samples, 22 (27.8%) were positive and 57 (72.2%) were negative for HBsAb. Detection of HBV- DNA from 45 out of 79 HBcAb positive samples (57.0%) yielded positive results for OBI which was negative for HBsAb. Genotyping of all hepatitis B virus showed that all 45 (100%) were genotype-D. Detection of OBI among chronic hepatitis patients suggests testing for OBI for management and treatment.

## INTRODUCTION

Infection of hepatitis B virus (HBV) poses a severe public health threat worldwide. There are 2 billion people currently infected with HBV, with 360 million having chronic infections related to HBV and 600,000 deaths annually from either HBV-related liver disease or hepatocellular carcinoma all over the world (Shepard *et al*., 2006). HBV infection is linked with a wide range of clinical manifestations, including acute or fulminant hepatitis to various forms of chronic infection, including asymptomatic carriers, chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Detection of hepatitis B surface antigen (HBsAg) in blood is routinely employed for diagnosis HBV infection. But HBsAg may not be detected from blood donors in sero-conversion stage, from chronic HBV carriers with low circulating HBsAg level as well as from donors infected with some mutant HBV. The presence of potentially infectious HBV DNA can be detected by sensitive molecular techniques in the liver, serum or both, in individuals without detectable HBsAg in circulation and this category is designated as "occult HBV infection" (OBI). OBI is a clinical class of HBV infection and can appear in two forms: sero-positive OBI and sero-negative OBI. In sero-positive OBI, serum HBV-DNA is detectable and both anti-HBc/ anti-hepatitis B surface (HBs) IgGs are positive or only anti-HBcIgG is positive, while in sero-negative OBI, only HBV-DNA is detectable in serum/or liver tissue, but anti-HBcIgG/anti-HBs IgGs are negative in serum (Hu KQ., 2002).There is increasing evidence that OBI is associated with chronic liver

disease and HCC (Ikeda *et al*., 2007; Pollicino *et al*., 2004) in addition to being a source of transmission of HBV by blood transfusion or orthotropic liver transplantation (Satake *et al*., 2007). The frequency of occult HBV infection varied considerably from different parts of the world according to the prevalence of HBV in the population. Prevalence of OBI is high in high HBV prevalence zone and low in low HBV prevalence zone of the world. The prevalence of OBIs among HBcAb-positive blood donors has been reported as 12.2% in Iran, 2.8% in Lebanon, and 2.9% in Pakistan (Memish *et al*., 2010; Al-Tawfiq *et al*., 2008; Alshabanat *et al*., 2013). Studies from other parts of India reported occult HBV infection ranging from 21% in Kolkata (Eastern India), 20.87% in New Delhi (Northern India) to 0% in Chandigarh (Northwestern India) (Dhawan *et al*., 2008; Duseja *et al*., 2003; Bhattacharya *et al*., 2007).

Some studies have reported the prevalence of HBV infection in Odisha, India which deal with the prevalence of HBsAg among blood donors (Panda and Kar, 2008; Panigrahi *et al*., 2010), thalassemia patients due to multiple blood transfusion (Sabat *et al*., 2015) and among certain community of Primitive tribes group (PTG) as general practice of tattooing (Dwibedi *et al*., 2014). Limited information is available about the occurrence of OBI in Odisha among the blood donors and PTG community. Although the need of reporting the OBI among chronic hepatitis patients is highly essential for treatment and management, no data is available in this context. Considering the clinical and epidemiological importance of detecting OBIs, we aimed to utilize molecular and the serological testing for the characterization and evaluation of the prevalence of OBIs among chronic hepatitis patients in Odisha.

## MATERIALS AND METHODS

The study was conducted in SCB Medical College, Cuttack and MKCG Medical College, Berhampur. These two medical colleges serve as the referral centre for various diseases from larger part of Odisha. Study individuals were selected from patients with clinical symptoms of acute/chronic hepatitis, cirrhosis of liver or hepatic carcinoma either from outpatients or indoor set ups, during the study period from March 2006 to December 2008. A total of 554 subjects were enrolled from these two medical colleges of Odisha, for this study. After detection of HBsAg in 172 cases, 353 HBsAg negative serum samples were included in this study to identify OBI by detecting HBcAb and HBV DNA.

**Serological analysis**

Various markers of HBV; HBsAg, antiHBs and antiHBc were tested by commercial ELISA Kit (Ranbaxy, India). All the AntiHBc positive samples were tested repeatedly and only those which have given repeated positive results were considered as antiHBc positive. All the antiHBc positive samples were subjected for detection of antiHBs. Those samples that showed negative for HBsAg and antiHBs and positive for antiHBc were selected for HBV-DNA extraction. The subjects with HIV and HCV infection were excluded from the studies. The study was approved by the institutional ethical committee. Informed consent was taken from each of the patients. Sera from patients were stored at -80°C, and are thawed once for serological examinations.

**Serum HBV DNA isolation, detection and sequencing**

DNA was extracted from the serum samples negative for HBsAg and positive for anti HBc. Extraction was carried out using an extraction kit (QIAamp blood kit, Qiagen, USA) and stored at –20ºC. Nested PCR was performed for amplification of part of the S gene according to previously described procedures (Arankalle *et al*., 2003)

The PCR products were purified using the Wizard DNA purification kit (Promega Corporation, Madison, WI, USA) and subsequently sequenced using the Big Dye Terminator cycle sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and automatic sequencer (ABI Prism310 Genetic Analyser, Applied Biosystems). Genotyping of HBV was carried

out on the basis of phylogenetic analysis of 363nt fragment of the S gene. Mega software was employed to determine the phylogenetic status of different HBV isolates.

## RESULTS

Investigation of 525 serum samples collected from 350 acute hepatitis, 43 chronic hepatitis, 90 liver cirrhosis cases, 17 HCC and 25 asymptomatic carrier patients revealed 172 were positive for HBsAg that confirmed HBV infection. Considering the analysis of chronic infection among the 175 serum samples, 86 (49.0%) were detected with HBsAg whereas 89 samples (51%) were negative (Fig. 1). Among the HBsAg-negative samples, 79 (88.8%) were positive for HBcAb and 10 (11.2%) were negative. All HBcAb positive samples were subjected for HBsAb and HBV-DNA testing. Of the 79 HBcAb-positive samples, 22 (27.8%) were positive for HBsAb, but all of them were negative for HBV-DNA (Figure 1). Moreover, of the 79 HBcAb-positive samples 57(72.2%) were negative for HBsAb and among these, 45(79%) were positive for HBV-DNA thereby confirmed as OBIs and 12(21%) were negative for HBV-DNA (Figure 1 and Table 1). Genotyping analysis of these 45 OBIs showed that all belonged to genotype D.

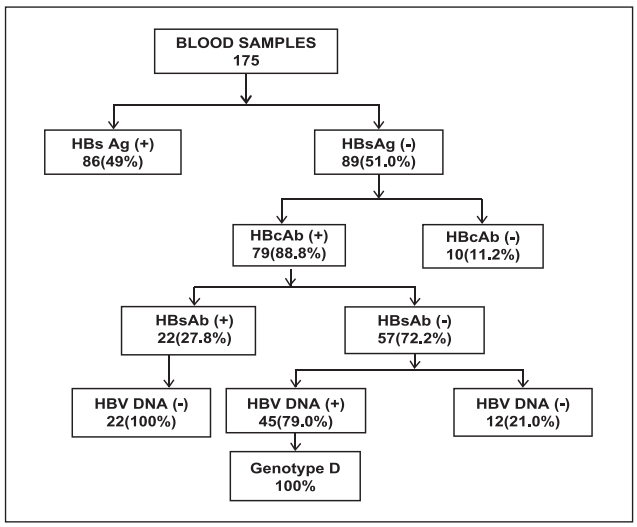


Figure 1. Distribution of serological and molecular markers of hepatitis B virus (HBV) infection among chronic hepatitis patients in Odisha, India.

Table 1. Distribution of HBsAg, antiHBsAg, Anti HBc and HBV DNA in serum sample of chronic Hepatitis patients

| Type of case | #cases | HBsAg positive | HBV DNA Positive in HBsAg Positive cases | HBsAg Negative | HBcAb positive | HBsAb positive | HbsAb Negative | HBV DNA in anti HBc positive cases |
|---|---|---|---|---|---|---|---|---|
| Chronic infection | 43 | 22 | 14 | 21 | 18 | 04 | 14 | 13 |
| Liver cirrus | 90 | 24 | 18 | 66 | 59 | 18 | 41 | 30 |
| HCC | 17 | 15 | 13 | 02 | 02 | 00 | 02 | 02 |
| Asymptomatic carrier | 25 | 25 | 18 | 00 | 00 | 00 | 00 | 00 |
| Total | 175 | 86 | 63 | 89 | 79 | 22 | 57 | 45 |

## DISCUSSION

This is the first report of OBI with genotype-D among chronic Hepatitis patients in Odisha. As a whole, the present study reported 75% HBV infection among Hepatitis patients. This includes 49% of HBsAg-positive and 26% of HBV-DNA-positive, but negative for HBsAg (OBIs). Previous studies in Odisha have documented the prevalence of HBV infection with the detection of HBsAg and HBV-DNA in HBsAg negative serum as OBI among primitive tribes, blood donors and thalassemia patients (Panigrahi *et al.*, 2010; Sabat *et al.*, 2015; Dwibedi *et al.*, 2014). Other studies demonstrated consistent results of HBsAg prevalence in India (Arankalle *et al.*, 2003; Ismail *et al.*, 2012) and in Bangladesh (Shah *et al.*, 2016).

The prevalence of OBI varies from region to region worldwide. The prevalence of OBI varies from 1% to 87% in different regions of the world. In the current study, 79 out of 89 samples (88.8%) were HBsAg-negative, but HBcAb-positive and these patients were suspected to have OBI. But detection of HBV-DNA in 45 samples with HBcAb confirmed them as OBI. However, 34 samples which were positive for HBcAb but negative for HBV-DNA were still considered as suspected HBV infection cases. The failure of detecting HBsAg and HBV-DNA could be due to viral mutation, or due to the limitation of the serological and molecular assays used. It has been suggested that such conditions may be observed during infection with mutant HBV or during the period when the levels of circulating HBsAg in the infected individuals are below the detection threshold (Liang *et al.*, 2009; Kwak and Kim, 2014).

It is well understood that OBI is an additional risk factor for progression of liver cirrhosis and HCC. In our study, liver diseases with different clinical phases demonstrate variable association of OBI. Among these 45 confirmed cases of OBI, 13 OBI (30.2%) were found in 43 chronic liver diseases, 30 OBI (30%) in 90 liver cirrhosis and 2 OBI (11.8%) in HCC cases (Table 1). Similar to our study, the prevalence of OBI in chronic liver disease varies from 3.88% to 55.6% (Hou *et al.*, 2001; Youssef *et al.*, 2009): in Brazil, 4.4% (Ferrari *et al.*, 2014); China, 28.3% (Fang *et al.*, 2009); Iran, 10% (Makvandi *et al.*, 2014) and India 9.5% (Srivastava *et al.*, 2015). The prevalence rates of OBI in cirrhotic patients have been reported: in Iran, 38% (Anvari *et al.*, 2014); India, 38% (Agarwal *et al.*, 2003); Italy, 27% (Sagnelli *et al.*, 2008); Brazil, 20% (Silva *et al.*, 2004); and China, 32% (Chan *et al.*, 2002); and China, 3.88% (Hou *et al.*, 2001). Several studies have documented that in patients with HCC who were negative for all HBV serum markers, including HBsAg, HBV DNA was detected in hepatocytes (Shafritz *et al.*, 1981,Paterlini *et al.*, 1993). The rate of cryptogenic liver diseases varies greatly in different regions of the world. Most findings described that OBI is an important risk factor for hastening the progression of liver disease and the development of cirrhosis and HCC (Shi *et al.*, 2012). In this connection, it is therefore suggested that for improving treatment and management, the sera and liver biopsy patients with cryptogenic hepatitis be screened for HBV-DNA by highly sensitive molecular means before developing signs of cirrhosis or HCC.

Hepatitis B virus genotype D infection is the most prevalent genotype in the world. The prevalence of genotype-D in general has been reported: in Saudi Arab 88.2% (Alsbayea *et al.*, 2016) and Bangladesh 73.7% (Shaha *et al.*, 2016). The prevalence of HBV, genotype D as a vast majority was reported among blood donors and primitive tribes in Odisha (Panigrahi *et al.*, 2010; Sabat *et al.*, 2015; Dwibedi *et al.*, 2014). It is demonstrated, Hepatitis B virus genotype A is more often associated with severe liver disease in northern India than is genotype D (Kumar *et al.*, 2005). However contrast to this document, in the current study all of our OBIs identified among chronic hepatitis patients were genotype D (100%).

In this study, the HBV-DNA detection assay was conducted only on serum samples from hepatitis patients who were positive for HBcAb. This is because HBcAb is considered as a pathognomonic marker of HBV infection especially in the absence of HBsAg. Identification of OBI among Hepatic disease patients based on the presence of HBV-DNA will suggest for antiretroviral

treatment and monitoring. However this type of approach should be discouraged as HBV-DNA negative with HBcAb carrying patients might have the earlier exposure to HBV.

In conclusion, this study is unique for two reasons (i) detection of OBI among chronic Hepatitis patients (ii) genotyping and reporting all OBI to be genotype-D among chronic Hepatitis patients. This study has the importance to direct clinicians for screening of blood from hepatitis patents with more sensitive methods, including nucleic acid testing along with serological assays, especially in endemic areas that can potentially encourage antiretroviral treatment and monitoring.

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