Brief Original Article

Occult hepatitis B infection among individuals belonging to the aboriginal Nicobarese tribe of India

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Abstract
Introduction: The long-lasting persistence of hepatitis B virus (HBV) genomes in the liver (with or without detectable HBV DNA) of individuals with negative for HBV surface antigen (HBsAg) is termed occult HBV infection (OBI). The present study is a part of the follow up on efficacy of vaccination, 10 years post inception, and was designed to understand the prevalence of Occult Hepatitis B infection (OBI) among the aboriginal Nicobarese tribal community.
Methodology: A total of 612 serum samples were collected and tested for various markers including HBsAg, Anti-HBs, Anti-HBc and HBV DNA. Part of S gene of the extracted HBV DNA was amplified by nested PCR. The amplified products were then subjected to sequencing. Genotyping was performed on the basis of phylogenetic relationship along with representative reference sequences from different sub genotypes.
Results: The study revealed OBI in 11.1% of the people belonging to the Nicobarese tribe. Phylogenetic analysis showed only one genotype, HBV/D circulating among the Nicobarese population with ayw3 was the major serotype detected. Single or multiple amino acids substitutions were found in 5 of 34 samples (14.7%) which includes I110T, P120T, P/T127I, A128P, M133L and G159V.
Conclusions: The detection of OBI among these aboriginal tribes is of great concern and stresses the need for the continuous surveillance as it may contribute to the progression of liver disease to a more advanced stage.

Key words: HBV; occult; Nicobarese; primitive; HCC; cirrhosis.

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Introduction
Hepatitis B is one of the most common infectious diseases with approximately 400 million chronic carriers around the world [1], of which more than 40 million are from India [2]. Occult HBV infection (OBI) is defined as the presence of circulating HBV DNA in the absence of serologically detectable hepatitis B surface antigen (HBsAg negative) [3-5]. The HBV DNA may be present in patients with serological markers of previous infection (anti-HBc and/or anti-HBs positive) or in patients without serological markers (anti-HBc and/or anti-HBs negative). Serological tests are useful in identifying different antigens and antibodies in various stages of infection. Anti-HBc antibodies appear in the acute phase of infection and persist for an indefinite time after virus clearance. The presence of antibodies against HBsAg (anti-HBs) is a marker of recovery and immunity. Anti-HBc coexisting with anti-HBs usually indicates a previous HBV infection, and when it coexists with HBsAg, a current infection [6]. The prevalence of OBI is variable depending on the level of endemicity, assays used, and the populations studied [7]. The Andaman and Nicobar Islands, a union territory of India, is situated in the Bay of Bengal and is home to six aboriginal tribes, constituting about 10% of the islands' population. Serological studies carried out earlier among the four accessible tribes revealed that HBV is widely prevalent among them. The Nicobarese, the largest

tribal group, had a prevalence of 23.3% [8]. Considering the high endemicity of HBV infection among the tribes, a pilot project of mass hepatitis B vaccination was initiated in 2000, in two of the 12 villages of Car Nicobar Island inhabited exclusively by the Nicobarese tribe [9]. The present study was conducted 10 years after the implementation of this vaccination program and was designed to understand the prevalence of OBI among this tribal community due to the high endemicity of hepatitis B.

Methodology

A cross-sectional study (n = 612) was conducted between September 2010 and July 2011, in which serum samples were collected from individuals living in the two villages of Car Nicobar Islands covered under the vaccination program implemented 10 years ago. The study was cleared by institutional ethical committee.

HBsAg, anti-HBs, and anti-HBc were tested by commercial enzyme-linked immunosorbent assay (ELISA) (General Biologicals Corporation, Hsin Chu Science Park, Taiwan).

All the anti-HBc positive samples were tested twice and only those that yielded repeated positive results were considered to be anti-HBc positive.

HBV DNA was isolated from 200 μL serum samples using phenol/isoamyl alcohol/chloroform after incubation with proteinase K [10]. Part of the S gene of the extracted HBV DNA was amplified by nested polymerase chain reaction (PCR) following standard protocol [10]. Primers used for first-round PCR were 5'-ACCCCTGCTCGTGTTACAGGC-3' (sense, nt 184–204) and 5'-AAAGCCAGACAGTGGGGGAAA-3' (antisense, nt 731–711). For second-round PCR, 1 μL of the first-round PCR product was subjected to PCR amplification of 25 cycles using primers 5'-GACTCGTGGTGGACTTCTCTC-3' (sense, nt 251–271) and 5'-TAAACTGAGCCAGGAGAAACG-3' (antisense, nt 679–659) to obtain a product of amplicon size 429 bp. The concentration of primers and PCR reactants was identical to first-round PCR. Guidelines for avoiding false positive results [11] were followed strictly. The amplified products were then subjected to sequencing in Genetic Analyzer (Applied Biosystems, Foster City, USA). Each amplicon was sequenced in both directions. All the nucleotide sequence data were derived from two independent readings. The forward and reverse sequences collected from the sequencer were checked and manually edited in the electro-pherograms using the SeqScape version 2.5 (Applied Biosystems) and MEGA5 software [12]. Genotyping was performed on the basis of phylogenetic relationship, taking 345 base pair sequences along with representative reference sequences from different sub genotypes as described earlier for hepatitis B virus [13,14]. Phylogenetic grouping using a bootstrap neighbour-joining (NJ) analysis with 1,000 replications was performed using the S gene sequence of HBV isolated from Nicobarese tribes and that of other representative sequences submitted in the NCBI database.

Table 1. Overall demographic, serological, and virological characteristics of anti-HBs (+)/anti-HBc (+) group among the individuals belonging to the Nicobarese tribe

Features	Total Vaccinated
HBsAg (-ve)	558
Age in years (mean ± SD)	41.2 ± 1.1
Male/Female	227/331
HBV DNA positive (%)	62/612 (10.1%)
Anti-HBc+anti-HBs (+ve) (%)	22/62 (35.5%)
Anti-HBc only (+ve) (%)	18/62 (29.0%)
Anti-HBs only (%)	12/62 (19.4%)
Anti-HBc+anti-HBs (-ve) (%)	10/62 (16.1%)
Positive for sequencing	47
HBV genotype by sequencing (%)	
D	47 (100%)
Serotype	
ayw3	38
ayw2	8
ND	1

Table 2. Details of the patients belonging to the Nicobarese tribe with OBI infection

Sl No.	Sample No.	Age (in years)/sex	SEROLOGY		Amino acid change (100–160aa)
			Anti-HBs (mIU/mL)	Anti-HBc	
1	LT0192	17/F	122	N	-
2	LT0248	48/F	391	N	-
3	LT0738	20/M	865.3	P	-
4	LT0742	56/M	69.5	P	-
5	LT0749	21/F	1055.2	N	-
6	LT0753	40/F	765.8	N	-
7	LT0759	16/M	418.6	N	-
8	LT0760	36/F	747.7	P	-
9	LT0761	38/F	700.8	P	-
10	LT0762	13/M	1091.5	N	-
11	LT0199	18/M	5	N	-
12	LT0256	72/F	6	P	-
13	LT0441	32/F	4.7	P	-
14	LT0132	28/F	152	N	-
15	LT0181	39/F	283	P	-
16	LT0184	20/F	44	N	-
17	LT0187	58/M	16	N	-
18	LT0195	59/F	214	P	-
19	LT0197	56/F	13	P	-
20	LT0202	65/M	37	P	-
21	LT0208	32/F	264	P	-
22	LT0209	35/F	40	P	-
23	LT0214	60/M	11	P	-
24	LT0223	49/F	45	P	-
25	LT0258	39/F	64	P	-
26	LT0260	39/F	13	P	-
27	LT0269	52/M	317	P	-
28	LT0291	38/M	24	P	-
29	LT0293	44/F	10	P	-
30	LT0347	35/M	15.3	P	-
31	LT0355	52/M	58	N	-
32	LT0373	23/F	440.4	P	-
33	LT0381	24/F	182.3	P	A128P
34	LT0411	45/M	31.1	N	-
35	LT0474	29/M	59.9	P	-
36	LT0609	6/F	446.5	N	-
37	LT0610	39/M	479.8	P	-
38	LT0069	48/F	9	P	-
39	LT0185	65/F	3	N	-
40	LT0194	71/M	4	P	-
41	LT0203	50/F	8	P	-
42	LT0206	11/F	5	N	-
43	LT0220	12/F	4	N	-
44	LT0263	26/F	3	P	-
45	LT0265	35/F	6	P	P120T
46	LT0294	45/M	9	P	I110T, G159V
47	LT0297	49/F	3	P	P/T127I
48	LT0312	52/F	5	P	M133L
49	LT0437	45/F	4.8	P	-
50	LT0438	55/F	4.2	P	-
51	LT0460	63/F	0.2	P	-
52	LT0212	22/F	8	P	-
53	LT0224	26/F	4	N	-
54	LT0225	35/F	5	N	-
55	LT0246	60/F	4	P	-
56	LT0331	14/F	3.2	N	-
57	LT0582	7/M	1.9	N	-
58	LT0592	25/F	0.9	N	-
59	LT0595	60/F	4.9	P	-
60	LT0596	19/F	1.3	N	-
61	LT0633	53/F	2	P	-
62	LT0647	67/M	4.3	P	-

Representative sequences belonging to each genotypes of hepatitis B (*i.e.,* A, B, C, D, E, F, and H) were extracted from the NCBI database and used in the phylogenetic analysis. The sequence of woolly monkey HBV was used as an outgroup to root the phylogenetic tree. Mutations present, if any, in the amino acids of the hepatitis B isolated from Nicobarese tribe members were detected using MEGA5 software by comparing them with other sequences submitted in the NCBI GeneBank. The representative sequences of each genotype are indicated in the phylogeny with their accession number along with their genotype.

Results

The demographic, serological, and virological characteristics are shown in Table 1.

Out of 612 serum samples collected, 558 (91.2%) were negative for HBsAg, which included samples from 227 males and 331 females (mean age 41.2 ± 1.1).

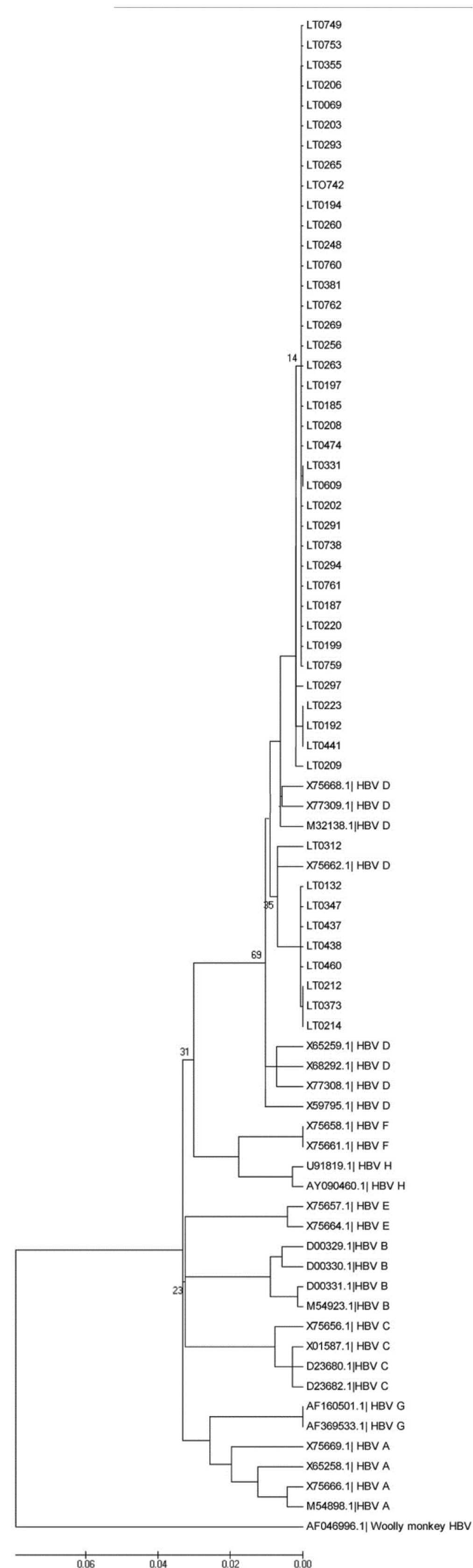
Among these HBsAg-negative samples, 62 were positive for HBV DNA by PCR, giving a prevalence of 10.1% (62/612) for occult hepatitis. Among these 62 cases of occult hepatitis, 52 (83.9%) were positive for either anti-HBs, anti-HBsAg, or both, indicating these patients probably had long-standing infections and low viral loads with undetectable levels of HBsAg. Twenty-two (35.5%) of the occult hepatitis B cases were positive for both anti-HBc and anti-HBsAg, while 18 (29.0%) were positive for only anti-HBc, and 12 (19.4%) were positive for only anti-HBsAg. Ten (16.1%) of the 62 cases of occult hepatitis were negative for all serological markers of hepatitis B that were screened for (Table 1).

Out of these 62 samples positive for HBV DNA, bidirectional sequencing of a partial S gene was successfully achieved for 47 samples. Phylogenetic analysis showed only one genotype, HBV/D, circulating among the vaccinated population (Figure 1).

Out of these 47 HBV isolates with D genotype, *ayw3* was the major (38/47, 80.9%) serotype, and 8 (8/47, 17%) isolates belonged to *ayw2* serotype (Table 1).

Serotype could not be detected for one isolate, LT0297, due to an amino acid change at the serotype determining position (Table 2). Among HBsAg-negative non-vaccinated samples, single or multiple amino acids substitutions were found in 5 of 47 samples (10.6%). The amino acid substitutions found

Figure 1. Phylogenetic analysis of the partial S gene showing the genotype of the HBV virus from OBI cases (LT)



within the S-gene region were I110T, P120T, P/T127I, A128P, M133L, and G159V (Table 2).

The nucleotide sequences determined in this study have been submitted to the GenBank database and assigned accession numbers KC433959, KC433966 and KC433962-64.

Discussion

Several reports exist demonstrating the persistence of HBV DNA in different populations or individuals carrying either anti-HBs or anti-HBc only, particularly in blood donors [15,16]. The present study revealed OBI in 10.1% of individuals belonging to the Nicobarese tribe. Varying prevalence of anti-HBc, a marker for exposure to HBV infection, has been reported from different parts of India, ranging between 8% and 18% [5]. Prevalence of occult HBV infection was also high among the anti-HBc positive individuals belonging to the vaccinated and non-vaccinated cohorts from the present study; 64.5% (40/62) of anti-HBc-positive cases were HBV DNA positive. According to an earlier report [17], about 20% of OBI sera are negative for all serological markers of HBV infection except HBV DNA, 50% are positive for anti-HBc (± anti-HBs), and 35% are positive for anti-HBs (± anti-HBc).

Different clinical contexts are reported behind the mechanism of OBI; these include undetectable HBsAg at the tail end of chronic carriage during the long term non-replicative phase, the occurrence of escape mutants interfering with HBsAg synthesis, co-infection with delta hepatitis virus (HDV), and, commonly in endemic areas, chronic carriage stage with HBsAg too low to be detected and recognized by the presence of anti-HBc as the only serological marker (referred to as "anti-HBc alone" or "isolated anti-HBc") [4].

The frequency of occult HBV infection varies considerably from different parts of the world according to the prevalence of HBV in the population [5]. 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 (north-western India) [18-20].

The prevalence of anti-HBs was found to be higher than prevalence of HBsAg among the Nicobarese before vaccination, and half of the HBsAg- and anti-HBs-negative people were positive for anti-HBc (54%) [9]. Earlier findings suggest that recovery from acute hepatitis B virus infection may not result in complete virus elimination; rather, the immune system keeps the virus at a very low level, leading to occult hepatitis B infection [20].

HBV genotype is also an important factor influencing the frequency of occult HBV. Occult HBV during the non-replicative phase has been found to be more frequent in areas where genotypes A, D, and E are prevalent rather than genotypes B and C [4,21]. Our report is in accordance with an earlier report [5] from India that revealed HBV/D as the prevalent genotype among OBI cases.

The P120T substitution reported in our study was earlier found to be linked with chronic hepatitis and liver cirrhosis patients [22], to possibly cause problems with diagnostic assays, and to also possibly cause vaccine escape and poor response to HBIG therapy [23]. The mutations (I110T, A128P, G159V, and M133L) observed here were reported earlier to be associated with diagnostic or immune escape and impaired virion secretion [24].

There is increasing evidence that OBI is associated with chronic liver disease and HCV progression and that it interferes with treatment response [5,25,26] in addition to being a source of transmission of HBV. The implications of occult HBV infection involve different clinical aspects, which include presenting potential risks of HBV transmission through blood transfusion, hemodialysis, and organ transplantation; causing cryptogenic liver disease; and contributing to acute exacerbation of chronic hepatitis B or even fulminant hepatitis. It is also associated with development of hepatocellular carcinoma and may affect disease progression and treatment response of chronic hepatitis B and C [27].

Conclusions

The detection of occult hepatitis B among these aboriginal tribes is of great concern. The study stresses the need for the continuous surveillance of patients with OBI, as it may contribute to the progression of liver disease to a more advanced stage. The present study indicates that routinely used serological markers of HBV infection do not rule out occult and ongoing hepatitis B virus infections, and emphasizes application of molecular methods for the detection of occult HBV infection. Our study advocates inclusion of HBV DNA testing along with anti-HBc test for screening of HBV infection as a routine practice.

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