

Characteristics of occult hepatitis B virus infection in the Solomon Islands

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Abstract. Hepatitis B virus (HBV) infection is highly endemic in the Solomon Islands. However, little is known about the status of occult HBV infection in the Solomon Islands. This study aimed to investigate the prevalence of occult HBV infection and its clinical and virological features in the community of Solomon Islands. Blood samples were collected from a total of 564 asymptomatic individuals aged over 18 years in the Western province. The samples used in the present study consisted of 200 samples from 108 males and 92 females (mean age, 37.4 years; range, 18-71 years) that were randomly selected among the hepatitis B surface antigen (HBsAg)-negative samples from all the participants enrolled in this study. HBV-DNA was detected by real-time PCR in 25 (12.5%) of the 200 HBsAg-negative samples. Most of the HBV-DNA-positive individuals were infected with wild-type HBV, and only 3 strains demonstrated specific amino acid substitutions (P121X, T123N, C138S, P142S and D144E) in the α determinant region. In conclusion, occult HBV infection was documented in 12.5% of individuals that demonstrated serologic evidence of resolved HBV infection in this study. The prevalence of occult infection was also influenced by ethnicity; it was more prevalent in Melanesians than Micronesians. In addition, occult HBV infection demonstrated a weak association with the S-variants.

Introduction

The diagnosis of hepatitis B virus (HBV) infection is usually supported by the detection of hepatitis B surface antigen

(HBsAg), and the clearance of HBsAg is considered to represent the disappearance of viremia and remission of the disease (1,2). However, occult HBV infection is characterized by the presence of HBV-DNA in HBsAg-negative patients and is associated with more severe liver damage and the development of liver cancer (3-6). Occult HBV infection is often explained by low levels of viral replicative activity and/or mutations in the α epitope of the S gene that encodes amino acid residues 124-147 of HBsAg. Recent epidemiological studies have detected occult HBV infection worldwide (7).

The Solomon Islands are located in the Southwestern Pacific Ocean, and its Western province, which has a multi-ethnic population, is approximately 380 km northwest of the capital. Previously, we reported an HBsAg positivity rate of 21.5% in a community-based survey of the Western province of the Solomon Islands (8). The aims of this study were to investigate the prevalence of occult HBV infection and its clinical and virological features in the highly endemic country, the Solomon Islands.

Materials and methods

Sample collection and serological assays. Blood samples were initially collected in September, 2004, from a total of 564 asymptomatic individuals aged over 18 years (mean age, 38.7±14.6 years; 281 males and 283 females) in the Western province of the Solomon Islands. All participants signed an informed consent and participated voluntarily in this study in response to an offer of free health examination. The study protocol was reviewed and approved by the Ethics Committee of Kobe University. All 564 serum samples were screened in 2004 for HBsAg by a reverse passive hemagglutination assay (R-PHA) (Mycell II HBsAg; Institute of Immunology Co., Ltd., Tokyo, Japan). A total of 121 samples (21.5%) were positive for HBsAg. Two hundred samples (mean age, 37.4±14.2 years; 108 males and 92 females), were randomly selected among the 443 HBsAg-negative samples and were used in this study. The prevalence of the antibody against the hepatitis B core antigen (anti-HBc) and of the antibody against the HBsAg (anti-HBs) were assessed by a PHA assay (Mycell II

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Table I. Clinical characteristics of occult HBV infection.

Characteristics	Total n	Occult (n=25)	Non-occult (n=175)
Gender			
Male	108	10 (9.3%)	98
Female	92	15 (16.3%)	77
Ethnicity			
Mela	114	20 (17.5%) ^a	94
Micro	86	5 (5.8%) ^a	81
Positive for anti-HBc	25	24	1
Positive for anti-HBs	25	19	6
Age (mean ± SD)		33.0±14.2	38.0±14.2
ALT (IU/l)		19.1±9.7	17.5±11.0
Albumin (g/dl)		4.2±0.2	4.2±0.3
Total bilirubin (mg/dl)		0.5±0.2	0.5±0.2

Mela, Melanesian ethnicity; Micro, Micronesian ethnicity. ^aP<0.05.

anti-HBs; Mycell anti-rHBc, Institute of Immunology). The samples were also examined for alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and total bilirubin (T-Bil). Although the prevalence of co-infection with HCV and HIV in our participants is unclear, a previous study reported that the prevalence of anti-HCV was 0.2% (9). Detailed data of HIV/AIDS is unavailable in the Solomon Islands.

Detection of HBV DNA. The sera were stored at -80°C until the assays were carried out. DNA was extracted from 100 µl of serum using a DNA extractor kit (Wako Pure Chemical Industries, Ltd., Japan). The presence of HBV DNA was assayed in the surface (S) and pre-core/core promoter (PC/CP) genes of the viral genome. The S gene was amplified by PCR with primers HB8F and HB6R for the first PCR, and HB1F and HB1R and/or HB2F and HB2R for the second PCR (10), and the PC/CP gene was amplified with primers es2 and CB2 for the first PCR, and is2 and CB4 for the second PCR (11). If the results of the S gene PCR were indeterminate, HBV-DNA was detected by single PCR using primers HBSF1 and HBSR1 and/or HBSF2 and HBSR2 (12). The primers HBSF2 and HBSR2 corresponded to a determinant region, which is involved in antigenicity.

Determination of the viral load. The viral load was assessed by real-time PCR using an AB 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA), which can distinguish between samples containing 5,000-10,000 DNA template copies, with a statistical confidence level of 99.7%. HBV DNA was amplified with a primer and probe set, as previously described (12).

Confirmation of the HBsAg status in HBV DNA-positive cases. To minimize the possibility of false-negative results for HBsAg and to confirm occult HBV infection, a highly

sensitive chemiluminescent immunoassay (CLIA) (Architect, Abbott Laboratories, North Chicago, IL, USA) was used for samples that were HBsAg-negative by R-PHA but HBV DNA-positive by real-time PCR. The sensitivity of the R-PHA for HBsAg (10-12 ng/ml) was less than that of CLIA (0.06-0.08 ng/ml) (13).

Sequence analysis. The amplified fragments were sequenced directly by dideoxy sequencing using the Taq DyeDeoxy Terminator cycle sequencing kit with a 3100-Avant genetic analyzer (Applied Biosystems). The amino acid and nucleotide substitutions (nt 124-147) in S genes were then analyzed.

Phylogenetic analysis. To examine the HBV genotype and sub-genotype, the two S gene sequences (950 bp) of the strains M37 and P163 found in this study were compared with those of 48 reference sequences retrieved from the DDBJ/EMBL/GenBank database and with the 4 sequences (M41, P71, R73 and R75) identified in subjects from the Solomon Islands during our previous study (8). The subtypes of the strains used for the comparison were obtained from published articles (14,15). The sequences were aligned using ClustalX software, and phylogenetic trees were constructed by the neighbor-joining (NJ) method (16). To confirm the reliability of the phylogenetic tree analysis, bootstrap re-sampling and re-construction were carried out 1,000 times. The nucleotide distance was estimated with the Tamura-Nei substitution model. These analyses were carried out using the Molecular Evolutionary Genetics Analysis (MEGA) software program (available at <http://www.megasoftware.net>) (17).

Statistical analysis. Statistical analysis was performed using the χ^2 test or the Fisher's exact test for categorical variables. The independent t-test was used for continuous variables, as appropriate. A P-value <0.05 was considered to be statistically significant.

Table II. Characteristics of the individuals with occult HBV infection.

ID	Age	Gender	Ethnicity	Anti-HBs	Anti-HBc	AST IU/l	ALT IU/l	PC/CP	S
M37	64	F	Mela	+	+	33	18	○	○
M76	56	F	Mela	+	+	77	10		○
P103	65	F	Mela	+	-	38	18		
P131	22	M	Mela	-	+	24	17	○	
P135	22	M	Mela	+	+	64	29		
P149	29	F	Mela	+	+	15	11	○	○
P152	35	F	Mela	+	+	35	31		○
P155	26	F	Mela	+	+	21	16	○	○
P158	31	F	Mela	+	+	34	18		○
P163	33	F	Mela	-	+	23	21	○	○
P165	64	F	Mela	+	+	31	18	○	○
P177	32	F	Mela	+	+	107	52	○	○
P181	23	F	Mela	+	+	23	22		
P182	19	F	Mela	+	+	21	13		
P183	38	F	Mela	+	+	37	15		
P184	32	M	Mela	+	+	26	11		
P190	32	M	Mela	+	+	36	21		○
P194	34	M	Mela	+	+	42	34	○	○
P209	27	M	Mela	+	+	18	12		
R74	20	M	Micro	-	+	24	28		
R123	18	M	Micro	+	+	24	11		
R141	22	F	Micro	-	+	15	8		
R144	26	F	Micro	+	+	30	18		
R148	32	M	Micro	-	+	21	12		
R163	22	M	Mela	-	+	15	13		

Mela, Melanesian ethnicity; Micro, Micronesian ethnicity; ○, presence of surface (S) or pre-core/core promoter (PC/CP) genes determined by PCR. HBsAg is negative for all samples. Viral load of all samples is <2.6 log copies/ml.

Results

Prevalence of occult HBV infection. The positivity of anti-HBc and anti-HBs was examined in 200 HBsAg-negative samples. HBV-DNA was also assessed using real-time PCR and a conventional PCR assay. In the real-time PCR assay, HBV-DNA was detected in 25 of the 200 individuals (12.5%), despite their low viral load (<2.6 log copies/ml). Serologically, 24 of the 25 cases (96%) were positive for anti-HBc, and 19 of the 25 cases (76%) were positive for anti-HBs (Table I). In addition, the prevalence of occult HBV infection was significantly higher in Melanesians (17.5%) than in Micronesians (5.8%) ($P < 0.05$). There was no significant difference in age, the level of ALT, albumin and T-Bil between the occult and non-occult groups. The characteristics of occult HBV infection are listed in Table II. The mean level of HBsAg in the samples confirmed by CLIA was 0.003 IU/ml. Because of limited sample volume, we were unable to examine the level of anti-HBs. Although the quantity of HBV-DNA detected was low, 12 cases demonstrated the presence of fragments of the S and PC/CP genes by conventional PCR analysis. Of

these, 8 cases were positive for the PC/CP promoter region, and 11 cases were positive for the S region.

Substitutions in the α determinant region. The HBV S region nucleotide sequences were translated to amino acids and aligned with the reference sequences. As to the putative amino acid analysis of the α determinant region (amino acids 124-147) of the S gene, we found amino acid substitutions as shown in Fig. 1. The specific amino acid substitutions detected in this region were I126, T131 and S143. In addition, strain P158 identified in this study, demonstrated a P143 substitution while strain P190 was shown to have undergone three substitutions: S138, S142 and E144 (Fig. 1).

Phylogenetic analysis. Phylogenetic analysis of the two HBV strains (M37 and P163) found in this study based on the nucleotide sequences of the S gene (nt 18-989) was conducted by comparing their sequences with those of 54 reference consensus sequences retrieved from the DDBJ/EMBL/GenBank database. As a result, we found that M37 was classified into HBV/C3 along with strains from the Solomon

		124											147																												
A1	AF297621	120	P	C	K	T	C	T	T	P	A	Q	G	N	S	M	F	P	S	C	C	C	T	K	P	T	D	G	N	C	T	C	I	P	I	P	S	S	155		
A2	AJ012207	120	155
A3	AB194952	120	.	.	R	155
B1	D23678	120	T	155	
B2	AF121251	120	T	155	
C	X75665	120	I	.	.	.	T	S	155	
D	AJ344117	120	.	.	R	.	.	M	.	T	T	.	.	Y	S	155	
E	X75657	120	.	.	R	.	.	.	L	T	S	.	S	155	
F	X75658	120	L	T	S	.	S	155	
G	AF160501	120	Y	S	155	
H	AY090457	120	L	T	S	155	
M37	this study	120	.	.	R	.	.	.	I	T	S	155	
M76	this study	120	T	155
P152	this study	120	T	S	155
P155	this study	120	I	T	S	155
P158	this study	120	.	.	I	N	T	P	155
P163	this study	120	155
P190	this study	120	I	T	S	.	S	S	E	155

Figure 1. S antigen α determinant amino acid sequence alignment. The first 11 sequences are the consensus sequences of the corresponding HBV genotype/sub-genotype reference strains retrieved from the DDBJ/GenBank database. Dots indicate positions with amino acids identical to the HBV/A1 consensus sequence.

Islands and other Pacific regions, and P163 was classified into HBV/A2, which predominates in North West Europe and North America, with a high bootstrap support (Fig. 2).

Discussion

The disappearance of HBsAg and the appearance of anti-HBs are considered to indicate the remission of hepatic infection. Recently, however, HBV-DNA has been detected in the sera of HBsAg-negative patients, so called occult HBV infection. In the present study, the prevalence of occult HBV infection was high (12.5%) in the Solomon Islands, a highly endemic region for HBV infection with an HBsAg carrier prevalence of 21.5%. A previous study indicated that the prevalence of occult HBV infection among the general population of China was 2.0-11.5% (18) and 8.1% in the Inuit (Eskimo) community (7). Thus, occult HBV infection is common in most regions of the world where HBV infection is endemic.

In the present study, 96% of the carriers with occult HBV infection were anti-HBc positive, and 76% were anti-HBs positive. Occult HBV infection is more common in individuals that demonstrate serologic evidence of recovery from a previous exposure to HBV (anti-HBc and anti-HBs positive) (4). Twenty-five of 200 individuals that demonstrated anti-HBc and anti-HBs positivity had viral DNA detected in their serum by real-time PCR, even though their viral load was very low. Our results suggest that HBV is able to persist as an occult infection for a long period after the loss of HBsAg, even during the anti-HBs positive phase. Therefore, the serological profile of a patient is not always reliable for determining their HBV infection status, and the clearance

of HBsAg followed by the emergence of antibodies does not necessarily indicate the termination of viremia. Occult HBV infection is clinically relevant not only with regard to perinatal transmission of the virus but also in a variety of other settings, including blood transfusion and treatment of patients who are immunocompromised. The most concerning clinical impact of occult HBV infection in the Solomon Islands, however, is the risk of HBV transmission through the perinatal route from a carrier mother with occult HBV to her child, because blood transfusion or medical practice is uncommon in the Solomon Islands.

Previous studies have suggested that point mutations, deletions, or re-arrangements in several genes of the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein may be responsible for the lack of HBsAg during occult infection (19). Accordingly, the amino acid mutations in a determinant region described in this study suggest that they are related to the lack of HBsAg in occult infection. However, the substitutions (P143, S138, S142 and E144) could possibly exist as variations, since these substitutions have not been described previously. The potential significance of the surface gene mutants for vaccination failure has been studied in several endemic countries (20-22). The Solomon Islands launched a nationwide hepatitis B universal childhood vaccination program in 1977, and the coverage in the Solomon Islands in 2009 was estimated by the World Health Organization/United Nation Children's Fund to be 81% (Solomon Islands: WHO and UNICEF estimates of national immunization coverage, 1997-2009 (available at http://www.who.int/immunization_monitoring/data/slb.pdf). Because none of the participants of this study were hepatitis B

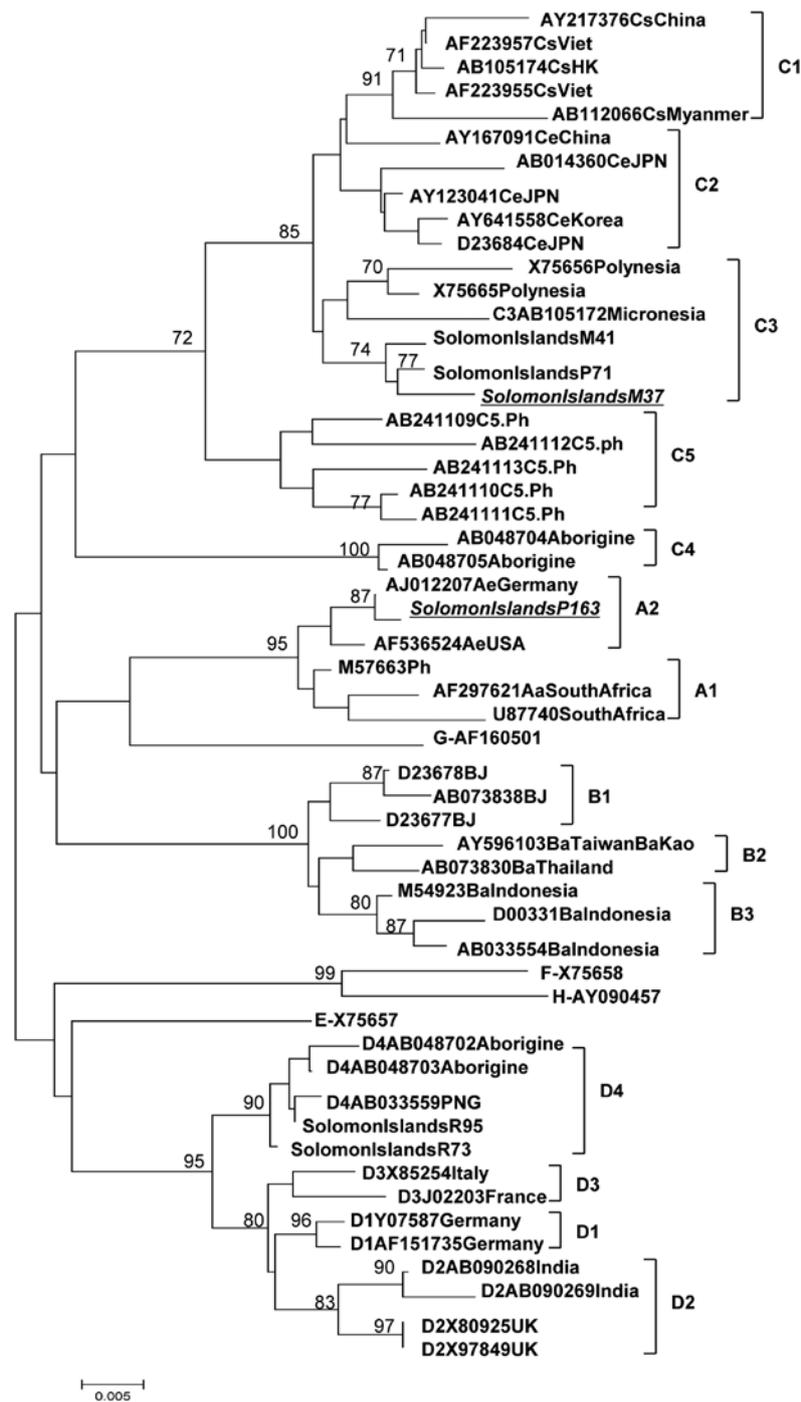


Figure 2. Phylogenetic analysis based on the partial nucleotide sequences of the S genes of two HBV strains (M37 and P163) was conducted by comparing them with those of 54 reference strains. Reference isolates from the database are indicated together with their accession number and the country in which they were found. Bootstrap values (as a percentage of 1000 replicates) are shown at the nodes of the tree. The lengths of the horizontal bars indicate the number of nucleotide substitutions per site.

vaccinated, the relationship between the level of HBV DNA and the vaccination status is uncertain, and the mutations in the α determinant region in this study are impossible to be vaccine escape mutant.

The prevalence of occult HBV infection was higher in Melanesians than in Micronesians, although no difference in HBsAg positivity (8) and the clinical characteristics between the two groups were found. In addition, HBV DNA encoding the PC/CP and HBs regions was only amplified in

samples obtained from Melanesians. The HBV genotype C is predominant in Melanesians, as previously reported (8). In this study, we used the set of the primers (SF1 and SR2, SF2 and SR2) in a real-time PCR assay which is frequently used in Japanese HBV isolates in which the predominant genotype is also C. Therefore, Melanesians may be more sensitive to these primers than Micronesians. Furthermore, the 13 samples detected only by real-time PCR and undetected by conventional PCR may have been due to the sensitivity of

the assay. Susceptibility to infection might also depend on the genotype of the virus, but due to the low HBV viral load, genotypes were only assessed in two individuals. However, little is known about the mechanisms involved in the absence of circulating HBsAg in this ethnic group. Although HBV/A is an uncommon genotype in the Solomon Islands, one of the strains (P163) found in this study was observed to be closely related to a strain from Germany (accession no. AJ012207) due to the low evolutionary distance between them (0.002 substitutions per-position). Another strain (M37) was found to belong to HBV/C, which contains other Solomon Island strains.

In summary, occult HBV infection was documented in 12.5% of individuals that demonstrated serologic evidence of resolved HBV infection. The prevalence of occult infection is influenced by ethnicity; the prevalence of infection in Melanesians is higher than that in Micronesians.

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