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Serologic and Molecular Characteristics of Hepatitis B Virus among School Children in East Java, Indonesia

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Abstract. Universal childhood hepatitis B vaccination was introduced in Indonesia in 1997; by 2008, coverage was estimated to be 78%. This study aimed to investigate the serologic status and virologic characteristics of hepatitis B virus (HBV) among the children in East Java. A total of 229 healthy children born during 1994–1999 were enrolled in this study. Overall, 3.1% were positive for hepatitis B surface antigen (HBsAg) and 23.6% were positive for antibody to HBsAg (anti-HBs). HBV DNA was detected in 5 of 222 HBsAg-negative carriers, which were suggested to be cases of occult HBV infection. A single amino substitution (T126I) in the S region was frequently found. HBV infection remains endemic, and the prevalence of anti-HBs remains insufficient among children in East Java, Indonesia.

INTRODUCTION

A safe and effective vaccine against hepatitis B has been available since 1982. The introduction of a childhood immunization program in many countries has dramatically reduced the carrier rate of hepatitis B virus (HBV) and significantly decreased the incidence of hepatocellular carcinoma (HCC).[1–3] However, HBV infection, which can lead to acute or fulminant hepatitis, chronic hepatitis, cirrhosis, and HCC,[4] remains endemic in many parts of the world.[5]

The potential significance of surface gene mutants for vaccination failure has been studied in several endemic countries.[6–8] Hepatitis B surface antigen (HBsAg) is a major component of the hepatitis virion envelope. The *a* determinant is a key region for HBV antigenicity, and it has been suggested that amino acid changes in this region affect immune responses.[9] Surface gene variants have been detected in vaccinated children, liver-transplant recipients treated with monoclonal and polyclonal antibodies to HBsAg (anti-HBs), and chronic carriers with occult HBV infection.[10,11] Moreover, previous studies have suggested that variations in the core region may be responsible for the lack of HBsAg.[12,13]

The status of HBV is of particular concern in Indonesia, an area with intermediate-to-high endemicity for HBV infection and a carrier rate of 5–20% in the general population.[5] Most chronic HBV carriers were infected in early infancy. Indonesia launched a nationwide hepatitis B universal childhood vaccination program in 1997, and the coverage (defined as the percentage of children receiving at least three doses of hepatitis B vaccine) in Indonesia in 2007 was estimated by World Health Organization/United Nations Children's Fund to be 78%.[14] However, the current serologic status of HBV in older children has not been fully investigated.

The aim of this study was to investigate the status of HBV infection and to elucidate the prevalence and significance of HBV variants in children in Lamongan, an area in East Java, Indonesia, with intermediate-to-high endemicity for HBV.

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MATERIALS AND METHODS

Study subjects. Four of 18 elementary schools in the Lamongan District, a rural area of East Java, were randomly selected. All children in grades 4–6 (n = 229, 113 boys and 116 girls 8–13 years of age) were enrolled in this study. Serum samples were obtained during November–December 2007 and stored at –20°C until use. The coverage rate among 8–10-year-old children (n = 118) was estimated to be 90% on the basis of local records for the childhood hepatitis B vaccination program during 1997–1999. Written informed consent was obtained from parents of all children. No individual hepatitis B vaccination records remained. The study protocol was reviewed and approved by the Ethics Committees of Kobe University in Japan and Airlangga University in Indonesia.

Serologic markers of HBV infection. All refrigerated samples were tested for HBsAg by Reversed Passive Hemagglutination (R-PHA) (Mycell II HBsAg; Institute of Immunology, Tokyo, Japan) and for anti-HBs by Passive Hemagglutination (PHA) (Mycell II anti-HBs; Institute of Immunology). To differentiate vaccine-induced antibody from naturally acquired antibody and to identify occult HBV infections, the prevalence of antibody to hepatitis B core antigen (anti-HBc) was assessed by PHA (Mycell anti-rHBc; Institute of Immunology).

Detection of HBV DNA and nucleotide sequence analysis. After being assayed for HBV serologic status, HBsAg-positive serum samples (n = 7) and serum samples negative for HBsAg but positive for either anti-HBs or anti-HBc (n = 89) were subjected to HBV genetic analysis to confirm infection and identify surface antigen and pre-core/core variants. After serologic testing, serum samples were stored at –80°C until genetic testing. DNA was extracted from 100 μL of serum samples by using a DNA extractor kit (QIAamp DNA Blood Mini Kit; QIAGEN, Tokyo, Japan). The presence of HBV DNA was assayed by two nested polymerase chain reaction (PCR) assays with primer pairs from the surface and pre-core/core promoter genes of the viral genome, as described.[15,16] Amplified fragments were directly sequenced by using the Big Dye Deoxy Terminator cycle sequencing kit with an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). Viral load was assessed by real-time PCR using an ABI Prism 7300 Real Time PCR System (Applied Biosystems). HBV was amplified by using a primer and probe set, as described previously.[17] The HBV nucleotide sequences

of the S gene were translated to amino acids and aligned with reference sequences.

Confirmation of HBsAg status in HBV DNA-positive children. To minimize the possibility of false-negative results for HBsAg and confirm occult HBV infection, an immunochromatography method based on the principle of an enzyme immunoassay (EIA) (Espline HBsAg; Fuji Rebio, Tokyo, Japan) was used for samples that were HBsAg negative by R-PHA but HBV DNA positive by PCR.

HBV genotyping. The HBV genotypes were determined by using the phylogenetic tree of the S region. Reference sequences were retrieved from the DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank database. Alignments were performed by using CLUSTAL X software (www.clustal.org), phylogenetic trees were constructed by using the neighbor-joining method, and bootstrap resampling was performed 1,000 times. Analyses were conducted by using the Molecular Evolutionary Genetics Analysis (MEGA) software program.[18]

Statistical analysis. Statistical analysis was performed by using the chi-square test or Fisher's exact test for categorical variables. A P value < 0.05 was considered statistically significant.

RESULTS

A total of 229 children were screened for serologic markers of HBV infection. Because of limited sample volumes, 208 of 229 samples were tested for anti-HBc. Overall, positivity rates for HBsAg and anti-HBs were 3.1% (7 of 229) and 23.6% (54 of 229), respectively. Among 8–10-year-old children (n = 118) born after introduction of the universal vaccination program, anti-HBs was found in 28 (23.7%). Of these children, anti-HBc testing was performed on 21 children; 16 children were negative. Of 54 anti-HBs-positive children, 28 were negative for anti-HBc. All 7 HBsAg-positive children (L17, L23, L69, L70, L74, L103, and L216) were negative for anti-HBs and had a high HBV viral load (4.9–7.4 logcopies/mL), as shown in Table 1.

Five cases of occult HBV infection were found in this study (Table 2). Nucleotide sequence analysis of the core promoter gene showed that the A1762T/G1764A mutation was observed in only one child (L44, Table 2). Children L29, L62, and L119 had two amino acid substitutions, T126I and T143S, in the *a* determinant region (Figure 1 and Table 2). The T143S substitution is invariably associated with T126I. Moreover, these 3 strains had other substitutions (S113T, Y161F, V177A, M213I, and T227I), which were specific to three strains that showed changes outside the *a* determinant region but within the S gene (Figure 1). Child L33 also had the substitutions K24R, Q56P, I57T, and C64S, as did children L29 and L119. No substitution was found in the *a* determinant region of child L44. Two (L44 and L62) of five children with occult HBV infections were positive for anti-HBs (Tables 1 and 2). The HBV viral load was detected in four of the five children and ranged from 4.5 to 6.1 log copies/mL (Table 1).

We found 12 HBV DNA-positive children in this study (Table 1). The HBV genotypes were successfully determined for 11 (91.7%) of the 12 HBV DNA-positive children (Figure 2). The HBV genotype B (HBV/B) was identified in 10 (83.3%) children, HBV/C was identified in 1 (8.3%) child, and was not identified in 1 child.

TABLE 1

Demographic, serologic, and virologic characteristics of 12 HBV DNA-positive children, East Java*

ID	Age, years	Sex	Genotype (subgenotype)	HBsAg	Anti-HBs	Anti-HBc	Viral load (log copies/mL)
L17	11	M	B3	+	–	+	7.2
L23	11	F	B3	+	–	NT	6.9
L69	9	M	B3	+	–	–	4.9
L70	9	M	B3	+	–	NT	NT
L74	9	M	B3	+	–	–	UD
L103	11	M	B3	+	–	NT	7.4
L216	11	M	UD	+	–	+	UD
L29	10	F	B3	–	–	+	4.8
L33	10	F	B3	–	–	+	4.8
L44	9	F	C6	–	+	–	UD
L62	9	M	B3	–	+	+	4.5
L119	11	F	B3	–	–	+	6.1

*HBV = hepatitis B virus; ID = identification; HBsAg = hepatitis B surface antigen; anti-HBs = antibody to HBsAg; anti-HBc = antibody to hepatitis B core antigen; NT = not tested; UD = undetermined.

TABLE 2

Genomic variability of HBV in HBsAg-negative carriers, East Java, Indonesia*

ID	Anti-HBs/ Anti-HBc	Amino acid mutation in the Pre-S/S region		Nucleotide mutation in the Pre-C/CP region	
		Pre-S region	S region	A1762T/ G1764A	G1896T
L29	–/+	K24R,Q56P,I57T,C64S, S113T	T126I, T143S	–	–
L33	–/+	K24R,Q56P,I57T,C64S	Wild	UD	UD
L44	+/–	K24R,Q56P,I57T,C64S	Wild	+	–
L62	+/+	S113T	T126I, T143S	–	–
L119	–/+	K24R,Q56P,I57T,C64S, S113T	T126I, T143S	–	–

*HBV = hepatitis B virus; HBsAg = hepatitis B surface antigen; ID = identification; anti-HBs = antibody to HBsAg; anti-HBc = antibody to hepatitis B core antigen; S = surface; C = core; CP = core promoter; UD = undetermined.

DISCUSSION

A universal childhood vaccination program was launched in Indonesia in 1997 and its coverage was estimated to be high (approximately 90%) on the basis of the local records for Lamongan in East Java, Indonesia. The Indonesian government provided hepatitis B vaccines produced in Indonesia to health centers called *puskesmas*. Most infants, especially in rural areas, were taken to *puskesmas* to be vaccinated. Plasma-derived HBV vaccine was produced in Indonesia until 1997, when it was replaced by a recombinant hepatitis B vaccine. In this study, some children had received the plasma-derived vaccine and others had received the recombinant vaccine during infancy. Although a higher loss rate of anti-HBs was detected when the recombinant vaccine was used, it may provide better protection than the plasma-derived vaccine.[19] In the initial vaccination program in Indonesia, three doses of recombinant vaccine were administered, with the first dose being given within 12 hours of birth to prevent perinatal HBV transmission, and the second and third doses being given at one and six months of age, respectively, in accordance with national guidelines. However, the government now recommends the first dose of HBV vaccine to given within seven days of birth.

Presently in Indonesia, an HBV-diphtheria-pertusis-tetanus combination vaccine is given at birth, followed by a monovalent HBV vaccine. In Indonesia, recombinant hepatitis B

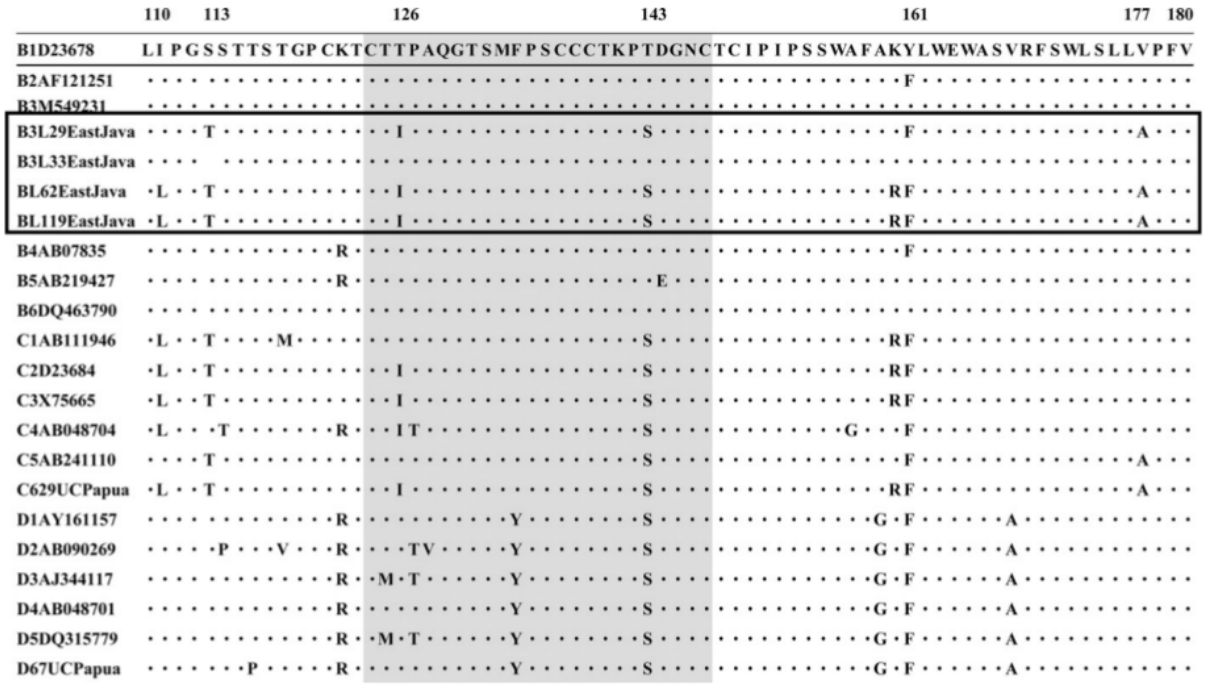

FIGURE 1. Surface antigen *a* determinant amino acid sequence alignment for hepatitis B virus (HBV). The first line sequence is the consensus sequence corresponding to an HBV subgenotype (B1; accession no. D23678) reference strain retrieved from the DNA Data Bank of Japan/GenBank database. Dots in the alignment indicate positions with amino acids identical to the HBV/B1 consensus sequence. The *a* determinant region in the S gene is indicated with a different color.

vaccines that include pre-S1 and pre-S2 regions currently unavailable. However, this new triple-antigen vaccine is more efficient than the single-antigen vaccine,[20,21] and prevents infection from vaccine-escape mutants.[22] The triple-antigen vaccine should be considered in our study area where the prevalence of anti-HBs remains insufficient among children.

This study was unable to assess the actual coverage rate because no individual vaccination records remained. For this reason, efficacy of vaccination was not evaluated in this study. However, this study did show that acquiring protective antibody against HBV infection was insufficient among children born after introduction of the universal vaccination program. There are two possible explanations for this finding. First, the first dose of vaccination may have been delayed until after birth. To prevent perinatal transmission, the first dose should be given as soon as possible after birth.[23] In reality, the first dose of HBV vaccine was often given several months after birth because the vaccinators and parents were fearful that the infant might have been killed by being immunized so soon after birth.[5] Moreover, because giving birth at home is still common, such children have less opportunity to receive the vaccine than those born in a hospital.[4] Failure to administer the vaccine in a timely manner will reduce the impact of vaccination in countries with a high prevalence of HBV infection,[5] such as Indonesia. Second, loss of protective antibody against HBV infection may have occurred in some children. In previous studies in Taiwan, protective anti-HBs titers gradually decreased by age 12; a higher loss rate of anti-HBs was demonstrated in children immunized with the recombinant vaccine.[24–26]

This study identified seven HBsAg-positive children, and we suggest that these children were naturally infected during infancy. The prevalence of HBsAg (3.1%) in this study was similar to that among the general population in Indonesia.[5]

Causes of the failure to detect HBsAg in serum may include mutations in the S regions of the virus genome, which is also known as occult HBV infection.[27] In this study, the R-PHA assay was used to screen for HBsAg positivity. This assay was useful but less sensitive (around 93%) than an EIA.[28] In addition, accuracy of the EIA assay was higher than that of the R-PHA.[29,30] Thus, we confirmed the presence of occult HBV infection by using the EIA.

We identified five HBsAg-negative children who were positive for HBV DNA and considered them to be children with occult HBV infections. The status of the five children with undetectable HBsAg was as follows: child L44 had recovered from wild-type HBV infection because of production of anti-HBs and a low viral load; child L62 had a vaccine-escape mutant; and children L29, L33, and L109 were naturally infected with vaccine-escape mutants and did not show production of anti-HBs after vaccination.[31] These results suggest that potential vaccine-escape mutants exist within normally infected HBV carriers and that such carriers do not produce detectable HBsAg.

Mutations in the *a* determinant region (amino acids 121–149) affect the structure of the S region and are known to cause vaccine-escape mutations during immunization. The G145R mutation is the most common vaccine-escape mutant after immunoprophylaxis and in nature.[32] Another amino

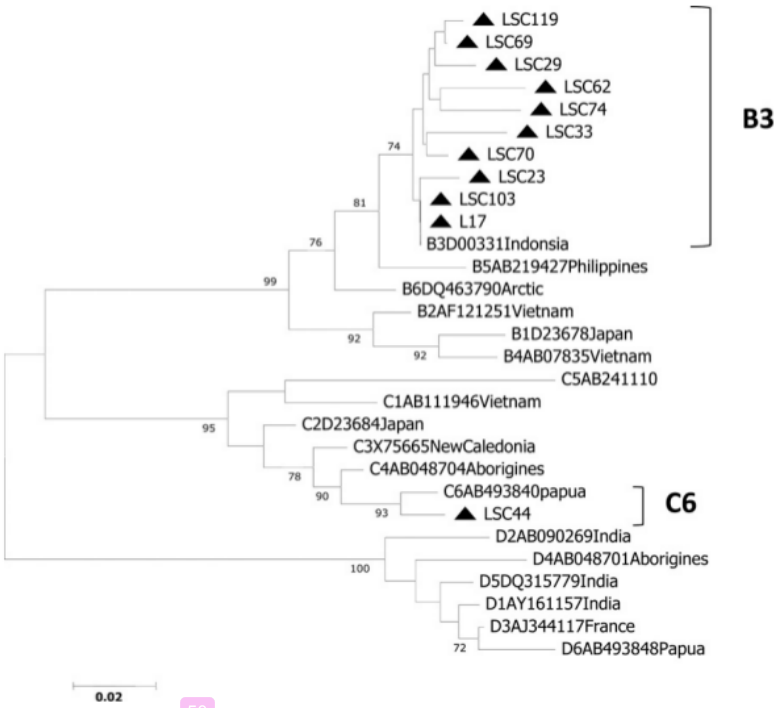

FIGURE 2. Phylogenetic trees of the surface gene in hepatitis B virus (HBV) strains isolated from 11 children in East Java, Indonesia, and 18 reference strains. Numbers in the tree indicate bootstrap reliability. Lengths of horizontal bars indicate number of nucleotide substitutions per site. Isolates from the database are indicated by their accession number, and relevant country names have been added to each HBV/B, HBV/C, and HBV/D strain.

acid substitution, T126I, which is unique to genotype C, has also been reported to affect the antigenicity of HBsAg.[33–35] T143S was invariably associated with T126I in this study, suggesting that amino acid substitutions (not T126I itself, but in combination with T143S) affect the antigenicity of HBsAg. In this study, T126I appeared in only HBV/B and was not specific to genotype C, in contrast to findings of a previous study. Because the T126I substitution involves the largest change in chemical properties, it is most likely to cause structural changes in HBsAg.[33–35] In addition, A1762T/G1764A was observed in only one child, who had recovered from wild-type HBV infection. Although previous studies have reported A1762T/G1764A mutations in occult HBV infection,[11–13] the correlation between these mutations and occult HBV infection remains unclear. No other mutations were found in the core region in this study.

All 10 HBV/B strains were classified into subgenotype B3 (HBV/B3), which is prevalent in Indonesia. One strain of HBV/C had high homology with HBV/C6, which was reported as a novel strain from Papua (accession no. AB493840).[36,37]

HBV infection in children is still endemic in East Java. We detected several variants in the *a* determinant region in children who were HBsAg negative, and T126I might be one of the viral mechanisms that help the virus to escape from current hepatitis B vaccines. Emergence of viruses capable of escaping neutralization by vaccine-induced antibodies poses a serious threat to our ability to control hepatitis B.[31] This pilot study indicates the presence of unique HBV among children in East Java. Because this study was small, an analysis of a larger population is necessary to confirm our findings. Empirical data are essential for future policy changes on the maintenance of the vaccination program.

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