Hepatitis B Virus Infection in Indonesia 15 Years after Adoption of a Universal Infant Vaccination Program: Possible Impacts of Low Birth Dose Coverage and a Vaccine-Escape Mutant

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Abstract. A universal hepatitis B vaccination program for infants was adopted in Indonesia in 1997. Before its implementation, the prevalence of hepatitis B surface antigen (HBsAg)–positive individuals in the general population was approximately 5–10%. The study aimed to investigate the hepatitis B virus (HBV) serological status and molecular profile among children, 15 years after adoption of a universal infant vaccination program in Indonesia. According to the Local Health Office data in five areas, the percentages of children receiving three doses of hepatitis B vaccine are high (73.9–94.1%), whereas the birth dose coverage is less than 50%. Among 967 children in those areas, the seropositive rate of HBsAg in preschool- and school-aged children ranged from 2.1% to 4.2% and 0% to 5.9%, respectively. Of the 61 HBV DNA–positive samples, the predominant genotype/subtype was B/adw2. Subtype adw3 was identified in genotype C for the first time in this population. Six samples (11.5%) had an amino acid substitution within the a determinant of the S gene region, and one sample had T140I that was suggested as a vaccine-escape mutant type. The low birth dose coverage and the presence of a vaccine-escape mutant might contribute to the endemicity of HBV infection among children in Indonesia.

INTRODUCTION

Indonesia experiences intermediate to high hepatitis B virus (HBV) endemicity. Before adoption of universal infant vaccination, the prevalence of hepatitis B surface antigen (HBsAg) seropositivity was estimated to be 5–10% in the general population.1,2 The perinatal transmission route has played an important role in the increasing number of infants and children in the general population.1,2 The perinatal transmission route has played an important role in the increasing number of infants and children in the general population.1,2 The perinatal transmission route has played an important role in the increasing number of infants and children in the general population.1,2

A universal hepatitis B vaccination program for infants was adopted in Indonesia in 1997. The HepB3 coverage (defined as the percentage of children receiving three doses of hepatitis B vaccine) in 2011 was estimated by the World Health Organization to be 63%; however, this number varies in each area in Indonesia.4,5

The widely distributed hepatitis B vaccine consists of recombinant HBsAg, which has a major antibody-neutralizing epitope region, termed the “a determinant.” Amino acid changes within this region could make HBV resistant to the neutralizing effect of anti-HBsAg (anti-HBs) and could threaten the effectiveness of the hepatitis B vaccination program.6 Moreover, the emergence of HBV variants with mutations within the small surface protein (S) after the implementation of the hepatitis B universal vaccination program has increased through the years and may cause vaccination failure.7,8

Nine HBV genotypes, from A to H and J, that are based on its surface gene, have been documented,3–12 and HBV genotypes B and C were predominant among Indonesian populations.3–16 Ten HBV serological subtypes, which are based on the amino acid sequences of HBsAg, have been identified.17–19 Indonesia has been geographically divided into four different HBV subtype zones: the adw, adr, ayw, and mix zones.20 It is important to record the S gene variant type in an endemic population because this information may be critical for determining policies for vaccine and diagnostic reagent design.21–23

Fifteen years after the implementation of the universal vaccination program, the serological status and molecular profile of HBV in children has still not been fully investigated. This study aimed to analyze the prevalence and significance of HBV variants, including emerging vaccine-escape mutants, among children in several districts in Indonesia based on HBV subtype and genotype zones.

MATERIALS AND METHODS

Study subjects. Serum samples were collected from preschool-aged (1–5 years) and school-aged (6–12 years) children during April–October 2012. The following four cities were included in this study: Kotawaringin Barat in Central Kalimantan—adw/mix zone (N = 258), Kupang in West Timor—ayw zone (N = 177), Sorong in West Papua—adr zone (N = 167), and Bau-bau in southeast Sulawesi—mix zone (N = 136). The data for Lamongan, East Java, which is an adw zone (N = 229), were obtained from a previous study conducted in 2007.14 Serum samples were sent to the Institute of Tropical Disease, Airlangga University, Surabaya, Indonesia.

Written informed consent was obtained from the parents of the children. However, some of the individual vaccination records were not available. The hepatitis B immunization coverage data of each area were provided by the Local Health Office. The study protocol was reviewed and approved by the ethics committees of Kobe University in Japan and Airlangga University in Indonesia.
Serological markers of HBV infection. All refrigerated samples were tested for HBsAg and anti-HBs using enzyme-linked immunoabsorbent assays (ELISAs) (Hepalisa HBsAg and New Hepalisa Anti HBs kits, respectively; Indec Diagnostic, Jakarta, Indonesia). To differentiate vaccine-induced antibody from natural infection-acquired antibody, the antibody to HBV core antigen (anti-HBc) was examined by ELISA (Hepalisa Anti HBc; Indec Diagnostic).

Molecular detection of HBV DNA. All HBsAg-positive (N = 24) and HBsAg-negative/anti-HBc-positive (N = 79) samples were examined for HBV DNA to confirm infection and identify the surface antigen. DNA was extracted from 200 μL of serum using a DNA extractor kit (QIAamp DNA Blood Mini Kit; QIAGEN, Tokyo, Japan). The presence of HBV DNA was then assessed by nested polymerase chain reaction (PCR) assays using primers P7 and P8, which target nucleotides 256–796 of the S gene, as the first primer pair. When the first PCR amplification was negative, a second round of PCR was carried out using primers HBS1 and HBS2, which target nucleotides 455–713 of the S gene. The amplification products were visualized on a 2% agarose gel stained with ethidium bromide.13

Nucleotide sequence analysis. Nucleotide sequencing of the amplified fragments was performed using the BigDye deoxy Terminator v1.1 cycle sequencing kit with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA), as described previously,13 to determine the HBV genotype and subtype and the possible presence of another HBV S gene variant (vaccine-escape mutant). HBV genotypes were determined based on homology (> 96%) to the S gene17,24 using Genetyx Win v9.0 (Genetyx Corporation, Tokyo, Japan). HBV nucleotide sequences were aligned using the Bioedit program and were analyzed further. HBV subtypes were then deduced on the basis of the predicted amino acid sequence substitution at positions 122, 127, 134, 140, 159, 160, and 177 of the S gene, as previously described.9,10,19,25 Other amino acid substitutions in the S gene related to the vaccine-escape mutant were also analyzed.

RESULTS

The subjects from East Java and South East Sulawesi included school-aged children who ranged in age from 8 to 13 years. The subjects from Central Kalimantan and West Timor included both preschool- and school-aged children. The subjects from West Papua included only preschool-aged children (Table 1).

The seropositive rates of HBsAg among the preschool-aged children in the five areas ranged from 2.1% to 4.2%, and those among school-aged children ranged from 0% to 5.9%. The prevalence of anti-HBs seropositivity among preschool-aged children was higher (61.4–65.8%) than among school-aged children (20.9–40.4%). The prevalence of anti-HBc seropositivity among preschool- and school-aged children ranged from 3.5% to 4.8% and from 5.2% to 50.4%, respectively. The highest prevalence of anti-HBc seropositivity was found in southeast Sulawesi.

In this study, HBV DNA was detected in 62 samples with either HBsAg positivity or HBsAg negativity/anti-HBc positivity from five areas (Table 1). The HBV genotypes that were found among children in Indonesia included genotype B (68.8%) and C (31.2%). Genotype B was predominant in East Java (90.9%), Central Kalimantan (100%), and southeast Sulawesi (60%), whereas both genotypes B and C were common in West Timor and West Papua (Table 2).

The HBV subtype prevalence of each area also varied. The HBV subtype adw was predominant among children in East Java and Central Kalimantan (90.9% and 100%, respectively) (Table 2). Meanwhile, subtype adr was predominant among children in West Papua, and two samples (PB 09 and PB 26) had the A159/A177 amino acid combination and were classified as adr indeterminate (Figure 1). In southeast Sulawesi, subtypes ayw and adr were commonly found among school-aged children (44.0% and 40%, respectively). In West Timor, HBV subtypes ayw and adw were common in prevalence, 33.3% and 44.4%, respectively, and subtype adw3 was identified for the first time in Indonesia.

In this study of 32 samples with genotype B, the number of samples identified as subtypes adw2 or ayw was 28 and 14, respectively. HBV genotype C with subtype adwr+ was predominant in Indonesia and was found in 15 of 19 samples. Other subtypes identified in HBV genotype C included adw2, adw3, and adwr(i).

Of the 61 HBV DNA-­–positive samples, six (11.5%) had an amino acid substitution within the a determinant of the S gene region (Table 3); all of these samples were genotype B and subtype adw2. Four samples were found among preschool-aged children (PB53, PB54, PB173, and NT43). However, the anti-HBc (PB173) status of one isolate with insufficient serum could not be determined. Two types

<table>
<thead>
<tr>
<th>Characteristic of subjects</th>
<th>Preschool-aged children</th>
<th>School-aged children</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Central Kalimantan</td>
<td>West Timor</td>
</tr>
<tr>
<td>No. of subjects (male, female)</td>
<td>143 (77, 66)</td>
<td>73 (34, 39)</td>
</tr>
<tr>
<td>Mean age ± SD (years)</td>
<td>2.7 ± 1.3</td>
<td>4.2 ± 0.9</td>
</tr>
<tr>
<td>Birth dose HB vaccination coverage (%)†</td>
<td>40.9</td>
<td>46.8</td>
</tr>
<tr>
<td>DPT-HB3 vaccination coverage (%)‡</td>
<td>94.1</td>
<td>73.9</td>
</tr>
<tr>
<td>No. of HBsAg positive (%)</td>
<td>3 (2.1)</td>
<td>2 (2.7)</td>
</tr>
<tr>
<td>No. of anti-HBs positive (%)</td>
<td>94 (65.7)</td>
<td>48 (65.8)</td>
</tr>
<tr>
<td>No. of anti-HBc positive (%)</td>
<td>5 (3.5)</td>
<td>3 (4.1)</td>
</tr>
<tr>
<td>Detectable HBV DNA§</td>
<td>5/6</td>
<td>3/4</td>
</tr>
</tbody>
</table>

*Preschool- and school-aged children groups were described as 1–5 and 6–12 years, respectively.
†Birth dose HB: complete dose of hepatitis B vaccine (three doses vaccines of combined diptheria, pertussis, and tetanus toxoid–Hepatitis B).
‡DPT/HB3: complete dose of hepatitis B vaccine (three doses vaccines of combined diptheria, pertussis, and tetanus toxoid–Hepatitis B).
§Detection of HBV DNA was performed for HbsAg-positive and/or anti-HBc-positive samples.

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HB = hepatitis B; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; SD = standard deviation.

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†‡Birth dose HB: complete dose of hepatitis B vaccine (three doses vaccines of combined diptheria, pertussis, and tetanus toxoid–Hepatitis B).
of single mutations were identified (T140I and S155F), and others had mixed mutations within the α determinant (P120S-A159V and I152T-F158L). Another sample had mixed mutations in both the inner and outer regions of the protein (M133L and S171F).

Three samples were HBV DNA positive but HBsAg negative, suggesting they resulted from occult hepatitis B infections (sample numbers NT43, KM61, and SS112). One of these isolates, NT43, which was anti-HBs positive, had a threonine-to-isoleucine amino acid substitution at position 140 (Figure 1).

**DISCUSSION**

Indonesia adopted a universal hepatitis B vaccination program for infants in 1997. Plasma-derived vaccine was produced until 1997, when it was replaced by a recombinant hepatitis B vaccine. In 1999, Indonesia introduced an innovative policy by using a prefilled single-use injection device of hepatitis B vaccine that is stable even outside the cold chain. This approach has improved hepatitis B vaccine coverage and reduced hepatitis B carrier rates. In 2000, Indonesia introduced a program for using the device for the birth dose in seven provinces. In 2003, the program expanded to target all of Indonesia’s 5 million annual births.

The ultimate goal of the national hepatitis B immunization program is an HBsAg prevalence of less than 1% in 5-year-olds born after the start of routine immunization. This study revealed that the prevalence of HBsAg among preschool- and school-aged children ranged from 2.1% to 4.2% and 0% to 5.9%, respectively (Table 1). These rates were lower compared with a previous study on adult (from 5 to 10%) and pregnant female (5.2%) populations in Indonesia. According to the first immunization project model of hepatitis B vaccination among children in Lombok in 1987–1991, the carriage rates decreased from 6.2% to 1.4% in Taiwan.
one of the highest hepatitis B–endemic countries to successfully implement a universal vaccination program, the prevalence of HBsAg decreased from 9.8% in 1984, the year when universal infant immunization began, to 0.7% in 1999, 15 years later.\textsuperscript{30,31}

Hepatitis B vaccines and immunoglobulin (HBIG) are highly effective methods (89–98%) of preventing perinatal transmission if given within 12–24 hours after birth.\textsuperscript{32–35} According to the national immunization policy in Indonesia, the birth dose of the hepatitis B vaccine should be given within 7 days after birth and should be followed by three doses of combination vaccines including diphtheria, pertussis, tetanus and hepatitis B within the 2nd, 3rd, and 4th months (DPT-HB3).\textsuperscript{27,36} The parents should bring their baby to the primary health center to receive the vaccine; however, many people in remote areas in Indonesia have difficulty reaching these centers due to geographical isolation.\textsuperscript{3} Furthermore, screening for hepatitis B to all pregnant women is not implemented as a national program in Indonesia,\textsuperscript{27} and most infants born to women with hepatitis B would therefore not have received HBIG. If the mother is infected and transmits the virus before the child is vaccinated, and no HBIG is given simultaneously within 24 hours of birth, vaccination would not protect the child.\textsuperscript{4}

According to the Local Health Office data, DPT-HB3 coverage in Central Kalimantan, West Timor, and West Papua is quite high (73.9–94.1%) (Table 1). In contrast, the birth dose coverage of the hepatitis B vaccine in those areas is less than 50%. Many people in Indonesia who live far from health service centers prefer giving birth at home and finding a traditional birth attendant to deliver the baby.\textsuperscript{5} These facts could delay the scheduling of the birth dose of hepatitis B vaccine.

In this study, anti-HBs seropositivity prevalence among preschool-aged children was higher (61.4–65.8%), than among school-aged children (20.9–40.4%) (Table 1). A previous study among vaccinated children in Taiwan had shown that the anti-HBs positivity rates for 2-year-old children were as high as 93–97%.\textsuperscript{36} The antibody titer then gradually fell to less than 10 mIU/mL by 10–15 years.\textsuperscript{38,39} The results of this study may reflect a similar decline in anti-HBs levels; however, the lower immunization coverage in Indonesia during earlier years of its implementation (before 2003)\textsuperscript{37} may also have contributed to these results.

This study demonstrated the current geographical distribution pattern of the HBV genotypes and subtypes among children 15 years after implementation of the universal vaccination program. A previous study identified four genotypes (A, B, C, and D) among adults in Indonesia; however, genotypes B and C showed predominant distributions.\textsuperscript{15} Furthermore, the four zones of HBV subtypes shared the pattern that was identified in Indonesia: adw, ayw, adr, and mix zones.\textsuperscript{20}

This study found that genotype B and subtype adw were predominant in western Indonesia, that is, East Java and Central Kalimantan (Table 2). There were no altered genotype or subtype patterns in Java, where most ancestors of the people migrated from Indo-Chinese areas where adw predominates. Subtype adw was also the main subtype in Central Kalimantan, possibly because the ancient people of Kotawaringin Barat mostly originated from Sumatra and Java Island.

Both genotypes B and C were significantly found in regions of eastern Indonesia, that is, southeast Sulawesi, West Timor, and West Papua. Genotype B mostly originated from China, Japan, and southeast Asia.\textsuperscript{18} Genotype C mainly originated from Far East Asia, Australia, New Zealand, and Polynesia, and it was predominant in eastern Indonesia, including North Sulawesi, Moluccas, and Papua.\textsuperscript{13,15,16} Both areas in southeast Sulawesi and West Timor showed a mixed pattern of HBV subtypes. On the other hand, there was an altered pattern of the main subtype in West Papua, from adr to both adr and adw subtypes. Currently, the demographic data reveal that many citizens who live in West Papua migrated from outside the island, mostly from Java, Sulawesi, and Moluccas.\textsuperscript{38}

This study found that genotype B and subtype adw were common among children in all areas of Indonesia. The genotype and subtype geographical distribution pattern findings in this study are a result of various ethnic groups living in Indonesia and of rapid interisland population movements within the last decades.

HBV subtype adw3, which was previously published as a new subtype,\textsuperscript{30} was identified in one sample (NA 08) from West Timor and was also determined to be a new subtype in this study. It has been known that amino acid positions 122 and 160 are responsible for the expression of \( d/v \) and \( w/r \) specificity;\textsuperscript{40} in addition, the presence of threonine at position 127 is necessary to distinguish \( w \) specificity.\textsuperscript{41} Interestingly, subtype adw3, which belonged to genotype C but had been officially recognized in genotype B and D, was first identified in Indonesia.\textsuperscript{19,39}

The major target of the neutralizing antibody produced by natural infection or vaccination is the \( a \) determinant within HBsAg.\textsuperscript{32} The \( a \) determinant region represents the immunodominant HBsAg, which was initially considered as a conformational cluster of epitopes located between residues 120 and 150 of HBsAg and has currently been expanded to residues 110–160 after the discovery of two more epitopes.\textsuperscript{43} Alteration of residues within the \( a \) determinant region can result in reduced antigenicity and protein expression, which can result in failure to neutralize viral infection and can
affect diagnostic assay detection.\textsuperscript{2,4,45} The previous study in East Java suggested T126I substitution, in combination with T143I, in genotype B could affect the antigenicity of HBsAg.\textsuperscript{14} The most common and widely recognized HBV vaccine-escape mutant type is the one with a change at position 145, from glycine to arginine.\textsuperscript{45,46} The G145R mutation was not found in this study and has not yet been reported in Indonesia.

Three samples were HBV DNA positive and anti-HBs positive, which had an amino acid mutation within the $\alpha$ determinant region (PB53, PB 54, and NT43). One of these samples, NT43, had a T140I amino acid substitution, which has also been potentially suggested as a vaccine-escape mutant type. In contrast, this mutation was also previously found in unvaccinated Taiwanese children.\textsuperscript{47} Several alterations in the $\alpha$ determinant region (positions 121–149), particularly those located within the highly hydrophilic residues 137–149 of the major HBsAg epitope, are known as vaccine-escape mutations during immunization.\textsuperscript{46} The amino acid substitution M133T, which was detected in sample PB54, was also previously reported in an adult patient with chronic liver disease in Korea.\textsuperscript{49}

The M133L mutation that was identified in this study, sample KM 61, was also previously reported in occult HBV infection among adult blood donors and vaccinated children in Indonesia.\textsuperscript{6,55} This mutation showed the least significant changes in antigenicity profile (compared with T143M/L, T123N, and M133IT/V), which may have slightly decreased the sensitivity of the HBsAg assay.\textsuperscript{52,53} Other mutation types (T123N, M133IT/V, S155F, P120S-A159V), respectively, should be studied further in the future to determine their association with vaccination failure.

Limitations to this study included the following: some of the individual vaccination records were not available, and the vaccination coverage rate used in this study was based on the Local Health Office data from a year before sample collection. Although our sampling strategy was nonrandom, our results from five representative areas might nonetheless describe the general situation of hepatitis B infection among children based on the HBV subtype zones in Indonesia.\textsuperscript{20}

In conclusion, this study finds that HBsAg seropositivity among populations of children in Indonesia has decreased but HBV still remains low to moderately endemic 15 years after adoption of a universal infant vaccination program. The low birth dose coverage of hepatitis B vaccination and the presence of an HBV vaccine-escape mutant may have contributed to these findings. Altered HBV genotype and subtype distribution patterns were observed in several areas, and the migration of populations is suggested as one of the associated factors. Further studies with larger-scale subject populations and complete individual vaccination records are necessary to obtain empirical data for evaluating the hepatitis B vaccination program in Indonesia.

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### References

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