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High Prevalence of Occult Hepatitis B Infection (OBI) and its Molecular Characteristics among Pregnant Women in Surabaya, Indonesia

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Perinatal transmission is the predominant mode of hepatitis B virus (HBV) transmission in countries where HBV infection is endemic. Newborns of HBV infected mothers have a high risk (up to 90%) of chronicity through perinatal transmission. HBsAg serology screening has been recommended to pregnant women, to prevent perinatal HBV infection. However, at present HBV DNA can be detected in serum with negative HBsAg (OBI - occult hepatitis B infection). The aim of this study was to determine the prevalence of occult hepatitis B infection (OBI) in pregnant women in Surabaya, Indonesia and its virological characteristics. A total of 50 HBsAg-negative and anti-HBc-positive sera were tested for anti-HBs and HBeAg. HBV DNA was isolated from these samples, analyzed by polymerase chain reaction (PCR) and sequenced. HBV DNA was detected in 9 (18%) samples, based on part of the S gene sequence. HBV/B3-*adw2* was found predominant in 7 of 9 (77.7%) samples, HBV/B9-*ayw1* in 1 (11.1%) sample, and HBV/C7-*adrq+* in 1 (11.1%) sample. Three samples had mutations (Q129H, T131N, M133S, T140I, T126I) in the '*a*' determinant region, which may play a role in the undetectability of the virus by the common HBsAg detection kit. The prevalence of OBI in pregnant women from Surabaya is high, but still in line with the general population in Asia. Application of anti-HBc antibody or HBV DNA detection in screening would be very beneficial and prevent perinatal transmission from OBI pregnant women.

Key words: HBV DNA, mutation, occult hepatitis B infection, pregnant woman, Surabaya

Transmisi perinatal merupakan salah satu bentuk penularan infeksi virus hepatitis B yang paling banyak terjadi di negara-negara dengan tingkat endemisitas infeksi VHB yang tinggi. Anak-anak yang lahir dari ibu yang terinfeksi VHB memiliki risiko mengalami infeksi kronis sampai 90% melalui transmisi perinatal. Pemeriksaan serologi HBsAg telah direkomendasikan bagi ibu hamil untuk mencegah infeksi VHB perinatal, namun saat ini DNA VHB juga dapat ditemukan dalam serum dengan HBsAg negatif (OBI). Tujuan dari penelitian ini yaitu untuk mengetahui prevalensi OBI pada ibu hamil di Surabaya, Indonesia dan karakteristik dari VHB.Total 50 serum dengan HBsAg negatif dan anti-HBc positif dilakukan pemeriksaan anti-HBs dan HBeAg. DNA VHB yang didapat dari sampel dengan HBsAg negatif dan anti-HBc positif dianalisa menggunakan *polymerase chain reaction* (PCR) dan disekuensing. DNA VHB didapat sebesar 9 (18%) sampel berdasarkan sekuen dari gen-S. VHB/B3-*adw2* dilaporkan predominan pada 7 (77,7%) sampel, VHB/B9-*ayw1* pada 1 (11,1%) sampel, dan VHB/C7-*adrq+* pada 1 (11,1%) sampel. Tiga sampel memiliki mutasi (Q129H, T131N, M133S, T140I, T126I) pada regio determinan *'a'*, yang mungkin berperan menyebabkan virus tidak dapat dideteksi oleh kit HBsAg yang umum digunakan. Prevalensi OBI pada ibu hamil dari Surabaya tinggi, namun hal tersebut sesuai dengan prevalensi OBI pada populasi umum di Asia. Pemeriksaan anti-HBc atau DNA VHB sebagai skrining akan sangat bermanfaat untuk mencegah transmisi VHB dari ibu hamil dengan OBI.

Kata kunci: DNA VHB, ibu hamil, mutasi, *occult hepatitis B infection*, Surabaya

Hepatitis B Virus (HBV) infection is still a major worldwide health problem, including in Indonesia. Indonesia has a moderate to high endemic level of hepatitis B infection. The carrier rates among voluntary blood donors in Indonesia were reported between 2.1% to 9.5%, with the exception of Papua province (10.5%) (Khan *et al*. 2004, Sastrosowignjo *et al*. 1991). Transmission of hepatitis B infection occurs mainly during infancy and early childhood in hyperendemic areas, where chronicity is up to 90% (Nie *et al*. 2011). To prevent the transmission of HBV in Indonesia, the

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Ministry of Health launched a nationwide hepatitis B universal childhood vaccination program in 1997; the coverage (defined as the percentage of children receiving at least three doses of hepatitis B vaccine) in Indonesia in 2013 was estimated 75.6% (Ministry of Health Republic of Indonesia 2013).

Screening for HBV infection during pregnancy is the most effective way to identify newborns that require prophylaxis with hepatitis B vaccine and HBIG (hepatitis B immunoglobulin), as well as pregnant women who require antiviral therapy (CDC 2011). Babies of mothers who are HBsAg and HBeAg positive should be vaccinated. In most developed countries, determining HBsAg status has been routinely undertaken for mothers during prenatal visits or before delivery using serological methods (Zuckerman *et al*. 2007). In developing countries such as in Indonesia, however, there is no uniform policy for HBV testing during pregnancy in the health care facilities.

Some studies reported that HBV can be transmitted by blood donors, who are positive for anti-HBc but negative for HBsAg (Levicnik-Stezinar *et al*. 2008). Occult hepatitis B infection (OBI) is defined as the presence of HBV DNA in serum which is HBsAg negative (Raimondo *et al*. 2008). OBI has important clinical implications, including; reactivation from an immunosuppressive state, a possible speeding up of the progression of liver fibrosis, an increased risk of developing hepatocellular carcinoma with co-existing causes of liver damage, and possible HBV transmission during blood transfusion and organ transplantation (Schmeltzer *et al*. 2010). Failure of HBsAg detection may be due to e.g. a delay between HBV infection and appearance of HBsAg during the window period, the presence of HBsAg mutants, and/or the low level of viral load (Allain 2004).

There is little information about OBI in pregnant women, and there is no data about the prevalence of OBI among pregnant women, either in Surabaya or Indonesia. The aim of this study is to determine the prevalence of OBI and to analyze the genotype/subgenotype, subtype and mutation in the *'a'* determinant of the S region among pregnant women in Surabaya.

MATERIALS AND METHODS

Serum Samples. Sera were collected from 193 pregnant women who visited Perak Public Health Center, Surabaya, East Java, Indonesia between January-August 2011. Sera were stored at -20 °C until further use. HBsAg and anti-HBc positivity were 4.14% and 76.2%, respectively (Utsumi *et al.* unpublished data). Fifty HBsAg negative and anti-HBc positive samples were selected for this study. All subjects signed an informed consent form and participated voluntarily in this study. The study and protocol were reviewed and approved by the Ethics Committees of the Faculty of Medicine, Airlangga University and Kobe University, Japan.

Serologic Markers of HBV Infection. Samples were tested for the presence of HBsAg by Reversed Passive Hemagglutination (R-PHA), antibody to hepatitis B core antigen (anti-HBc antibody) by Passive Hemagglutination (PHA), anti-HBs antibody by PHA and HBV-e antigen (HBeAg) by EIA (Enzyme Immunoassay).

Detection of HBV DNA and Nucleotide Sequence Analysis. After being assayed for their HBV serologic status, serum samples which were HBsAg negative but anti-HBc positive (n=50) were subjected to HBV genetic analysis to confirm infection and identify surface antigen variants. DNA extraction and amplification were done as described previously (Utsumi *et al*. 2010).

Confirmation of HBsAg Status in HBV DNA-Positive Serum Samples. To minimize the number of false-negative results for HBsAg and to confirm occult hepatitis B infection, an enzyme-linked immunosorbent assay (ELISA) was used for samples that were HBsAg-negative and anti-HBc positive by R-PHA. HBV DNA positivity was confirmed 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 the Japanese/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 (Tamura *et al*. 2007).

RESULTS

A total of 50 samples which were HBsAg negative and anti-HBc positive, were screened for anti-HBs and HBeAg. Eleven (22%) of 50 samples had detectable anti-HBs and all samples were negative for HBeAg.

HBV DNA was detected in 9 (18%) of 50 samples. All HBV DNA-positive samples were negative for anti-HBs and HBeAg. Demographic and serologic data of pregnant women with HBV DNA are shown in Table 1.

Nine cases of occult hepatitis B infection were found in this study. Nucleotide sequence analysis of the surface gene showed that the HBV DNA isolated from a pregnant woman sample, PH-17, had three amino acid mutations, Q129H, T131N and M133S in the *'a'* determinant region. The HBV DNA isolated from PH-29 showed a T140I mutation, and the HBV DNA from PH-24 showed T126I and T143S mutations.

HBV genotype B (HBV/B) was identified in 8 of 9 (88.8%) samples and HBV/C was identified in 1 (11.1%) sample (Fig 1).

Fig 2 depicts the multiple alignment of amino acid sequences at positions 114 to 180 in HBsAg of the HBV isolates analyzed in this study and the 34 reference sequences of the nine HBV genotypes (A to I) obtained from the international DNA data bank. Based on the amino acid substitutions at positions 122, 127, 134 and 160, it was found that subtype *adw2* was predominant (77.7%), followed by *adrq+* (11.1%) and *ayw1* (11.1%). The isolates of *adw2* belong to HBV/B (subgenotype B3), *ayw1* belongs to HBV/B (subgenotype B9), and *adrq+* belongs to HBV/C (subgenotype C7). One isolate of HBV/C had alanine at position 159 (A159) and valine at position 177 (V177) in HBsAg. This combination is considered important for the expression of the *q* determinant (Norder *et al*. 1994). Therefore, this isolate was classified as subtype *adrq+*.

DISCUSSION

In developing countries such as Indonesia, there is no uniform policy for HBV testing during pregnancy. A significant proportion of births in Indonesia takes place at home, with limited access to trained maternity care providers. At community health centers (Puskesmas) and small hospitals, there is no HBsAg testing policy for pregnant women as yet. Recently, some studies reported that the currently available commercial assays may not recognize HBsAg in serum (Raimondo *et al*. 2010). Individuals who are HBsAg-negative but positive for HBV DNA in their serum are defined as having an occult hepatitis B infection (OBI) (Raimondo *et al*. 2010). Walz *et al*. (2009) reported that the incidence of HBV transmission from women (who are HBsAg-negative but anti-HBc positive) to their infants was 6.7%. The risk of perinatal transmission is similar to the risk of transmission associated with blood transfusion and organ transplantation (Walz *et al*. 2009).

The prevalence of OBI varies significantly between geographical regions (Hollinger and Sood 2010). In this study, HBV DNA was detected in 18% individuals who were HBsAg-negative and anti-HBc-

Table 1 Demographic, serologic, and virologic characteristics of HBV DNA-positive pregnant women in Surabaya

Sample	Age (years)	Sampling time of gestational period (month)	Ethnicity	Anti-HBs	Genotype/ Subgenotype	Subtype	Mutations in the *'a'* determinant
PH-9	20	4	Madura	Negative	B3	*adw2*	*wt
PH-17	35	6	Madura	Negative	B3	*adw2*	Q129H, T131N, M133S
PH-24	20	1	Madura	Negative	C7	*adrq+*	T126I, T143S
PH-29	20	7	Java	Negative	B3	*adw2*	T140I
PH-61	28	8	Java	Negative	B3	*adw2*	wt
PH-63	24	5	Java	Negative	B3	*adw2*	wt
PH-65	38	4	Java	Negative	B3	*adw2*	wt
PH-76	18	5	Java	Negative	B3	*adw2*	wt
2PH-33	22	9	Java	Negative	B9	*ayw1*	wt

*wt=wildtype

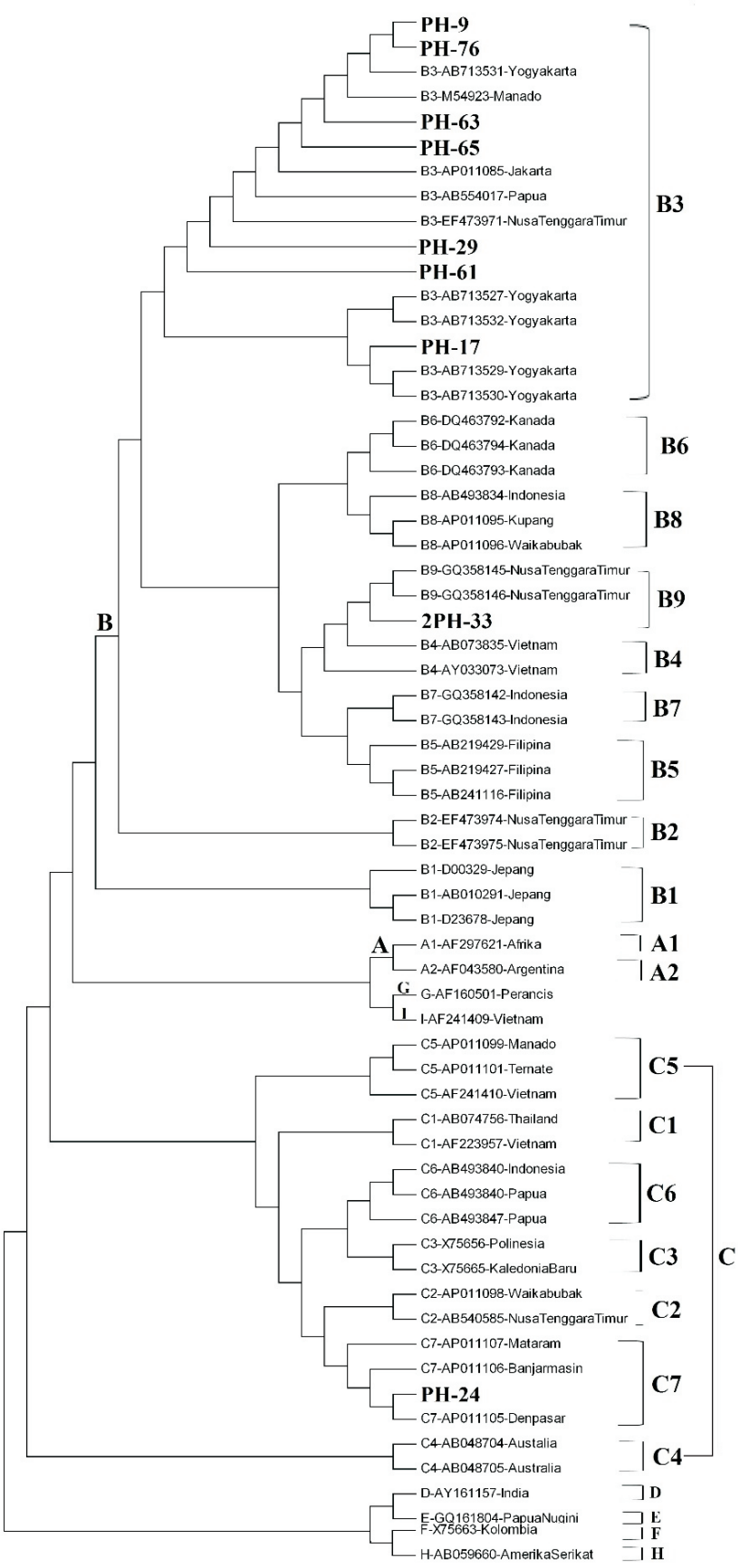

Fig 1 Phylogenetic tree of the surface gene in hepatitis B virus (HBV) strains isolated from 9 pregnant women in Surabaya, Indonesia, and 54 reference strains on the basis of partial S gene sequences (nt 494-694). Isolates from the database are indicated by their accession number, and relevant country names have been added to each HBV strains. The genotypes are indicated on the branches, and the subgenotypes are on the right.

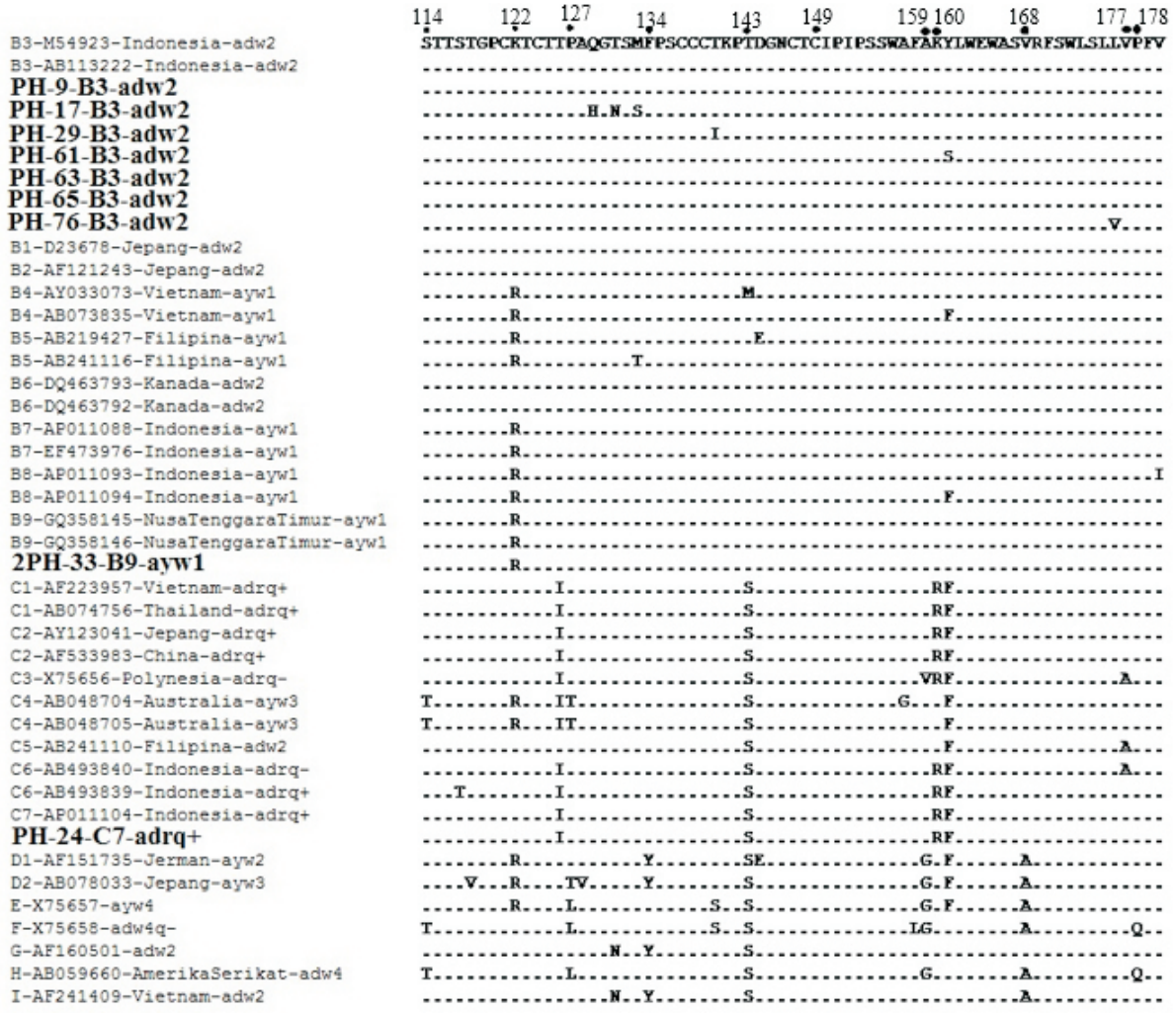

Fig 2 Multiple alignment of amino acid sequences of HBsAg (positions 114-180) and *'a'* determinant region (positions 121-149) of HBV isolates from pregnant women in Surabaya, Indonesia (code PH; shown in bold) and those from the international DNA data bank (indicated with accession numbers and countries of origin). Genotypes, subgenotypes, and subtypes are also indicated. • , residues that determine subtypes.

positive, suggesting OBI. This corresponds well with the 18.7% OBI prevalence in blood donors in Indonesia (Thedja *et al*. 2010).

HBV genotyping within the S gene sequence is, in general, consistent with genotyping of the full genomic sequence, and therefore, HBV genotypes can be assigned based upon S gene sequence (Mizokami *et al*. 1999; Norder *et al*. 1994; Okamoto *et al*. 1988). HBV genotype B was found predominant in this study. Eight (88.8%) out of nine HBV DNA positive samples were identified as genotype B and 11.1% as genotype C. Previously, Lusida *et al*. (2003) reported 54 HBV strains in Surabaya belonging to genotype B. Lusida *et al*. (2003) studies documented the distribution of HBV in Indonesia: genotype B was predominant in Java; Surabaya is located on Java (Lusida *et al*. 2003).

Seven of the eight HBV/B isolates were classified as subgenotype B3 (HBV/B3), which is prevalent in Indonesia. Interestingly, one isolate isolated from an individual with Javanese ethnicity was classified as subgenotype HBV/B9, which was reported to be the specific subgenotype among Malayu-Polynesians in East Nusa Tenggara (Thedja *et al*. 2011). The HBV/C isolate classified as subgenotype HBV/C7. HBV/C-*adrq+* has been found uniquely among Papuans in the easternmost part of Indonesia. However, in this study, the HBV/C-*adrq+* was isolated from a Maduranese in Surabaya. Further study to find more samples with an HBV infection among Maduranese is needed to elucidate the issue. Further analysis of the entire genome of the HBV isolates for subgenotype determination is required and currently underway in our laboratory.

Amino acid substitutions in the HBV S gene, especially in the *'a'* determinant region (amino acids 121-149) have been described in vaccinated children

and patients treated with hepatitis B immunoglobulin. Many studies pointed out that such substitution could affect the antigenicity of HBsAg, resulting in the loss of recognition by antibodies (Fuji *et al*. 1992). In this study, the most commonly found mutations G145R, K141R and T131I (Seddigh-Tonekaboni *et al*. 2000) were not detected. Utsumi *et al*. (2010) reported that T1261I frequently appeared in HBV/B only and that it was not specific to genotype C among school children in East Java (Utsumi *et al*. 2010). Variants T123A, M133L, and T143M were found in the *'a'* determinant in OBI samples of Indonesian blood donors (Thedja *et al*. 2010).

Three isolates (33.3%) have amino acid mutations within *'a'* determinant T126I, which was associated with T143S (HBV/C7) in one isolate, T140I (HBV/B3) in a second, and Q129H, T131N, M133S (HBV/B3) in the third. All isolates were HBsAg-negative, anti-HBc-positive, anti-HBs-negative and HBeAg-negative. This suggests that a mutation in the *'a'* determinant is more frequent in HBsAg-negative and anti-HBc positive samples. This results corroborates a study by Thedja MD *et al*. (2010) with OBI in blood donors. Ren *et al*. (2006) have reported amino acid substitution T126I, which is unique to genotype C, and which may cause structural changes in HBsAg. This T126I substitution in the first loop may be more important than the T143S in the second loop, which is not unique to genotype C. The large difference in chemical properties means that the T126I substitution may have a major impact on the antigenicity of HBsAg. A T131N substitution, reported by Asahina *et al*. (1996), was associated with acute exacerbations of chronic hepatitis B (CHB). Q129H has been reported as a variant that caused diagnostic failure and as a vaccine escape mutant in HBIG therapy (Jolivet-Reynaud *et al*. 2001). The prevalence of diagnostic escape mutants among anti-HBc positive individuals varies between 0.7 and 1% (Alhababi *et al*. 2003; Weber *et al*. 2001). Further protein model prediction, based on these amino acid substitution patterns, might explain the conformational changes of HBsAg; assays to confirm the reduction in binding affinity of the altered epitopes to monoclonal anti-HBs have been suggested (Thedja *et al*. 2010).

In this study, 6 of 9 (66.7%) pregnant women with OBI had HBV DNA with the wild-type S gene. The detection of wild-type HBV strains in OBI cases implies that other characteristics, beside antigenic properties, may be involved (Schmeltzer and Sherman 2010). A low viral load would provide an alternative explanation for the undetectability of wild-type HBV by the HBsAg serological assay (Thedja *et al*. 2010). This is supported by the fact that HBV DNA in these samples was detectable only in the second-round of nested PCR. The finding of ccc-DNA, RNA transcripts, and pregenomic replicative RNA intermediates in the liver, peripheral blood mononuclear cells (PBMC) and/or blood of a large proportion of infected patients suggest that most occult infections are caused by low-level replication of wild-type virus (Raimondo *et al*. 2013). The reasons why low levels HBV DNA persist in the absence of detectable HBsAg, remain largely undefined; however, it is suggested that both host and viral factors are important in suppressing viral replication and keeping the infection under control (Hollinger and Sood 2010).

Once HBV infection occurs, even as an OBI, it can be transmitted, and it is not easy to prevent vertical transmission to the offspring during childbirth, even by the use of the most potent anti-HBV drugs presently available. Thus, the only chance to win the war against HBV is to perform neonatal vaccination, especially to those born to infected mothers. Studies involving babies born to mothers with OBI and involving more samples from various regions in Indonesia are important to investigate the risk of vertical transmission from pregnant women with OBI to their newborn baby. In conclusion, occult hepatitis B infection (OBI) was detected among pregnant women in Surabaya, East Java with 18% prevalence. Amino acid mutations in the *'a'* determinant region were found in several samples; these mutations may alter HBsAg antigenic properties. Anti-HBc testing in pregnant women is recommended, in addition to HBsAg testing, especially in a highly endemic country such as Indonesia.

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