Table V. Mutations of BCP, PC and core according to the HBV DNA level in anti-HBe(+) samples.

	HBV DNA level	
Mutations of HBeAg	Low [<5 log copies/ml (%)]	High [≥5 log copies/ml (%)]
BCP T1762/A1764	3/7 (42.9)	4/7 (57.1)
PC A1896	7/14 (50)	7/14 (50)
PC A1899 + A1896	2/3 (66.7)	1/3 (33.3)

BCP, basal core promoter; PC, precore; HBV, hepatitis B virus; (+), positive.

In the PC region, the predominant mutation is a G-to-A change at nt 1,896 (A1896), which creates a premature stop codon and eliminates the synthesis of HBeAg (29). A previous study demonstrated that HBeAg may be a target antigen on HBV-infected hepatocytes (30) and failure to produce a target antigen may be a means of evading immune clearance. It was reported that there is a significant association between PC mutations and remission of liver disease (31). However, other studies reported a high prevalence of PC mutations in patients with severe liver disease (32-34). The results of this study have demonstrated that in the PC region, the A1896 mutant was predominant in patients with HCC (100%) and those with LC (60%), although it was also found in UC patients (60%) (Table I). The variability in the prevalence of the A1896 mutant in different geographical regions is associated with the predominant HBV genotype, since this mutant is restricted to the HBV genotypes with T at nt 1,858 and is not encountered in those with C at nt 1,858 (13,35). The A1896 and T1858 tighten the stem structure by forming a T-A pair (12), although T1858 may also form a wobble pairing with G1896 (11). The PC A1896 is found only in patients infected with HBV genotypes B, D, E and in a minority of those infected with the C and F strains that bear a variant T at nt 1,858 (36,37). In accordance with previous studies (23,38), this study has demonstrated that HBV genotype B was predominant, detected in all 24 serum samples collected mainly from individuals of Java origin (88%), as well as of Sulawesi, Aceh-Batak and Flores origin (4% each) who resided in Surabaya. The HBV genotypes also appear to be associated with ethnic origin. However, the association between the A1896 in the PC region and other genotypes could not be elucidated in this study. As regards clinical outcomes, our results were in accordance with those previously reported (39), stating that A1896 alone may have no direct pathogenic role, particularly in HBV isolates with genotypes harbouring T1858. The other PC mutation, A1899, was detected in three serum samples combined with A1896. These combined mutations may enhance the stability of the stem loop which is essential for viral replication (40). However, in this study, in two out of the three samples, low HBV DNA levels were detected (Table V). Additional studies are required to confirm the significance of these mutations on viral replication.

In the BCP region, the most common mutations involve a two-nt substitution: A-T at nt 1,762 and G-A at nt 1,764 (T1762 and A1764) (17). Previous transfection studies demonstrated that the T1762 and A1764 mutations decrease the level of pre-C mRNA by 50-70% and lead to reduced HBeAg synthesis (14,41). The BCP mutants may enhance HBV virulence by increasing host immune response to HBV-infected hepatocytes, increasing viral replication or altering the coding region for the X (14,15,42,43). The BCP T1762/A1764 were found in patients of the HCC (25%) and UC (20%) groups, although the majority was encountered in the LC group (50-60%) (Table I). This finding was in agreement with a previous study which reported that BCP mutations were found mainly in cirrhotic tissues with a lower incidence in HCC tissues (44). Of note, the majority of isolates (55.5%, 5/9) with BCP T1762/A1764 were not accompanied by PC A1896. It was suggested that BCP mutations frequently emerge during the late HBeAg phase of infection, whereas PC mutations usually emerge later, at the height of the anti-HBe immune response (9). In this study, the two HBeAg-positive serum samples with BCP mutations exhibited no concomitant PC mutation; however, four out of the seven anti-HBe-positive samples with BCP mutations also exhibited PC mutations.

Mutant HBV may also exhibit enhanced virulence with alteration of epitopes which is critical for the host immune response. Mutations in B- and T-cell epitopes are associated with viral persistence, affecting the host immune response (45-47). The inflammatory activity produced by viral adaptive mechanisms may persist in up to 15% of cases, leading to the development of cirrhosis (48). A previous study by Hosono *et al* (20) suggested that core mutations in HBV accumulated more errors in tumors compared to non-tumors. The aa 120-140 in the core region exposed on the surface of mature HBeAg and HBcAg are related to the recognition of helper T cells (49-51) and the immunodominant B-cell recognition sites within the HBcAg have also been found around residues 126-135 (45,46). One of the frequent core mutations is at aa 130, predominated by Thr130 [67 out of 96 (70%)]. It was noted that Thr130 was frequently associated with the occurrence of Leu97 [50 out of 67 (75%)], although it may occur *per se* [17 out of 67 (25%)] (52). The Ser130 mutation was also reported to affect cellular and humoral immunity, since the codon is part of a domain which is recognized by B and T cells (53). The Leu97 of HBcAg was reported to enable the virus to secrete an excessive amount of immature genome with nascent incomplete single-strand DNA in an envelope-dependent manner, leading to attenuation of the total yield of mutant virus production (52,54,55). However, this excessive secretion of incomplete virions may be offset by an additional mutation at codon 130 (Pro to Thr/Ser) (52,53). In

this study, the Thr/Ser130 mainly occurred in the HCC group (2/4, 50%), followed by the LC (30%) and UC (10%) groups. The Thr/Ser130 was mostly detected in combination with Leu97 (Table I).

Certain combined mutations in the BCP, PC and core regions are of higher clinical significance regarding the severity of liver disease. In this study, patients with LC and HCC carried HBV with mutations of at least BCP or PC. Some patients with UC also carried the HBV mutation; however, more mutations were likely to be associated with LC and HCC (Table II). The combination of mutations rather than a single mutation was associated with the development of progressive liver disease (56).

The most frequently encountered variant of chronic HBV infections is HBeAg-negative chronic hepatitis B. It may follow seroconversion from HBeAg to anti-HBe antibodies during the immune reactive phase or develop after years or decades of inactive carrier state. The patients are HBeAg-negative and harbour a predominance of HBV virions with nt substitutions in the PC and/or the BCP regions that lead to absent or low expression levels of HBeAg (5,57). HBeAg-negative mutants frequently become the predominant virus population in chronic HBsAg carriers, possibly indicating a selection advantage against the wild-type (10,17). This study has demonstrated that HBeAg-negative/anti-HBe -positive was the predominant type (71%) in all the groups, particularly in the HCC group (100%) (Table III). Following seroconversion, PC A1896 was predominant and the number of the BCP T1762/A1764 mutations was increased (Table IV). In the majority of anti-HBe-positive serum samples, the BCP T1762/A1764 was mostly correlated with a high viral load ($\geq 10^5$ copies/ml), but PC A1896 was not correlated with viral load level (Table V). This finding was in agreement with previously reported results (58), suggesting that patients with the BCP T1762/A1764 mutant exhibited significantly higher serum HBV DNA levels compared to those with the BCP A1762/G1764 wild-type strain, regardless of the PC 1896 status. The BCP mutations were found in the dominant viral species at the late HBeAg-positive and early anti-HBe phases of HBV infection. Thus, chronic hepatitis B patients infected with the T1762 and A1764 mutants may have a longer duration of active replication (58). The number of the core mutation Thr130 was lower following seroconversion compared with prior to seroconversion (Table IV). The duration of seroconversion from HBeAg to anti-HBe may be attributed to the extent of immunological attacks against the HBV core region.

Of note, two serum samples collected from one patient of the UC (7RS) and one of the LC group (17RS) were HBeAg-positive in the presence of the PC A1896 mutation. This observation may be due to the presence of a mixed infection by the mutant- and wild-type viruses. The presence of the wild-type virus is required by certain mutants for the infection of hepatocytes. It is likely that HBV exists as a quasi species of wild-type and mutant clones, even in the HBeAg-positive phase (7). To determine any mixed infection (59), the two samples were examined by the INNO-LiPA assay and it was confirmed that they had mixed strains of mutant and wild-type PC. A heterogeneous virus population circulating in patients with chronic HBV infection may thus determine the outcome of infection.

In conclusion, the PC mutation A1896 was predominant in all the groups and the BCP mutations T1762/A1764 were only predominant in patients with chronic hepatitis B and LC. The BCP mutations may be considered as a more efficient indicator of a poor outcome compared to the PC mutations. Additional studies are required, including a larger population, to determine which type of specific mutations or combined mutations is associated with liver disease severity and may thus be involved in the pathogenetic process.

Acknowledgements

The authors are grateful to Slamet Riyadi, Nur Achmad Tjipto and Nasronudin for their cooperation and to Mochamad Amin and Koen Poedijati for their technical assistance. We are also grateful to the patients at Dr Soetomo General Hospital, Surabaya and the blood donors at the Blood Transfusion Unit-Indonesia Red Cross, Surabaya, who provided the blood samples for this study. This study was supported by a grant from the Directorate General of Higher Education, Department of National Education, Indonesia and by the Japan Initiative for Global Research Network on Infectious Diseases, the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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