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A cautionary note to hepatitis B *e* antigen (HBeAg)-negative test results in pregnant women in an area prevalent of HBeAg-negative chronic hepatitis B

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Abstract

Maternal hepatitis B *e* Antigen (HBeAg) positivity poses a risk for hepatitis B virus (HBV) mother-to-child transmission (MTCT). In resource-constrained settings, HBeAg testing is recommended as an alternative to HBV DNA testing to establish antiviral prophylaxis eligibility. Nevertheless, the high prevalence of HBeAg-negative chronic hepatitis B (e-CHB) in many countries should not be overlooked. We studied HBV characteristics and explored the potential MTCT risk among HBeAg-negative/HBsAg-positive expectant mothers in an area prevalent of e-CHB. Among 1348 pregnant mothers screened for HBV infection, 81 (6.0%) were HBsAg-positive. These women were examined for HBeAg, HBV DNA, and cord blood HBV DNA. Sixteen (19.8%) of the HBsAg-positive mothers were HBeAg-positive, whereas 65 (80.2%) were HBeAg-negative, including eight inactive carriers (HBsAg <100 IU/ml, HBV DNA ≤ 2000 IU/ml, and ALT < 40 IU/L). Of the remaining 57 HBeAg-negative mothers, ten revealed HBV Basal Core Promoter or Precore mutations, with three having high viremia (HBV DNA > 200 000 IU/mL), which is associated with a high MTCT risk and therefore qualifies them for antiviral prophylaxis. This pilot study provides a cautionary note to the interpretation of negative HBeAg test results when determining eligibility for MTCT antiviral prophylaxis in situations with limited resources and in regions where e-CHB is prevalent.

KEYWORDS

hepatitis B virus, mutation, vertical transmission

Abbreviations: "a" determinant, antigenic determinant; AC, active carrier; ALT, alanine aminotransferase; anti-HBe, antibody to hepatitis B se antigen; anti-HBs, antibody to hepatitis B surface antigen; BCP, basal core promoter; CHB, chronic hepatitis B; DNA, deoxyribonucleic acid; e-CHB, HBeAg-negative chronic hepatitis B; GPs-CHB Study Group, Good Practice in using HBsAg in Chronic Hepatitis B Study Group; HBeAg, hepatitis B e antigen; HBIG, hepatitis B immune globulin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; IC, inactive carrier; IT, Immune-tolerant; LR, low-replicative; MTCT, mother-to-child transmission; OR, odds ratio; PC, precore; PCR, polymerized chain reaction; RT-PCR, real-time polymerized chain reaction; STROBE, Strengthening the Reporting of Observational Studies in Epidemiology; WHO, World Health Organization.

1 | INTRODUCTION

A significant proportion of the world population has been infected by hepatitis B virus (HBV). In 2019, the World Health Organization (WHO) estimated that 296 million people lived with chronic hepatitis B (CHB) with approximately 820 000 deaths, mostly from cirrhosis and hepatocellular carcinoma.[1] More than half of these chronically infected people live in the Asia Pacific including Indonesia, which has hepatitis B surface antigen (HBsAg) prevalence of 7.1%.[2] The majority of people with HBV infection are infected perinatally or during early childhood by mother-to-child transmission (MTCT). Infection in this period is associated with a higher risk (>90%) of chronicity and is a key element in maintaining the reservoir of the infection, particularly in some endemic regions.[3] The risk of MTCT is increased in women who have high HBV DNA levels, particularly those who are positive for hepatitis B *e* antigen (HBeAg), despite vaccine and hepatitis B immunoglobulin (HBIG) prophylaxis.[4]

The recent WHO 2020 guidelines recommend pregnant women testing positive for HBsAg with a high HBV DNA viral load (≥200 000 IU/ml or ≥10^5 log_{10} IU/ml) receive antiviral (tenofovir) prophylaxis to prevent MTCT of HBV, in addition to the mandatory birth dose and three dose hepatitis B vaccination in all infants. In situations where HBV DNA testing is not available, HBeAg can be used as an alternative to determining eligibility for this peripartum prophylaxis, thus reducing the incidence of MTCT in low-income settings.[5]

The disease spectrum and natural history of chronic hepatitis B (CHB) are diverse and variable. It is recognized HBeAg as a reliable marker of HBV replication and infectivity. After acquiring the infection perinatally, CHB patients enter the immune-tolerant (IT) phase in the initial few decades of life, with high levels of HBV DNA and positive HBeAg, but with minimal hepatic damage. In most individuals, successful immune clearance will lead to the low-replicative (LR) phase, also known as the "inactive carrier" (IC) phase, which is characterized by seroconversion from HBeAg-positive to HBeAg-negative, with or without the appearance of antibody to HBeAg (anti-HBe), suppression of HBV DNA, and normalization of alanine aminotransferase (ALT) levels. This phase confers a good prognosis with survival rates comparable to noninfected populations.[3,6–8] Among some patients, for a yet uncertain reason, the immune pressure associated with seroconversion selects for HBV variants that express little or no HBeAg. Most of these patients harbor HBV variants in the precore (PC) and/or the basal core promoter (BCP) regions that impair or abolish HBeAg production. This phase, referred to as HBeAg-negative chronic hepatitis B (e-CHB), has continuing active HBV DNA replication with various grades of liver diseases manifested by variable ALT levels, with or without clinical and histological evidence of cirrhosis.[6–8]

Currently, e-CHB has become the most common type of CHB with marked variations in the prevalence of associated HBV variants across different geographical regions, including Southeast Asia.[7,9] The risk of HBV MTCT among HBsAg-positive/HBeAg-negative women has been quoted in the literature as 5%–30% in Asia and 4.8% in sub-Saharan Africa in the absence of prophylaxis measures.[9,10] Meanwhile, there is a scarcity of data from population-based studies, which may preclude definitive conclusions on the true prevalence and risk of HBV perinatal transmission, particularly in areas where e-CHB is prevalent.[11–13] Hence, we performed this pilot study to investigate HBV DNA characteristics in HBsAg-positive/HBeAg-negative pregnant women and to explore the potential risk of HBV MTCT in an area with high prevalence of e-CHB.

2 | METHODS

2.1 | Study population

This study was part of longitudinal research on HBV infection among pregnant women and the outcome of hepatitis B immunoprophylaxis in infants born to HBsAg-positive mothers in Indonesia. Within the framework of this project, which is anticipated to be completed by the middle of 2022, we carried out this cross-sectional study with the main emphasis on the HBV viral load and the characteristics of HBV DNA in HBeAg-negative pregnant mothers.

From December 2017 to December 2019, 1348 pregnant women who attended antenatal care units at Hasanuddin University Hospital, Wahidin Sudirohusodo General Hospital, Khadijah Mother and Child Hospital (MCH), Fatimah MCH, Pertiwi MCH, and several other maternity clinics in Makassar, Indonesia, were screened for HBV infection and counseled by trained doctors and midwives. Of these women, 81 (6.0%) were HBsAg-positive and included in this study. The inclusion criteria were HBsAg-positive for more than 6 months without prior antiviral medication and consent to participate in this study. The exclusion criteria included pregnancy complications, co-infection with hepatitis A virus, hepatitis C virus, or HIV, and severe hepatitis or liver cirrhosis. Before delivery, maternal serum samples were collected, divided into several aliquots, and stored at −80°C until use. Soon after birth, the umbilical cord blood was collected aseptically and transferred into a blood collection tube, then stored at −80°C after centrifugation.

All participants gave written informed consent according to the Declaration of Helsinki. This study was approved by the Research Ethics Committees of Hasanuddin University, Faculty of Medicine, Wahidin Sudirohusodo Hospital, and Hasanuddin University Hospital (Authorization No. 0942/H4.8.4.5.31/PP36-KOMETIK/2017), and adhered to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline for observational studies.[14]

2.2 | Serological examination

Serum samples were tested for HBsAg with VIDAS HBsAg Ultra (BioMérieux SA), while HBeAg and anti-HBe status were determined using Monolisa™ HBeAg Ag–Ab PLUS (Biorad). HBsAg titer was measured with Elecsys HBsAg Quant II immunoassay (Roche Diagnostics) using Roche Cobas® e411 Immunoanalyzer.

2.3 | HBV DNA detection and analysis

HBV DNA level in maternal sera and corresponding cord blood was determined from 500 µl of serum using quantitative real-time polymerase chain reaction (RT-PCR; CobasTaqman™ HBV Test, Roche Diagnostics) with a linearity range of 6–1.1 × 10^8 IU/ml. For molecular analysis, HBV DNA was extracted from 140 µl of serum, then eluted in 60 µl of elution buffer (QIAamp DNA Minikit, Qiagen). The DNA fragment containing the "a determinant" encoding region of HBV S gene was amplified by nested PCR using two specific primer sets: S2-1 (nt 455–474; 5′-CAAGGTATGTTGCCCGTTTG-3′) and S1-2 (nt 704–685; 5′-CGAACCACTGAACAAATGGC-3′) as outer primers, and S88 (nt 462–481; 5′-TGTTGCCCGTTTGTCCTCTA-3′) and S2-2 (nt 687–668; 5′-GGCACTAGTAAACTGAGCCA-3′) as inner primers.[15] PCR products were purified using PCR purification column (QIAGEN) and subjected to direct sequencing reaction on DNA sequence analyzer ABI 3130xl (Applied Biosystems).

HBV genotype was determined by phylogenetic analysis of S gene nucleotide sequences, compared with 70 reference sequences of known genotypes (A–J) retrieved from GenBank. Phylip 3.68 software with Kimura-2 parameter, neighbor-joining algorithm, and 1000 bootstrapping was used in this analysis.

2.4 | Determination of HBV basal core promoter and precore mutants

HBV DNA fragments containing basal core promoter (BCP) and precore (PC) regions were amplified by nested PCR with two specific primer sets PC1 (nt 1653–1672; 5′-CATAAGAGGACTCTTGGACT-3′) and PC2 (nt 1949–1972; 5′-AAAGAAGTCAGAAGGCAAAAAAGA-3′) as outer primers, and S012 (nt 1679–1699; 5′-AATGTCACCGACCGACCTTG-3′) and S013 (nt 1919–1941; 5′-TCCACAGAAGCTCCAAATTCTAA-3′) as inner primers. Successfully amplified products were purified and sequenced as previously described.[15] A sequence comparison against a wild-type reference sequence from Indonesia (GenBank Accession No. M54923) was performed to identify BCP and/or PC mutations. HBV nucleotide numberings were based on the start of the *EcoRI* restriction site within the HBV genome.

2.5 | Definitions of HBV infection phases among HBeAg-negative pregnant women

According to the Good Practice in using HBsAg in Chronic Hepatitis B Study Group (GPs-CHB Study Group), HBV infection activity among HBeAg-negative pregnant women was classified into IC, intermediate carrier, or active carrier (AC) based on biochemical and virological profiles.[16] IC was defined as having HBsAg < 100 IU/ml, HBV DNA ≤ 2000 IU/ml, and ALT < 40 IU/L.[7,8] Subjects with HBsAg 100–1000 IU/ml, HBV DNA ≤ 2000 IU/ml, and ALT < 40 IU/L were classified as intermediate carriers (or HBV remission), whereas those with HBsAg > 1000 or HBV DNA > 2000 IU/ml and any ALT levels were classified as ACs. This classification was used to differentiate between subjects in the IC state from those who may be in remission but should be grouped with the AC since HBV activity may still be present.

2.6 | Statistical analysis

Clinical and laboratory data were extracted from medical records at respective antenatal clinics and entered into a database specifically designed for this study in an anonymous way. Baseline data were descriptively summarized, and the differences of each variable between groups were calculated using one-way analysis of variance (ANOVA) and Tukey's HSD post hoc test. Continuous and categorical variables were compared between groups using the Mann–Whitney test and Chi-square/Fisher's exact test, respectively. Risk factors associated with HBV transmission were analyzed, and outcomes were reported as odds ratios (OR) with 95% confidence intervals (CI). Significant values were determined at $p < 0.05$. All statistical analyses were two-sided and performed using the Statistical Program for Social Sciences (IBM SPSS 22).

3 | RESULTS

Of 81 HBsAg-positive pregnant women, 16 (19.8%) were HBeAg-positive and 65 (80.2%) were HBeAg-negative (Figure 1). ALT, HBsAg, and HBV DNA levels were significantly higher in HBeAg-positive than in HBeAg-negative subjects ($p < 0.001$). The median HBsAg and HBV DNA levels were higher in HBeAg-positive subjects (3.99 [range 2.41–4.91] $\log_{10}$ IU/ml and 7.43 [range 1.54–8.46] $\log_{10}$ IU/ml, respectively) than in HBeAg-negative subjects (2.81 [0.7–4.23] $\log_{10}$ IU/ml and 1.7 [0.78–6.5] $\log_{10}$ IU/ml, respectively). The proportion of HBV DNA ≥ 5.3 $\log_{10}$ IU/ml (levels associated with high risk for MTCT) was higher in HBeAg-positive than in HBeAg-negative subjects (75.0% vs. 4.6%, $p < 0.001$).[5,17] After delivery, HBV DNA detection in cord blood was higher in the former than in the latter (43.8% vs. 10.8%, $p = 0.005$). Distributions of age, HBV genotype, and method of delivery were comparable between the two groups (Table 1).

In the HBeAg-negative group, eight (12.3%) subjects were determined as ICs, 23 (35.4%) as intermediate carriers, and 34 (52.3%) as ACs, as shown in Figure 1 and Table 2.[16] Thus, 57 (87.7%) mothers did not fit within the IC category and were regarded as subjects as having HBV activity and were further examined for the possibility of carrying mutants unable to produce HBeAg.

Sequencing of BCP and PC regions of HBV was successfully performed in 12 of the 57 subjects. The sequences were deposited at GenBank (accession numbers MW039565-MW039571, and MW048090-MW04809494). Ten subjects carried HBV variants with the following mutations: five with BCP (T1753A/C and/or A1762T/G1764A) mutations, three with PC (A1814C or G1896A) mutations,

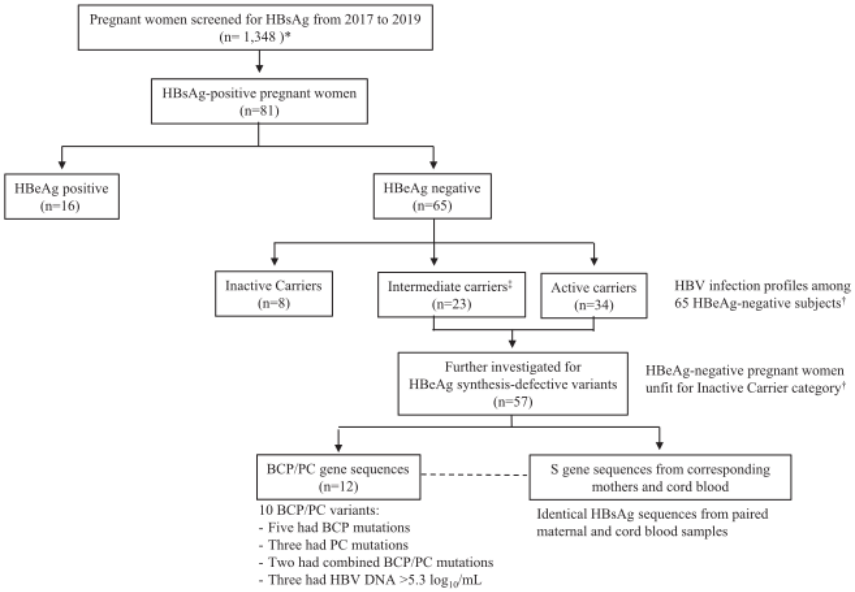

FIGURE 1 Flowchart of study and findings. *This study is part of a nationwide research project on the outcome of universal infant HBV prophylaxis in Indonesia. †Based on the Good Practice in using HBsAg in Chronic Hepatitis B Study Group (GPs-CHB Study Group) criteria.[16] ‡Also referred to as "HBV remission." BCP, basal core promoter; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; PC, precore.

and two with combined BCP and PC mutations (Figure 2). All BCP mutations, either alone or in combination with PC mutation, were observed in samples with HBV genotype C only. Among subjects with BCP and/or PC mutations, HBsAg levels ranged from 2.507 to 4.234 $\log_{10}$ IU/ml and HBV DNA levels ranged from 0.778 to 6.481 $\log_{10}$ IU/ml, with three subjects having HBV DNA levels >5.3 $\log_{10}$ IU/ml.

Further investigation was performed to characterize HBV nucleotide sequences from maternal and cord blood samples. S gene sequences encoding HBsAg from seven paired maternal and cord blood samples were generated and deposited in GenBank (accession No. MW039551-MW039564). The genotypes of all seven mother and cord blood HBV sequence pairs were identical, with five having genotype C and two having genotype B. As shown in Figure 3, near-identical sequences were observed within five pairs of deduced HBsAg sequences (MS/CB 177, 253, 384 with C genotype; and MS/CB 167 and 898 with B genotype). Clinical data, serological profile, and virological characteristics of the 12 subjects are shown in Table 3.

Overall, 80.2% (65/81) of HBsAg-positive pregnant women were HBeAg-positive and 87.7% (57/65) were considered to have HBV activity, including some with BCP or PC mutations and a high viral load associated with MTCT risk.

4 | DISCUSSION

This study revealed HBsAg prevalence of 6.0% among pregnant women attending antenatal clinics in South Sulawesi Province. This figure was slightly lower than the national HBsAg prevalence (7.1%) and that in the same province (7.6%) in 2013.[2] Of these women, 16 (19.8%) were HBeAg-positive and 65 (80.2%) were HBeAg-negative.

In many parts of the world, e-CHB has become the most prevalent form of disease in recent decades.[18] The geographical variation in the prevalence of e-CHB has been linked to the distribution of HBV genotypes in addition to differences in the age of infected subjects and the natural history of chronic HBV infection, as was previously expected.[12,13,19] Individuals infected with certain HBV genotypes (e.g., genotypes A and B) tend to undergo earlier HBeAg seroconversion than those infected with some other genotypes (e.g., genotypes C and D).[20] In southeast Asia, where HBV genotype C is predominant, the IT phase is longer and HBeAg, with seroconversion occurring at the later age of 30-35 years.[13] However, the 80.2% prevalence of HBeAg negativity at relatively younger ages in our cohort is higher than the 30% HBeAg-negative prevalence reported in other Southeast Asian countries.[13]

In the natural history of CHB, the differential diagnosis between IC and e-CHB phases is problematic because e-CHB is characterized by wide fluctuations in biochemical activity with variation in serum HBV-DNA and HBsAg levels.[21] According to the Asian-Pacific, European, and American guidelines on the management of HBV infection, LR or inactive HBeAg-negative infection is characterized by low (<2000 IU/ml) or undetectable HBV DNA levels with persistently normal ALT. However, HBeAg-negative-CHB is associated with HBV DNA fluctuations followed by long-term remissions, with levels that may decrease transiently to <2000 IU/ml, and exacerbations of infection leading to liver deterioration.[6–8] Meanwhile, ALT level is not a specific marker of viral-induced liver damage; the fluctuations in an

TABLE 1 Characteristics of HBsAg-positive pregnant women according to HBeAg status

Parameter	Overall (*n* = 81)	HBeAg status		
		Positive (*n* = 16)	Negative (*n* = 65)	*p* value*
Age (years)	30 (18–42)	28.50 (22–42)	30 (18–41)	0.646
ALT	24 (10–88)	38.50 (18–77)	23 (10–88)	<0.001
≥40 IU/L, *n* (%)	15 (18.5)	8 (50)	7 (10.8)	<0.001
<40 IU/L, *n* (%)	66 (81.5)	8 (50)	58 (89.2)	
HBsAg level[a]	3.04 (0.7– 4.91)	3.99 (2.41–4.91)	2.81 (0.7–4.23)	<0.001
>1000 (IU/ml), *n* (%)	41 (50.5)	15 (93.9)	26 (40.0)	0.001
100–1000 (IU/ml), *n* (%)	31 (38.3)	1 (6.3)	30 (46.2)	
<100 (IU/ml), *n* (%)	9 (11.1)	0 (0.0)	9 (13.8)	
HBV DNA level	2.18 (0.78–8.46)	7.43 (1.54–8.46)	1.7 (0.78–6.5)	<0.001
Viremia level[a]				
>2000 IU/ml, *n* (%)	58 (71.6)	2 (12.5)	56 (86.2)	<0.001
≤2000 IU/ml, *n* (%)	23 (28.4)	14 (87.5)	9 (13.8)	
MTCT-risk-associated HBV DNA level[b]				
≥200 000 IU/ml (5.3 $\log_{10}$ IU/ml), *n* (%)	14 (17.3)	12 (75.0)	3 (4.6)	<0.001
<200 000 IU/ml (5.3 $\log_{10}$ IU/ml), *n* (%)	67 (82.7)	4 (25.0)	62 (95.4)	
HBV Genotype)				
B, *n* (%)	16 (19.8)	4 (25)	12 (18.5)	0.820
C, *n* (%)	47 (58.0)	9 (56.3)	38 (58.5)	
Undetermined	18 (22.2)	3 (18.8)	15 (23.1)	
HBV DNA in cord blood, *n* (%)				
Positive, *n* (%)	14 (17.3)	7 (43.8)	7 (10.8)	0.005
Negative, *n* (%)	67 (82.7)	9 (56.3)	58 (89.2)	
Method of delivery, *n* (%)				
Vaginal, *n* (%)	61 (75.3)	12 (75)	49 (75.4)	0.602
Cesarean, *n* (%)	20 (24.7)	4 (25)	16 (24.6)	

Note: Data are presented as median (range) or number (percentage).

Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; CHB, chronic hepatitis B; HBeAg, hepatitis B *e* antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; MTCT, mother-to-child transmission; WHO, World Health Organization.

*ANOVA or Chi-square/Fisher exact test.

[a]Classified according to the Good Practice in using HBsAg in Chronic Hepatitis B Study Group (GPs-CHB Study Group) criteria to define phases of infection in HBeAg-negative patients (described in Table 2).[16]

[b]Classified according to Prevention of mother-to-child transmission of hepatitis B virus: guidelines on antiviral prophylaxis in pregnancy.[5]

TABLE 2 Phases of HBV infection among HBeAg-negative pregnant women (*N* = 65)

Phenotype	Biochemical and HBV virological profile[a]	*n* (%)
Inactive carriers	HBsAg <100 IU/ml and HBV DNA ≤ 2000 IU/ml and ALT < 40 IU/L	8 (12.3)
Intermediate carriers[b]	HBsAg 100–1000 IU/ml and HBV DNA ≤ 2000 IU/ml and ALT < 40 IU/L	23 (35.4)
Active carriers	HBsAg >1000 IU/ml or HBV DNA > 2000 IU/ml and any ALT levels	34 (52.3)

Abbreviations: ALT, alanine aminotransferase; HBeAg, hepatitis B *e* antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

[a]Based on the Good Practice in using HBsAg in Chronic Hepatitis B Study Group (GPs-CHB Study Group) criteria to define phases of infection in HBeAg-negative patients.[16]

[b]Also referred to as "HBV remission."

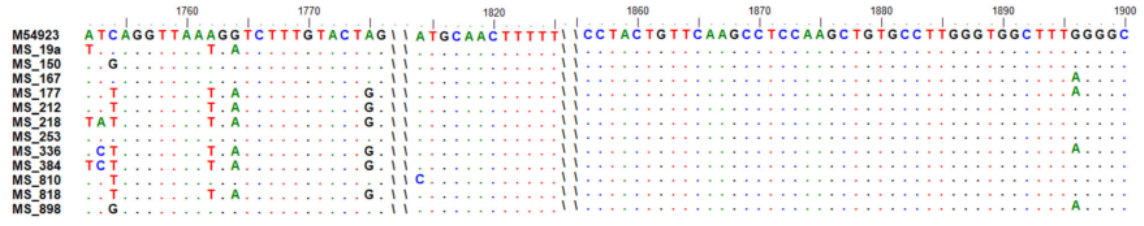

FIGURE 2 BCP and PC sequences of 12 HBeAg-negative pregnant women. Ten samples had HBV BCP or PC mutations: Five had BCP mutations T1753A/C and/or A1762T/G1764A, three (MS_167; MS_263, and MS_810) had PC mutations A1814C or G1896A, and two (MS_ and MS_336) had combined BCP and PC mutations. Shown are fragments of BCP (nucleotide [nt] 1752-1825 and PC (nt 1814-1900). HBeAg, hepatitis B *e* antigen; HBV, hepatitis B virus; MS, mother's serum; BCP, basal core promoter; PC, precore.

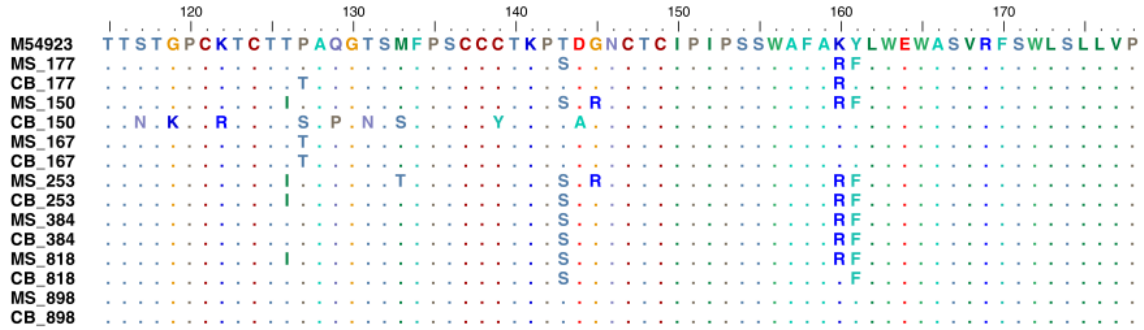

FIGURE 3 Paired HBsAg sequences from mothers and cord blood samples. Seven amino acid sequences of HBsAg "a" determinant generated from seven HBeAg-negative pregnant women (MS_177, MS_150, MS_167, MS_253, MS_384, MS_818, and MS_898) and corresponding cord blood (CB_177, CB_150, CB_167, CB_253, CB_384, CB_818, and CB_898). MS, mother's serum; CB, cord blood; HBeAg, hepatitis B *e* antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus.

TABLE 3 Characteristics HBeAg-negative pregnant women examined for BPC/PC mutation

	Clinical data			Serological profile		Virological characteristics			
Patient ID	Age (years)	Mode of delivery	ALT (IU/L)	HBsAg level (log_{10} IU/ml)	HBeAg/ anti-HBe	HBV DNA level (log_{10} IU/ml)	Genotype	Mutation type	HBV DNA in cord blood
MS 19a	20	Vaginal	15	2.507	-/+	0.778	C	BCP	-
MS 150	26	Vaginal	27	3.995	-/+	2.616	C	-	+
MS 167	35	Vaginal	45	2.691	-/-	5.548	B	PC	+
MS 177	34	Cesarean	40	3.461	-/-	6.481	C	BCP/PC	+
MS 212	25	Vaginal	15	3.548	-/+	0.778	C	BCP	-
MS 218	27	Vaginal	45	3.556	-/+	3.290	C	BCP	-
MS 253	33	Vaginal	33	3.054	-/+	1.356	C	-	+
MS 336	30	Cesarean	15	3.739	-/+	2.697	C	BCP/PC	-
MS 384	23	Vaginal	88	3.037	-/+	6.083	C	BCP	+
MS 810	29	Vaginal	10	2.698	-/+	3.500	C	PC	-
MS 818	36	Cesarean	40	4.234	-/+	1.344	C	BCP	+
MS 898	28	Vaginal	23	3.041	-/+	1.310	B	PC	+

Abbreviations: ALT, alanine aminotransferase; Anti-HBe, anti-hepatitis B *e* Antigen; BCP, basal core promoter; PC, pre-core; -, no BCP/PC mutation.

inactive carrier may be caused by nonviral liver diseases.[22] Patients with normal ALT levels may have no or minimal disease progression, while a substantial proportion of Asian patients with minimally elevated ALT levels have significant histological diseases.[23,24] Other studies also reported that ALT levels are lower during pregnancy and viral load is more likely to increase due to the natural immune suppression related to pregnancy.[25] Therefore, accurate discrimination between IC and e-CHB is crucial for assessing prognosis and potential infectivity.

The GPG-CHB Study Group recently defined inactive carriers as those with HBV DNA levels ≤2000 IU/ml and HBsAg levels <100 IU/ml, which showed 98% specificity and 97% positive predictive value for all HBV genotypes.[16] Based on this definition, eight (13.8%) of the 65 HBeAg-negative pregnant women in our study were categorized as LR or inactive HBV carriers with a high chance of HBsAg loss. This implies that the remaining 57 (87.7%) subjects could still have the possibility of progression to e-CHB, associated with the risk of liver deterioration and further complications.

Ten of 12 HBV nucleotide sequences generated from the 57 subjects had BCP or PC variants either alone or in combination. Among BCP variants, T1753A/C and the common A1762T/G1764A were identified. These variants have been associated with lower levels of precore mRNA and HBeAg synthesis.[26] Among PC variants, an A-to-C substitution at nt 1814, known to encode a noncanonical start codon that reduces HBeAg synthesis, or an A-to-G substitution at nt 1896, known to encode a stop codon that prematurely terminates HBeAg synthesis, were found. The dominance of this PC A1896G variant could account for HBeAg seronegativity.[27] Our result is consistent with numerous reports that associate HBV genotype C with a greater prevalence of HBeAg negativity and the high (8/10) occurrence of BCP/PC variants.[13] However, our finding could be unusual, since the PC mutation A1814C is more prevalent in HBV genotype A and rare in other genotypes.[28] Notably, among the 10 subjects with BCP or PC variants, three had a high viral load (≥5.3 $\log_{10}$ IU/ml). These women were at a high risk of MTCT and also eligible for antiviral prophylaxis.[5,18]

Five of these 10 pregnant women had HBV DNA in their cord blood, with two having HBV DNA ≥ 5.3 $\log_{10}$ IU/ml This finding is significant, particularly in the context of negative HBeAg status and intrauterine transmission of HBV.[17,29] The paired sequences of HBV DNA from the maternal and cord blood samples exhibited a high degree of HBsAg sequence similarity, raising more concerns about the risk of MTCT.

Prevention of MTCT of HBV is one of the five core strategies in the Global Health Sector Strategy to eliminate hepatitis B as a public health threat by 2030 with a goal of less than 0.1% HBsAg prevalence in children aged 5 years.[30] However, according to the latest WHO estimates, the proportion of children under the age of five infected with HBV dropped to just under 1% in 2019, down from around 5% in the pre-vaccine era in the 1980s.[31] Epidemiological and modeling studies suggest that infant vaccination alone would not be sufficient to reach this target, and peripartum prophylaxis may be needed.[5,32] On World Hepatitis Day 2020, WHO responded to eliminate this gap by issuing a new recommendation for HBV-infected pregnant women with high HBV DNA levels to receive antiviral prophylaxis, and the use of the HBeAg test as an alternative for HBV viral load testing in resource-limited settings to determine the eligibility for antiviral prophylaxis.[5,18]

The WHO 2020 recommendations can be viewed as a step forward in the efforts to attain the 2030 goal.[33] However, there remain challenges as we embark on this journey to eliminate hepatitis B by halting the MTCT of this virus, particularly regarding HBV-infected pregnant women who have negative HBeAg status but high levels of viremia.[34] These women still pose the risk to transmit HBV to their neonates, especially those with variants defective for HBeAg production. In addition, individuals carrying these mutations with high HBV DNA levels are still at risk of developing severe liver diseases.[35] It is also worth noting that compared to HBV DNA, HBeAg has a high sensitivity (99.1% [95% CI 61.8–100]), but low specificity (55.7% [95% CI: 34.0–75.5]) for predicting the risk of MTCT.[5,36] According to three major liver societies—the Asia Pacific Association for the Study of the Liver, the European Association for the Study of the Liver, and the American Association for the Study of Liver Diseases, e-CHB, referred to as "phase 4" or "immune-active CHB," is characterized by the absence of serum HBeAg, persistent or fluctuating moderate to high levels of serum HBV DNA, and persistent or fluctuating elevated ALT values (6. 7. 8). With forethought, it would be necessary to develop further strategies to identify and rescue mothers with negative HBeAg status who are at risk of MTCT and also eligible for antiviral prophylaxis.[7] This is especially important for pregnant women living or born in regions where e-CHB is prevalent.[11,34,37]

Several limitations were present in this study. First, the limited number of pregnant women led to an inadequate number of HBV nucleotide sequences for broader analysis. Wider clinical and community-based studies are needed from different geographical regions where variant-related genotypes are predominant.[11,13,37] Second, the cross-sectional design of this study did not make it possible to assess the fluctuating ALT profile in these HBeAg-negative pregnant women. Serial ALT level measurements are required to accurately identify pregnant women at risk of HBV MTCT, followed by HBV DNA level measurements in subjects with fluctuating ALT levels.[7,38] This appears to be a feasible option soon, as the test for quantifying HBV DNA near or at point-of-care is becoming available.[39,40]

In conclusion, this pilot study observed a high prevalence of HBeAg-negative among HBsAg-positive pregnant women in Makassar, some with variants defective for HBeAg synthesis and high levels of viremia. Negative HBeAg test results in HBsAg-positive pregnant women should be interpreted with caution when determining eligibility for MTCT antiviral prophylaxis, especially in situations with limited resources and in areas where e-CHB is prevalent. Further population-based studies and HBV DNA variant investigation are needed to ascertain the true prevalence of e-CHB and the contribution of BCP and PC to e-CHB among pregnant women in each geographical region. Adding ALT measurement for HBeAg-

negative pregnant women in subsequent antenatal visits could be an option for identifying individuals at risk of MTCT.

AUTHOR CONTRIBUTIONS

Maisuri T. Chalid and David H. Muljono conceived and designed the study. Rizalinda Sjahril, Ridha Wahyuni, and Turyadi contributed to data acquisition and data management. Maisuri T. Chalid and Ridha Wahyuni contributed to sample collection and management, Ridha Wahyuni, Rizalinda Sjahril, Turyadi, and Susan I. Ie contributed to laboratory work, Maisuri T. Chalid, Turyadi, Susan I. Ie, M. Nasrum Massi, and David H. Muljono contributed to data interpretation and statistical analysis. Maisuri T. Chalid, Susan I. Ie, and David H. Muljono, drafting, and revision of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data generated and analyzed in this study are included in this article. The original datasets used for this study are not publicly presented but are available upon request.

ETHICS STATEMENT

This study was approved by the Research Ethics Committees of Hasanuddin University – Faculty of Medicine, Wahidin Sudirohusodo Hospital, and Hasanuddin University Hospital (Authorization No. 0942/H4.8.4.5.31/PP36-KOMETIK/2017), and fully complied with the Declaration of Helsinki and the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline for observational studies. Informed consent was obtained from all study participants.

CONSENT TO PUBLISH

All authors approved the final version of the article including the authorship list and consented to publication in Journal of Medical Virology.

CLINICAL TRIALS REGISTRATION

It is an observational study and not registered.

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