RESEARCH ARTICLE

Intrauterine Transmission of Hepatitis B Cannot Be Ruled Out by A Single Negative Hepatitis B e Antigen (HBeAg) Result among Hepatitis B Surface Antigen (HBsAg) - Positive Pregnant Women

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Abstract

BACKGROUND: The risk factors for intrauterine transmission of hepatitis B virus (HBV) in hepatitis B surface antigen (HBsAg)-positive pregnant women are poorly understood. Numerous factors are considered to be involved, including placental barrier, obstetric environment, high viral load, and positivity of hepatitis B e antigen (HBeAg). This study was conducted to investigate the role of placenta barrier, clinical, and viral factors in intrauterine transmission of HBV.

METHODS: A cross-sectional study was conducted involving 1,353 pregnant women who underwent HBsAg screening. Eighty-four (6.2%) women were detected as HBsAg positive and were examined for HBsAg level, anti-HBs, anti-HBc, HBeAg/hepatitis B e antibody (anti-HBe) status, and HBV DNA presence in cord blood. Quantitative HBV DNA was analyzed using real-time polymerase chain reaction (PCR).

RESULTS: Eighty-four of 1,353 subjects were HBsAg-positive. HBV DNA was positive in 28/84 (33.7%) maternal sera, 19/79 (24.05%) placental specimens, and 9/83 (10.84%) in cord blood. There were significant associations between HBV DNA in maternal serum (p=0.000) and placental tissue (p=0.000) with HBV DNA in the cord blood. No clinical factors were associated with HBV DNA transmission in cord blood. Sixty percent of viral load >5.3 $\log_{10}$ copies/mL were found in the cord blood, of which 43.8% HBeAg positive and 3.1% HBeAg negative.

CONCLUSION: Reduced transmission via compartments established the placenta's barrier function in mother-to-child transmission. A high maternal viral load and positive HBeAg were risk factors for intrauterine transmission, while negative HBeAg still has the possibility of transmission.

KEYWORDS: mother-to-child transmission, hepatitis B virus, intrauterine

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Introduction

Chronic hepatitis B (CHB) is a public health burden affecting approximately one-third of the world's population and has an impact on more than 900,000 deaths annually. (1-3) Indonesia is classified as endemic for hepatitis B with a prevalence was 7.1%.(4) Despite the nationwide infant hepatitis B vaccination program that has been implemented since 1997, approximately 2 to 4% of children born to hepatitis B virus (HBV)-infected mothers remain infected, which indicates the failure of immunoprophylaxis. HBV infection in neonates might raise the chance of CHB by 90%.(5,6) This chronic disease increases the risk of liver

cancer and cirrhosis in future adulthood. This situation became the cause of the persistent endemicity of hepatitis B in Indonesia.(6-8)

The highest possibility of mother-to-child transmission (MTCT) occurs during childbirth or the perinatal period. This occurs predominantly due to the exposure of newborns to HBV-containing maternal fluid or blood during passage through the birth canal. Prior to birth, however, a small amount of maternal blood may enter fetal circulation via the placenta, amniotic fluid, and cord blood, which means that the fetus would be exposed to HBV throughout intrauterine life. Meanwhile, newborn vaccines are only administered immediately after birth. Therefore, the presence of HBV DNA in the neonatal cord blood has been considered as a marker for intrauterine exposure to infection. In such cases early neonatal active and passive immunization (given less than 12 hours after birth) could be the cause of immunoprophylaxis failure.(6,9-11) Knowing the mechanism of transmission between compartments, interactions with the clinical environment (including maternal and obstetrics factors) and the role of viral factors (hepatitis B virus infection indicators) are essential in understanding intrauterine transmission. Therefore, this study was conducted to evaluate the association of placenta barrier, clinical environment and viral factors in intrauterine exposure to HBV.

Methods

Study Design and Subjects

From January 2019 to March 2021, a cross-sectional study was conducted at Wahidin Sudirohusodo Hospital, Universitas Hasanuddin Hospital, Khadijah Mother and Child Hospital, Pertiwi Mother and Child Hospital, Fatimah Mother and Child Hospital, Makassar. The Faculty of Medicine's Health Research Ethical Committee of Universitas Hasanuddin, Wahidin Sudirohusodo Hospital, and Universitas Hasanuddin Hospital had approved the study protocol in accordance with the Helsinki Declaration and Nuremberg Code (No. 1216/UN4.6.4.5.31/PP36/2019). All enrolled subjects also had signed informed consent prior to the study.

A total of 1,353 pregnant women underwent testing for HBV, and 84 (6.2%) were HBsAg-positive. Inclusion criteria for subjects were women detected as HBsAg-positive for 6 months or longer without prior antiviral therapy. The exclusion criteria encompassed complications related to pregnancy, concurrent infection with hepatitis A, C, or human immunodeficiency viruses (HIV), as well as severe hepatitis or cirrhosis. The ACON Hepatitis B Surface Antigen Rapid Test (ACON Laboratories Inc. San Diego, CA, USA) was used to test for HBV infection during the first visits. The remaining sera were divided into aliquots and kept at -80°C for future use. A number of molecular examination of stored biological material was carried out continuously until the beginning of the year 2022.

Among 84 HBsAg-positive maternal sera which were enrolled, they corresponded with 79 specimens of the placenta and 83 specimens of cord blood that were available for HBV DNA testing (Figure 1).

Specimen Collection

A 10 mL vial of maternal blood was obtained from the antecubital vein, while the umbilical vein cord blood was taken after delivery by an 18G needle. The specimen was centrifuged for 10 minutes at 4000 rpm, split into aliquots, and kept at -80°C until further examination. The 2x2 cm portion of central placental tissue was stored at -80°C in a 10% formaldehyde buffered solution.

Serological Examination

The maternal samples that yielded positive results for HBsAg during the initial screening were further confirmed using an HBsAg immunoassay (VIDAS HBsAg BioMérieux SA, Marcy l'Etoile, France). Detection of HBeAg and anti-HBe was determined using the MonolisaTMHBeAg Ag–Ab PLUS immunoassay (Biorad, Marnes-la-Coquette, France). The HBsAg titer was quantified according to the manufacturer's procedure using Elecsys HBsAg Quant II (Roche Diagnostics, Indianapolis, IN, USA) on a Roche Cobas® e411 Immunoanalyzer (Roche Diagnostics).

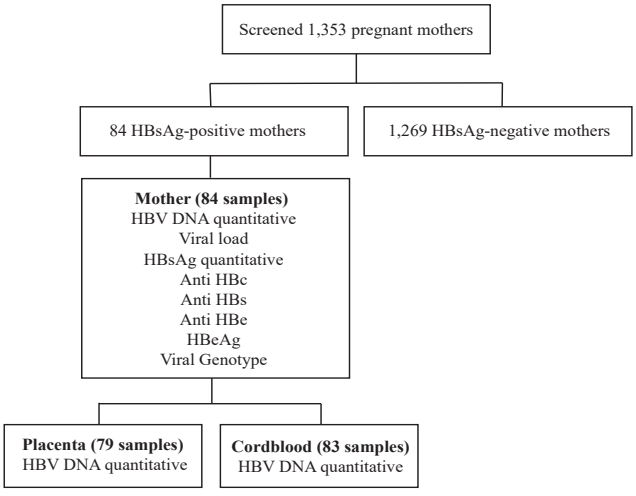

Figure 1. Flow diagram of study subjects recruitments.

HBV DNA Detection and Analysis

The HBV DNA titer was performed using quantitative real-time polymerase chain reaction (PCR) CobasTaqmanTM HBV Test (Roche Diagnostics) on a volume of 500 µL of serum with a linearity range of 6 to 1.1 x 10^8 IU/mL. HBV DNA was extracted for molecular analysis from 140 µL of serum using the QIAamp DNA MiniKit (Qiagen, Valencia, CA, USA) according to the manufacturer's guidelines. DNA was extracted and eluted in 60 µL of elution buffer. The DNA fragment encoding the 'a determinant' region of the S gene was amplified using nested PCR with specific primer sets S2-1 (nt 455-474, sense; 5'-CAA GGT ATG TTG CCC GTT TG-3') and S1-2 (nt 704-685, antisense; 5'-CGA ACC ACT GAA CAA ATG GC-3') as outer primers and S88. Purification of amplification products was performed using a PCR purification column (Qiagen) and direct sequencing was performed on a DNA sequence analyzer ABI 3130XL (Applied Biosystems, Waltham, MA, USA).

The HBV genotype was ascertained using phylogenetic analysis of nucleotide sequences from the S gene. This technique involved comparing the sequences with 70 reference sequences of known genotypes (A–J) obtained from GenBank. The analysis employed Phylip 3.68 software, utilizing the Kimura-2 parameter, neighbor-joining technique, and 1000 bootstrapping.

Statistical Analysis

The statistical analysis was perfomed using SPSS version 22 (IBM Corporation, Armonk, NY, USA). All statistical significance values were assessed at $p<0.05$.

Results

Subject Characteristics

Out of a total sample size of 1,353 pregnant women who gave birth in several hospitals and maternity clinics located in Makassar, a prevalence rate of 6.2% (n=84) tested positive for HBsAg. The characteristics of the subjects studied were shown in Table 1. Of these 84 subjects, 19% were HBeAg positive and 77.4% were HBeAg negative, the mean HBsAg titer was 3.03 log10 IU/mL, viral load was 2.99 $\log_{10}$, which was more common with genotype C (56%), while genotype B was 21.4%.

Table 1. Baseline characteristics of study participants.

Parameter	n (%)	Mean±SD
Maternal Characteristic		
Age (years) (n=84)		29.9±5.87
<20	1 (1.2%)	
21-34	63 (75%)	
>35	20 (23.8%)	
First trimester body mass index (kg/m^2) (n=84)		
Normal weight (18.5-24.9)	49 (58.3%)	
Overweight (25.0-29.9)	35 (41.7%)	
Liver function test (n=83)		
ALT (u/L)		27.54±9.26
AST (u/L)		28.31±14.64
Gestational age (n=84)		
<37 weeks	2 (2.4%)	
>37 weeks	82 (97.6%)	
Parity (n=84)		
Nulliparous	33 (39.3%)	
Multiparous	51 (60.7%)	
Virological Characteristic		
HBsAg level ($\log_{10}$ IU/mL) (n=83)		3.03±1.047
HBeAg status (n=84)		
Positive	16 (19.0%)	
Negative	65 (77.4%)	
Undefined	3 (3.6%)	
Anti-HBc status (n=84)		
Positive	82 (97.62%)	
Negative	1 (1.19%)	
Equivocal	1 (1.19%)	
HBV DNA level ($\log_{10}$ IU/mL) (n=82)		2.99±2.34
HBV DNA genotype (n=84)		
Genotype B	18 (21.4%)	
Genotype C	47 (56%)	
Indeterminate	18 (21.4%)	
HBV subtype (n=36)		
Adr	18 (50%)	
Adw	12 (33.3%)	
Ayw	3 (8.3%)	
Indeterminate	3 (8.3%)	

AST: aspartate aminotransferase; ALT: alanine aminotransferase; HBsAg: hepatitis B surface antigen; Anti-HBc: anti-hepatitis B core; HBeAg: Hepatitis B e antigen; HBV: hepatitis B virus.

Intrauterine Transmission from Maternal Serum, Placenta to The Cord Blood

The presence of HBV DNA in the newborn cord blood provided evidence of intrauterine exposure to HBV. Twenty-eight out of 84 (33.7%) mothers had positive HBV DNA in the serum. The HBV DNA detection rate was 19/79 in placental tissue (24.05%), and 9/83 in the umbilical cord (10.84%).

Further examination was conducted to identify the presence of HBV DNA in both maternal serum and placenta compartments, and to establish an association between its presence with intrauterine exposure to HBV infection, as confirmed by the detection of HBV DNA in cord blood (Table 2). The transmission process from maternal blood and

Table 2. Association of HBV DNA presence in obstetrics compartment of maternal serum, placenta, and HBV DNA in cord blood (intrauterine transmission).

Compartment	HBV DNA in Cord Blood [n (%)]		OR (95% CI)	*p*-value
	HBV DNA Positive	HBV DNA Negative		
Maternal serum (n=83)				
HBV DNA positive (n=28)	9 (32.1%)	19 (67.9%)	undefined	0.000
HBV DNA negative (n=55)	0 (0.0%)	55 (100%)	reference	
Placenta (n=79)				
HBV DNA positive (n=19)	8 (42.1%)	11 (57.9%)	42.9 (4.87-378.1)	0.000
HBV DNA negative (n=60)	1 (1.7%)	59 (98.3%)	reference	

Chi-square/Fisher exact test.

placenta to the cord blood (intrauterine) was significantly influenced by the placenta, which plays an essential part. If the virus has reached the placenta, the risk of transfer to the cord blood is 42.9 times higher.

Clinical Environment and Viral Factors Contribution to Transmission

The contributions of clinical and viral factors related to HBV transmission were analyzed concerning HBV detection in the cord blood (Table 3). Several maternal and obstetric variables, including BMI, parity, onset of labour, premature rupture of the membrane, mode of delivery, and history of miscarriage, were not shown to have a significant contribution on the risk of intrauterine transmission. Meanwhile, viral factors such as hepatitis B infection indicators with viral load levels >5.3 $\log_{10}$, HBsAg titer >4 $\log_{10}$, and HBeAg positive status play a significant role in the transmission (Table 3).

Maternal viral load (3-5.29 $\log_{10}$ IU/mL) status had very strong associations with transmission to the placenta and cord blood, and the highest viral load >5.3 $\log_{10}$ had reached the cord blood (intrauterine transmission) (Figure 2). Of these, 9 subjects who have intrauterine exposure to infection (HBV DNA in the cord blood), 6 subjects (66.7%) have genotype/subtype C/adr, 2 (22.1%) with B/adw, and only 1 subject (11.1%) with B/ayw.

According to the maternal HBeAg status, the distribution of HBV DNA detection was higher in HBeAg-positive than in HBeAg-negative mothers with decreasing frequencies from maternal serum, placenta, to cord blood. Two samples of negative HBeAg status were found positive HBV DNA in the cord blood of corresponding babies (Figure 3). These 2 subjects had normal and slightly increased ALT levels and no clinical signs of liver disease, but had a viral load of more than 5.3 $\log_{10}$ IU/mL, genotype C, and subtype adr.

Discussion

In this study, there are 84 (6.2%) positive HBsAg of out 1,353 pregnant women in labour. This result is significantly higher than the previous antenatal screening results performed in 2018 throughout South Sulawesi (2.51 %) and the national average figure (1.88 %), with a very wide range (0.9 %-5.53 %) between 34 provinces.(12) The difference in these results could be due to the hospital-based nature of our study, the difference in cultural and health service practices, geographical profiles, and laboratory methods of HBV detection.

Nineteen percent of samples tested positive for HBeAg placing these subjects at an extremely high risk of MTCT in the absence of immunoprophylaxis.(5) Around 56% of these subjects had HBV genotype C, which was associated with a high prevalence of HBeAg positive and a higher risk of advanced liver disease and liver cancer.(13)

During pregnancy, the maternal immune system adapts to prevent fetal rejection.(14) This condition also causes pregnant women to be in the immune tolerant phase of CHB infection, with normal levels of ALT and AST, despite high viremia or active viral replication.(15) This study found that the majority of participants were fully-term, had normal liver function, and had no hepatitis B symptoms.

HBV DNA detected in the cord blood proves intrauterine transmission occurs before labour.(9,10) In comparison to peripheral venous blood, previous study demonstrated that the presence of HBV DNA in cord blood has a 100% sensitivity and an 80% positive predictive value for diagnosing fetal infection.(9) Another study found similar levels of HBV detection in the cord blood and femoral venous blood of neonates prior to immunoprophylaxis, despite the difficulty of definitively differentiating the period of infection *in utero* from the period of infection

Table 3. Association between maternal clinical factors and hepatitis B infection indicators with the presence of HBV DNA in the cord blood (intrauterine transmission).

Clinical and Viral Factors	Positive [n (%)]	Negative [n (%)]	*p*-value	OR (95% CI)
Clinical Factors				
First trimester body mass index (BMI)				
Overweight	4 (11.8%)	30 (88.2%)	0.546	1.17 (0.29-4.71)
Normal	5 (10.2%)	44 (89.8%)		reference
Parity				
Multiparous	4 (7.8%)	47 (92.2%)	0.225	0.46 (0.11-1.86)
Nulliparous	5 (15.6%)	27 (84.4%)		reference
Labour phase				
Active	7 (10.1%)	62 (89.9%)	0.471	0.68 (0.13-3.67)
Latent	2 (14.3%)	12 (85.7%)		reference
Premature rupture of the membrane (PROM)				
Yes	3 (23.1%)	10 (76.9%)	0.144	3.2 (0.69-14.9)
No	6 (8.6%)	64 (91.4%)		reference
Mode of delivery				
Vaginal	7 (11.3%)	55 (88.7%)	0.592	1.21(0.23-6.33)
Caesarean section	2 (9.5%)	19 (90.5%)		reference
History of miscarriage				
Yes	1 (11.1%)	8 (88.9%)	0.664	1.03 (0.11-9.34)
No	8 (10.8%)	66 (89.2%)		reference
Hepatitis B Infection Indicators				
Viral load				
≥5.3 $\log_{10}$ IU/mL	9 (60%)	6 (40%)	0.000	undefined
<5.3 $\log_{10}$ IU/mL	0 (0%)	67 (100%)		reference
HBs Ag level				
≥4 $\log_{10}$ IU/mL	5 (35.7%)	9 (64.3%)	0.006	9.03 (2.04-40)
<4 $\log_{10}$ IU/mL	4 (5.8%)	65 (94.2%)		
HBe antigen				
Positive	7 (43.8%)	9 (56.3%)	0.000	24.5 (4.39-136.8)
Negative	2 (3.1%)	63 (96.9%)		reference

Chi-square/Fisher exact test.

during delivery.(16) Based on HBV DNA detection in the cord blood, our study found the intrauterine transmission was 10.84%. It is worth an emphasized that the cord blood sampling in this study, was performed during childbirth, before the administration of immunoprophylaxis. In China, the rate of intrauterine infection was reported to range from 3.7% to 9.9%.(17-19) The term intrauterine infection was established if the marker of infection was found within 24 hours after birth.(19) On the contrary, other studies stated that infants whose HBV DNA or HBsAg remained positive for more than 7-14 months were included as having intrauterine HBV infection.(16, 20)

The presence of HBV DNA or positive serological marker in the cord blood cannot be defined as intrauterine infection, but indicating that neonates were exposed to the hepatitis B virus.(16,21) 'Transient' HBV DNA could

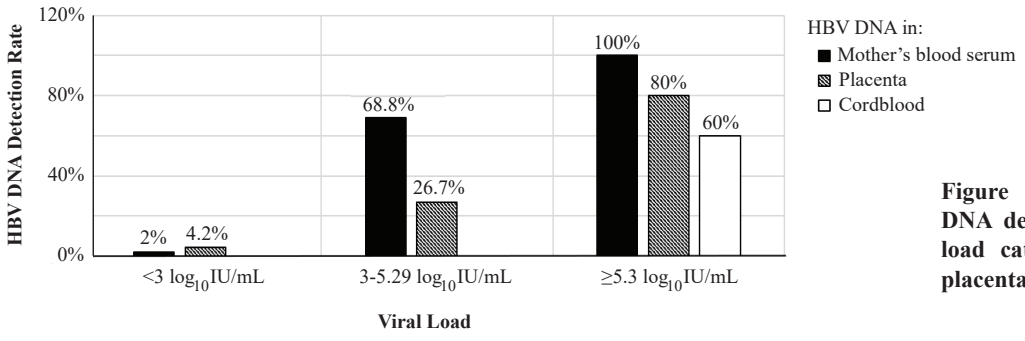

Figure 2. Comparison of HBV DNA detection rate based on viral load category in maternal serum, placenta, and cord blood.

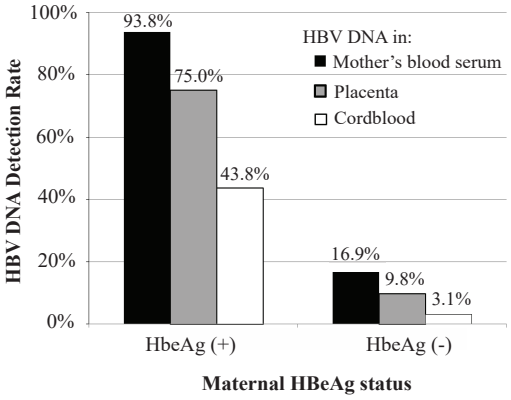

Figure 3. Comparison of HBV DNA detection rate in maternal serum, placenta, and cord blood according to maternal HBeAg status.

be cleared by fetal immunity or/and immunoprophylaxis after vaccination is completed.(22) Therefore, it is more appropriate to use the term intrauterine 'transmission' or exposure rather than infection.

The placental barrier serves as a protective barrier in mitigating the risk of intrauterine transmission of HBV.(21) This study found a decline in the proportions of sequential HBV DNA in the maternal compartment, placenta, and cord blood. A study showed the function of the placental barrier in an *in vitro* experiment and a clinical intervention involving HBV infection that reached the fetal side via placental trophoblast cells.(23) Another study also demonstrated the transcytosis of infectious HBV across placental trophoblasts by *in vitro* study.(24) The incidence of HBV infection in placental tissue decreased progressively from the maternal to the fetal side, while the correlation between placental and intrauterine transmission increased in gradient.(18,25)

Intrauterine infection refers to a transmission that arises preceding the onset of labour or when contraction arises during pregnancy which enables transplacental leakage by a tear in the placental barrier.(23,26,27) Our study revealed that increased uterine contractions during the active phase of labour and a history of miscarriage were found to have no correlation with HBV detection in the placenta or cord blood. Unfortunately, this study was limited by a lack of information regarding a past miscarriage, either before to or following infection with hepatitis B.

Mother to child transmission was not associated with premature rupture of the membrane (PROM) or mode of delivery. Research encompassing 174 pregnant women with CHB, revealed no significant correlation between intrauterine infection with parity, number of abortions, gestational age, and method of delivery.(25)

The controversy between vaginal delivery and the caesarean section regarding intrapartum transmission risk is still under discussion. Numerous studies have been published to support elective caesarean section for the purpose of reducing MTCT.(24,28,29) However, the studies required randomization and did not specify the risk group. Other meta-analysis study has found that the benefit of cesarean delivery in protecting against transmission has not been established.(30)

A gradual decrease of HBV DNA percentage, sequentially from the maternal, placenta, and cord blood, displayed the mechanism of compartments in reducing the transmission from mother to fetus (Figure 2). It was also clear that >5.3 $\log_{10}$ IU/mL is a threshold for intrauterine transmission, which was proven by HBV DNA detection in the cord blood only in the >5.3 $\log_{10}$ IU/mL group (Figure 2). This cut off refers to the World Health Organization's (WHO) recommendation threshold in providing antiviral prophylaxis.(31) An antenatal HBV DNA level greater than 6 $\log_{10}$ copies/mL (>200,000 IU/mL) was the most significant predictor of MTCT.(6) The American Association for the Study of Liver Diseases (AASLD) recommends antiviral therapy for perinatal transmission prevention when the viral load is greater than 6 $\log_{10}$ copies/mL (>2 x 10^5 IU/mL). (32) This study supports the World Health Organization's (WHO) recommendation to provide antiviral therapy tenofovir prophylaxis to mothers with a viral load of 5.3 $\log_{10}$ IU/mL (200,000 IU/mL^2) or higher from the 28th week of pregnancy until at least birth, in order to prevent MTCT of HBV.(31)

A similar description of compartments in reducing transmission to the fetus (cord blood), was shown in the HBeAg positive group. It has been known that HBeAg status was an additional factor besides viral load that contributes to vertical transmission. A positive correlation between cord blood HBV DNA and maternal HBV DNA and HBeAg positivity was discovered. Placental leakage can facilitate HBeAg, which are small enough particles, making them able to pass through.(26)

Similar to our findings, a higher prevalence of HBeAg seropositivity has been reported in genotype C carriers compared to genotype B carriers, indicating that this genotype may play a role in perinatal transmission.(33) This study provides evidence that high viremia and HBeAg positivity can penetrate the feto-maternal (placental) barrier, demonstrating the importance of antiviral therapy to prevent mother-to-child transmission. Nevertheless, it is crucial to take into account the occurrence of intrauterine transmission in pregnant women who are negative for the HBeAg, have

a high viral load, despite absence of clinical signs. This is particularly relevant during the immunological tolerance period of pregnancy.(34)

There are several limitations to this study. Although umbilical blood sampling at birth is thought to be the most practical and safe for infants, it is acknowledged that it does not fully explain the mechanism of intrauterine transmission, beside further constraint was the lack of data on the infants' serologic outcomes. Further studies are necessary to explore more possibilities regarding this mechanism.

Conclusion

This study provided evidence for reduction of transmission mechanisms through feto-maternal compartments, particularly the placenta as a barrier in MTCT, with the rate of intrauterine transmission being 10.8%. A maternal high viral load of >5.3 $\log_{10}$ copies/mL (>200 000 IU/mL) was the most important predictor of MTCT. Although the positivity of HBeAg was an additional risk factor for transmission, pregnant women with negative HBeAg and high viral load might transmit, which is providing a notable message to the interpretation of negative results of HBeAg testing.

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Authors Contribution

All authors contributed to this work. MTC and MNM designed and conceived the study. DBF, TT, RS contributed to data acquisition and management. RM, RW, DBF contributed to sample collection and management. RS, RW, TT contributed to laboratory work. MTC, TDJ, MNM contributed to data interpretation and statistical analysis. MTC, RM, and TDJ contributed to drafting, revision of the manuscript.

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