Detection of Hepatitis B Viral DNA in Pregnant Woman Sera by PCR
การตรวจหาดีเอ็นเอไวรัสตับอักเสบบีในซีรัมหญิงตั้งครรภ์ด้วยวิธีพีซีอาร์

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นำตัวอย่างซีรัมหญิงตั้งครรภ์จากโรงพยาบาลจุฬาลงกรณ์ 512 ตัวอย่าง มาตรวจหาดีเอ็นเอบริเวณ S gene ของไวรัสตับอักเสบบี (HBV-DNA) ซึ่งเป็นแถบดีเอ็นเอขนาด 431 คู่เบส ด้วยวิธีพีซีอาร์ (PCR, Polymerase Chain Reaction) ศึกษาควบคู่กับการตรวจหาดัชนีในซีรัม (seromarkers) อื่น ๆ ด้วยวิธีทางอิมมิวโนแอสเสย์ ได้แก่ แอนติเจนชนิดผิวของไวรัสตับอักเสบบี (HBsAg) ภูมิต้านทานต่อแอนติเจนชนิดผิวของไวรัส (anti-HBs) และ ภูมิต้านทานต่อส่วนแกนกลางของอนุภาคไวรัส (anti-HBc) การตรวจหา HBsAg ใช้ 2 วิธี คือ Chemiluminescent immunoassay : Abbott prism HBsAg ของบริษัท Abbott ประเทศเยอรมัน และ วิธี ELISA (Enzyme-Linked Immunosorbent Assay) ซึ่งพัฒนาโดยกรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข นนทบุรี วิธี PCR สามารถตรวจพบ HBV-DNA ทั้งหมด 14 ตัวอย่าง จาก 512 ตัวอย่าง (ร้อยละ 2.73) เมื่อเปรียบเทียบผลการทดลองของวิธี PCR กับ Chemiluminescence พบ HBV-DNA ในกลุ่มตัวอย่างซีรัมที่ให้ผล HBsAg เป็นลบซึ่งถือว่าเป็นกลุ่มปกติ 4 ตัวอย่าง จาก 482 ตัวอย่าง (ร้อยละ 0.83) และในกลุ่มที่ให้ผล HBsAg บวกซึ่งคิดว่าเป็นพาหะของโรค 10 ตัวอย่าง จาก 30 ตัวอย่าง (ร้อยละ 33.33) ความไวของวิธี PCR เมื่อเทียบกับวิธี Chemiluminescence คิดเป็นร้อยละ 33.33 ความจำเพาะร้อยละ 98.96 และความถูกต้องร้อยละ 95.31 เมื่อเปรียบเทียบวิธี PCR กับ ELISA พบว่า วิธี PCR ตรวจพบ HBV-DNA ในกลุ่มที่ให้ผล HBsAg เป็นบวก 13 ตัวอย่าง จาก 34 ตัวอย่าง (ร้อยละ 38.24) ในกลุ่มที่ให้ผล HBsAg เป็นลบ 1 ตัวอย่าง จาก 478 ตัวอย่าง (ร้อยละ 0.21) ความไวของวิธี PCR เมื่อเทียบกับวิธี ELISA คิดเป็นร้อยละ 38.24 ความจำเพาะร้อยละ 99.79 ความถูกต้องร้อยละ 95.70 แม้ว่าวิธี PCR จะมีข้อจำกัดในการใช้ตรวจหาไวรัสตับอักเสบบีในซีรัม แต่เป็นวิธีที่ให้ประโยชน์มากหากใช้ควบคู่ไปกับวิธีทางอิมมิวโนแอสเสย์ในการตรวจหาผู้ที่เป็นพาหะของโรค เพื่อป้องกันการเกิดผลลบลวง เพราะสามารถตรวจหาไวรัสตับอักเสบบีได้แม้ในกรณีที่ตรวจไม่พบ HBsAg

คำสำคัญ : ไวรัสตับอักเสบบี แอนติเจนชนิดผิว การวินิจฉัย พีซีอาร์ อิมมิวโนแอสเสย์

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สถาบันวิจัยวิทยาศาสตร์สาธารณสุขแห่งชาติ กรมวิทยาศาสตร์การแพทย์ กระทรวงสาธารณสุข นนทบุรี

Abstract : Sattaporn Sirotamarat and Kruavon Balachandra. 2002. Detection of hepatitis B viral DNA in pregnant woman sera by PCR. Thai J Hlth Resch 16(1) : 51-64.

Five hundred and twelve sera from pregnant women at King Chulalongkorn Memorial Hospital were screened for hepatitis B viral DNA (HBV- DNA) by PCR (Polymerase Chain Reaction). The PCR products of DNA samples were investigated for DNA band 431 base pairs size, using specific primers from S gene conserved region of HBV. This study was compared with the detection of other seromarkers such as HBsAg, anti-HBs and anti-HBc. There were two methods used to investigate for HBsAg, namely Chemiluminescent immunoassay : Abbott prism HBsAg of Laboratories, Co. Ltd., Germany and ELISA (Enzyme-Linked Immunosorbent Assay) developed by the Department of Medical Sciences, Ministry of Public Health, Nontaburi. HBV-DNA was detected in 14 out of 512 samples (2.73%) of total pregnancy. Comparing the PCR technique with chemluminescence, HBV-DNA were detected in 4 out of 483 samples (0.83%) of $HBsAg^-$ (normal) group and 10 out of 30 samples (33.33%) of $HbsAg^+$ (active carriers) group. The sensitivity, specificity and accuracy of PCR technique comparing with Chemiluminescence were 33.33%, 98.96% and 95.31%, respectively. Comparing the PCR technique with ELISA, the PCR technique could detect HBV-DNA in 1 out of 478 samples (0.21%) of $HBsAg^-$ group and 13 samples out of 34 samples (38.24%) of $HBsAg^+$ group. The sensitivity, specificity and accuracy of PCR technique comparing with ELISA were 38.24%, 99.79% and 95.70%, respectively. Although PCR technique has limited in effectiveness for HBV detection but HBV-DNA by PCR was a useful marker for additional screening HBV in pregnant woman sera since it could be directly detected the HBV active carriers even in the case of $HBsAg^-$. It is concluded that PCR is a reliable method if it is used in conjunction with HBsAg immunoassay to eliminate the false negative.

Key words : Hepatitis B virus, HBsAg, diagnosis, PCR, immunoassay

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Introduction

Hepatitis B is one of the most common infectious diseases in the world. It has been estimated that 350 million people world-wide are chronic hepatitis B virus (HBV) carriers and 75% of all chronic carriers live in Asia and Western Pacific. The predominant routes of transmission vary according to the endemicity of HBV infection. In area of high endemicity, perinatal or vertical transmission is the main route of transmission, in area of low endemicity, sexual contact among high-risk adults is the predominant route. Between one quarter to one third of people infected chronically with HBV are expected to develop progressive liver disease, cirrhosis and primary liver cancer or hepatocellular carcinoma after the 40 years of age (Meddrey,2000).

Thailand had a high prevalence of HBV infection, the HBV carriers were 4.36-10% or about 6 million persons (Bhuvanath *et al.*, 1999; Chalobon, 1993). Ten percent of blood donors at Thai Red Cross Society and 6.3% of pregnant women at King Chulalongkorn Memorial Hospital were identified as HBV carriers (anonymous, 2002; Yong, 1996). Chronically infected pregnancy, defined as those who carry HBsAg for more than 6 months are represented as the major source of HBV infection (Meddrey,2000). Moreover, it was reported that perinatal transmission of HBV be high as 65-90% in mothers who were HBV carriers with no symtom but HBsAg and HBeAg could be detected in sera (Mondelli and Eddleston,1984). Although the detection for HBsAg has high value in the diagnosis of current HBV infection and increasing the safety of the blood supply but HBV cases were still high as 7-13% of the post-transfusion hepatitis cases (Brechot *et al.*, 1985). PCR technique for detection of HBV-DNA is the another method for screening of heptitis B carriers in laboratory diagnosis. This technique is highly sensitive and specific method for detection of DNA in serum samples that have small amount of DNA. It was reported that PCR was able to detect as few as three to five genomes in a serum sample (Kaneko *et al.*, 1989). In addition, PCR technique could detect HBV-DNA faster than the detection of HBsAg by immunoassay 2-3 weeks in patient sera (Kekesi *et al.*, 1993). The detection of serum HBV-DNA in a routine diagnosis laboratory by quantitative PCR, using Amplicor HBV Monitor Test and a non-radioactive hybridization, showed the excellent sensitivity and adequate reproductivity for antiviral treatment (Kessler *et al.*, 1998). Therefore, this study is to detect HBV-DNA as one of the seromarkers for indication of hepatitis B carriers in pregnant women.

Materials and Methods

Serum samples

Five hundred and twelve of blood samples of pregnant women, from King Chulalongkorn Memorial Hospital, were separated by clotting at room temperature. The sera were centrifuged at 2000 rpm for 5 minutes at 4°C and inactivated in water bath at 56°C for 30 minutes. All sera, free of HIV antibody, were kept for further study at −40°C.

Detection of seromarkers of HBV by immunoassay

1. Detection of HBsAg by chemiluminescent immunoassay (ChLIA) : All 512 serum samples were screened for HBsAg using Abbott prism HBsAg, an automate chemiluminescent test kit of Abbott Laboratories, Co. Ltd., Germany.

2. Detection of HBsAg by ELISA : All 512 serum samples were screened for HBsAg using one-step ELISA test kit, developed by the Department of Medical Sciences , Ministry of Public Health, Nontaburi (Nongluk,2000).

3. Detection of anti-HBs by ELISA : All 512 serum samples were screened for anti-HBs using one-step ELISA test kit, developed by the Department of Medical Sciences, Ministry of Public Health, Nontaburi. (Panadda, 1997)

4. Detection of anti-HBc by ELISA : Serum samples were screened for anti-HBc using Monolisa anti-HBc plus kit of Sanofi Diagnostics Pasteur, USA. (We detected anti-HBc for some samples to confirm HBV-DNA positive or HBsAg positive because the test kit is very expensive about 10,000 bath/kit)

Detection of HBV-DNA by PCR

Preparation of DNA samples : 200 µl of serum were incubated at 55 °C for 2 hours in 200 µl of proteinase K (100 µg/ml of Sigma) in 2.5x premix buffer (20 µl 20 mg/ml glycogen, 500 µl 20% SDS, 100 µl 0.5 M EDTA, 170 µl 3M CH_3COONa pH 6.5 and 7.2 ml distilled water). The solution was extracted with 1 volume of phenol and chloroform (1:1). DNA was precipitated with 2 volumes of absolute ethanol and 0.1 volume of 3 M sodium acetate pH 5. Then, the solution was centrifuged at 15,000 rpm 4 °C for 10 minutes. The precipitate was washed with 2 volumes of 70% ethanol and centrifuged at 15,000 rpm at 4 °C for 5 minutes. The precipitate was dried and dissolved in 10 µl of

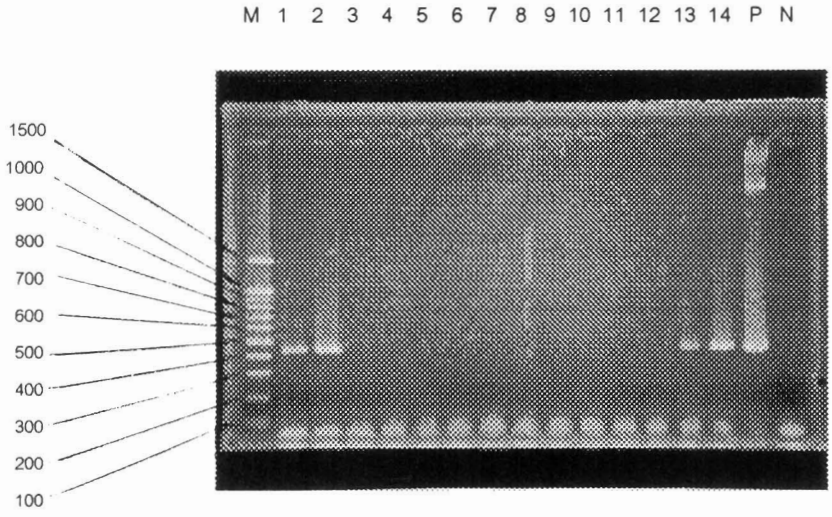

Figure 1 431 base pairs size of PCR products of HBV- DNA from pregnant women sera (M= DNA marker, P = positive HBV- DNA control , N = negative serum control)

Table 1 Comparison of HBV-DNA detection by PCR with HBsAg detection by Chemiluminescent immunoassay in pregnant woman sera

HBV-DNA detection	HBsAg detection		Total
	+	-	
+	10 (TP)	4 (FN)	14
-	20	478 (TN)	498
Total	30	482	512

TP = true positive, TN = true negative, FN = false negative

Sensitivity = true positive/total positive
= (10/30) = 33.33%
Specificity = true negative/total negative
= (478/498) = 98.96%
Accuracy = (TP + TN)/total samples
= (10+478)/512 = 95.31%

Table 2 Comparison of HBV-DNA detection by PCR with HBsAg detection by ELISA in pregnant woman sera

HBV-DNA detection	HBsAg detection		Total
	+	-	
+	13 (TP)	1 (FN)	14
-	21	477 (TN)	498
Total	34	478	512

TP = true positive, TN = true negative, FN = false negative

Sensitivity = true positive/total positive
= (13/34) = 38.24%
Specificity = true negative/total negative
= (477/478) = 99.79%
Accuracy = (TP + TN)/total samples
= (13+477)/512 = 95.70%

Tris-EDTA buffer (10 mM Tris HCl pH 8, 0.1 mM EDTA) and then kept at $-20^{\circ}C$ for PCR amplification.

PCR amplification of serum DNA : The method for PCR amplification was modified from Kruavon's procedure (Kruavon, 1994). Oligonucliotide primers, specific for HBV surface gene (S gene) sequence, were synthesized in DNA synthesizer (Applied Biosystems). Two specific primers of which the nucleotides were numbered from EcoRI site were used (Okamoto *et al.*, 1989). They were primer 262, 5' GGACTTCTCTCAATTTTC TAGGG 3' , beginning at map position 262 and primer 693R (from the complementary or reverse DNA strand), 5' CAAATGGCACTAGTAAACTGAGC 3', beginning at map position 693 of the HBV genome. Amplification of serum HBV-DNA was performed by using Taq polymerase under processing in DNA amplification machine. Target sequences were amplified in 50 µl reaction volume containing 3 µl of the serum DNA sample, 1 µl or 1 unit of Taq polymerase (Promega), 0.5 µl of 25 mM dNTP mixture, 0.6 µl (250 ng) of primer 262 and 0.75 µl (250 ng) of primer 693R, 5.0 µl 10x PCR reaction mixture, 3 µl of 25 mM $MgCl_2$ and 36.15 µl of distilled water. The reaction was performed for 40 cycles in a programmable thermal cycler (Perkin-Elmer Cetus). The samples for each cycle were heated to denature of DNA at $96^{\circ}C$ for 30 seconds, cooled to $55^{\circ}C$ for 30 seconds for primer annealing and incubated for 1 minute at $74^{\circ}C$ for primer extension. To eliminate sources of DNA contamination, all reagents were prepared and put into new containers and the experiments were performed in parallel with positive and negative control sera.

Analysis of amplified DNA : Each serum samples, 10 µl of amplified DNA reaction mixture was fractionated in 2% agarose gel electrophoresis, after staining with ethidium bromide, DNA was visualized by UV fluorescence.

Results

In this study, the serum samples taken from pregnant women at King Chulalongkorn Memorial Hospital were detected for HBV-DNA by PCR (Polymerase chain reaction). The PCR products of DNA samples were investigated for DNA band at 431 base pairs size, using specific primers from S gene conserved region of HBV. PCR detection was compared

with the other seromarkers detection, namely HBsAg, anti-HBs and anti-HBc by ELISA and Chemiluminescent immunoassay.

Figure 1 showed the results of HBV-DNA being seen in 4 (no. 1, 2, 13 and 14) out of 14 serum samples and in positive HBV-DNA control (lane P). The HBV-DNA bands were not seen in negative serum control (lane N) and 10 negative samples (no. 3 to 12).

Table 1 summarized the results of HBV-DNA detection by PCR as compare to HBsAg detection by the commercial test kit, Chemiluminescent immunoassay. The results was shown that HBV-DNA were detected in 10 out of 30 samples (33.33%) of HBsAg positive group and 4 out of 482 samples (0.83%) of HBsAg negative (normal) group. Total samples of HBV-DNA detection by PCR were 14 out of 512 samples (2.73%). The sensitivity, specificity and accuracy of PCR technique comparing with Chemiluminescence were 33.33%, 98.96% and 95.31%, respectively. From Table 1 the result of HBV-DNA and/or HBsAg positive seromarkers were shown that the pregnant women were infected with HBV.

Comparison of HBV-DNA detection by PCR with HBsAg detection by ELISA test kit were shown in Table 2. HBV-DNA was detected in 1 out of 478 samples(0.21%) of HBsAg negative group and 13 out of 34 samples (38.24%) of HBsAg positive group. The sensitivity, specificity and accuracy of PCR technique comparing with ELISA were 38.24%, 99.79% and 95.70%, respectively.

More studies, these serum samples were detected for other two HBV seromarkers, anti-HBs and anti-HBc, demonstration in Table 3. As the results, HBV-DNA was detected by PCR , HBsAg by Chemiluminescence, anti-HBs and anti-HBc by ELISA. It was found that pregnant women from group 1 to group 3, 24 out of 512 samples (4.69%), were chronic HBV infection because they had HBV-DNA or HBsAg and anti-HBc positive but anti -HBs was negative. Especially the pregnancy in group 2, 4 out of 512 samples, HBV-DNA and anti-HBc were positive but HBsAg was negative. In this case PCR was true positive result and HBsAg immunoassay was false negative result. Pregnant women with HBsAg positive but anti-HBc negative in group 4, 10 out of 512 samples (1.95%), were in stage of early HBV infection so they did not have the immune response to HBV. The result was also shown that the pregnant women with anti-HBs positive in group 5, 161 out of 512 samples (31.45%) had immune response to HBV because they were infected with HBV and recovered from this disease or no symtoms or HBV vaccination. Lastly, pregnant

women with negative seromarker tests in group 6, 317 out of 512 samples (61.91%), had never been infected with HBV. They had chance to infect with HBV if they did not have HBV vaccination. The pregnant woman sera in group 5 and 6 were not necessary to detect anti- HBc because this detection was used to confirm that HBV-DNA and HBsAg were true positive tests or not. The other reason, anti- HBc test kit was expensive for screening massive specimens.

Table 3 The results of four seromarker detections (HBV-DNA/PCR, HBsAg/Chemiluminescence, anti-HBs and anti- HBc/ELISA) in pregnant woman sera

Group	Seromarkers	No. of serum samples	Percentage(%)
1	DNA^+ $HBsAg^+$ Anti-HBs^- Anti-HBc^+	10	1.95
2	DNA^+ $HBsAg^-$ Anti-HBs^- Anti-HBc^+	4	0.78
3	DNA^- $HBsAg^+$ Anti-HBs^- Anti-HBc^+	10	1.95
4	DNA^- $HBsAg^+$ Anti-HBs^- Anti-HBc^-	10	1.95
5	DNA^- $HBsAg^-$ Anti-HBs^+	161	31.45
6	DNA^- $HBsAg^-$ Anti-HBs^-	317	61.91
Total		**512**	**100**

From Table1 and Table 2, we concluded that the infection of HBV in pregnancy at King Chulalongkorn Memorial Hospital were classified as HBV active carriers, they were 2.73% (14/512 samples) of HBV-DNA detection by PCR and 5.86% (30/512 samples) and 6.64% (34/512 samples) detection for HBsAg by Chemiluminescence and ELISA, respectively. As the result, the positive tests of HBV infection were diffferent for HBV-DNA detection by PCR, HBsAg by Chemiluminescence and ELISA. Thus, to confirm positive results of HBV infection of three methods, correlation or difference of those results were demonstrated in Table 4. The result was shown that HBV-DNA was detected in 9 samples of Chemiluminescence and ELISA positive group and 9 out of 14 samples of total HBV-DNA detection.

From the questionairs of pregnant women about HBV vaccination before pregnancy, the result was shown that only 8 out of 512 of pregnant women (1.56%) were vaccinated by HBV vaccine and the anti-HBs were positive in 5 out of 8 persons (62.5%). From data in Table 3, the pregnant women 317 persons with negative all seromarkers, have chance to be infected by HBV and transmit this virus to their infants.

Table 4 Demonstration of HBV-DNA by PCR and HBsAg detections by Chemiluminescence and ELISA

HBsAg	HBV-DNA		Total
	+	-	
Chemiluminescence^{+}, ELISA^{+}	9	10	19
Chemiluminescence^{+}, ELISA^{-}	1	8	9
Chemiluminescence^{-}, ELISA^{+}	4	10	14
Total	14	28	42

Discussion

From Table 1, HBV-DNA were detected by PCR in 2.73% of total pregnancy and were 33.33% and 38.24% of hepatitis B carrier group by Chemiluminescene and ELISA, respectively. The sensitivity of PCR technique was low when compared with immunoassay because of the low concentration of HBV-DNA in chronic HBV infection of the pregnant woman sera. It was reported that a low level of HBV-DNA was rarely detected in the serum of chronic persistent infection but often detected in liver tissues (Brechot *et al.*, 1985). In addition, HBV-DNA could be detected in sera when there were active viral replication and had dane particles in sera (Nuanchawee *et al.*, 1996). Our result was consistent with the results from the studies by Nuanchawee *et al.* and Kruavon *et al.* that HBV-DNA could be detected in 22% of HBsAg positive group of blood donors and they found that HBV-DNA at least 1 ng concentration could be detected by PCR technique (Brechot, 1981; Kruavon, 1994). The other reasons, there are many factors in effectiveness of PCR technique such as process of extraction of DNA, activity of Proteinase K enzyme and Taq polymerase enzyme. Moreover, the low sensitivity of PCR did not cause by the primers that we used because S gene conserve region was specific for whole genome of HBV. The primers were covered all subtypes of HBV especially ad (adr and adw) subtype which had high prevalence in Thailand (Duanthanorm *et al.*, 1994).

In our study, we found that the false negative test was 0.83% by chemiluminescent immunoassay and 0.21% by ELISA (Table 1 and Table 2). Although the result indicated the presence of HBV-DNA in HBsAg carriers and normal persons, PCR for HBV diagnosis could not finally replace HBsAg immunoassay in clinical specimens. However, the detection of HBV-DNA by PCR was a useful method for additional screening HBV in pregnant woman sera since it can directly detect of HBV active carriers even in the case of HBsAg

negative. If we screen these samples only HBV-DNA marker, we will miss some HBV positive samples. Therefore, it is more useful if PCR technique is used in conjunction with other serological tests to detect a false negative. Usually, HBV can transmit from the infected mothers to their infants when they give birth by touching of blood or feeding their children at early stage of life. Administration of vaccine and specific immunoglobulin to neonates of infected mothers can reduce transmission (Gillespie and Bamford, 2000). Besides, hepatitis B carriers of pregnant women in our study in year 2000 were 5.86% detection for HBsAg by Chemiluminescence and 6.64% by ELISA. The results were consistent with King Chulalongkorn Memorial Hospital report in year 2000 showing that HBV carriers of pregnant women were 6.3% (Anonymous, 2002). It is indicated that HBV infection be important problem of public health in Thailand. If we can screen of HBV carriers out of normal persons by using powerful method, the cause of HBV infection will be finally decrease.

From Table 4, the commercial HBsAg test kits are different in sensitivity, we do not know which one is real positive result. In this study, we should confirm screening of HBV-DNA negative samples with the third HBsAg test kit or others seromarkers such as HBeAg and anti-HBe.

It was reported that Chemiluminescent immunoassay (Abbott prism) also was excellent sensitivity, specificity and ease automation to detect HBsAg, anti- HBc, hepatitis C virus, HIV and others (Khalil *et al.*,1991; Cheng *et al.*, 1999). For Abbott prism HBsAg by Chemiluminescent immunoassay has estimated specificity of 99.69% and the sensitivity for detection of ad and ay subtypes are approximately 0.1 and 0.08 ng/ml, respectively. Nowadays, Chemiluminescence is a gold standard method for screening of HBV infection because of a high sensitivity and specificity. The screening of HBV infection of blood donors and pregnant woman sera at Thai Red Cross Society also used this method.

It was reported that the ELISA test kit of the Department of Medical Science was developed by using monoclonal antibody derived from adr subtype of HBV vaccine and used as probe to develop the one-step ELISA test. Adr subtype of HBV often transmits in Thailand. It has estimated sensitivity, specificity and accuracy 95.1%, 100% and 97.3%, respectively (Nongluk, 2000). In our study, ELISA test kit of Department of Medical Science was more sensitive than Chemiluminescence and this test kit was available because the prize of that is cheaper than commercial ELISA test kit for massive HBV serum

screening. In addition, the process of ELISA test kit was simple for diagnosis and can detect the target faster than PCR. Especially, this test kit was produced in our country.

Table 5 Demonstration of approximately cost of test kit for detection of HBV infection.

Method	Prize/test kit/96 wells	Cost/samples (bath)
Chemiluminescence	-	30
Commercial ELISA	4,500	100
Developed ELISA	2,800	50
PCR	-	100

From table 5, Although the cost per sample of Chemiluminescence is cheaper than other methods but the Chemiluminescent automate diagnosis equipment or machine is expensive and we should detect a large number of specimens such as 1000-1500 specimens/time/day. It will be benefit for the diagnosis otherwise we will lost the reagents. The cost of ELISA and PCR are nearly the same depend on the trademark but the immunoassay is simple and faster than PCR technique but it still miss HBV positive detection. If we can develop PCR technique to be high effectiveness, it will be beneficial for our country in the future.

In conclusion, PCR technique could not replace the conventional HBV immunoassay because of it's low sensitivity but it is useful to be additional test for increasing the sensitivity of HBV for diagnosis especially for prevention of HBV transmission from mothers to their infants.

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