

Detection of Hepatitis B Virus Pre-core Mutant by Allele Specific Polymerase Chain Reaction

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ABSTRACT

Introduction: Mutation in pre-core region is characterized by negative HBeAg and positive anti-HBe despite active replications of the virus. The mutation has diagnostic and prognostic implications. Therefore, detection of pre-core mutant is important. Standard diagnosis approach for detecting pre-core mutant is through DNA sequencing of hepatitis B virus (HBV) pre-core region. Unfortunately, DNA sequencing is not available in most centers. Hence, a simpler diagnostic approach is necessary.

Method: An observational-analytic design study was performed. Detection of pre-core mutant was conducted in individuals with positive HBsAg and HBV DNA that had various patterns of HBeAg and anti HBe. HBsAg, HBeAg and anti-HBe was detected using immunochromatography technique. The HBV DNA was evaluated by using qualitative polymerase chain reaction (PCR) testing. PCR was done by three rounds of amplification with primers derived from wild type pre-core and mutant pre-core.

Results: Of 25 sera with HBeAg negative, anti-HBe positive and HBV DNA positive, allele specific (AS) PCR pre-core mutant was detected in 20 (80%) sera. Two sera with HBeAg negative, anti HBe negative and HBV DNA positive were negative for pre-core mutant. Of 8 sera with HBeAg positive, anti HBe negative and HBV DNA positive, pre-core mutant was detected in 2 (25%) sera.

Conclusion: Most of individuals with HBV DNA positive, HBeAg negative and anti-HBe positive have harbored pre-core mutant. The finding indicated that all patients with HBsAg positive, HBV DNA positive and HBeAg negative, but anti-HBe positive should be examined for the presence of pre-core mutant. Pre-core mutant is also found in HBeAg positive individual.

Keywords: HBV, pre-core mutant, polymerase chain reaction

ABSTRAK

Pendahuluan: Mutasi di daerah pre-core ditandai oleh HBeAg yang negatif dan anti-HBe positif pada kasus-kasus replikasi aktif virus. Mutan pre-core menyebabkan masalah diagnostik dan prognostik karena itu deteksi mutan pre-core sangat penting. Diagnosa standar untuk mutan pre-core adalah pengurutan DNA pada daerah pre-core virus hepatitis B (VHB). Namun fasilitas untuk pengurutan DNA sulit didapatkan karena itu diperlukan diagnostik yang lebih sederhana.

Metode: Dalam penelitian ini digunakan desain observasional analitik. Deteksi mutan pre-core dilakukan pada sera HBsAg positif dan DNA VHB positif dengan berbagai pola HBeAg dan anti HBe. HBsAg, HBeAg dan anti HBe dideteksi dengan teknik imunokromatografi. DNA VHB dideteksi dengan PCR kualitatif. PCR spesifik-alel diperiksa oleh tiga PCR dengan primer-primer yang berasal dari region pre-core tipe liar dan pre-core mutan.

Hasil: Dari sejumlah 25 sera HBeAg negatif dan anti HBe positif serta DNA VHB positif mutan pre-core didapatkan pada 20 (80%) sera. Dua sera dengan HBe negatif dan anti HBe negatif serta HBV positif keduanya tidak terdeteksi mutan pre-core. Dari 8 sera HBsAg positif dan anti HBe negatif serta HBV positif mutan pre-core didapatkan pada 2 (25%) sera.

Simpulan: Sebagian besar individu dengan DNA VHB positif dan HBeAg negatif dan anti HBe positif menunjukkan mutan pre-core. Dari hasil penelitian ini semua penderita dengan HBsAg positif, DNA VHB positif dan HBeAg negatif serta anti HBe positive seharusnya diperiksa untuk mengetahui adanya mutan pre-core. Mutan pre-core juga didapatkan pada penderita HBeAg positif.

Kata kunci: HBV, mutan pre-core, PCR

INTRODUCTION

Mutation in pre-core region was first reported several decades ago by Carman et al.¹ It was reported that the mutation affects hepatitis B virus (HBV) replication. One of the widely known consequences is the inability to produce HBeAg and positivity of anti HBe despite active replication of the virus. Thus, the presence of pre-core mutant may cause inability to predict HBV replication based on previous knowledge on HBeAg and anti HBe status only. The mutation commonly takes place in the pre-core region, which is in the nucleotide number 1386 causing classic HBeAg negative and anti HBe positive cases in patients with active viral replication.^{1,2} The pre-core mutation is usually found in patients with chronic hepatitis, liver cirrhosis, hepatocellular carcinoma and it is reported in acute fulminant hepatitis cases of certain region. Moreover, it is also reported in asymptomatic carrier.^{2,3,4,5,6}

Several study reports has shown that it is correlated with relative diminished sensitivity to anti viral treatment.^{7,8} Many reports have suggested that a special approach is required in the management of patients with pre-core mutant.^{8,9,10} Therefore, detection of pre-core mutant has clinical importance for predicting prognosis and selecting appropriate management.

The most common approach for detecting pre-core mutant is DNA sequencing of pre-core region.¹¹ DNA sequencing is an advanced and relatively complicated molecular laboratory test, which is only available in large-scale industrial research laboratories. Meanwhile, such test is not available commercially and it is intended only for clinical use in our country. Therefore, a more practical and non-sequencing approach is necessary. In this study, we report the utilization of allele specific polymerase chain reaction (PCR) for detecting pre-core mutant. This method can be done more easily

in laboratories with limited molecular laboratory equipment. It is sensitive enough for detecting DNA mutation in a specific known position. In our study, we evaluated the presence or absence of pre-core mutation in HBV DNA positive sera, which had various HBeAg/anti HBe patterns, in HBsAg positive individuals using allele specific PCR.

METHOD

HBsAg was detected by immunocromatography technique of (entebe) Mataram. The remaining sera were collected and examined for HBeAg and anti-HBe using immunocromatography stick (Acon USA). The sera were frozen in minus 20°C temperature. Finally, the sera were examined in Biomedika laboratory, June 2012, for HBV DNA using qualitative PCR method with primers derived from S gene. All sera with HBV DNA positive sera were examined for the presence or absence of mutation in 1,396 nucleotide using allele specific PCR.

Because the portion of mutated virus maybe very small in size, the allele specific PCR is preceded by amplification phase, in which the pre-core sequence for both wild and mutant viruses were amplified using three kinds of primers. The PCR product was then used for allele specific PCR. The PCR method and primers were advised by Tilmann et al.⁴

Polymerase Chain Reaction

There were three rounds of PCR amplification, which were done on each sample using five primers. The three PCR rounds are described as follows: PCR 1 and PCR 2 are concerning the pre-core/core region. PCR 3 is allele specific PCR using primer with mutation in base 83.⁴

The product of PCR 1 was re-amplified in PCR 2. In PCR 2, a forward primer derived from wild type virus was used. The amplification product of PCR 2

was utilized in PCR 3. In PCR 3, the primer derived from pre-core mutated virus was used. The conditions set for allele specific PCR was denaturation at 94°C for 1 minute, annealing process at 72°C for 1 minute, and primer extension at 72°C for 1 minute.

PCR1

Pre-C F 5'GGAGGC TGTAGG CAT AAA TTG GTC
Pre-C R 5'GAT CTTCTG CGACGC GGC GATTGAGA

PCR2

Pre-C R 5'GAT CTTCTG CGACGC GGC GATTGAGA
OHB 5' TGT GCC TTG GGT GGC TTT G

PCR 3

Pre-C R 5'GAT CTTCTG CGACGC GGC GATTGAGA
MHB 5' TGT GCC TTG GGT GGC TTT A

Positive findings of 450 bp band in electrophoresis of PCR 2 indicated that there was wild type virus; while positive band seen in electrophoresis of PCR 3 products demonstrated the presence of pre-core mutant type virus.

RESULTS

A total of 44 HBsAg positive sera can be recollected and 23 of those were HBV DNA positive. The remaining sera were sufficient for AS PCR in 35 (79.5%). The correlation of HBV DNA and HBeAg/anti HBe is shown in Table 1.

Table 1. HBV DNA in HBsAg positive individuals according to HBeAg/ Anti-HBe status

HBeAg	Anti-HBe	Total	HBV DNA positive (%)
Positive	Negative	11	11 (100%)
Negative	Positive	26	11 (42%)
Negative	Negative	7	1 (14.3%)
Total		44	23 (52.3%)

We performed AS PCR in 35 HBV DNA positive with various patterns of HBeAg and anti HBe. As the negative control, we also performed AS PCR in 8 sera, which were positive for HBsAg but negative for HBV DNA.

Of 25 sera with HBeAg negative, anti-HBe positive and HBV DNA positive, pre-core mutant was detected in 20 (80%) sera by using AS PCR and pre-core mutant was negative in 5 (20%) sera. Both pre-core mutant and normal pre-core sequences were detected in 10 (50%) sera with pre-core mutant positive. There was indicated a mixed population of pre-core sequence in

those sera. Moreover, in other 10 (50%) sera, the whole part of the pre-core sequence was mutated.

Two sera with HBeAg negative, anti HBe negative and HBV DNA positive were negative for pre-core mutant. Of 8 sera with HBeAg positive, anti HBe negative and HBV DNA positive, the pre-core mutant was detected in 2 (25%) sera. Both of them had mixed normal pre-core and mutant pre-core sequences. The summaries of AS PCR results are shown in Table 2, Table 3, and Table 4.

Table 2. The results of pre-core AS PCR in HBeAg positive and anti HBe negative of HBV DNA positive sera in HBsAg positive patients

HBeAg/anti-HBe status	Pre-core sequence	Number of sera (%)
HBeAg – anti-HBe positive	Normal pre-core only	5 (20%)
	Pre-core mutant only	10 (40%)
	Mixed pre-core	10 (40%)
Total		25 (100%)

Table 3. The result of pre-core AS PCR in HBeAg negative and anti HBe negative of HBV DNA positive sera in HBsAg positive patients

HBeAg/anti-HBe status	Pre-core sequence	Number of sera (%)
HBeAg – anti-HBe negative	Normal pre-core only	2 (100%)
	Pre-core mutant only	0 (0%)
	Mixed pre-core	0 (0%)
Total		2 (100%)

Table 4. The result of pre-core AS PCR in HBeAg positive and anti HBe negative of HBV DNA positive sera in HBsAg positive patients

HBeAg/anti-HBe status	Pre-core sequence	Number of sera (%)
HBeAg – anti-HBe negative	Normal pre-core only	6 (75%)
	Pre-core mutant only	0 (0%)
	Mixed pre-core	2 (25%)
Total		8 (100%)

Of 8 sera with positive HBsAg and negative HBV DNA, we found that all sera were negative for both normal and mutant pre-core sequence.

DISCUSSION

Point mutation of HBV gene mutations is one of important factors for developing of chronic and malignant liver disease.¹¹ One of important mutations is mutation in nucleotide number 1,396 at pre-core region. The pre-core mutations increase virus pathogenicity and decrease sensitivity to anti-viral agents. Several studies has demonstrated the clinical importance of pre-

core mutant.^{9,12} The prognosis of patients with pre-core mutant is worse compared to those without pre-core mutant.⁷ Due to limitations in laboratory techniques, only minority of patients with possible pre-core mutations can probably be examined for detecting the presence of such mutations. Our study results implied that the mutation can be detected by an easier and faster non-sequencing method. It is expected that the method can detect mutation with wider coverage as needed and it can be used routinely to detect pre-core mutations.⁴ The presence of pre-core mutant can also be detected from histological sample.¹³ Pre-core mutation causes HBeAg negativity in replicative phase of the virus. It decreases the immunotolerance effect of HBeAg resulting in greater immune response and may affect clinical manifestation or severity of the disease.¹²

Theoretically, like other DNA mutations, pre-core mutation is caused by host immune pressure exerted. It is a viral effort to escape from the host's immune response.^{2,5,7}

Pre-core mutant causes negative HBeAg and positive anti-HBe during replicative phase; however, the mutant is not only found in HBeAg negative individuals. It has also been reported in minority of

patients with HBeAg positive and anti HBe negative individuals as also shown in our study. Several other studies have also reported the presence of pre-core mutant in HBV DNA positive with HBeAg negative and anti HBe negative individuals.^{2,14} Such findings indicate that pre-core mutant detections in patients other than those with negative HBeAg and positive anti-HBe are questionable. One of study reports demonstrates that the presence of pre-core mutant in HBeAg positive patients is often accompanied by relapse after treatment with anti-viral agent.¹⁵ The importance of pre-core mutant detections is important in Indonesia, where the majority of the patients has genotype B that are more frequently accompanied by pre-core mutant.^{16,17,18} A study conducted in Surabaya showed that 62.5% of chronic hepatitis B without hepatoma and 85.7% patients who had chronic hepatitis B with hepatoma had demonstrated positive findings of pre-core mutant.¹⁶

CONCLUSION

Eighty percent (80%) of sera with HBV DNA positive and negative for anti-HBe had harbored pre-core mutant and most of the samples had mixed population of normal and mutated pre-core sequence and only 20 % of the sample had pure mutated pre-core. This finding indicates that all patients with HBsAg positive, HBV DNA positive and HBeAg negative, but anti-HBe positive should be examined for detecting the presence of pre-core mutant. Pre-core mutation is also detected in sera with positive HBeAg and negative HBeAg. Therefore, pre-core mutant is not exclusively associated with HBeAg negative and anti HBe positive cases. Allele specific PCR can detect mutant or wild type pre-core sequence in a simple and relatively easy method, so that detection of pre-core mutant can be performed with wider coverage for appropriate cases, which are suspected of having the condition including those cases with positive HBV DNA and negative HBeAg but positive for anti-HBe.

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Figure 2. Gel electrophoresis of allele spesific PCR product using primers derived from HBV DNA pre-core sequence. Primer O (ordinary type), primer M (mutant type at base No. 83); O: ordinary type; M: mutant type; Ladd: molecular weight marker. 450 bp bands were seen in O which indicates original type (wild type). The same bands are seen in M indicating mutant type (pre-core mutant)

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