The Journal of Microbiology (2010) Vol. 48, No. 1, pp. 111-116
DOI 10.1007/s12275-009-0282-8

Designing Primers from Multiple Sequences Using Matchup Program to Improve Detection of Hepatitis B Virus by Polymerase Chain Reaction

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(Received September 15, 2009 / Accepted October 15, 2009)

Traditionally primers for PCR detection of viruses have been selected from genomic sequence of single or representative viral strain. However, high mutation rate of viral genomes often results in failure in detecting viruses in clinical and environmental samples. Thus, it seems necessary to consider primers designed from multiple viral sequences in order to improve detection of viral variants. Matchup is a program intended to select universal primers from multiple sequences. We designed using Matchup program primer pairs for HBV detection from 691 full genomic HBV DNA sequences available from NCBI GenBank database. Thousands of primer candidates were initially extracted and these were sequentially filtered down to 5 primer pairs. These primer pairs were tested by PCR using 5 HBV Korean HBsAg(+) patient sera, and eventually one universal primer pair was selected and named MUW (multiple-universal-worldwide). This primer pair, 3 HBV reference primer pairs reported by others and 1 commercial primer pair were compared using 86 HBV HBsAg(+) sera from Korean and Vietnamese patients. The detection rate for MUW primer pair was 72.1%, much greater than those obtained by reference and commercial primers (32.5 to 40.7%). The superiority of MUW primer pair appeared to be correlated with the conserved sequences of the forward primer binding sites and primer quality score. These results suggest that the universal primers designed by the Matchup program from multiple sequences could be useful in detecting viruses from clinical samples.

Keywords: HBV, PCR, primer, Matchup

Hepatitis B virus (HBV) is a member of *Hepadnaviridae* and causes chronic liver diseases in millions of people globally. Globally, at least 2 billion people or one third of the world population have been infected with HBV, approximately 400 million remain chronically infected, and an estimated 1 million die each year from acute and chronic sequelae secondary to HBV infection (Lee, 1997; Ocama *et al*., 2005; Zanetti *et al*., 2008). In addition, approximately 4.5 million new HBV infections occur worldwide each year, of which a quarter progresses to liver disease. In Western countries, the disease is relatively rare due to extensive vaccination and acquired primarily in adulthood, whereas in Asia and most of Africa, chronic HBV infection is common and usually acquired early in life.

In addition to a symptomatic, serologically evident infection, occult persistent HBV infection has been identified (Allain, 2004; Mulrooney-Cousins and Michalak, 2007; Raimondo *et al*., 2007). Occult HBV infection can be defined as the long-lasting persistence of viral genomes in the liver tissue (and in some cases also in the serum) of individuals negative for the HBV surface antigen (HBsAg). Accumulated evidence indicate that occult HBV can be both a source of virus contamination in blood and organ donations, as well as the reservoir from which full blown hepatitis can arise (Larsen *et al*., 1990; Liu *et al*., 2006; Mulrooney-Cousins and Michalak, 2007). Identification of occult HBV infection has been possible since nucleic acid amplification techniques (NAT) such as polymerase chain reaction (PCR) of enhanced sensitivity became introduced for detection of hepadnaviral genomes and their replicative intermediates (Mulrooney-Cousins and Michalak, 2007).

In most of the clinical laboratories and blood centers, HBV infection is generally determined by enzyme immune assays (EIA) detecting antibodies to HBs or HBc antigens in the blood of infected people. EIA is simple and cost-effective but suffers from several drawbacks. During the initial phase of infection, viraemia precedes the development of antibodies. This period is called window period and may last up to 8 weeks for HBV, and the chances for false-negative result from HBV-positive bloods are average of 6.9 per million donations (Allain, 2004). In addition, the relatively high frequency of blood donors with circulating HBV DNA in the absence of other markers of HBV infection suggests that not all such individuals are in the window period, but rather may be infected with HBV variants or experiencing chronic viraemia without detectable antibodies (Loriot *et al*., 1997; Allain, 2004).

Therefore, in order to overcome the problems encountered by occult infection, windows period and HBV variants, designing efficient NAT or PCR protocols with primers targeted to sequences in highly conserved regions are merited. In this study, we designed primers using Matchup primer

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design tool from all available HBV full genomic sequences registered in NCBI GenBank database for better detection of HBV from blood.

Materials and Methods

Construction of primers for HBV detection using Matchup program

Full genomic DNA sequences of all HBV registered in NCBI GenBank database (n=691) were obtained and multiple-aligned using CLUSTAL X program (ver. 1.83, Thompson *et al*., 1997). The aligned sequences were imported to Matchup program developed by us (http://plaza.snu.ac.kr/~jchun/matchup/). Primer selection conditions were adjusted to generate primers with the following properties: size between 18 bp and 25 bp, melting temperature (Tm) between 50 and 60, maximum Tm difference 5, GC% between 45 and 60 and product size between 100 and 900. Further option values were selected in order to minimize nonspecific binding of primers, self-annealing of the primers, and possibility of primer-dimers between forward and reverse primers. Finally, the primer pairs suggested by Matchup program were filtered by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and NetPrimer programs (http://www.premier-biosoft.com/netprimer/index.html).

HBV DNA sample preparation

Total of 86 serum samples including 65 from Korean HBV patients and 21 from Vietnamese patients were used in this study. The 65 Korean serum samples were obtained from two general hospitals located in Cheongju, Korea: 35 from hospital CB and 30 from general hospital SM. HBV DNA was extracted from serum samples using QIAamp® DNA Mini kit (QIAGEN, Germany). Twenty microliter of buffer Al was added, mixed by vortexing for 15 sec and incubated for 10 min at 56°C. Then 200 μl of absolute ethanol was added to the reaction mixture, vortexed for 15 sec and transferred to QIAamp spin column. After centrifugation at 8,000 rpm for 1 min, supernatant was discarded, 500 μl of AW1 buffer was added to column, and centrifuged at 8,000 rpm for 1 min. The supernatant was discarded, 200 μl of AW2 buffer was added to column, and centrifuged at 14,000 rpm for 1 min. Finally, the DNA sample was transferred to QIAamp spin column, added 50 μl elution buffer for 2 min at room temperature, and spun for 1 min at 8,000 rpm. The DNA samples were stored at -70°C.

Polymerase chain reaction (PCR)

The HBV DNA samples were subjected to PCR using MUW primer pair designed by Matchup program, 3 pairs of reference primers (409.703A, 686.457, Hamasaki) and 1 pair of commercial primer (Company B, Korea). The PCR mixture contained 10× buffer (20 mM $MgCl_2$) 5 μl, 25 mM dNTP 4 μl, sample DNA solution 3 μl, forward and reverse primer 2 μl each (10 pmol), Taq polymerase (5 U/μl) 0.5 μl, and distilled water up to 50 μl. The sequences and PCR conditions of the primer pairs used in this study were as follows. MUW (CTCGACTCGTGGTGGACT/AAACTGAGCCAGGAGAAACG, this study): predenaturation at 94°C for 5 min, 35 cycles of 94°C for 20 sec, 59.3°C for 30 sec, 72°C for 40 sec. 409.703A (CATCCTGCTGCTATGCCTCATCT/CGAACCACTGAACAAATGGCACT, Nainan *et al*., 1996) and 686.457 (GGCACTAGTAAACTGAGCCA/GGTATGTTGCCCGTTTGTCCTCT, Nainan *et al*., 1996): predenaturation at 95°C for 5 min, 45 cycles of 95°C for 90 sec, 47°C for 45 sec, 72°C for 2 min. Hamasaki (CAAGCTGTGCCTTGGGTGGCCTT/CCCGATACAGAGCTGAGGCGGTGTC, Hamasaki *et al*., 1994): predenaturation at 94°C for 5 min, 30 cycles of 94°C for 60 sec, 60°C for 60 sec, 72°C for 60 sec. BION (sequence unavailable, commercial): predenaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 50 sec. All PCR reactions received final extension step at 72°C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, visualized by short-wave UV trans-illuminator and photographed.

Genetic distance analysis

The genetic distances among the primer binding sites or the distances from the primer sequences to the primer binding sites were estimated by calculating the nucleotide sequence diversities. Genetic distances are indicative of sequence conservation or evolutionary relatedness of the sequences under study. Nucleotide sequences of the primer binding sites were extracted from 691 HBV full genome sequences, and multiple-aligned with CLUSTAL X. Aligned sequences were compared with SeqAid program developed by us to get genetic distances among sequences belonging to different groups according to Jukes and Cantor's method (Jukes and Cantor, 1969). Then, mean and standard deviation values for each pair of comparison were calculated. Statistical analysis was performed with SPSS (ver. 10) in order to get statistical significance of the data when needed.

Results

MUW primer pair efficiently detects HBV from serum samples

From 691 full genomic HBV sequences, 22 primer pairs were selected using Matchup and BLAST programs. Examination using NetPrimer program yielded 5 primer pairs which do not generate undesired secondary structure. These primer pairs were tested against 5 pre-confirmed HBV DNA(+) serum samples and plasmid pAM containing HBV strain *adw* in pBR322. Finally a primer pair with better detection by PCR was selected and named MUW.

Total of 86 (65 Korean from two sources and 21 Vietnamese) HBV DNA extracted from sera of HBsAg(+) patients were subjected to PCR detection using MUW, 3 reference and 1 commercial primer pairs. The results are summarized in Table 1. In general, Vietnamese (VN) samples were detected better than Korean (KOR) samples by all 5 primer pairs used in this study. Among the primer pairs, MUW primer pair (72.1%) was better in detecting HBV from serum samples than reference or commercial primer pairs (34.9%-43.0%). Most of all, MUW primer pair was significantly more efficient in detecting HBV by PCR than any other primer pairs ($p<0.05$). Furthermore, MUW primer pair was the most efficient and least variable in detecting HBV by PCR regardless of the HBV sources (Table 1).

Detection efficiency is related with virus load

The reason for difference of detection efficiency by PCR among the clinical samples was investigated in terms of virus load in the samples. The amounts of HBV in 30 Korean samples from hospital SM (KOR-SM) were predetermined by COBAS Amplicor system (Roche Diagnostic, USA). The viral loads of these samples were plotted against detection efficiency by 5 primer pairs. As shown in Fig. 1, there was a linear relationship between the amount of virus and detection

Table 1. The rate of detection by PCR using 5 different primer pairs

Sample source	Number	Detection rate (%)					
		MUW	BION	Hamasaki	409.703A	686.457	Average
KOR-Total	65	67.7	29.2	32.3	32.3	27.7	38.7
KOR-CB[a]	35	62.8	25.7	31.4	22.8	20.0	33.8
KOR-SM[b]	30	73.3	33.3	33.3	46.6	36.6	44.4
VN	21	85.7	61.9	57.1	71.4	57.1	69.0
Total	86	72.1	43.0	38.4	43.0	34.9	47.3

[a] Samples obtained from general hospital CB located in Cheogju, Korea
[b] Samples obtained from general hospital SM located in Cheogju, Korea

efficiency of the primer pairs with regression coefficient (R^2) of 0.90 ($p=6\times10^{-6}$). Thus, the higher efficiency of primers in detecting HBV from patient's sera appears to be related with the amount of virus particles in the sera. When the amount of HBV was greater than 5 $\log_{10}$IU/ml, all were detected by PCR regardless of the primer used. When the virus load was lower than 3.5 $\log_{10}$IU/ml, only MUW primer pair was able to detect HBV in sample (data not shown).

Since the COBAS Amplicor HBV Monitor system of Roche Molecular Systems has been standard method in detecting viruses including HBV in clinical samples, we compared Amplicor system and MUW-1 system developed by us in this study. Of the 30 samples, 20 were detected by both Amplicor system and MUW, 6 were not detected by either system, 2 were detected by Amplicor system but not by MUW, and 2 were detected by MUW but not by Amplicor system (Table 2). Chi-square test suggested that MUW system is not different from Amplicor system in detecting HBV ($p<0.001$). Therefore, MUW primer system appeared as efficient as COBAS Amplicor system in detecting HBV from patient's serum used in this study.

Detection efficiency is related with sequence conservation and primer quality score

The difference in efficiency among the primer pairs to detect HBV by PCR might be explained by the difference in conservation of the sequences recognized by the primers. Thus, we estimated the nucleotide sequence diversity by calculating the evolutionary distances among the primer binding sites and distances from the primer sequences to the primer binding sites. Since there are two primer binding sites, one for forward primer and another for reverse primer, forward primer binding sites were considered first. As shown in Fig. 2A, there was a somewhat linear inverse relationship between the rate of HBV detection by PCR and sequence diversity among the forward primer binding sites ($R^2=0.6729$, $p=0.012$) or the distance from the forward primers to the primer sequences to the primer binding sites ($R^2=0.6144$, $p=0.012$). On the contrary, we were not able to observe statistically significant correlation between the detection efficiency and the nucleotide sequence diversity of the reverse primer biding sites, with very low coefficient of correlation ($R^2<0.1$, Fig. 2B). Thus, the efficiency of HBV detection by PCR using primer pairs tested in this study appears to correlate with the sequence conservation in the forward primer binding sites, but not in the reverse primer binding sites.

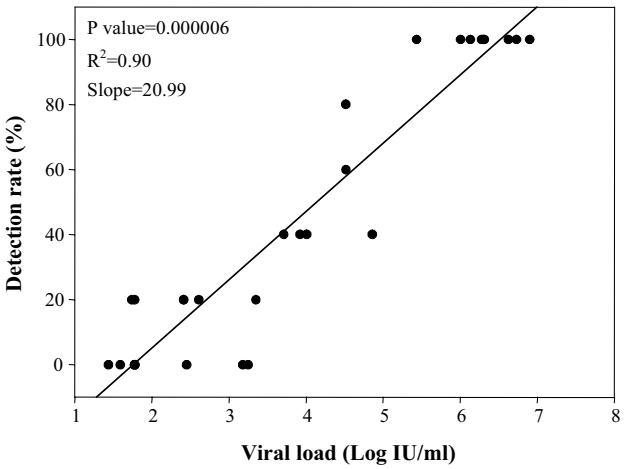

Fig. 1. Relationship between virus load and detection rate. Relationship between virus load and detection rate. The amount of HBV in serum samples were plotted against the rate of detection by PCR using each of the 5 primer pairs. Detection rate is 100% if detected by all 5 primer pairs, and 0% if detected by none of the primer pairs.

Next we wanted to know the reason for the higher detection efficiency of the MUW primer pair in relationship with the quality of the primers. Chen *et al*. (2003) developed a method to calculate the primer quality scores considering melting temperatures, formation of hairpin and dimer structure and free energies of the primers: $R = 100 - dT_m + dG_{forward}(3'\text{-}5') + dG_{reverse}(3'\text{-}5')$ + Hairpin score + Dimer score, where $dT_m = | T_m \text{ (forward)} - T_m \text{ (reverse)} |$, $dG = dH - 298.15 \times dS$. We previously developed a program to automatically calculate the primer quality score R using the primer sequences (UPSR program, Jang *et al*., 2008). There are many methods to calculate the melting temperature T_m, and three most widely used methods were considered in this study. The primer quality scores for MUW and three reference primers were calculated and arranged in the order of higher detection rate (Table 3). The MUW primer pair exhibited the highest primer

Table 2. Comparison of MUW primer system and Roche Amplicor system in detecting HBV from Korean patient's sera

		Amplicor		Sum
		+	-	
MUW	+	20	2	22
	-	2	6	8
	Sum	22	8	30

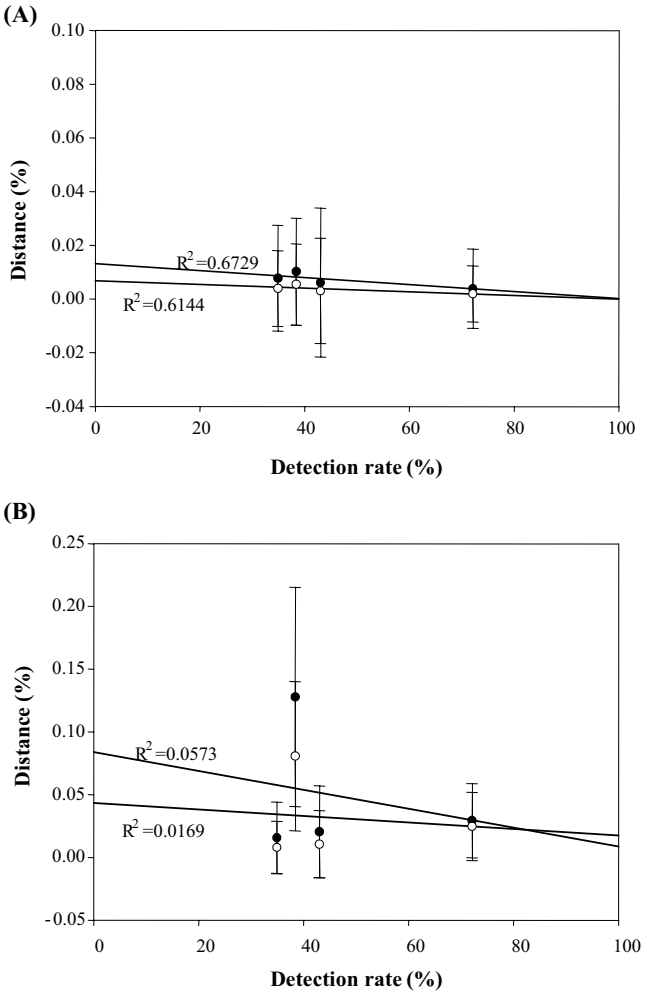

Fig. 2. Relationship between sequence conservation and detection rate. Relationship between sequence conservation and detection rate. Sequence diversities were obtained by calculating the genetic distances among the primer binding sequences (●, sequence diversity) or the distances between the primers and their binding sites (○, distance from primers). (A) forward primer; (B) reverse primer.

Table 3. Primer quality scores of the 4 primer pairs according to three most widely used Tm methods

Name	Detection rate (%)	Quality score		
		T_{m1}[a]	T_{m2}[b]	T_{m3}[c]
MUW	72.1	101.4	101.4	100.0
409.703A	43.0	96.7	96.7	97.8
Hamasaki	38.4	91.0	94.9	96.1
686.457	34.9	88.7	94.6	91.7

[a] T_{m1}=4(G+C) + 2(A+T), where G, C, A, and T are the numbers of corresponding bases in the primer.
[b] T_{m2}=81.5 + 16.6 log[Na+] + 41(G%+C%) - 500/L - 0.62F, where [Na+]: concentration of the salt (50 mM), G%+C%: mole fractions of G and C in the primer, L: primer length, and F: the percentage of formamide in the hybridization solution.
[c] T_{m3}=(1000dH/A + dS + Rln(C/4)) - 273.15 + 16.6 log[Na+], where dH: sum of the nearest-neighbor enthalpy changes for hybrids, dS: sum of the nearest-neighbor entropy changes, A: constant for helix initiation (-10.8), R: gas constant (1.987 cal/°C mol), C: concentration of the primer (1 nM) and [Na+]: concentration of the salt (50 mM).

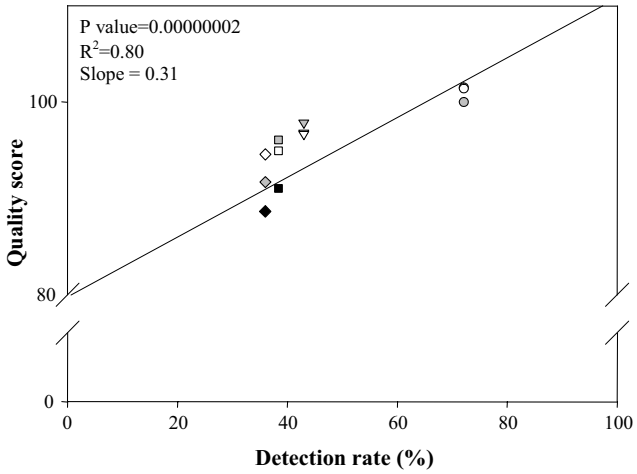

Fig. 3. Relation between the primer quality score and detection rate. Relation between the primer quality score and detection rate. Primer quality was scored using UPSR program (Jang *et al*., 2008) and plotted against the rate of detection by PCR using each of the 4 primer pairs. Commercial primer pair BION was not included since the nucleotide sequences are not available.

quality scores regardless of the T_m calculation methods. Furthermore, the primer quality scores were correlated with the HBV detection rates with statistical significance (R^2=0.80, p=2×10^{-8}, Fig. 3). Therefore the higher detection efficiency of the MUW primer pair could be explained by its higher primer quality.

Mutation at primer binding sites affects detection by PCR

Analysis of PCR detection of HBV from serum samples revealed that the primer pair with higher quality score was, in general, able to detect all samples that were detected by the primer pair with lower quality score as well as some samples that were not detected by the primer pair with lower quality score. However, there were exceptions for this general observation. For examples, two samples SM#63 and CB#14 were detected by the Hamasaki or 686.457 primer pair but not by the 409.703A primer pair, although the detection rate of the Hamasaki or 686.457 primer pair was lower than that of the 409.703A pair (Table 1). In order to explain this paradoxical observation, primer binding sites of the samples

	409 409.703A(F) 431	682 409.703A(R) 704
Consensus	CATCCTGCTGCTATGCCTCATCT	AGTGCCATTTGTTCAGTGGTTCG
SM#39	CATCCTGCTGCTATGCCTCATCT	AGTGCCATTTGTTCAGTGGTTCG
SM#65	CATCCTGCTGCTATGCCTCATCT	AGTGCCATTTGTTCAGTGGTTCG
SM#68	CATCCTGCTGCTATGCCTCATCT	AGTGCCATTTGTTCAGTGGTTCG
SM#63	CATCCTGCTGCTATGCCTCATAT	AGTGCCATTTGTTCAGTGATTCG
CB#14	CATCCTGCTGCTATGCCTCATCT	AATGCCATTTGTTCAGTGGTTCG

Fig. 4. Mutations at the primer binding sites of 409.703A. Mutations at the primer binding sites of 409.703A. The nucleotide sequences of forward and reverse primer binding sites to 409.703A primer pair were determined from 5 HBV samples. The sequences were aligned for comparison. Two substitutions at the primer binding sites of SM#63, and one substitution at reverse primer binding sites of CB#14 are indicated.

SM#63 and CB#14 were sequenced. For control, three randomly selected samples that were detected by all primer pairs were also sequenced. As shown in Fig. 4, the sequences of 409.703A primer binding sites were found to be mutated. In SM#63 sample, two substitutions were observed, one at the forward primer binding site and one at the reverse primer site. In CB#14 sample, one substitution at reverse primer binding site was observed. Interestingly, all mutations were found at near the 3′ or 5′ ends of the primer binding sites and substitutions were G or C to A substitutions. Thus, the failure of the 409.703A primer pair to detect SM#63 and CB#14 samples appeared to be due to the mutation at the primer binding sites.

Discussion

The aims of good primer design are to maximize both specificity and efficiency of the amplification reaction, which are determined by multiple factors (Newton, 1995). These include primer positions with respect to target, base composition of primers, primer length, melting temperature, the degree of degeneracy, mismatches within and at the ends of the primers, and complementarity between the ends of primers. Computer programs such as Primer3 and its modified versions have been developed to take these factors into account for better primer design (Rozen and Skaletsky, 2000; Koressaar and Remm, 2007; Untergasser *et al*., 2007). Matchup program was developed based on Primer3 (http://plaza.snu.ac.kr/~jchun/matchup/). One of the advantages of Matchup program over the conventional primer-designing programs is to design universal primer pairs from multiple sequences. This feature is important especially in designing primers to detect viruses whose genomic sequences are highly variable compared to prokaryotic and eukaryotic genomes.

We designed primer pairs to detect hepatitis B virus (HBV) from serum samples using Matchup program. One of such primer, MUW, was highly efficient in detecting HBV from serum samples. The efficiency of the MUW primer pairs was comparable to Amplicor system which has been a gold standard in clinical labs to detect HBV. In clinical labs, the detection and quantification of HBV genomes in molecular biology-based assays appear to be the most reliable methods for monitoring HBV infection and assessing responses to antiviral treatment (Pawlotsky *et al*., 2000). Many methods have been developed including a solution-hybridization assay based on hybrid-capture (Digene Hybrid-Capture, Murex Diagnostics, UK); a signal-amplification assay based on branched-DNA (bDNA) technology (Urdea *et al*., 1991; Hendricks *et al*., 1995); and a target-amplification assay based on competitive polymerase chain reaction (Ranki *et al*., 1995). The last assay was significantly more sensitive than both the hybrid-capture and bDNA methods and is available in standardized format (Amplicor HBV Monitor™, Roche Molecular Systems) (Gerken *et al.*, 1998; Kessler *et al*., 1998). Although limited in the number and diversity of clinical samples, comparison of the Amplicor system and our PCR system using MUW primer pair suggested that MUW system is compatible to Amplicor system in the efficiency of detecting HBV in serum samples. MUW system does not require specialized instrument or complicated protocol required for Amplicor system.

Since the MUW primer pair was designed by Matchup program using multiple (n=691) sequences, we expected that the higher detection rate is correlated with the higher conservation of the sequences of the primer binding sites. We found that the detection rate was correlated with the sequence conservation of the binding sites to forward primer but not those to reverse primer. Currently, the reason for this observation is not known. However, a clue may be found from the observation that the forward primer binding sites were more conserved among 691 isolates than the reverse primer binding sites for the primer pairs used in this study (data not shown).

Efficiency of the primers is also determined by the primer quality which depends on the primer sequence. Chen *et al*. (2003) developed a method to calculate the primer quality considering melting temperatures, formation of hairpin and dimer structure and free energies of the primers. Primers with higher quality score are more effective in detecting targets by PCR (Jang *et al*., 2008). In this study, we were able to find a strong correlation between the primer quality and detection rate among the primer pairs in HBV detection by PCR. Primers with higher quality scores were more effective than primers with lower quality scores in detecting HBV by PCR. There were exceptions for this observation where the primer with higher quality score failed to detect HBV from samples that were detected by the lower primer quality score. In these cases, substitutions at near the 3′ or 5′ ends of the primer binding sites were observed. The 3′ end of the primer is important for successful amplification of PCR and mis-pairing at the 5′ end of primer often results in wrong PCR products (Kwok *et al*., 1990; Dieffenbach *et al*., 1993; Cantor and Smith, 1999). Moreover, all these mutations are substitution of G or C to A. Thus, binding of primer oligonucleotide to its binding site becomes less stable.

In conclusion, we were able to design a good primer pair for HBV detection by PCR using Matchup program as effectively as COBAS Amplicor HBV Monitor system. Further refinement and standardization of the primer will help to detect HBV effectively with low cost. And Matchup program will prove to be useful to design efficient primers for viruses whose genomes are highly variable.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (Ministry of Education, Science and Technology). (The Regional Research Universities Program/Chungbuk BIT Research-Oriented University Consortium)

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