SUBTYPING OF THAI HEPATITIS B VIRUS DNA BY POLYMERASE CHAIN REACTION

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ABSTRACT

Polymerase chain reaction was modified and applied for subtyping of 200 sera of Thai hepatitis B virus(HBV) carriers. Four pairs of oligonucleotide primers which derived from certain amino acids coded by the S gene were utilized for subtyping. First 2 pairs which contained the variations at amino acid residues 68 and 122 of the S gene product were used to subtype for d *and* y *determinants, while the latters which had the variations at residues 47 and 213 were used for r and w determinants. Southern-blot hybridization was used to confirm the amplified products as due to HBV-DNA. Furthermore, an immunological subtyping with monoclonal antibodies (immunodiffusion) was also conducted to verify the PCR approach. The study revealed that all these Thai HBV-carriers were* ad *determinant positive which no* ay *were found at all, and they could be segregated as* adr *and* adw *subtypes as 81.0% and 19.0%, respectively.*

INTRODUCTION

The surface antigen (HBsAg) of hepatitis B virus (HBV) carries the common group specific antigenic determinant a. In addition, it contains one member of each of the two pairs of mutually exclusive subtype determinants d/y and w/r. These result in dividing the virus into four major subtypes adw, adr, ayw and ayr which are proposed to represent the phenotypic expression. These subtypes differed in their epidemiological distribution and were used in tracking the route of HBV infection among populations or among individuals[1]. In practice, subtypes of HBV have been distinguished by differential reactivity to monoclonal and monospecific polyclonal antibodies against the surface (S) antigen[2,3,4,5]. Recently, it has been demonstrated that the polymerase chain reaction (PCR) is a powerful technique for directly amplifying short segments of the genome. The application of PCR has improved as a sensitive detection of DNA or genome of hepatitis B virus (HBV-DNA) in clinical samples[6,7,8,9,10].

The specificity of HBsAg subtype is attributed to variation in the amino acid sequence of the polypeptide of 226 residues encoded by the S gene[11,12]. Thus,the antigenic characteristics

of HBsAg can be correlated to point mutations within the S gene[13]. The key amino acid residues for specifying *d/y* determinants have been shown to be positioned at 68 and 122, while for *w/r* determinants at 4, 47, 110, 113, 126, 160, and 207[14]. By using these correlations, the PCR has been previously applied to determine HBV subtypes[15,16,17].

Epidemiological studies of HBV subtypes in Thailand have not been seriously done due to lacking of appropriate method, thus, the PCR technique was modified and used as a tool to study the distribution of HBV subtypes among HBV carriers in Thailand.

MATERIALS AND METHODS

Serum samples

Two hundred human sera which contained HBsAg and e antigen of HBV were selected from those blood specimens obtained from HBV carriers donated to The Thai Red Cross. All blood specimens had been screened and found to be freed from non-A, non-B viral hepatitis, HIV antigen and antibody to HIV virus and only those HBsAg positive specimens were screened for HBeAg by reverse passive hemagglutination assay (RPHA).

DNA extraction

HBV DNA was extracted with phenol chloroform from 200 ml of serum sample treated for 2 hours with 200 mg/ml of proteinase K in 10 mM Tris-HCl pH 8.3, 0.5 % SDS at 50 °C. The DNA was then precipitated with ethanol and dissolved with 10 ml of 10 mM Tris-HCl pH 7.5, 0.1 mM EDTA.

Oligonucleotide primers

Four specific primer pairs of oligonucleotide primers were used. The nucleotides of each primer were selected from those nucleotide fragments that located at unique positions from the EcoRI site. The nucleotides were numbered according to method described by Okamoto, H., *et al.*[14] where the sequences were aligned with the nucleotide sequence using the first A residue of the EcoRI site as nucleotide 1.

1. P_1, 5'-CAACCTCTTGTCCTCCAAT-3' and P_2, 5'-CAGGAATCGTGCAGGTCTT-3' were the primer pair used to determine *d* determinant.

2. P_3, 5'-CAACCTCTTGTCCTCCAAC-3' and P_4, 5'-CAGGAATCGTGCAGGTTCT-3' were the primer pair used to determine *y* determinant.

P_1 and P_3 were nucleotide fragments that located at positions between 339 - 357 while P_2 and P_4 were located at positions between 518 - 536.

3. P_5, 5'-CTAGGGGGAGCACCCAC-3' and P_6, 5'-AAAAGAAAATTGGTAAGAGA-3' were the primer pair used to detect *r* determinant.

4. P_7, 5'-CTAGGGGGAACACCCGT-3' and P_8, 5'-AAAAGAAAATTGGTAAGAGG-3' were the primer pair used to detect *w* determinant.

P_5 and P_7 were nucleotide fragments that located at positions between 278 - 294 while P_6 and P_8 were located at positions between 796 - 815.

PCR amplification

An aliquot of 2 ml of the extracted DNA sample was added to 48 ml of a reaction mixture containing 10 mM Tris-HCl pH 8.3, 30 mM KCl, 1.5 mM $MgCl_2$, 125 mm each of dNTP (dATP, dGTP, dCTP, dTTP), 300 ng of each primer and 1.0 unit Taq polymerase (AmpliTaq, Perkin-Elmer Cetus, USA). Finally, 100 ml of mineral oil was laid over the reaction mixture. The reaction was performed in a programmable DNA thermal cycler for 40 cycles. One amplification cycle was consisting of denaturation for 30 seconds at 96 °C, annealing for 30 seconds at 55 °C and extension of the annealed oligonucleotide primers which was allowed for 1 minute at 74 °C. An annealing with the primers P_5 - P_6 and P_7 - P_8 was performed at 47 °C and extension was made at 66 °C.

After the amplification, an aliquot of 10 ml of the PCR reaction mixture was subjected to gel electrophoresis on 3% agarose in Tris - acetate - EDTA buffer. The gel was stained with ethidium-bromide for photographing.

Southern-blot hybridization

The amplified DNA of PCR product on agarose gel was blot-transferred onto a nylon membrane according to the method described by Southern[18]. The blot was hybridized with non-radioisotope labelled (ECL oligonucleotide labelling with an aid of a detection kit, Amersham, UK) oligonucleotide probe (Takara, Japan) which could specifically detect HBV-DNA. The hybridization was determined through chemiluminescent method on autoradiographed X-ray film.

HBsAg subtyping by serological immunodiffusion.

The samples of human serum and the monoclonal antibodies which raised against r or w epitopes of HBsAg were loaded into 2% agarose gel in 0.08 M barbitone buffer pH 8.2 in a manner of double-diffusion[19]. The gel was incubated overnignt at room temperature and then stained with coomassie-blue in order to detect the precipitin band.

RESULTS

HBV - DNA were successfully extracted from human sera obtained from blood donors that carried HBsAg and HBeAg. The HBV DNA was firstly amplified with the given primer pairs that used to determine *d* and *y* determinants (P_1 - P_2, and P_3 - P_4, respectively). It was found that all DNA samples subjected to amplification with those differentiating primer pairs were positive to *ad*-detection primers of 198 base pairs PCR product but negative to the other set of primers. Some of positive reactions are shown in Figure 1.

The DNA samples obtained from amplification with *ad*-detection primers were subsequently amplified to both *r* and *w* - determinant detection primers (P_5 - P_6 for *r* , and P_7 - P_8 for *w*) in order to discriminate between *r* and *w* determinants. It was found that some were positive to *w* primers (no. 28, 44) while some of the others (no. 49, 55, 78, and 93) were positive to *r* primers, and the size of products was approximately 538 base pairs. Some of the positive reaction are shown in Figure 2A. In order to confirm the existence of HBV genome due to these subtype - specific amplifications, the PCR products were Southern-

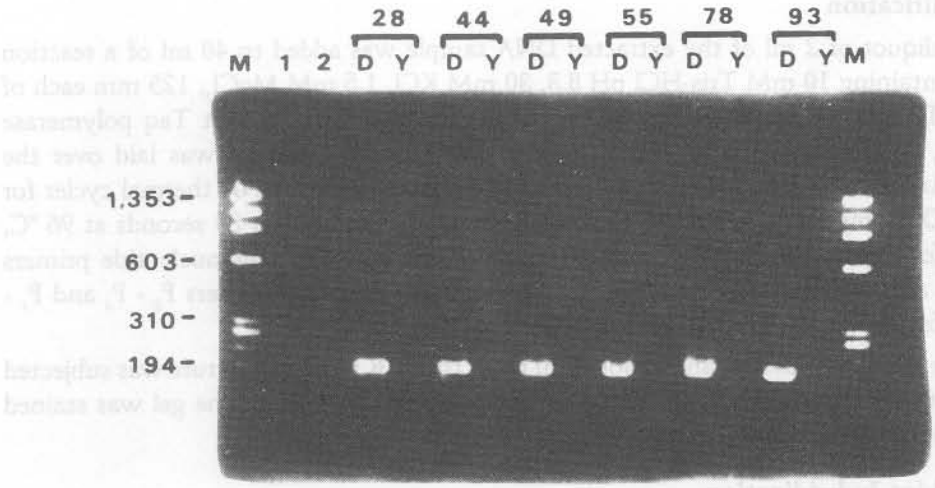

Fig. 1. Agarose gel electrophoresis of the PCR products after DNA preparations had been amplified with *d* (D) and *y* (Y)-determinant detection primers. Lanes: 1&16(M) show DNA molecular size markers in base pairs; 2 & 3 (1, 2), DNA preparations from normal human serum used as negative control, 4 to 15, DNA preparations from DNA sample no. 28, 44, 49, 55, 78, and 93.

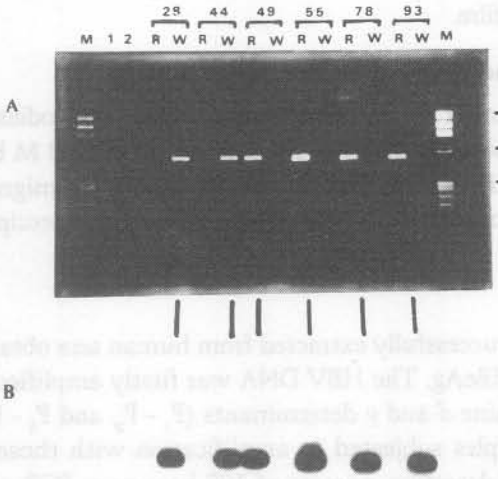

Fig. 2. Agarose gel electrophoresis of PCR products that had been amplified with *ad*-detection primers and were subsequently amplified to both *r* and *w* - determinant detection primers. Figure 2A shows DNA sample no. 28 and 44 were positive to w primer and DNA sample no. 49, 55, 78 and 93 were positive to *r* primer. Lanes 1 and 16 (M) show DNA molecular size markers in base pairs. Figure 2B demonstrated Southern-blot hybridization of PCR products in 2A with the oligonucleotide probe specific for HBV-DNA.

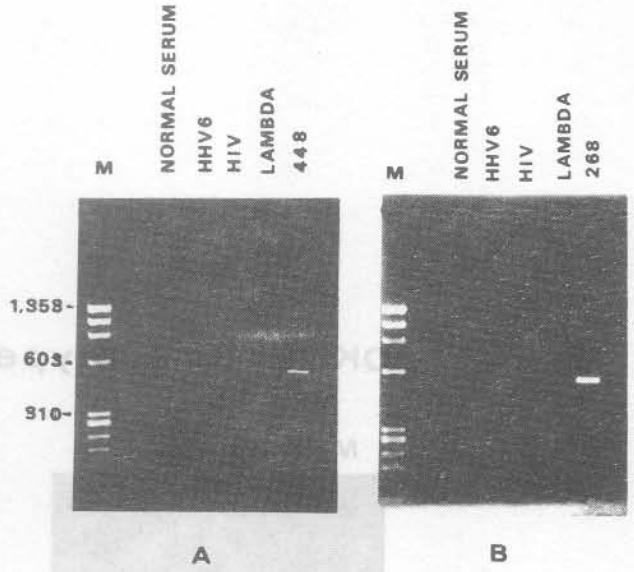

Fig. 3. Agarose gel electrophoresis of PCR products that had been amplified with *r* (A) and *w* (B) - determinant detection primers using DNA samples derived from normal human serum, human herpes virus 6, human immunodeficiency virus, and bacteriophage lambda . Lanes: M shows DNA molecular size markers in base pairs. Figure A, sample no.448 and figure B, sample no. 268 were positive against *adr* and *adw* by PCR, respectively.

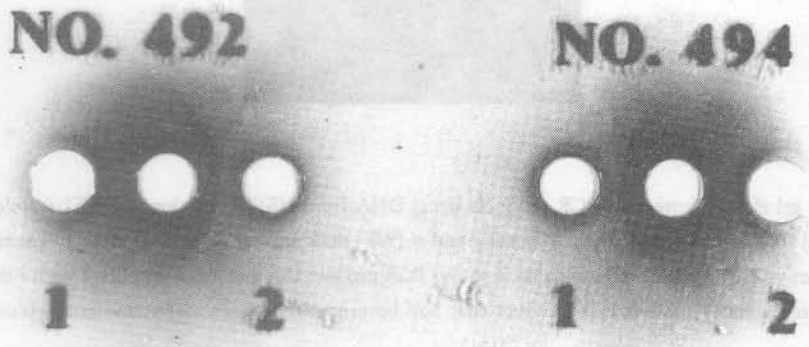

Fig. 4. Gel diffusion test of serum samples (in the middle well) no. 492 and 494 which were positive against *adw* and *adr* by PCR, respectively. Serum samples were tested against 1:20 dilution of the anti - *r* (1) and the anti - *w* (2) monoclonal antibodies. Serum no. 492 gave positive reaction to anti-w where serum no. 494 gave positive reaction to anti-*r*.

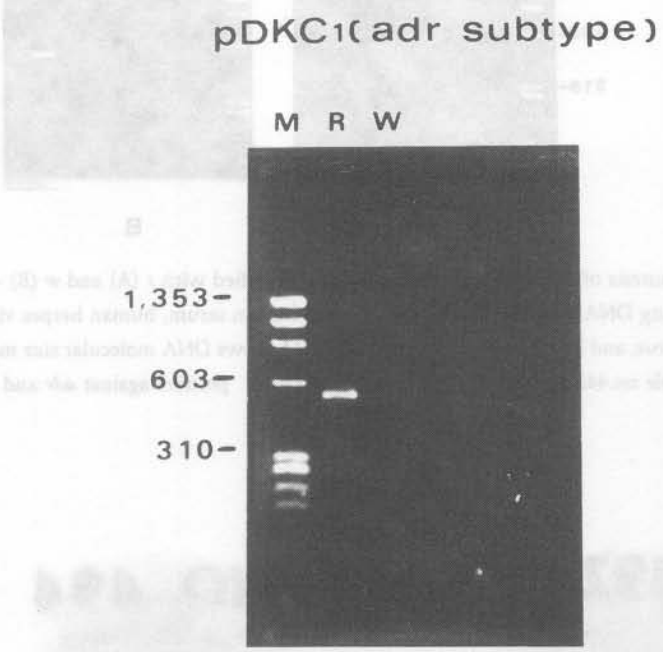

Fig. 5. Agarose gel electrophoresis of PCR products using DNA from HBV-DNA clone pDKC1 (serologically *adr* subtype) that had been amplified with *r* (R) and *w* (W) - determinant detection primers. Lanes: M shows molecular weight markers in base pairs; R shows PCR product that had been amplified with *r*-determinant detection primer; W shows PCR product that had been amplified with *w*-determinant detection primer.

blot hybridized to commercially supplied oligonucleotide probe which was specific to HBV genome. It was found that all of the amplified fragments were HBV-DNA. Some of positive sample are shown in Figure 2B. In this study, all 200 sera gave positive reaction to *d* - determinant and 162 samples were discriminated as *r* subtype (or equivalent to 81%) while the remaining 38 samples gave positive reaction to *w* determinant (or equivalent to 19%).

In order to rule out any cross-amplification of *adr/adw* subtype discriminating primers used with non-hepatitis B virus DNA, DNA from various sources, i.e., normal human serum that free from HBsAg and anti HBs, human herpes virus 6 (HHV6), human immunodeficiency virus (HIV), and bacteriophage lambda were used as control. Results showed no cross-reactivity between the primers and the DNA from the given sources. Some of examples are shown in Figure 3.

Two groups of samples where each group consisting of 20 serum samples were randomly selected from those sera that gave positive reaction to r determinant and to *w*, respectively, by the PCR technique, and analysed for *r/w* determinants by a simple serological immunodiffusion method, against anti - *r*, and anti - *w* monoclonal antibodies. Results of serological reaction correlated very well with PCR subtyping. As shown in Figure 4, the serum No. 492 and 494 which were positive to w and r by the PCR technique, respectively, produced precipitin band when reacted with the anti - *w* and the anti - *r* monoclonal antibodies, respectively. In addition, all samples of both groups showed no cross reaction with the antibody against the heterologous epitope, and the subtype specificity gave a positive correlation with those of the PCR technique.

The study on the specificity of the primers used for subdeterminant detection was done against known DNA. Some examples of results are shown in Figure 5. The pairs of primers P_5 - P_6 and P_7 - P_8 were tested against a known serologically subtype adr, HBV-DNA clone pDKC1 (Balachandra, K. *et al.*, Section of Biotechnology, Department of Medical Sciences, Bangkok, Thailand) using PCR reaction. It was found that only primers P_5 - P_6 which were used to determine the *r* determinant in this study gave a specific reaction. Unfortunately, due to unavailability of known *adw* DNA, the specificity of the latter primers was not tested.

DISCUSSION

In this study HBV-DNA was prepared from the serum containing HBsAg and HBeAg based on the fact that the DNA was accompanied by the presence of HBeAg [20]. It has been demonstrated that PCR is the most sensitive technique available so far for detecting HBV-DNA sequences which 10^{-5} pg of the DNA can be detected[9], especially in subjects which are negative for all serological markers for HBV or hepatitis C but has evidence of hepatitis[21,22], or individuals of chronic hepatitis B that give negative reaction by dot-blot hybridization[21,22].

On the basis of additional minor determinants, nine subtypes of HBsAg were defined, i.e., ayw_1, ayw_2, ayw_3, ayw_4, *ayr*, adw_2, adw_4, and *adr*[1].

The PCR has been previously applied to determine HBV subtypes by using the correlations between HBsAg subtype and the S gene sequence. It was shown that PCR was

successfully used to discriminate between *d/y* determinants[16], or to directly differentiate HBV subtypes[15,23,24] and to discriminate HBV genotypes in the approach of HBV transmission detection[17].

At present, the general practice in identifying subtypes among Thai HBV-carriers is that the blood samples are firstly discriminated between *d* and *y* determinants. The samples are then subtyping against *w* or *r*. In terms of specificity of the *d*-determinant primers used, the sequences of both primers (P_1 and P_2) have been checked for the homology in whole DNA of subtype *ayw*[25,26] and *ayr*[14], and it is shown that there are no sequence similarities among these genomes. These results suggest that there is no false positive occurred from the sera containing *y* epitope.

There are 2 amino acid sequence variations in the *d* or *y* detection primers used in this study that coded by the S gene which are subjected to determinant and subtype consideration, i.e., residues 68 and 122 which occurred in the forward and reverse primers, respectively. In these primers, the amino acids that can be coded by the sequences are isoleucine and lysine at residues 68 and 122, respectively, for the *d* determinant, while they are threonine and arginine at the same residues for the y determinant. It has been shown that there are some homology among 4 subtypes with some amino acid variations[14]. Among these variations, isoleucine is found in residues 68 of 6 adr and 2 *adw*-genomes, while threonine is found in 1 *ayr* and 2 *ayw*. At residue number 122 of the same genomes, lysine is found for the subtypes *adr* and *adw*, whereas arginine is found for *ayr* and *ayw*.

In this study, all 200 sera of HBV carriers were found to carry *d*- determinant by PCR. They could be segregated to *r* or *w* determinant by the same method in which 81% were *r*-determinant and 19% were *w* - determinant. The nucleotide sequences that coded for amino acids number 47 and 213 of the S gene were emphasized for the consideration of subtyping. According to *r* or *w* determinant detection amino acid number 47 was either threonine or valine, and 213 was either leucine and isoleucine, respectively. It was shown by Okamoto *et al.*[14] that a similar evidence was seen in residues 47 and 213 of 6 *adr*-DNA clones, 1 *ayr*, 2 *adw*, and 3 *ayw*, due to *r* or *w* determinants. The complete amino acid sequences of HBsAg of 88 HBV strains had been compared by Norder, *et al.*[27] and the conservation of certain residues could be observed. It was shown that at the amino acid number 47, all of 13 strains of *adr* were threonine, while all of 19 strains of adw_2 (subdeterminant of *adw*) were valine. On the other hand, at the number 213 all of the *adr* were leucine, while 13 of the 19 *adw* strains were isoleucine.

In addition to this HBV subtyping which manipulated by PCR, a serological test (immunodiffusion) was also tested as a referenced method. It was shown that both methods gave the same results for the investigations of *r* and *w* determinants.. Furthermore, a referenced HBV-DNA (*adr* subtype) gave a positive confirm to the specificity of the PCR method. Unfortunately, there was no *adw*-DNA to reconfirm this result.

It is demonstrated that the group of hepatitis B virus carriers can be subtyped by PCR and this technique is shown to be reliable to a certain extent.

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REFERENCES

1. Courouce-Pauty, A.M., Plancon, A., Soulier, J.P. (1983). Distribution of HBsAg subtypes in the world. Vox. Sang. **44**: 197-211.
2. Ben-Porath, E., Wands, J., Marciniak, R., Wang, M., Hornstein, L., Ryder, R., Canlas, M., Lingao, A., Issel-Bacher, K. (1985). Structural analysis of hepatitis B surface antigen by monoclonal antibodies. J. *Clin. Invest.* **76**: 1338-1347.
3. Courouce, A.M., Holland, P.M., Muller, J., Soulier, J. (1976). HBs antigen subtypes. *Bibl. Haematol.* **42**: 31-41.
4. Courouce, A.M., Lee, H., Drouet, J., Canavagio, M., Soulier, J. (1982). Monoclonal antibodies to HBsAg: a study of their specificities for eight different HBsAg subtypes. *Dev. Biol. Stand.* **54**: 527-534.
5. Usuda, S., Tsuda, F., Gotanda, T., Tachibana, K., Nomura, M., Okamoto, H., Imai, M., Nakamura, T., Miyakawa, Y., Mayumi, M. (1986). A solid phase immunoassay for the common and subtypic determinants of hepatitis B surface antigen with monoclonal antibodies. *J. Immunol. Methods* **87**: 203-210.
6. Larzul, D., Guige, F., Sninsky, J., Mack, D.H., Brechot, C., Guesdon, J.L. (1988). Detection of hepatitis B virus sequences in serum by using in vitro enzymatic amplification. *J. Virol. Methods* **20**: 227-237.
7. Thiers, V., Nakajima, E., Kremsdorf, D., Mack, D., Schellekens, H., Driss, F., Godeau, A.A., J.L., Wands, J., Sninsky, J., Tiollais, P., Brechot, C. (1988). Transmission of hepatitis B from hepatitis B seronegative subjects. *Lancet ii.*(special issue): 1273-1276.
8. Sumuzaki, R., Motz, M., Wolf, H., Heinig, J., Jilg, W., Deinhardt, F. (1989). Detection of hepatitis B virus in serum using amplification of viral DNA by means of the PCR. *J. Med. Virol.* **27**: 304-308.
9. Kaneko, S., Miller, R.H., Feinstone, S.M., Unoura, M., Kobayashi, K., Hatori, N., Purcell, R.H. (1989). Detection of serum heatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. *Proc. Natl.Acad. Sci. USA.* **86**: 312-316.
10. Keller, G.H., Huang, D.P., Shih, J.W.K., Manak, M.K. (1990). Detection of hepatitis B virus DNA in serum by polymerase chain reaction amplification and micro-titer sandwich hybridization. *J. Clin. Microb.* **128**: 1411- 1416.
11. Cold, J.W.M., Shih, J.M.K., Purcell, P.H., Gerin, J.L. (1976). Characterization of antibodies to the structural of HBsAg : evidence for subtype - specific determinants. *J. Immunol.* **117**: 1404-1406.
12. Shih, J.W.K., Tan, P.L., Gerin, J.L. (1978). Antigenicity of the major polypeptides of hepatitis B surface antigen(HBsAg). *J. Immunol.* **120**: 520-525.
13. Okamoto, H., Tsuda, F., Sakugawa, H., Sartrosoewignjo, R.I., Imai, M., Miyakawa, Y., Mayumi, M. (1988). Typing hepatitis B virus by homology in nucleotide sequence : comparison of surface antigen subtypes. *J. Gen. Virol.* **69** : 2575-2583.
14. Okamoto, H., Imai, M., Shimozaki, M., Hoshi, Y., Iizuka, H., Gotanda, T., Tsuda, F., Iyakawa, Y., Mayumi, M. (1986). Nucleotide sequence of a cloned hepatitis B virus genome, subtype ayr: comparison with genomes of the other three subtypes. *J. Gen. Virol.* **67**: 2305-2314.
15. Norder, H., Hammas, B., Magnius, O. (1990). Typing of hepatitis B virus genomes by a simplified polymerase chain reaction. *J. Med. Virol.* **31**: 215-221.
16. Nicholson. W.J., Black, S.H., Simmonds, P., Chung, C.W., Aw, D., Peutherer, J.F. (1992). Comparison of hepatitis B virus subtyping of *d/y* determinants by radio-immunoprecipitation assay and the polymerase chain reaction. *J. Med. Virol.* **36**: 21-27.

17. Repp, R., Rhiel, S., Heermann, K.N., Schaeffer, S., Keller, C., Ndumbe, P., Lampert, F., Gerlich, W.H. (1993). Genotyping by multiplex PCR for detection of endemic hepatitis B virus transmission. J. *Clin. Microbiol.* **31**: 1095-1102.

18. Southern, E.M. (1975). Detection of specific sequences among DNA fragments by gel electrophoresis. *J. Mol. Biol.* **98**: 503.

19. Ouchterlony, O., Nilsson, L.A. (1978). Immuno-diffusion and immuno-electrophoresis. In D.M. Weir (ed). Handbook of experimental Immunology 3e, 19. Blackwell Scientific Publicatios, Oxford.

20. Hoofnagle, J.H., Duscheiko, G.M., Seeff, L.B., Jones, E.A., Waggoner, J.G., Bales, Z.B. (1981). Seroconversion from hepatitis B e antigen to antibody in chronic types B hepatitis. *Ann. Intern. Med.* **94**: 744-748.

21. Paterlini, P., Gerken, G., Nakajima, E., *et al.* (1990). Polymerase chain reaction to detect hepatitis B virus DNA and RNA sequences in primary liver cancers from patients negative for hepatitis B surface antigen. *N. Engl. J. Med.* **323**: 80-85.

22. Wirth, S., Mollers, U., Keller, K.M., Winterpacht, A. (1992). Use of the polymerase reaction to demonstrate hepatitis B virus DNA in serum of children with chronic hepatitis B. *J. Pedriatrics* **120**: 438-440.

23. Yotsumoto, S., Okamoto, H., Tsuda, F., Miyakawa, Y., Mayumi, M. (1990). Subtyping hepatitis B virus DNA in free or integrated forms by amplification of the S-gene sequences by the polymerase chain reaction and single track sequencing for adenine. *J. Virol. Methods* **28**: 107-116.

24. Shih, J.W.K., Cheung, L.C., Altser, H.J., Lee, L.M., Gu, J.R. (1991). Strain analysis of hepatitis B virus on the basis of restriction endonuclease analysis of polymerase chain reaction product. *J. Clin. Microbiol.* **29**: 1640-1644.

25. Galibert, F., Mandart, E., Fitousi, F., Tiollais, P., Charnay, P. (1979). Nucleotide sequence of the hepatitis B virus genome (subtype *ayw*) cloned in *E. coli. Nature* **218**: 646-650.

26. Bichko, V., Pushko, P., Dreilina, D., Pumpen, P., Gren, E. (1985). Subtype ayw variant of hepatitis B virus DNA primary structure analysis. *FEBS.* **185**: 208-217.

27. Norder, H., Hammas, B., Dee, S., Bile, K., Courouce, A.M., Mushahwar, I.K., Magnius, L.O. (1993). Genetic relatedness of hepatitis B viral strains of diverse geographical origin and natural variations in the primary structure of the surface antigen. *J. Gen. Virol.* **74**: 1341-1348.