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Detection of low level viral genome (HBV DNA) by Polymerase Chain Reaction in nonresponders of plasma-derived hepatitis B vaccine produced at the Department of Medical Research (Lower Myanmar)

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Myanmar is an area of high endemicity for hepatitis B with a 35-60% infection rate and 10-15% HBsAg carrier rate. Several vaccine trials carried out at the Department of Medical Research (Lower Myanmar) using plasma-derived hepatitis B vaccine have shown that there was approximately 10% non-responder rate. Out of 337 apparently healthy persons who were initially screened for HBsAg, antiHBs and antiHBc by using the Abbott ELISA test kits (USA), 137 persons were negative for all markers (HBsAg, AntiHBs and AntiHBc). They were immunized with three doses of locally produced plasma-derived hepatitis B vaccine (10μg/ml) by intramuscular route in the deltoid region. One month after the completion of three doses of hepatitis B vaccine, 38 subjects who did not respond were vaccinated again using the same dose, site and route. Out of 38 vaccinees, 23 subjects did not have detectable levels (10 m IU/L) at two months after the booster vaccination. HBV DNA was extracted from all serum samples of nonresponders by using the phenol, chloroform and ethanol method. These extracted samples were tested for HBV DNA by using the Polymerase Chain Reaction (PCR). After the amplification of 40 cycles, HBV DNA was detected by 2% agarose gel electrophoresis. The short nucleotide of the HBV DNA (< 200bp) of the positive two samples were amplified successfully by nested PCR and sequenced by Taq Dye Deoxy TM Terminator Cycle Sequencing Kit. These two nonresponders (8.69%) may be due to silent HBV infection.

INTRODUCTION

Myanmar is an area of high endemicity for hepatitis B with 35-60% infection rate [1] and 10-15% HBsAg carrier rate [2]. In Myanmar, the control of viral hepatitis is one of the top priority programmes in the National Health Plan (NHP). Hundreds of millions of plasma-derived hepatitis B vaccines have been used worldwide since 1981 with an outstanding record of safety and efficacy. Several vaccine trials carried out at the Department of Medical Research (Lower Myanmar) using plasma-derived hepatitis B vaccine have shown that there were approximately 10% non-responder rates after three doses of vaccine. Genetic factors may play a role in non-responsiveness to HB vaccine in some instances and low level HBV related viral infection may be another possible mechanism to explain this phenolmenon [3]. Hepatitis B virus is a member of a family of DNA virus called Hepadnaviridae [4]. Its genome consists of approximately 3200 base pairs and contains various genes: PreC/ Cgene codes for two viral components, polypeptides making up the nucleocapsid

and containing the HB core antigen (HBcAg) and a protein which becomes measurable in the blood after post translation process as HBeAg. PreS1/PreS2/S gene make up the viral envelope which contains HBsAg. The P gene codes for the viral DNA polymerase which is central for viral reproduction. X gene is thought to exert various regulatory functions for the virus [5].

Although the serological markers have been shown to be sensitive and convenient for detecting the HBV infection, they are not always good indicators of viral activity. Viral DNA is a more reliable and direct evidence of the presence of the virus [6, 7, 8, 9]. Polymerase Chain Reaction is a highly sensitive and specific method, which can detect a single copy of a gene (HBV DNA) [10]. It is at least 10^4 times more sensitive than dot blot hybridization assays for HBV DNA [11].

Objective

- To detect low levels of (HBV DNA) in non-responders of plasma-derived heaptitis B vaccine produced at Department of Medical Research (Lower Myanmar) using the Polymerase Chain Reaction technique.

MATERIALS AND METHODS

A total of 668 apparently healthy persons comprising of 428 males and 240 females were initially screened for liver function test and HBsAg, antiHBs and antiHBc. Among them, 289 subjects were negative for HBsAg, antiHBs and antiHBc. They were immunized with three doses of plasma-derived hepatitis B vaccine (10 µg/ml) produced at the Department of Medical Research (LM) by intramuscular route in the deltoid region. One month after the completion of three doses, 251 persons developed antibody to hepatitis B (>10 IU/L of antiHBs) as tested by Abbott ELISA kit and the remaining 38 vaccinees who did not respond to vaccination were immunized with 10µg/ml of the same vaccine as booster dose intramuscularly at the same site. Twenty-three vaccinees out of 38 did not have detectable level (10 IU/L) at two months after the booster vaccination. HBV DNA was extracted from these 23 serum samples by the phenol, chloroform and ethanol method. Briefly,100 µl of serum was incubated at 70ºC for 3 hours in 300 µl of 1% SDS containing 15mM Tris HCl (pH = 8), 10mM EDTA (pH = 8) and 500 µg/ml of proteinase K (Merk, Darmstadt, Germany).

The nucleic acids were purified by phenol, chloroform extractions followed by precipitation with isopropanol. The resulting pellet was resuspended in 50 µl of RNase-free water, then 4 µl of this solution was used as a template for HBV DNA amplification. The 40 µl of reaction buffer was made up for PCR1 which contained 10 x Gold buffer (1 x buffer containing 1.5mM MgCl2), 5 U Ampli Taq Gold DNA polymerase (Perkin-Elmer, Norwalk, CT, USA), dNTP mix (10mM) and two pairs of PCR primers (MD24 and MD26 / HBX1 and HBX2). Thermocycler was programmed first to incubate the samples for 50 min at 37ºC, then preheat at 95ºC for 10 min to activate Ampli Taq Gold followed by 40 cycles consisting of 94ºC for 20 seconds, 55ºC for 20 seconds and 72ºC for 7 minutes using a Perkin-Elmer 9700 Thermal Cycler (Perkin-Elmer). For the PCR 2 reaction, 2 µl of the first PCR product was mixed with same PCR buffer and second set of each inner primer. Thermocycling for 40 cycles was done as above but the annealing temperature was set at 60ºC instead of 55ºC for the second round of PCR [12].

The PCR products were electrophoresed on 2% agarose gels staining with ethidium bromide and evaluated under UV light. The sizes of PCR products were estimated according to the migration pattern of 50bp DNA ladder (Pharmacia Biotech, Piscata-way, NJ, USA). The two serum samples (NR 16 & 18) which were positive for HBV DNA were purified by using the QIA quick PCR purification kit

(Germany). The recovered PCR products were subjected to direct sequencing from both directions using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer). Sequenses of amplified cDNA were determined using a sequencer (ABI model 310 Genetic Analyzer) according to computer programme.

Primer sequences used for nested PCR

Region of gene for HBV DNA	Level of PCR	Name of primer	Nucleotide sequences	Product size
X region	PCR 1	MD 24	5'TGCCAACTGGATCCTTC GCGGGACGTCCTT-3' (nt 1392-1421)	233bp
X region	PCR 1	MD 26	5'GTTCACGGTGGTCTCC ATG-3' (nt 1625-1607)	233bp
X region	PCR 2	HBX1	5'GTCCCCTTCTTCATCTG CCGT-3' (nt 1487-1507)	117bp
X region	PCR 2	HBX2	5'ACGTGCAGAGGTGAAG CGAAG-3' (nt 1604-1584)	117bp

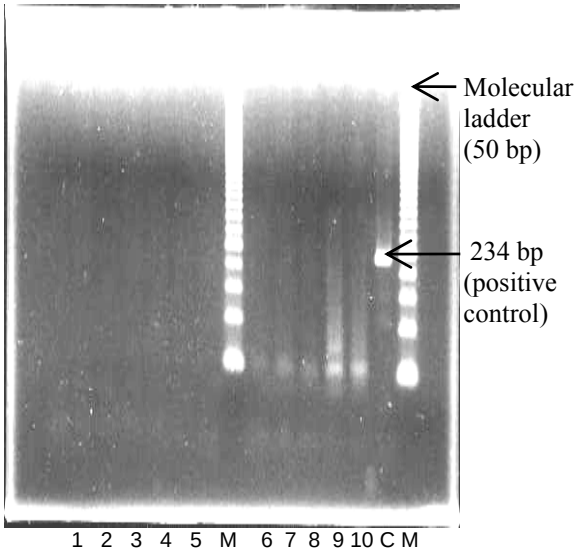

Fig. 1. Detection of HBV DNA in 10 samples from nonresponders amplified by PCR1 reaction, analyzed by 2% gel electrophoresis with ethidium bromide staining

In all samples, the multiple non-specific bands were detected according to 50 bp marker ladder. HBV DNA in positive control band was specifically detected at 234bp (arrow) (Fig.1). At 7 and 8 samples, a distinct band at the same level as positive control (118bp) was seen. The other samples of nested PCR products did not show the specific band (Fig.2).

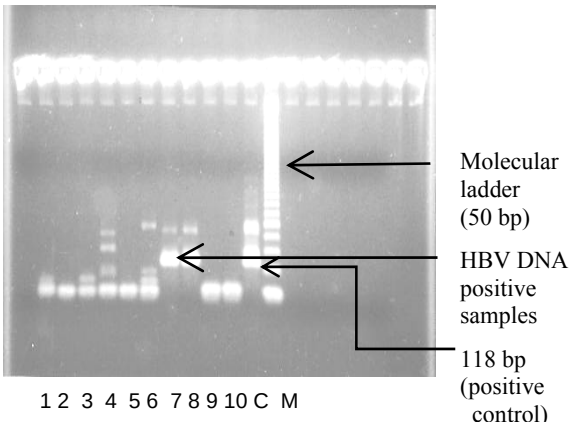

Fig. 2. Detection of HBV DNA in 10 samples of amplified nested PCR products analyzed by 2% agarose gel electrophoresis with ethidium bromide staining

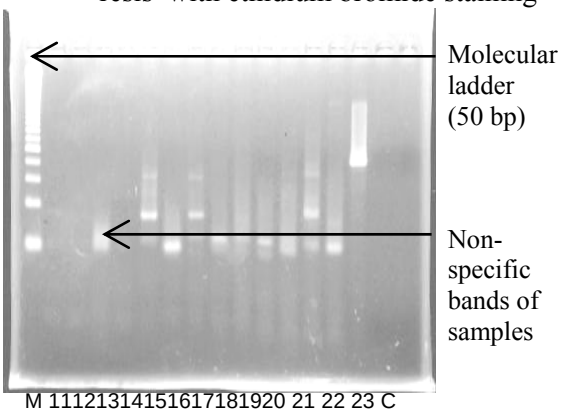

Fig. 3. Detection of HBV DNA in the last 13 samples of amplified PCR 1 products analysed by 2% gel electrophoresis with ethidium bromide staining

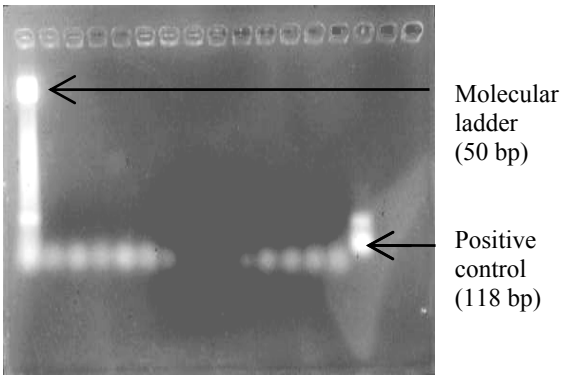

Fig. 4. Analyzing of last 13 samples of nested PCR 2 products by 2% agarose gel

In all serum samples were detected multiple nonspecific band at different levels according to the 50 bp marker ladder. The typical band of 234 bp was only detected in the positive control in this study (arrow) (Fig.3) The specific band was not detected in all 13 samples of non-responders at the same level of positive control (118bp) according to the marker ladder (50bp) (arrow) (Fig.4).

RESULTS

Two hundred and eighty nine apparently healthy persons received three doses of DMR plasma-derived hepatitis B vaccine, out of which 38 persons did not respond to this vaccine at one month after the completion of three doses and 23 non-responders did not have detectable level (10mIU) at two months after the booster vaccination. Persistence of non-responders rate (%) was highest in > 61 years age group (100%) and 51-60 years age group (85.71%). Lowest nonresponders rate was found in 11-20 year age group (0%) and 21-30 years age group (40%). The non-responders rate was 57.41% in 31-40 years age group and 60% in 41-50 years age group (Table 1). The non-responder persistence rate was 64.51% in male and 42.85% in female after booster vaccination (Table 2). After extraction of DNA from each serum sample of nonresponders by using the phenol/ chloroform method, non specific bands of first 10 samples were found in PCR 1 which was analyzed by 2% agarose gel electrophoresis stained with ethidium bromide. HBV DNA positive control band (234bp) was detected according to 50bp marker ladder (Fig.1). The distinct bands of two serum samples (NR 16 and NR 18) were found at the same level of positive control band (118bp) after amplification of nested PCR 2 using 2% agarose gel electrophoresis stained with ethidium bromide. The other samples did not show specific band on gel (Fig. 2). The remaining DNA extraction of 13 serum samples showed nonresponders bands according to the marker ladder (50bp) but positive control band (234bp) was found in 2% agarose gel electrophoresis stained with ethidium bromide after amplification of PCR1 (Fig. 3). The product of second nested PCR2 did not show the specific bands in 2% agarose gel, although 118bp positive control was detected according to 50bp marker ladder (Fig. 4).

The short nucleotide sequence of the HBV DNA (>200bp) of the positive two samples (NR 16 and NR 18) were amplified successfully by nested PCR and directly sequenced by using the Taq Dye Dexoy™ Terminator cycle kit. This different four colors pattern of nucleotide sequences (adenine, guanine, cytosine and thiamine) of two amplified nested PCR 2 products came out of from sequencer (ABI model 310 Genetic Analyzer) according to the primer pairs used at PCR 2 (HBX 1 and HBX 2).

Table 1. Non-responders of DMR plasma-derived hepatitis B vaccine in relation to age

Age (years)	Non-responders before booster vaccination			Non-responders after booster vaccination			
	M	F	Total	M	F	Total	% of non-responder
10-20	1	-	1	-	-	-	0
21-30	3	2	5	2	-	2	40.00
31-40	12	2	14	7	1	8	57.14
41-50	9	1	10	6	-	6	60.00
51-60	6	1	7	5	1	6	85.70
>61	-	1	1	-	1	1	100.00
Total	31	7	38	20	3	23	

M=Male F=Female

Table 2. Non-responders of DMR plasma-derived hepatitis B vaccine in relation to sex

Sex	Total number of non-responders	Non-responders after booster vaccination	% of non-response
Male	31	20	64.51
Female	7	3	42.85

DISCUSSION

In this study, out of 38 nonresponders, twenty-three subjects did not respond to booster vaccination of plasma-derived

hepatitis B vaccine after interval of six months from first vaccination. The results showed that the lowest antibody response was found in > 50 years age group and highest response was seen in < 30 years age group. Neonates and young infants generally respond very well to the vaccine, in contrast to older patients who tend to have a diminished immune response [13].

There was a significant correlation between the antibody response and age of the vaccinees in this study. In a previous human immunogenicity trial, using plasma-derived hepatitis B vaccine, the immune response as evidenced by presence of anti HBs, was seen in 90.16% in males and 89.74% in females vaccinees [14]. There was no significant correlation between antibody response and sex in this study (64.51% in males and 42.85% in females).

Certain risk factors such as increasing age, male gender, and obesity, history of smoking and administration of vaccine in the buttock rather than deltoid muscle were some of the risk factors associated with decreased rates of sero-conversion [15]. Low level HBV DNA was detected in the sera of two out of 23 nonresponders, by the Nested Polymerase Chain Reaction (Nested PCR) although hepatitis B markers (HBsAg, antiHBs and antiHBc) were not detected in all the sera samples of the nonresponder when tested by the ELISA method (Abbott EIA). ELISA assay could detect the HBsAg 100-200 pg/ml of serum, corresponding to roughly $3x10^7$ particles /ml. This method is not a perfect indicator for hepatitis B infection especially in endemic region. The calculated weight of HBV DNA equivalent to one virion is approximately $3 \_10^6$ pg. Dot blot assay may detect at least 10^{3-5} virions of one genome equivalent per sample. (3_10^{-6}pg) [16]. Serum hepatitis B virus (HBV DNA) was detected in eight subjects (7.3%) out of 107 HBsAg negative normal Chinese subjects by using poly-merase chain reaction which showed that serological markers were not adequate to rule out HBV infection [17].

In this study, the short nucleotide sequence (117bp) of X gene was detected in two subjects (NR 16 and NR 18) by nested PCR method and sequenced by Taq Dye Deoxy ^{TM} Terminator cycle sequencing kit and ABI^{TM} gene analyser model 310. HBV mutant was responsible for the pathogenesis of "silent" hepatitis B in patients who were negative for both hepatitis B surface antigen (HBsAg) and anti-hepatitis B core antibody (antiHBc) [18]. HBV DNA alone was detected in silent hepatitis B infection in the absence of hepatitis B markers. These two nonresponders (NR16 and NR18) may be due to silent hepatitis B virus infection in this study. Silent HBV infection could be explained by the 8 nucleotide deletion and DR 2 mutation in the X gene coding region. These mutations suppressed the replication and expression of HBV DNA, leading to low levels of HBsAg and AntiHBc which could not be detected by conventional immunological assay techniques [19].

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